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Immunohistochemistry in the diagnosis and classification of neuroendocrine neoplasms: what can brown do for you?☆

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Summary

This review is based on a presentation given at the Hans Popper Hepatopathology Society companion meeting at the 2019 United States and Canadian Academy of Pathology Annual Meeting. It presents updates on the diagnosis and classification of neuroendocrine neoplasms, with an emphasis on the role of immunohistochemistry. Neuroendocrine neoplasms often present in liver biopsies as metastases of occult origin. Specific topics covered include 1. general features of neuroendocrine neoplasms, 2. general neuroendocrine marker immunohistochemistry, with discussion of the emerging marker INSM1, 3. non-small cell carcinoma with (occult) neuroendocrine differentiation, 4. the WHO Classification of neuroendocrine neoplasms, with discussion of the 2019 classification of gastroenteropancreatic neoplasms, 5. use of Ki-67 immunohistochemistry, 6. immunohistochemistry to assign site of origin in neuroendocrine metastasis of occult origin, 7. immunohistochemistry to distinguish well-differentiated neuroendocrine tumor G3 from poorly differentiated neuroendocrine carcinoma, 8. lesions frequently misdiagnosed as well-differentiated neuroendocrine tumor, and 9. required and recommended data elements for biopsies and resections with associated immunohistochemical stains. Next-generation immunohistochemistry, including lineage-restricted transcription factors (e.g., CDX2, islet 1, OTP, SATB2) and protein correlates of molecular genetic events (e.g., p53, Rb), is indispensable for the accurate diagnosis and classification of these neoplasms.

Keywords

Neuroendocrine; World Health Organization Classification; Carcinoma of Unknown Primary; Immunohistochemistry; Differential Diagnosis; INSM1; Ki-67

1. General Features of Neuroendocrine Neoplasms

Neuroendocrine neoplasms (NEN) include well-differentiated neuroendocrine tumor (NET), poorly differentiated neuroendocrine carcinoma (NEC), pheochromocytoma (PHEO), and paraganglioma (PARA). All of these are characterized by **general neuroendocrine marker**

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expression and the production of **peptide hormones and/or biogenic amines**. NET/NEC (neuroendocrine epithelial neoplasms) are distinguished from PHEO/PARA (neuroendocrine non-epithelial neoplasms) by the expression of keratin in the former. Of note, the most frequently expressed keratins in neuroendocrine epithelial neoplasms are K8 and K18, one or both of which are recognized by most broad-spectrum keratins including OSCAR, MAK6, AE1/AE3, and CAM5.2 [1]. Neuroendocrine epithelial neoplasms are usually CK7/CK20-double negative (40% of bronchopulmonary NETs are CK7+; up to one quarter of gastroenteropancreatic [GEP] NETs are CK20+), and it must be emphasized that CK7/CK20-double negativity does NOT equate with broad-spectrum keratin negativity [2]. Occasionally, I have to resort to using antibodies to EpCAM (i.e., MOC-31, Ber-EP4) to confirm the epithelial nature of a keratin very weak-to-negative NET or NEC. NENs, especially well-differentiated examples, typically express **somatostatin receptors**. Somatostatin receptor subtype 2A (SSTR2A) expression is the basis of somatostatin receptor functional imaging (e.g., Ga 68-DOTATATE) and somatostatin analogue therapy—both cold peptide (e.g., octreotide acetate) and peptide receptor radionuclide therapy (e.g., Lu 177-DOTATATE) [3,4]. General neuroendocrine marker expression confers the “neuro” character to these neoplasms and distinguishes them from “just endocrine” epithelial neoplasms (e.g., follicular thyroid tumors, gonadal sex cord-stromal tumors).

Confirming the epithelial nature of a NET is “almost mandatory,” especially in a liver metastasis—to avoid misdiagnosing PHEO/PARA as NET. Confirming the epithelial nature of a NEC serves to distinguish it from other small round blue cell tumors in the differential. Most NETs and PARAs and the vast majority of NECs are non-functional and peptide hormone/biogenic amine immunohistochemistry is not routine. I do use serotonin as a “second tier” midgut NET marker and tyrosine hydroxylase (the rate determining step in catecholamine biosynthesis) as a PHEO and sympathetic-PARA marker. I occasionally perform pancreatic peptide hormone immunohistochemistry to distinguish neuroendocrine hyperplasia/islet aggregation (which should contain a mixture of insulin, glucagon, somatostatin, and pancreatic polypeptide-expressing cells) from a pancreatic NET (which may either express a predominance of one these markers or not express any of them). I also perform immunohistochemistry on rare functional tumors, especially in patients with MEN1, to suggest that the biopsied or resected tumor might be the one responsible for the patient's syndrome. I routinely perform SSTR2A immunohistochemistry (clone UMB-1) (Figure 1A) [5]. This correlates with the results of somatostatin receptor functional imaging and has been shown to be prognostically significant (Figure 1B) [6-11]. I have also found it useful in NET site of origin assignment (ubiquitously expressed by midgut NETs; expressed by 80–90% of pancreatic NETs; often weakly or not expressed by bronchopulmonary NETs) and, especially, in the distinction of NET G3 (usually strongly positive) from NEC (only one third are positive, with expression typically weaker than in NET) [12].

2. General Neuroendocrine Marker Immunohistochemistry

Traditional general neuroendocrine markers include synaptophysin and chromogranin A, with the former generally considered more sensitive and the latter more specific. Like broad-spectrum keratin immunohistochemistry, the demonstration of general neuroendocrine marker positivity is “almost mandatory,” especially in (presumed) liver metastases. NET/

PHEO/PARA should essentially always demonstrate diffuse, strong chromogranin A and/or synaptophysin-positivity, and staining weaker than this calls the diagnosis into question (see the section “Lesions Apt to be Misdiagnosed as Well-Differentiated Neuroendocrine Tumor”). Rectal, L-cell appendiceal, and gastrin-expressing NETs and gangliocytic paragangliomas are often chromogranin A weak-to-negative, which occasionally leads to diagnostic confusion; some of these will express alternative granins, including chromogranin B and secretogranins II, III, and V [13]. Unfortunately, around one quarter of NECs are chromogranin A/synaptophysin-negative, and chromogranin A-positivity is often “under-interpreted.” Synaptophysin is associated with synaptic-like vesicles, while chromogranin A is associated with dense core and chromaffin granules. NECs often have few dense core granules per cell, which frequently results in punctate rather than more diffuse cytoplasmic chromogranin A-positivity (Figures 2A-B). Until recently, in a suspected NEC, surrogate neuroendocrine markers have included dot-like keratin-positivity, TTF-1-positivity (seen in 85% of small cell lung and 40–50% of large cell lung and extrapulmonary visceral NECs), and Rb loss. I have found pulmonary pathologists to be fond of CD56 as a general neuroendocrine marker; I never use it for this purpose due to lack of specificity [14-16]. Insulinoma-associated protein 1 (INSM1) has emerged as an additional general neuroendocrine marker, which I have found especially useful in the diagnosis of NEC (Figures 3A-F).

The zinc-finger transcription factor INSM1 was initially discovered by “genomic subtraction” of a glucagonoma from an insulinoma cDNA library [17]. It was subsequently found to participate downstream of neurogenin 3 in β-cell development [18]. In situ hybridization experiments demonstrated INSM1 mRNA in brain, olfactory epithelium, retina, thymus, thyroid, pancreas, and neuroendocrine cells of the gastrointestinal (GI) tract [18-20]. Introduction of INSM1 into a human pancreatic cancer cell line induced transcription of the islet-associated transcription factors PAX6 and NKX6.1, and combined introduction of INSM1, NeuroD, and PDX-1 resulted in transdifferentiation into insulin-producing cells [21]. INSM1 was initially advanced as a potential diagnostic marker in 2015 by my medical school classmate, Jason Rosenbaum, who in his previous graduate work had studied the role of this transcription factor in the development of olfactory neuroepithelium [22]. Rosenbaum and colleagues reported INSM1-positivity in 88% of 129 NENs from diverse anatomic sites and only 1 of 24 non-NENs (likely representing “occult neuroendocrine differentiation”—see below) [23]. Since then there have been a bevy of “positive” studies in the cytology and organ-specific pathology literature [24-29]. Even the couple “negative” studies found it to be more specific, though less sensitive, than traditional general neuroendocrine markers. I was especially excited by reports of increased sensitivity for the diagnosis of NEC. For example, Rooper and colleagues reported INSM1-positivity in 95% of 39 small cell (average H-score 154) and 91% of 23 large cell neuroendocrine lung carcinomas (average H-score 114), compared to 62% and 61% for synaptophysin (H-scores 60, 97) and 49% and 48% for chromogranin A (H-scores 85, 114) [25]. In my own laboratory validation, I found INSM1-positivity (clone A-8) to be 95% sensitive in a cohort of 93 NECs, compared to 83% and 82% for chromogranin A and synaptophysin, respectively; at an H-score threshold of ≥50, INSM1 was 88% sensitive and 99% specific (87 metastatic non-neuroendocrine carcinomas from diverse anatomic sites studied

concurrently) (unpublished observation). Given these results, I have substituted INSM1 for chromogranin A and synaptophysin as my first-line marker to support the morphologic impression of NEC.

3. Non-Small Cell Carcinoma with (Occult) Neuroendocrine Differentiation

Ten to twenty percent of non-neuroendocrine carcinomas show at least some degree of general neuroendocrine marker expression. I refer to this phenomenon as “occult” neuroendocrine differentiation. Often this takes the form of “scattered cells staining,” but tumors can demonstrate diffuse, strong expression for one or more of the general neuroendocrine markers. For chromogranin A and synaptophysin, this phenotype is more typically seen with adenocarcinoma than with squamous cell carcinoma [30,31]. Although best described in lung and colon cancer, this finding probably extends to all sites of origin. While some studies have found occult neuroendocrine differentiation to be associated with adverse histologic features, unfavorable prognosis, and increased chemoresponsiveness, the preponderance of studies, including the largest studies, have not [32-35]. I often quote Ionescu and colleagues who titled their paper on this topic “Nonsmall cell lung carcinoma with neuroendocrine differentiation—an entity of no clinical or prognostic significance.” [34]

I aggregated results of studies in non-small cell lung cancer, applying “strict” (occult neuroendocrine differentiation defined by chromogranin A-positivity) and “permissive” (occult neuroendocrine differentiation defined by any general neuroendocrine marker-positivity) criteria and found occult neuroendocrine differentiation in 11.8% (386/3283) and 22.6% (751/3317) of tumors, respectively [30,31,33-45]. I similarly aggregated results of studies in colon cancer (most of which defined neuroendocrine differentiation as any chromogranin A-positivity) and found a rate of 23.5% (229/974) [32,46-54]. I stained tissue microarrays (TMA) of breast cancer and found diffuse, strong chromogranin A and synaptophysin-positivity in 3.8% (4/105); no case demonstrated less extensive general neuroendocrine marker expression (unpublished observation) (Figures 4A-D).

This phenomenon may lead to diagnostic and clinical confusion, typically when general neuroendocrine markers are applied to poorly differentiated carcinomas in which the line of differentiation is uncertain on the H&E (e.g., solid adenocarcinoma, non-keratinizing squamous cell carcinoma). Scattered neuroendocrine marker expression in these tumors should not be taken as “proof” of the diagnosis of small cell or large cell NEC. More commonly, such cases are signed out with the ambiguous descriptive diagnosis of “poorly differentiated carcinoma with neuroendocrine differentiation” or “features,” leaving clinicians with the question “Is this small cell carcinoma?” (Implicit in this question is another: “Should I give small cell-chemotherapy [i.e., platinum/etoposide]?”) The vast majority of squamous cell carcinomas (>95%) express p40, and most solid adenocarcinomas will express site-specific differentiation markers (generally transcription factors). The diagnoses of small cell NEC and large cell NEC should initially be based on a strong morphologic impression, though small cell NEC demonstrates a greater range of morphologies than typically depicted in textbooks, and large cell neuroendocrine carcinoma shows so much overlap with “large cell undifferentiated carcinoma” that demonstration of

neuroendocrine differentiation is required to make the diagnosis. Given the frequency of occult neuroendocrine differentiation, I do not even begin to consider general neuroendocrine marker expression in support of a diagnosis of NEC unless ≥30% of cells demonstrate at least moderate staining (an arbitrary threshold that I borrowed from the definition of mixed neuroendocrine-non-neuroendocrine carcinoma, in which the minor component must represent at least 30% of the tumor). The more extensive and intense the expression, the more confident I am in the diagnosis. As discussed above, dot-like keratin-positivity, TTF-1-positivity (for visceral NECs; CK20-positivity for Merkel cell carcinoma), and Rb loss are corroborative.

4. WHO Classification of Neuroendocrine Neoplasms

The WHO Classification of Tumours, published as a series of "Blue Books," represents the international "gold standard" for tumor classification. The 4th edition was composed of 12 organ-system-based volumes published between 2007 and 2018. Each Blue Book has "jurisdiction" over the classification of NENs within its organ-system scope.

The 5th edition of the *WHO Classification of Tumours of the Digestive System* (the first volume in the 5th series) was published in August 2019 [55]. It has jurisdiction over all GEP-NENs, the classification of which I refer to as "WHO 2019" (Table 1). WHO 2019 supplants the classification from the 4th edition GI Blue Book (WHO 2010). It entirely adopted the classification from the 4th edition Endocrine Blue Book (WHO 2017), which technically only had jurisdiction over pancreatic NENs, but which I began applying to all GEP-NENs as soon as it was published because it improved upon deficiencies in WHO 2010. Specifically, WHO 2019 closes the G1/G2 Ki-67 "hole," it introduces the diagnostic category "NET G3," and it provides a new diagnostic term for tumors composed of both neuroendocrine and non-neuroendocrine elements. The classification of lung NENs, contained in the *WHO Classification of Tumours of the Lung, Pleura, Thymus, and Heart* (4th edition) is distinct (Table 2); specifically, Ki-67 immunohistochemistry is not a component of that classification [56].

In WHO 2010, G1 GEP-NET was defined as having a Ki-67 proliferation index ≤2%, while G2 GEP-NET was defined as having a Ki-67 proliferation index from 3–20%. There was no accounting for tumors with proliferation indices >2% but <3%, an oversight rooted in the bygone "eyeball estimate" era. In WHO 2019, G1 GEP-NET is defined as having a Ki-67 proliferation index <3%, while the definition of G2 GEP-NET is unchanged.

As soon as WHO 2010 was published, pathologists recognized that there were rare neuroendocrine epithelial neoplasms with well-differentiated morphology but with Ki-67 proliferation indices in the G3 range (i.e., mitotic count >20 per 10 HPF and/or Ki-67 proliferation index >20%), while in this classification G3 neoplasms were "definitionally" NEC. In WHO 2019 these tumors are now classified as NET G3 (Figures 5A-F). Basturk and colleagues assembled a multi-institutional cohort of 19 such pancreatic tumors, which had outcomes (median survival 54.1 months; 5-year-survival 29.1%) in-between that of cohorts of stage-matched pancreatic NET G2s (n = 50; median survival 67.8 months; 5-year survival 62.4%) and pancreatic NECs (n = 42; median survival 11 months; 5-year-survival

16.1%) [57]. Tang and colleagues published on an overlapping cohort of 33 NET G3s from the institutional cohorts of Memorial Sloan Kettering Cancer Center (MSKCC) and Cedars-Sinai Medical Center, including 21 pancreatic, 6 small intestinal, 2 bile duct, and 2 rectal tumors. The G3 component was seen in the primary in 12 (39%), regional metastasis in 3 (9%), and distant metastasis in 16 (48%); it was synchronous in 74% and metachronous in 26%. The authors noted that in most cases the G3 component constituted ≥20% of the total tumor and demonstrated greater cytoarchitectural abnormality than the background NET G1/2, acknowledging that while none of the G3 areas resembled small cell NEC, “there was some degree of histologic overlap between the high-grade portions in WD-NET with large cell NEC.” [58] They reported that none of the NET G3s demonstrated abnormal p53 or Rb staining. To get a sense of the frequency of NET G3, I analyzed the University of Iowa cohort. From the Fall of 2014 to 2015, we saw 178 NET G1/2s and 10 NET G3s (5.3%), including 5 pancreatic, 4 ileal (5% of all ileal NETs), and 1 rectal tumor. The current lung NEN classification does not recognize NET G3, though I have seen a couple examples and a series of 12 cases was recently published; I suspect it occurs at a similar 5% rate [59].

Unlike in the lung, in which most NECs arise de novo, GEP-NECs arise from non-neuroendocrine precursors and/or may co-exist with a non-neuroendocrine carcinoma component (although the NEC often “overgrows” these). WHO 2010 referred to tumors composed of mixtures of neuroendocrine and non-neuroendocrine elements as mixed adenoneuroendocrine carcinomas (MANEC). This term reflected a Western bias, as esophageal tumors in the East are more likely to arise in association with squamous lesions. In a series of 42 Chinese esophageal NECs, 50% arose from squamous cell carcinoma in situ and 31% had an associated invasive squamous component, while only 16% showed a minor component of adenocarcinoma [60]. In contrast, in the MD Anderson Cancer Center cohort of 40 esophageal NECs, an adenocarcinoma component was present in 15 (38%), while a squamous cell carcinoma component was present in only 1 (2.5%) [61]. Anal NECs may also arise in association with squamous cell carcinoma and, like uterine cervical NECs, are high-risk human papillomavirus (HR-HPV)-driven [62]. The term MANEC also failed to reflect the fact that, rarely, the neuroendocrine component in mixed tumors is well-differentiated. [63-67] In the WHO 2019 classification the term “mixed neuroendocrine-non-neuroendocrine neoplasm” (MiNEN) is substituted, with the continued requirement that the minor component comprise ≥30% of the tumor (Figures 6A-F).

5. Ki-67 Immunohistochemistry—When and How

The discovery of Ki-67 (pronounced “key-67,” as it was named for both its site of discovery —Kiel, Germany—and the clone's position in a 96-well plate) was a happy accident [68]. Gerdes and colleagues were attempting to generate monoclonal antibodies to nuclear antigens of the Hodgkin lymphoma cell line L428, when they discovered one clone that reacted exclusively with proliferating (i.e., non-G_0) cells. They immediately understood the magnitude of their discovery, noting that “Ki-67 may be a potent tool for easy and quick evaluation of the proportion of proliferating cells in a tumour.” While the original Ki-67 monoclonal antibody was only reactive in frozen sections, several monoclonal antibodies (e.g., MIB1, 30–9, SP6, K2) may be used in formalin-fixed, paraffin embedded material. Ki-67 expression increases steadily throughout the cell cycle, peaking in G_2/M, and any

discernable staining (nucleolar or diffuse; weak or strong) should be counted [69,70]. It took thirty-three years to discover Ki-67's function as a "biological surfactant to disperse mitotic chromosomes;" in the meantime, Ki-67 immunohistochemistry had become indispensable to the grading of GEP-NETs [71,72]. Up to one third of tumors have discordant grades based on mitotic counting and Ki-67 proliferation index, with the grade in these discordant cases nearly always (1 grade) higher based on Ki-67; the higher grade "counts," as it dictates prognosis [73-75]. There are also compelling reasons to perform Ki-67 immunohistochemistry in non-GEP-NETs (e.g., bronchopulmonary) and in NECs, though staining in these cases is not considered "mandatory."

I perform Ki-67 immunohistochemistry on biopsies and resections of all GEP-NETs. In resections, I test blocks of primary, regional, and distant disease. I only "eyeball estimate" cases well below the 3% G1/G2 threshold. In most cases, I manually count a camera-captured image of a Ki-67 "hotspot," including at least 500 tumor cells. In my laboratory, we are currently validating image analysis for Ki-67 quantification (I'm not sure what I'll do with all the resulting free time!). I started simultaneously testing blocks of primary, regional, and distant disease after reading a 2012 paper from Dhall and colleagues, in which they showed that a Ki-67 proliferation index >2% at either the primary or a metastatic site was the only significant predictor of progression-free survival in a cohort of 57 ileal NETs. I call this the "Any G2 Trumps Rule." As suggested by the discussion above, any G3 is even worse. Shi and colleagues analyzed a cohort of 27 small intestinal NETs in which at least 2 liver metastases had been resected [76]. While all the primary tumors were G1/2, 8 (30%) patients had at least 1 G3 liver metastasis; progression-free survival in these patients was 7 months versus 38 months for patients without a G3 liver metastasis. We analyzed the Iowa experience, which included 79 jejunoileal and 21 pancreatic tumors (64% G1 and 36% G2 primaries) [77]. Grades were concordant between matched primary-metastasis (1°-M) in 66%, the grade was higher in the metastasis in 24%, and the grade was higher in the primary in 10%. Patients with any G2 had inferior progression-free and overall survival (median overall survival in G1 1°/M = 11.3 years, G1 1°/G2 M = 7.3 years, G2 1°/M = 5.6 years), with increased grade in the metastasis the factor with the highest hazard ratio on a multivariate analysis of overall survival. With an eye toward pragmatism, I only stain one block each of primary, regional, and distant disease; I generally select the largest focus of tumor from each of these categories, as studies have shown a positive correlation between tumor size and Ki-67 proliferation index [76,78]. If tumor in any block looks morphologically "worse," that supersedes the "size rule".

Our multidisciplinary group is interested in knowing the Ki-67 proliferation index in all well-differentiated NENs, which figures broadly (along with many other factors including site of origin, anatomic distribution of disease, pace of disease) into treatment decisions (e.g., whether to give chemotherapy, a biologic, octreotide, or nothing at all; whether to surgically debulk, deploy liver-directed therapy, or give peptide-receptor radionuclide therapy). Although the Ki-67 proliferation index is not formally part of the classification of bronchopulmonary NETs, typical carcinoid (TC) tumors are roughly equivalent to G1 GEP-NETs and atypical carcinoid (AC) tumors are roughly equivalent to G2 GEP-NETs [79,80a]. Most TCs should have a Ki-67 proliferation index <5% and most ACs should have a Ki-67 proliferation index ≥5%. If the H&E classification and the Ki-67 proliferation index are

discordant, the case is worth a closer look. In a cohort of 114 bronchopulmonary carcinoids, 5 pulmonary pathologists unanimously agreed on a diagnosis of TC or AC in only 55.3% (κ = 0.316), while 4 agreed in 25.4%, and only 3 agreed in the remaining 19.3% [80b]. The classification of TC versus AC was not prognostically significant based on this consensus classification (P = .11). When Ki-67 was used to reclassify the 22 cases in which only 3 pathologists agreed (<5% = TC, ≥5% = AC), the classification became highly prognostically significant (P = .0004). Ki-67 immunohistochemistry is also useful in small biopsies, which often lack the requisite $2mm^2$ for mitotic counting (Figures 7A-B). In addition, Ki-67 immunohistochemistry is useful in crushed biopsies, in which NET may be mistaken for NEC [80]. The reason NET G3 is underrecognized in the lung is that Ki-67 immunohistochemistry has not been routinely performed. Even if Ki-67 remains out of the lung NEN classification, it should be strongly considered, especially at metastatic sites. Rekhtman and colleagues very recently published that—in a cohort of 66 lung carcinoid patients with stage IV disease—a hot spot Ki-67 proliferation index >20% was seen in 13% of primaries and 27% of metastatic samples, and at least one sample was mitotically or Ki-67-based G3 in 42% of patients [81]. Recapitulating the GEP-NET G3 story, tumors lacked Rb and p53 alterations and were associated with a median survival (2.7 years) superior to that for stage IV NECs (<1 year).

Ki-67 immunohistochemistry is also potentially applicable to NECs, in which higher proliferation indices are associated with adverse prognosis, better responses to platinum-based chemotherapy, and worse responses to temozolomide chemotherapy. The first two conclusions are based on the results of the NORDIC NEC study, which included 305 advanced G3 GEP-NENs, including 115 small cell and 148 non-small cell tumors (in which histology was specified in pathology reports) [82]. (Of note, this study preceded widespread recognition of NET G3 and, thus, the non-small cell group is expected to contain a mixture of NET G3 and large cell NEC.) A Ki-67 proliferation index of 55% was found on receiver operating characteristics-analysis to best stratify response to platinum-based therapy (response rate at <55% = 15%, ≥55% = 42%). When this same cutpoint was applied to prognosis, patients with a Ki-67 proliferation index <55% survived longer (14 versus 10 months). Small cell versus non-small cell histology and degree of chromogranin A staining were not prognostically significant. A subset of this study's authors had previously suggested that second-line temozolomide (after progression on platinum/etoposide) was more effective in G3 NENs with a Ki-67 proliferation index <60%, though detailed statistics were not presented [83]. Milione and colleagues categorized 136 G3 GEP-NENs into three groups based on differentiation and Ki-67 proliferation index; well-differentiated tumors with Ki-67 proliferation indices <55% (n = 24), poorly differentiated carcinomas with Ki-67 proliferation indices <55% (n = 30), and poorly differentiated carcinomas with Ki-67 proliferation indices ≥55% (n = 82) had median overall survivals of 43.6, 24.5, and 5.3 months, respectively [84]. In a multinational European cohort of 313 G3 GEP-NENs (again, care was not taken to distinguish NET G3 from NEC), Ki-67 proliferation index (this time thresholded at 80%—optimized to predict overall survival rather than treatment response) formed part of a nomogram also including the presence of liver metastasis, alkaline phosphatase, lactate dehydrogenase, and performance status to predict overall survival [85].

I'm often asked by pathology colleagues about the significance of this “55% threshold.” My response is that there are no “magic numbers.” The Ki-67 proliferation index is a continuous variable and absolute thresholds (e.g., 2, 3 or 5% for G1 versus G2; 20% for G2 versus G3) are inherently arbitrary and not necessarily generalizable between tumor types (i.e., NET versus NEC, various sites of origin) and applications (prognosis or prediction). Specifically, an absolute Ki-67 threshold is not applicable to the distinction of NET G3 from NEC. In a recent series of G3 pancreatic NENs, NETs and NECs had substantially overlapping proliferation indices with a mean; median (range) of 49%; 50% (30–80%) for NET and 70%; 80% (26–95%) for NEC [86]. Thirty-two percent of NET G3s had a Ki-67 proliferation index >55%, while 23% of NECs had an index <55%.

6. Immunohistochemistry to Assign Site of Origin in Metastasis of Occult Origin

6.1. Neuroendocrine Tumor

Ten to twenty percent of NETs present as metastasis of occult or origin, typically to liver and/or bone, and site of origin assignment is prognostically and therapeutically significant [2,87]. For example the median survival of stage IV jejunoileal, pancreatic, and bronchopulmonary tumors is 65, 27, and 17 months, respectively [87]. Everolimus and capecitabine/temozolomide are often used in pancreatic NETs but almost never in midgut tumors [88]. NET primaries are often resected, even in the face of widespread metastatic disease; this is especially the case for tumors of jejunoileal origin, in which unresected tumors are associated with significant bleeding and obstruction risk [89].

There are often morphologic clues to site of origin (Figures 8A-D). Soga and Tazawa described 4 main architectural patterns in NETs: nested (type A), trabecular (type B), pseudoglandular (type C), and diffuse (type D) [90]. Enterochromaffin (EC)-cell NETs demonstrate predominantly nested architecture with a variably prominent secondary pseudoglandular pattern, which tends to manifest around the periphery of individual tumor nests. Tumor cells show variable eosinophilic cytoplasmic granularity, which reflects serotonin content. Nuclei are round to oval, centrally placed, and evenly spaced; they are mostly monomorphous, though occasionally punctuated by “endocrine atypia.” This is the phenotype of Oberndorfer's “classic ileal carcinoid tumor” but also that of distal jejunal, Meckel's diverticulum-associated, and many appendiceal tumors and of so-called “insular carcinoid tumors” of ovarian origin; it is an uncommon morphology in tumors of gastric or pancreatic origin and is not uncommonly seen in the rare primary tumors arising in the testis or kidney. Of all NET morphologies, this one is the most stereotypical—so much so that, when faced with a metastasis of occult origin, the first question I ask myself is “Is this tumor almost certainly, plausibly, or almost certainly not of midgut origin?” (Implied in this question is the fact that the vast majority of metastatic EC-cell NETs are of ileal origin.) Rectal NETs are typically type B, with a tendency for the trabecula to fold back upon themselves (like a paperclip); tumor cells are more columnar, and nuclei tend to be somewhat fusiform. Rectal tumors recapitulate enteroglucagon-producing L-cells, and appendiceal L-cell tumors (which are uniformly benign) demonstrate similar morphology. Type C-predominant tumors tend to occur at the ampulla and are apt to be mistaken for

adenocarcinoma in small biopsies; these tumors are somatostatin-expressing and may contain psammomatous calcifications. Pancreatic and bronchopulmonary NETs demonstrate a wide range of morphologies. Pancreatic tumors may simultaneously display multiple morphologic patterns and metastatic bronchopulmonary tumors often show spindle cell morphology.

The Iowa NET Site of Origin Classifier is presented in Fig. 9. This algorithm assumes positivity for one or more general neuroendocrine marker and for a broad-spectrum epithelial marker. Most NET metastases of occult origin are of jejunoileal (especially) or pancreatic origin, and the classifier's “first round” of stains is geared toward detecting these [91,92]. CDX2 is the “tier-one” midgut neuroendocrine marker. It is 90% sensitive with expression typically diffuse and strong [2]. Up to 15% of pancreatic NETs express CDX2, which ranges from weak to strong, though they will co-express one or more markers of pancreatic origin; I have not found these 15% of tumors to demonstrate any distinctive features [93]. Islet 1 is the most sensitive pancreatic NET marker (70% sensitive in the aggregated published literature but in my experience 85%), though it is also typically expressed by rectal NETs, medullary thyroid carcinoma, and PHEO/PARA and is positive in up to 10% of lung NETs [94]. I had initially intended to employ polyclonal PAX8 as a “tier-one” pancreatic neuroendocrine marker. It is 55% sensitive in the aggregated published literature, though when I tested it in my own laboratory, tumors were non-reactive [95]. I substituted PAX6, which is the main PAX-family transcription factor that polyclonal PAX8 cross-reacts with in pancreatic NETs; I have found it to be 70% sensitive [96]. As many laboratories are migrating from polyclonal to monoclonal PAX8 assays, it is worth noting that monoclonal PAX8 is never positive in pancreatic NETs. EC-cell tumors are essentially never positive for these pancreatic markers.

Based on results of the initial panel, tumors are assigned to presumed midgut origin (CDX2+/ISL1−/PAX6−), “pancreatic pattern” (any ISL1 and/or PAX6+), or “indeterminate pattern” (pan-negative). Of note, the “pancreatic pattern” is also typical of rectal and duodenal NETs. As discussed above, rectal NETs recapitulate enteroglucagon-expressing L-cells and are driven by some of the same transcriptional machinery that determines glucagon-expressing α-cell-lineage. A rectal origin is suggested by morphology and can be confirmed with SATB2-positivity (strongly positive in nearly all [96%] rectal NETs and never strongly expressed by pancreatic tumors); incidentally, SATB2 is also expressed by most (79%) appendiceal NETs [97]. Duodenal and pancreatic enteroendocrine cells are of common embryologic origin and tumors arising at these sites are not readily distinguished, though it would be exceptional for a duodenal NET to present as a distant metastasis of occult origin. For tumors of confirmed pancreatic origin, I perform ATRX, which is prognostically significant (loss of expression is prognostically adverse in patients with locoregional disease, though prognostically favorable in patients with distant metastasis) [98-101].

The second round of the classifier is geared toward identifying tumors of bronchopulmonary origin, as well as the 10% of CDX2-negative midgut and 5–10% of PAX6/ISL1-negative pancreatic tumors. Although TTF-1 is the most widely utilized bronchopulmonary NET marker, OTP is the clear first choice. This homeodomain-containing transcription factor,

which is normally only expressed by specific hypothalamic nuclei, was discovered through gene expression profiling of lung carcinoids. While TTF-1 is only 30–40% sensitive, OTP is twice as sensitive, without sacrificing specificity. Papaxoinis and colleagues reported OTP-positivity in 89% of 132 TCs and 62% of 34 ACs (compared to 52% and 34% for TTF-1, with no OTP-/TTF-1+ tumors) [102]. I found OTP-positivity in 82% of 77 TCs, 50% of 12 ACs, and only 1 of 603 GEP-NETs (a pancreatic tumor that co-expressed PAX6 and islet 1) [103].

I suspect CDX2-negativity in some midgut tumors may reflect CDX2 promoter methylation, and I have occasionally seen “clonal loss” of CDX2 (Figure 10A). In other instances it may reflect lack of robustness of CDX2 immunohistochemistry in specimens subjected to prolonged cold ischemia time or poor fixation (Figure 10B). I validated serotonin as a “tier-two” midgut NET marker. In TMAs of 430 midgut, 142 pancreatic, and 44 lung NETs, I found serotonin-positivity in 81% of midgut, 3% of pancreatic, and 0% of lung tumors. The addition of serotonin to CDX2 increased sensitivity for the diagnosis of midgut NET from 90 to 96%; the 4 serotonin-positive pancreatic NETs all expressed islet 1 and/or PAX6 [93]. Before I added serotonin to the classifier, I had used absence of clusterin expression in a NET to indicate jejunoileal origin; I had found positivity in 82% of 148 non-midgut (median H-score 183) and only 8.4% of midgut (median H-score 31) NETs [104]. Before SATB2, PrAP was the best rectal NET marker (≥80% sensitive); I found it was also expressed by up to half of midgut tumors, though of more limited extent than in rectal tumors [95].

Though it has not been extensively studied, PR is the most widely available pancreatic NET marker. Viale and colleagues reported positivity in 58% of 96 pancreatic, 0% of 29 tubal gut, and 7% of 15 lung NETs [105]. When I formally studied it, results were nearly identical, with positivity in 67% of 70 pancreatic, 5% of 107 jejunoileal, and 5% of 20 lung tumors [95]. Frequent ATRX inactivation (10–18%) was identified in recent studies defining the molecular genetic landscape of pancreatic NETs [106,107]. Although I am not aware of a peer-reviewed comprehensive immunohistochemical survey of the role of ATRX immunohistochemistry in assigning NET site of origin, ATRX inactivation was not seen in studies defining the molecular genetic landscape of ileal and bronchopulmonary tumors, though it was identified in 13% of 103 PHEO/PARAs [108-110]. I have not formally studied it to date because ATRX immunohistochemistry is very sensitive to prolonged cold ischemia time and poor fixation, and, thus, it is not amenable to examination in TMA. Although ATRX inactivation is selected for in metastasis, the rate of combined ATRX and DAXX (found to be inactivated in 20–25% of primary tumors) immunohistochemical loss probably does not exceed 60% [99,100]. I validated ATRX in my laboratory for its use as a mainstream neuropathology marker—inactivation is seen in ≥50% of diffuse astrocytomas, is lineage-defining in the differential with oligodendroglioma, and is prognostically favorable; these pancreatic NET applications are an added bonus [111,112]. I have yet to validate DAXX due to unclear added value as a diagnostic marker. ATRX and DAXX inactivation are both associated with the alternative lengthening of telomeres (ALT) phenotype, which may be detected by fluorescence in situ hybridization [113].

For the occasion of the Hans Popper Hepatopathology Society companion meeting, I created an alternative, simplified algorithm (Fig. 11). It utilizes 4 or 5 widely available markers,

applied simultaneously. In the event these are negative, sendout for islet 1, OTP, and serotonin immunohistochemistry could be considered. Finally, alternative diagnoses should be considered in a presumed NET for which site of origin assignment is uncertain.

6.2. Neuroendocrine Carcinomas

The vast majority of NECs arise in the lung. In a recent analysis of SEER data, among visceral primaries, a lung origin outnumbered an extrapulmonary one on the order of 10:1 [114]. The most common extrapulmonary sites in descending order included urinary bladder, colon, pancreas, uterine cervix, and prostate. Extrapulmonary visceral tumors were evenly divided between those arising in the GI tract, other sites, and of unknown primary. Extrapolating from the annual incidence of small cell lung cancer and the SEER 10:1 ratio, the estimated annual incidence of extrapulmonary visceral NEC is 2850 [115]. The incidence of Merkel cell carcinoma is climbing sharply, with 1500 new cases in 2007 and 2835 estimated new cases in 2020 (i.e., nearly identical to that of extrapulmonary visceral NEC [116].

Historically, site of origin assignment in metastatic NEC of unknown primary was a mainly academic exercise, as first-line therapy was platinum/etoposide regardless of origin [117]. Two things have changed. First, checkpoint inhibitor therapy has been shown to be highly effective in Merkel cell carcinoma (and at most modestly so in visceral NECs) and has rapidly emerged as first-line therapy in this tumor type [118-120]. Second, extrapulmonary visceral NECs are increasingly treated with regimens active in site-specific non-neuroendocrine carcinomas (e.g., FOLFOX, FOLFIRI in GEP-NECs) or with novel treatment regimens (e.g., platinum/irinotecan, CAPTEM) [121,122].

Unlike the situation in NET, immunohistochemistry has a more limited role in assigning NEC site of origin. Aside from TTF-1 (visceral origin) and CK20 (cutaneous origin), there are a handful of additional useful markers to suggest a cutaneous (neurofilament, CM2B4, SATB2) or visceral (ASCL1) origin. Aside from in situ hybridization for HR-HPV (anogenital and rarely head and neck origin), to date there are no useful markers to distinguish among sites of origin in visceral NEC [123,124].

When I had aggregated the published literature back in 2013, I found TTF-1-positivity in 83% of 846 small cell lung cancers, 36% of 283 large cell lung NECs, 36% of 550 extrapulmonary visceral NECs, and 0.8% of 260 Merkel cell carcinomas [2]. CK20-positivity had been reported in 88% of 472 Merkel cell carcinomas, 63% of poorly differentiated NECs of major salivary gland (typically parotid) origin, 6% of 331 extrapulmonary visceral NECs of non-major salivary gland origin, and 5% of 383 lung NECs. When TTF-1 and CK20 are aberrantly expressed by cutaneous and lung NECs, respectively, expression is weak and patchy.

Neurofilament is frequently expressed by Merkel cell carcinoma (75%) and less commonly by tumors of lung or extrapulmonary visceral origin [2,125]. When I examined neurofilament in my laboratory (clone 2F11), I found staining in 67% of 39 Merkel cell, 17% of 24 small cell lung, and 6% of 18 extrapulmonary visceral NECs [126]. Up to 80% of Merkel cell carcinomas are driven by Merkel cell polyomavirus, with the remainder UV

light-associated [127-129]. Polyomavirus-associated tumors can be identified with immunohistochemistry to the virus's large T antigen (clone CM2B4), while widely utilized pan-polyomavirus immunostains directed against SV40 are non-reactive (Figures 12A-D) [97,130]. Although I was initially very excited by this, most Merkel cell polyomavirus-associated tumors are CK20-positive. While several series have reported CM2B4-positivity to be restricted to CK20-positive tumors, among their cohort of 36 cases, Busam and colleagues found 2 CM2B4+/CK20− tumors, and addition of CM2B4 increased sensitivity for the diagnosis of Merkel cell carcinoma from 89% to 94% [131]. I have found CM2B4 immunohistochemistry to be most useful in the head and neck in the distinction of Merkel cell carcinoma from major salivary gland primaries. SATB2 was serendipitously discovered to be expressed by Merkel cell carcinoma in a follow up of the Human Protein Atlas study that had initially described SATB2 as a lower GI tract-specific marker [132]. Kevarrec and colleagues subsequently found diffuse, strong staining to be among the best discriminators between Merkel cell carcinoma (n = 98) and visceral NEC (n = 57): 64% sensitive, 98% specific. I recently published similar results—SATB2-positivity thresholded at an H-score of ≥150 was 69% sensitive and 90% specific for Merkel cell carcinoma [97].

Achaete-scute complex-like 1 (ASCL1; aka m/hASH1) is preferentially expressed by visceral NECs, though data are scant. Ralston and colleagues reported ASCL1-positivity in 85% of 59 small cell lung cancers and 0% of Merkel cell carcinomas, while LaRosa and colleagues reported positivity in 82% of 34 pulmonary, 44% of 137 extrapulmonary visceral, and 22% of 23 cutaneous NECs [133]. I recently found it (clone 24B72D11.1) to be expressed by 83% of 29 pulmonary (median H-score 180), 42% of 19 extrapulmonary visceral (median H-score 230), and 9% of 43 cutaneous (median H-score 4) NECs (unpublished observation). I found ASCL1 and TTF-1 expression to be highly correlated, and ASCL1 immunohistochemistry did not increase the sensitivity for the diagnosis of lung NEC, though it did increase the sensitivity for the diagnosis of extrapulmonary visceral NEC from 42% to 53%.

Unlike in NET (and adenocarcinoma), beyond the rare examples discussed above (TTF-1 and ASCL1-positivity for visceral origin; strong SATB2-positivity for Merkel cell carcinoma), transcription factor immunohistochemistry has no role in assigning the site of origin of metastatic NEC of unknown primary. In fact, NECs have a tendency to express multiple transcription factors, independent of site of origin, a phenotype I dubbed “marked transcription factor lineage infidelity” (Figures 13A-F). Several years ago, Jason Hornick and I stained NEC TMAs for every transcription factor we ran between our two laboratories. NECs expressed a median of 8 different transcription factors out of 38 examined (range 0–18); the median number of transcription factors expressed did not differ among lung, extrapulmonary visceral, and cutaneous tumors. Although expression of individual transcription factors is often patchy, it can be rather intense and multifocal to fairly diffuse. Even SATB2 is subject to this phenomenon. When any staining was considered (rather than thresholding at an H-score of ≥150), I found positivity in 79% of cutaneous, 60% of extrapulmonary visceral, and 33% of lung NECs (i.e., specificity for Merkel cell carcinoma drops to 56%).

7. Immunohistochemistry to Distinguish Well-Differentiated Neuroendocrine Tumor G3 from Poorly Differentiated Neuroendocrine Carcinoma

Although in most instances NETs and NECs are readily distinguished on morphologic grounds, in some cases, particularly in small biopsies (often liver biopsies of metastatic tumor), the distinction of NET G3 from large cell NEC on the H&E is impossible. As an example, in a recent study of 33 G3 pancreatic NENs from MSKCC, in which 3 reviewers were asked to assign a diagnosis of NET, large cell NEC, small cell NEC, or uncertain, there was unanimous agreement on a diagnosis of NET or NEC in only 14 (42%) [86]. Using a combination of morphologic clues from additional histologic sections (i.e., presence of a clear cut NET component = NET; presence of a ductal adenocarcinoma component = NEC) and immunohistochemistry, a conclusive diagnosis was reached in 18 of 19 (95%) initially ambiguous cases; their immunohistochemistry panel was informative in 11 of these 19 (58%).

The Iowa NET G3 versus large cell NEC algorithm is presented in Fig. 14. Immunohistochemistry for p53 and total Rb (clone G3-245) represents the lynchpin of the classifier, which is predicated on NEC genetics (Figures 15A-F). Biallelic inactivation of *TP53* and *RB1* is the molecular genetic hallmark of small cell lung cancer, while mutations in these tumor suppressors are rarely to never seen in NET—even NET G3 [134]. In the session I had quipped that “small cell lung cancer is the id of cancers.” Genetically, large cell lung NEC is composed of both small cell lung cancer-like (40% of tumors; biallelic inactivation of *TP53* and *RB1*) and non-small cell lung cancer-like (60%; *TP53* and *STK11* and/or *KEAP1*-mutant) subsets [135,136]. Rb inactivation appears to be permissive to the development of the NEC phenotype (Figures 16A-B). It is seen as a resistance mechanism in *EGFR* mutant lung adenocarcinomas that transform into small cell NEC, is often demonstrated in the non-small cell carcinoma components occasionally identified adjacent to large cell lung NEC, and is also typical of castration-resistant prostate cancer—a tumor type that often shows NEC morphology [137,138]. Data on Rb inactivation in extrapulmonary NECs is scant and highly variable. Yachida and Tang's groups reported loss of Rb expression in 26% of 19 and 58% of 12 pancreatic NECs, respectively, while Jesinghaus and Shamir's groups reported loss in 11% of 19 and 78% of 18 colonic NECs [62,86,139,140]. Across these 4 studies, there was p53 mutant-pattern staining in 69% of cases. For USCAP 2019, I performed p53 and Rb immunohistochemistry on TMAs of 30 small cell lung, 21 extrapulmonary visceral, and 21 CM2B4-negative cutaneous NECs, finding mutant-pattern p53 in 76% of both lung and extrapulmonary visceral and 57% of CM2B4-negative cutaneous; Rb loss in 87% of lung, 55% of extrapulmonary visceral, and 76% of CM2B4-negative cutaneous; and abnormal p53 and/or Rb staining in 97% of lung, 81% of extrapulmonary visceral, and 90% of CM2B4-negative cutaneous NECs [141].

Although p53 and Rb appear to be sufficient in most cases, I supplement these with clusterin, SSTR2A, and CXCR4. While strongly expressed by most (80%) non-jejunoileal NETs, clusterin is only occasionally, weakly expressed by NECs (19%; median H-score 36) [104]. While SSTR2A is nearly always strongly expressed by ileal and is strongly expressed

by 80–90% of pancreatic NETs, it is expressed by only one third of NECs, with expression generally both less intense and less extensive; anecdotally, we have also seen less intense, less extensive staining in lung NET [12]. Our multidisciplinary group is currently investigating the biotheranostic target C-X-C motif chemokine receptor 4 (CXCR4) in NEC. In TMA, I found it to be expressed by 84% of 95 NECs (median H-score 104) and only 4.5% of 66 GEP-NETs (median H-score 3) [142]. Anecdotally, we have also seen it expressed by up to half of ACs of lung origin.

8. Lesions Apt to be Misdiagnosed as Well-Differentiated Neuroendocrine Tumor

Several tumors are consistently mistaken for NET. All of them may express synaptophysin, while only a few of them also express chromogranin A. Although subsets of NETs are consistently synaptophysin-positive/chromogranin A weak-to-negative, anytime I see a "synaptophysin+ only" tumor, I am on "red alert." Most of these tumors are frequently encountered in the metastatic setting.

Glomus tumor is mistaken for NET based on its monomorphous, epithelioid cytomorphology and variable synaptophysin-positivity, which is weaker and less extensive than in NET; chromogranin A is uniformly negative (Figures 17A-C). Most tumors arise in the skin or superficial soft tissue, but, like NET, they may arise at virtually any visceral site. Gastric tumors predominate in the GI tract. Glomus tumors demonstrate very well-defined cell borders and display prominent subendothelial growth. Malignant examples, characterized by marked cytologic atypia and increased mitotic activity (>5 per 50 HPF) or the presence of atypical mitotic figures, are exceptional. Diffuse, strong smooth muscle actin-positivity secures the diagnosis.

Before diagnosing a pancreatic NET one should always consider the differential of solid pseudopapillary neoplasm (SPEN), acinar cell carcinoma (ACC), and pancreatoblastoma. SPEN demonstrates variable synaptophysin-positivity, qualitatively similar to that seen in glomus tumor, while, again, chromogranin A is uniformly negative (Figures 17D-F). Broad-spectrum epithelial markers are also weak-to-negative. Clues to diagnosis are the presence of a large solid and cystic tumor presenting in a woman of childbearing-age, a distinctive perivascular growth pattern reminiscent of "ependymal rosettes," and frequent hyaline globules. Nuclear beta-catenin-positivity distinguishes SPEN from NET; LEF1, a mainstream chronic lymphocytic leukemia marker, is also consistently expressed [143,144]. ACC may demonstrate acinar but often shows diffuse-pattern growth; it is best recognized by its uniformly prominent nucleoli and eosinophilic cytoplasmic granularity. It demonstrates a continuum of chromogranin A and synaptophysin expression from 0–100% cells staining. The WHO classification regards cases with fewer than 30% cells staining as ACC, while those with ≥30% cells staining are considered mixed acinar-neuroendocrine carcinoma (Figures 17G-I). In a recent series of 62 acinar cell neoplasms, 58% contained no chromogranin A or synaptophysin-positive cells, 23% contained 1–30%, and 19% contained ≥30%. The cases designated mixed acinar-neuroendocrine carcinoma were not morphologically or clinically distinctive. Two recent genomic analyses of acinar neoplasms

(containing 16% and 42% mixed acinar-neuroendocrine carcinomas) failed to reveal meaningful differences between pure and mixed tumors (e.g., both tumor types showed occasional *RAF* fusions and *PRKAR1A* loss of function and frequent inactivation of DNA repair genes) [145,146]. Thus, in the absence of morphologically discrete areas of ACC and NET or NEC (which has certainly been reported), in most cases this appears to represent the "occult neuroendocrine differentiation" discussed above [147]. Trypsin is the most sensitive diagnostic marker; an antibody to the COOH-terminus of BCL10 (which cross-reacts with carboxyl ester hydrolase) may also be used [148]. The vast majority of pancreatoblastomas present in the pediatric setting (Figures 17 J-L). These polyphenotypic tumors demonstrate simultaneous acinar (trypsin-positive), neuroendocrine (general neuroendocrine marker-positive), and ductal differentiation. The presence of squamoid nests is the key diagnostic feature. Tumors show alternating areas of membranous and nuclear beta-catenin expression, with the latter preferentially localizing to the squamoid nests.

Adrenal cortical carcinoma is among the "great mimickers." Tumors may demonstrate low-grade, monomorphous cytomorphology simulating NET or present as pleomorphic malignant neoplasms with a broad differential (Figures 17 M-O). Sixty percent of adrenal cortical carcinomas are synaptophysin-positive, which is often diffuse and strong, though chromogranin A is always negative. Low-grade examples have Ki-67 proliferation indices in the 5% range, while high-grade examples are often around 15% [149]. They are frequently weak-to-negative for multiple broad-spectrum epithelial markers, occasionally raising the additional differential of PHEO. If you consider the differential, SF1 is the best diagnostic marker (≥95% sensitive, very specific) followed by melan A (80% sensitive, but also expressed by melanoma and translocation renal cell carcinoma); calretinin and inhibin A are similarly sensitive but much less specific. Incidentally, 15% of adrenal cortical carcinomas demonstrate nuclear beta-catenin-positivity (which once caused me to misdiagnose one as SPEN), and up to 5% are mismatch repair deficient (which once caused me to almost misdiagnose one metastatic to the colon as undifferentiated colon cancer) [150,151].

PHEO/PARAs are probably the tumor types most often mistaken for NET. They have neuroendocrine morphology (neuroendocrine chromatin; zellballen architecture, though sometimes more diffuse) and are diffusely, strongly positive for synaptophysin and chromogranin A. The differential of PHEO/PARA is why I consider the demonstration of broad-spectrum epithelial marker-positivity to be "almost mandatory" before diagnosing NET, especially in the metastatic setting. As discussed above, most PHEO/PARAs are islet 1-positive, which I have seen lead to incorrect diagnoses of pancreatic NET. PHEO/PARA is widely known for having sustentacular cells, which can be demonstrated with either S-100 or SOX10, but not widely known is that NETs can have sustentacular cells, too, especially tumors of bronchopulmonary and appendiceal origin [152,153]. For this reason, the best widely available positive marker for PHEO/PARA is GATA-3, which Miettinen and colleagues found to be positive in 87% of 46 PHEO/PARAs and 0% of 43 NETs in their large-scale immunohistochemical survey of 2500 tumors [154]. As a note of caution, in these tumor types I have found GATA-3 to be especially susceptible to delayed and poor fixation, though this is rarely a problem in biopsy material. Additional "positive" PHEO/PARA markers in this differential include tyrosine hydroxylase (diffusely, strongly positive in 100% of PHEOs and 40% of PARAs and quite robust) and loss of SDHB (seen in 30% of

thoracoabdominal and ≥ 15% of head and neck PARAs and 5% of PHEOs) (Figure 18A-F) [155,156].

9. Putting It All Together

Table 3 summarizes required and recommended data elements for biopsies and resections of NENs and associated required and recommended immunohistochemistry. For resections, use of College of American Pathologists Cancer Protocols is recommended, and tumors are staged according to the 8th edition of the *AJCC Cancer Staging Manual*. Because of their especially poor prognosis, NECs are reported and staged using the same site-specific protocols/criteria used for non-neuroendocrine carcinomas.

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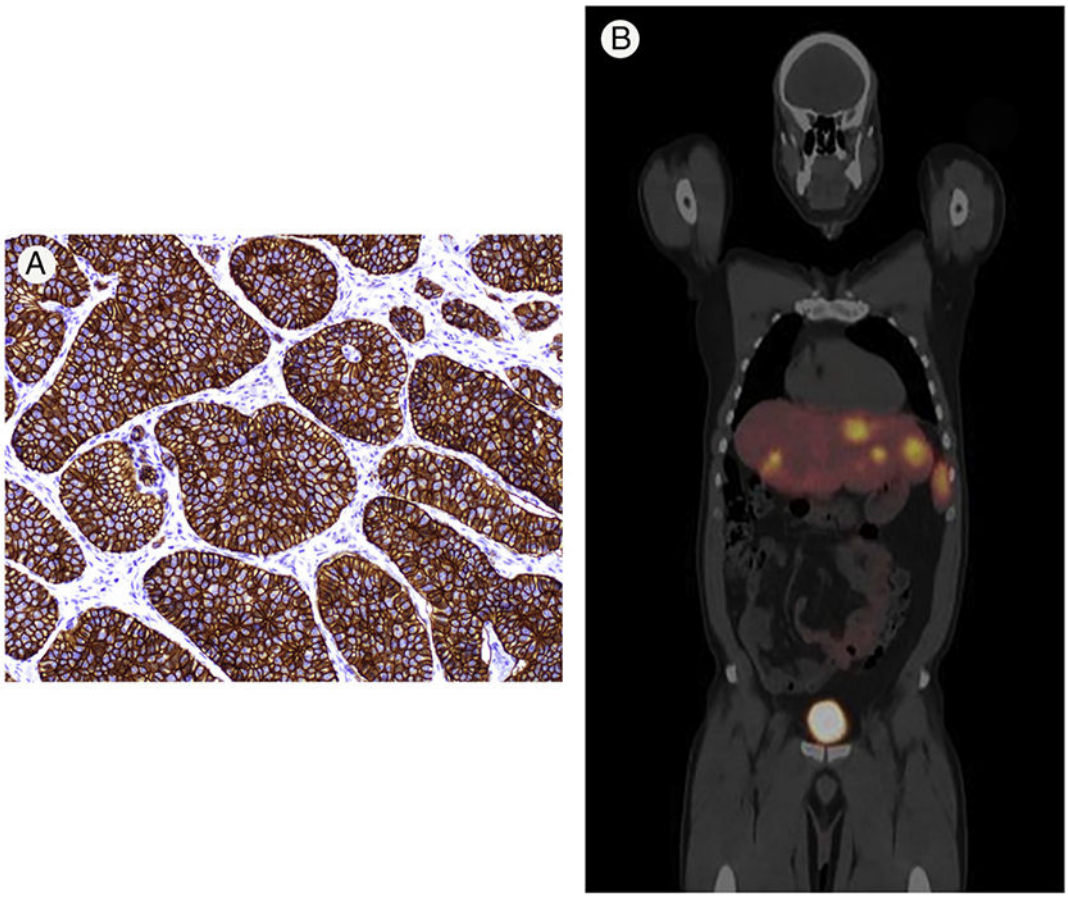

Figure 1. Somatostatin Receptor (SSTR) Expression by Neuroendocrine Neoplasms:
Along with general neuroendocrine marker expression and the production of peptide hormones and/or biogenic amines, somatostatin receptor expression is a characteristic feature of neuroendocrine neoplasms (NEN). (A) At Iowa, we have been routinely performing SSTR2A immunohistochemistry on NENs since the Fall of 2014. Diffuse, strong membrane expression is seen in nearly all enterochromaffin-cell tumors (depicted here), 80–90% of pancreatic tumors, and fewer bronchopulmonary tumors and poorly differentiated neuroendocrine carcinomas. (B) Somatostatin receptor expression is the basis of NEN functional imaging and octreotide and peptide receptor radionuclide therapy. This DOTATATE scan demonstrates several liver metastases.

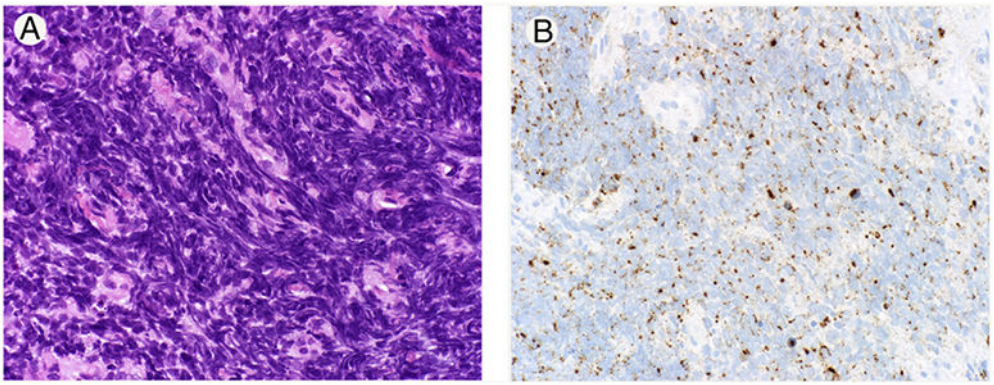

Figure 2. Chromogranin A (CgA) Expression by Small Cell Neuroendocrine Carcinoma:
(A) This crushed small cell neuroendocrine carcinoma demonstrates (B) rather extensive, though punctate CgA expression. CgA is a component of neuroendocrine dense core granules, which are often few in number in this tumor type. When assessing CgA-positivity, I pay more attention to the proportion of cells with signal than the total cross-sectional area occupied by signal.

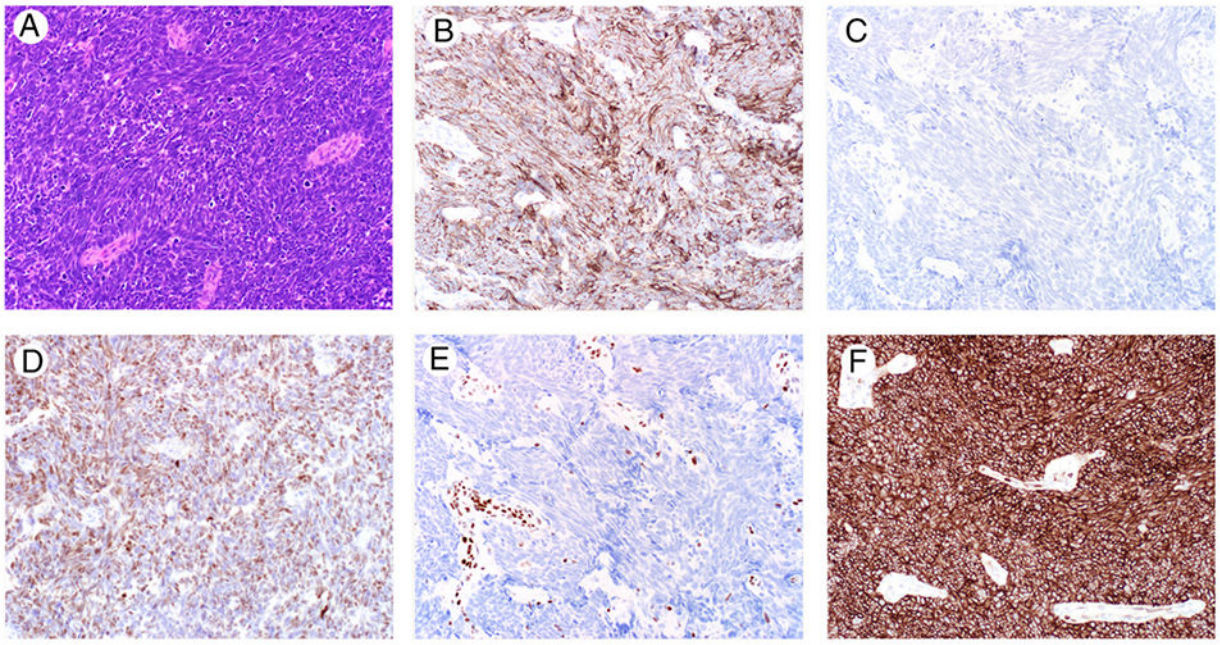

Figure 3. Extended Immunohistochemistry to Support a Diagnosis of Small Cell Neuroendocrine Carcinoma (NEC):

(A) The morphologic impression in this metastasis from a patient with a lung mass was small cell NEC. (B) Keratin AE1/AE3 is positive, while both (C) chromogranin A (depicted) and synaptophysin were entirely negative. (D) INSM1 demonstrates multifocal positivity, (E) Rb expression is lost (with intact staining in endothelial cell nuclei), and (F) CXCR4 shows diffuse, strong membrane staining. INSM1 is more sensitive than traditional general neuroendocrine markers in NEC; Rb inactivation, a molecular genetic hallmark of small cell lung cancer, is a sensitive NEC marker; CXCR4 overexpression is seen in ≥80% of NECs; in my anecdotal experience these latter two markers are reasonably specific for NEC.

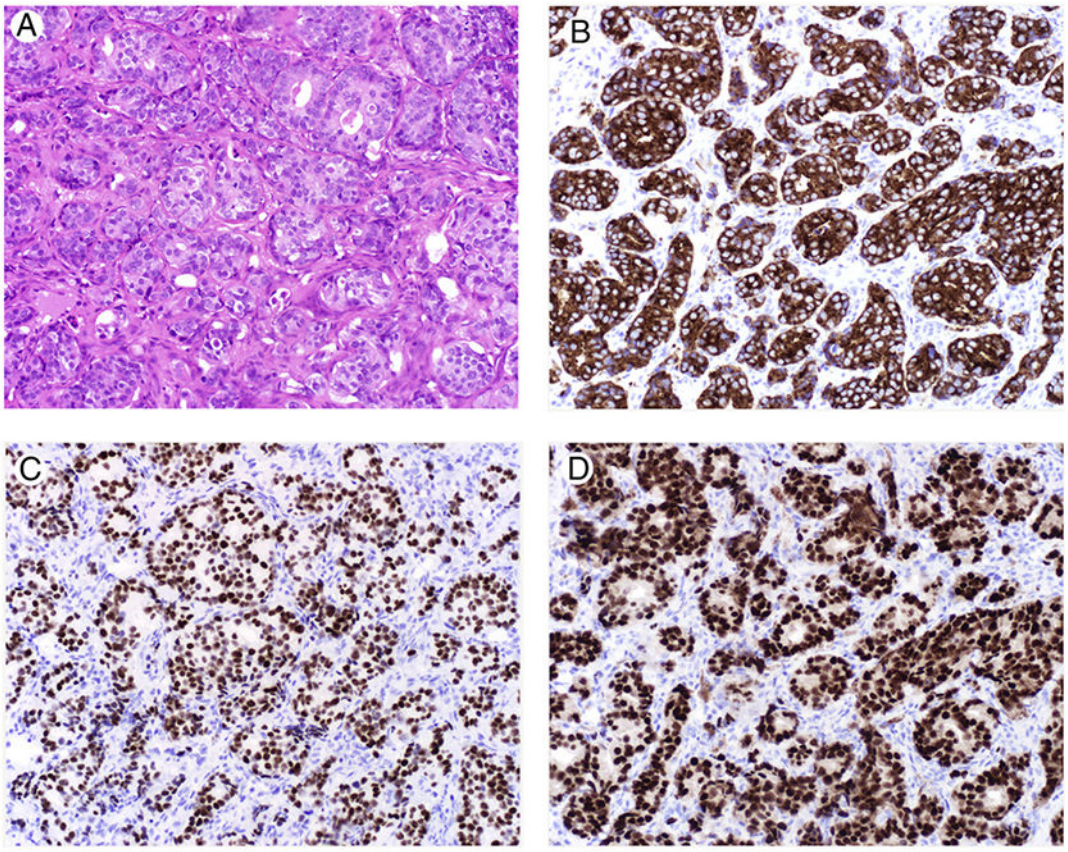

Figure 4. Non-Small Cell Carcinoma with (Occult) Neuroendocrine Differentiation:

(A) This metastasis from a woman with a history of breast cancer simultaneously expresses (B) synaptophysin, (C) GATA-3, and (D) ER. Ten to twenty percent of non-neuroendocrine carcinomas from diverse anatomic sites demonstrate some degree of general neuroendocrine marker expression, ranging from rare cells to diffuse, strong staining (as in this example). Occult neuroendocrine differentiation has no clear prognostic or therapeutic implication. The diagnosis of “poorly differentiated carcinoma with neuroendocrine differentiation” is strongly discouraged, as it engenders confusion in the treating clinician. Breast cancers with occult neuroendocrine differentiation are typically of luminal A type and are treated as such.

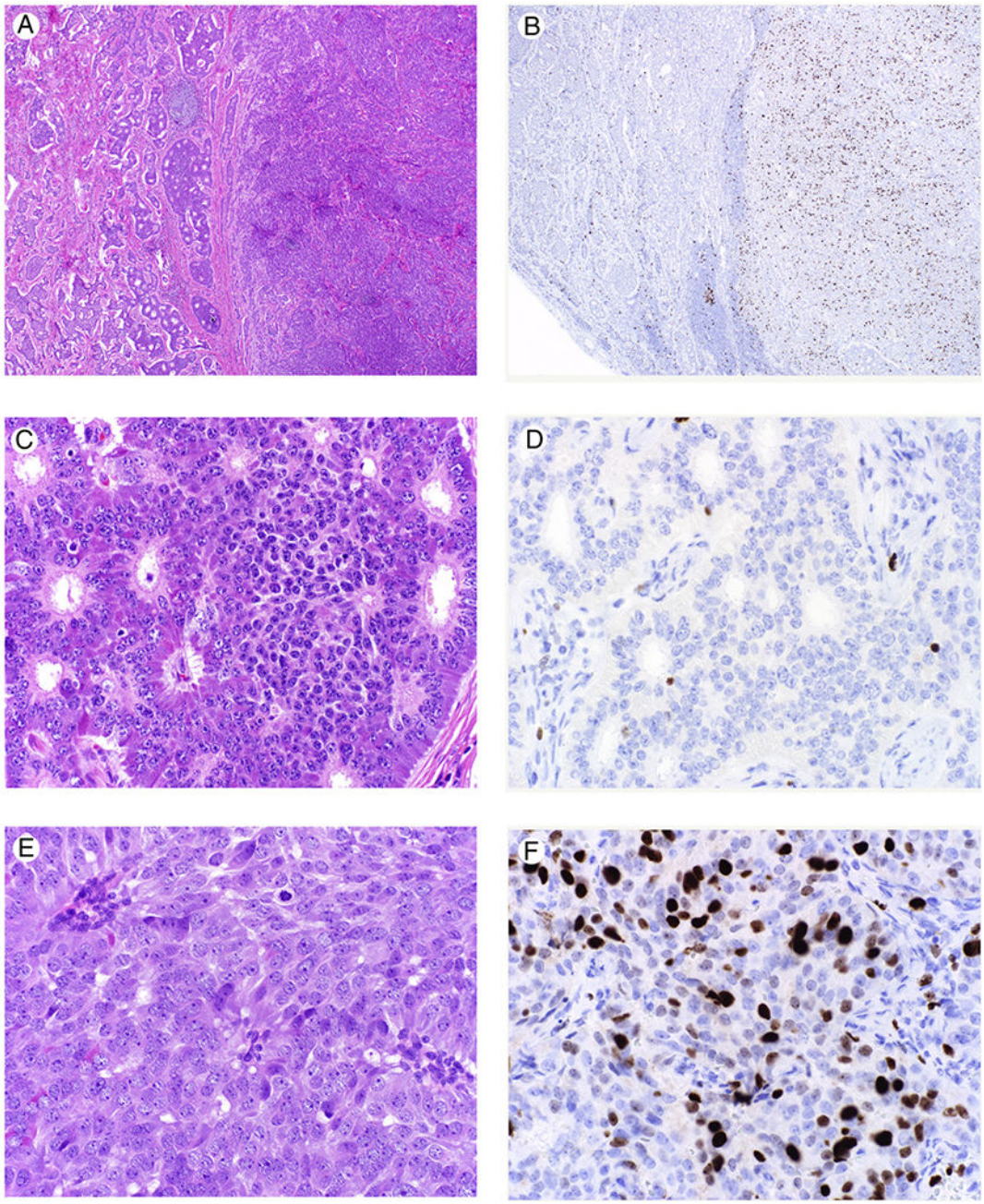

Figure 5. Well-Differentiated Neuroendocrine Tumor (NET) G3:

(A) Metastatic tumor demonstrating juxtaposition of well- (left) and “less well”-differentiated (right) components with (B) corresponding Ki-67. (C) At higher power the well-differentiated component demonstrates extensive polarized eosinophilic cytoplasmic granularity and prominent pseudoglands (i.e., a readily recognizable midgut NET) with a (D) corresponding Ki-67 proliferation index of 0.75%. (E) The “less well”-differentiated component demonstrates a (F) Ki-67 proliferation index of 35%. Five percent of NETs are morphologically well-differentiated but with “G3 range”-proliferation indices and/or mitotic counts. Some examples demonstrate substantial morphologic overlap with large cell

neuroendocrine carcinoma. Presence of an adjacent G1/G2 NET supports the diagnosis of NET G3. In this case, I had also performed p53 (wild-type), Rb (intact), and CDX2 (expressed throughout, though with diminished intensity in the G3 component), which further support the diagnosis of NET G3 (not depicted).

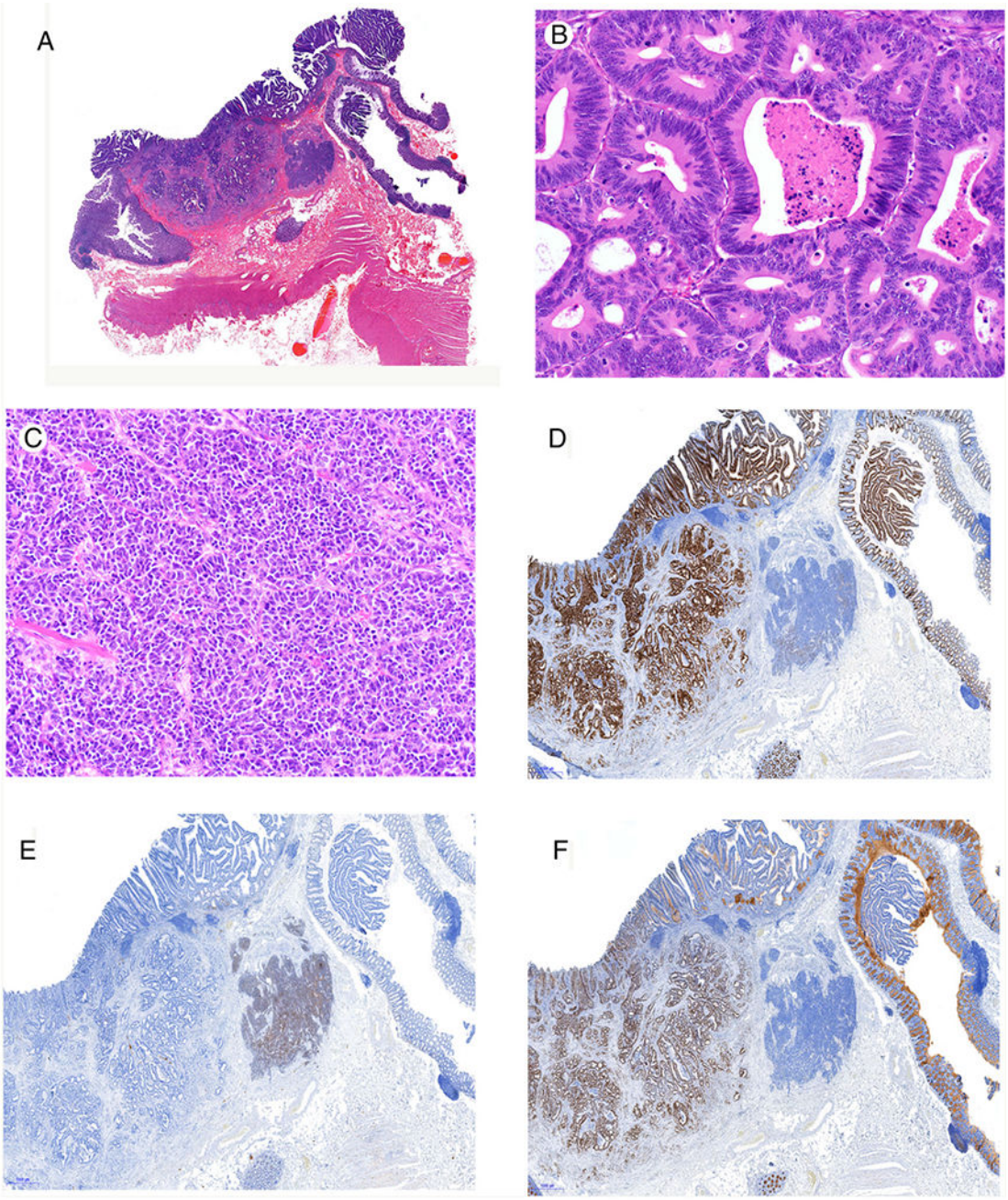

Figure 6. Mixed Neuroendocrine-Non-Neuroendocrine Neoplasm (MiNEN):

(A) This colon cancer arising in association with an adenoma is composed of (B) conventional adenocarcinoma and (C) poorly differentiated neuroendocrine carcinoma (NEC) components. (D) CDX2 is expressed by the background colonic mucosa, adenoma, and adenocarcinoma but is nearly absent in the NEC, while (E) synaptophysin shows the opposite pattern. (F) Rb loss is confined to the NEC component. The WHO 2019 term "MiNEN" replaces the term "mixed adenoneuroendocrine carcinoma (MANEC)," as the non-neuroendocrine component of mixed tumors may be squamous and the neuroendocrine

component may be NET, though the combination depicted in this example is the most common one in the West.

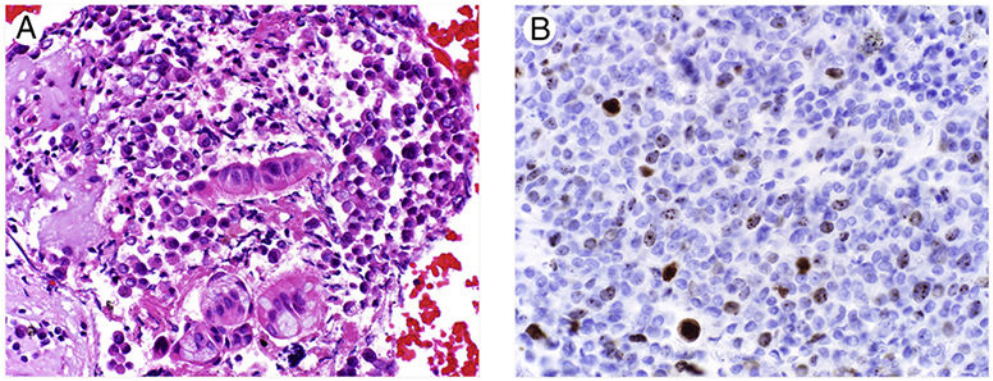

Figure 7. Use of Ki-67 Immunohistochemistry Beyond Gastroenteropancreatic Well-Differentiated Neuroendocrine Tumor:

(A) Fine-needle aspiration of a lung mass reveals carcinoid tumor but there are only a couple HPF for mitotic counting. The medical oncologist asked us to perform a Ki-67 to get a better sense of whether this was typical carcinoid or atypical carcinoid tumor, which would impact treatment planning. (B) The Ki-67 proliferation index is 15%; although Ki-67 is not part of the WHO Classification of lung neuroendocrine neoplasms, this result is essentially diagnostic of atypical carcinoid tumor. Subsequent DOTATATE scan revealed extensive hilar and mediastinal adenopathy. This Ki-67-immunostained slide reveals weak and strong, nucleolar and diffuse-pattern staining, all of which are considered positive.

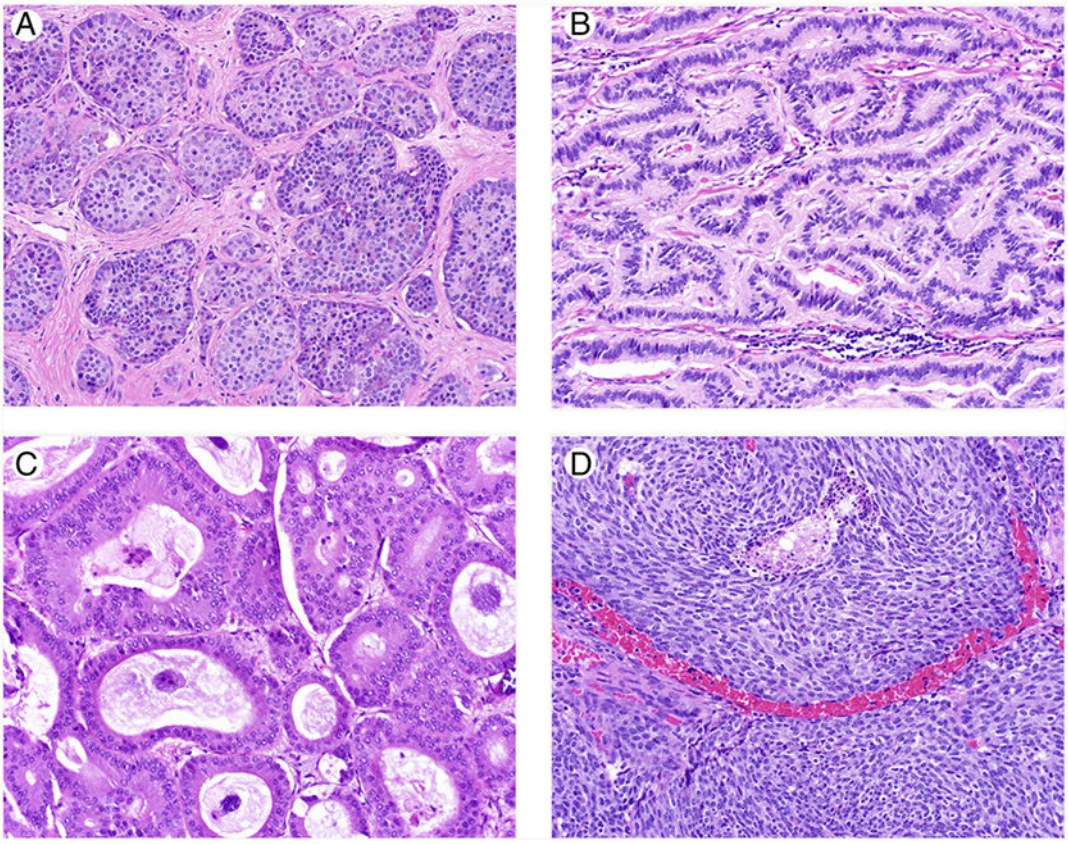

Figure 8. Morphologic Clues to Well-Differentiated Neuroendocrine Tumor Site of Origin:
(A) Tumor with predominantly nested architecture, minor pseudogland component, and heavy eosinophilic cytoplasmic granularity diagnostic of an enterochromaffin-cell tumor; tumors with this morphology typically arise in the ileum, though this is actually a testicular primary. (B) Trabecular architecture is most commonly seen in tumors of rectal origin. (C) Pseudoglandular architecture is typical of ampullary tumors, which are apt to be mistaken for adenocarcinoma in forceps biopsy. (D) Spindle cell morphology is often seen in lung tumors that metastasize; small foci of necrosis like this are more common in lung (in this case diagnostic of atypical carcinoid tumor) than gastroenteropancreatic tumors.

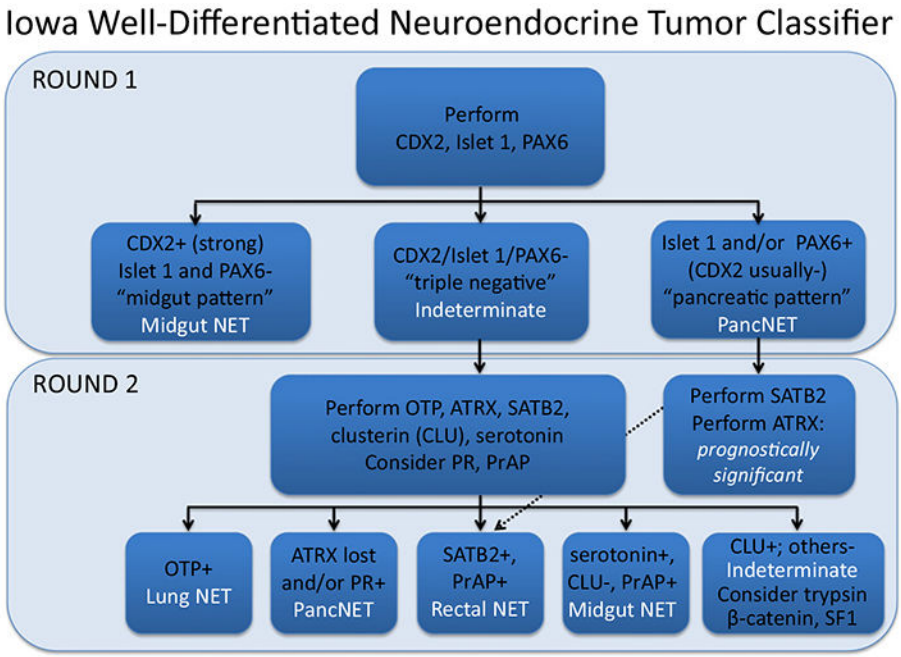

Figure 9. University of Iowa Immunohistochemical Algorithm for Well-Differentiated Neuroendocrine Tumor Site of Origin.

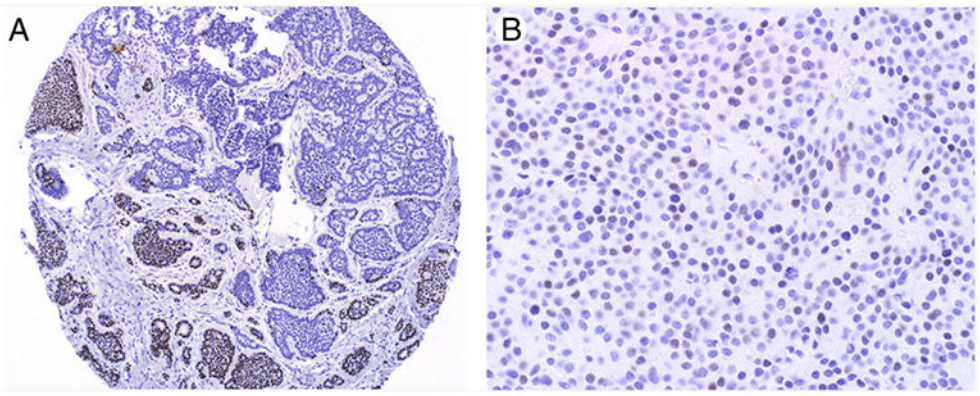

Figure 10. CDX2-Negativity in Enterochromaffin (EC)-Cell Well-Differentiated Neuroendocrine Tumors (NET):

CDX2 is the best widely available EC-cell NET marker, expressed by 90% of tumors of midgut origin. (A) Clonal loss of CDX2 in a primary tumor, possibly due to promoter methylation. (B) Weak, patchy CDX2-positivity in a whole section from a liver resection; this tumor appeared to be CDX2-negative in tissue microarray.

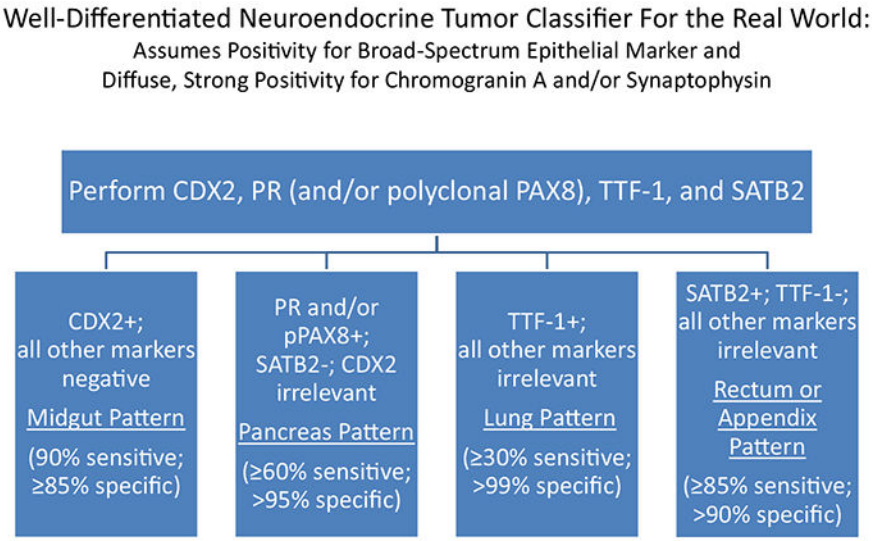

Figure 11. Simplified Immunohistochemical Algorithm for Well-Differentiated Neuroendocrine Tumor Site of Origin.

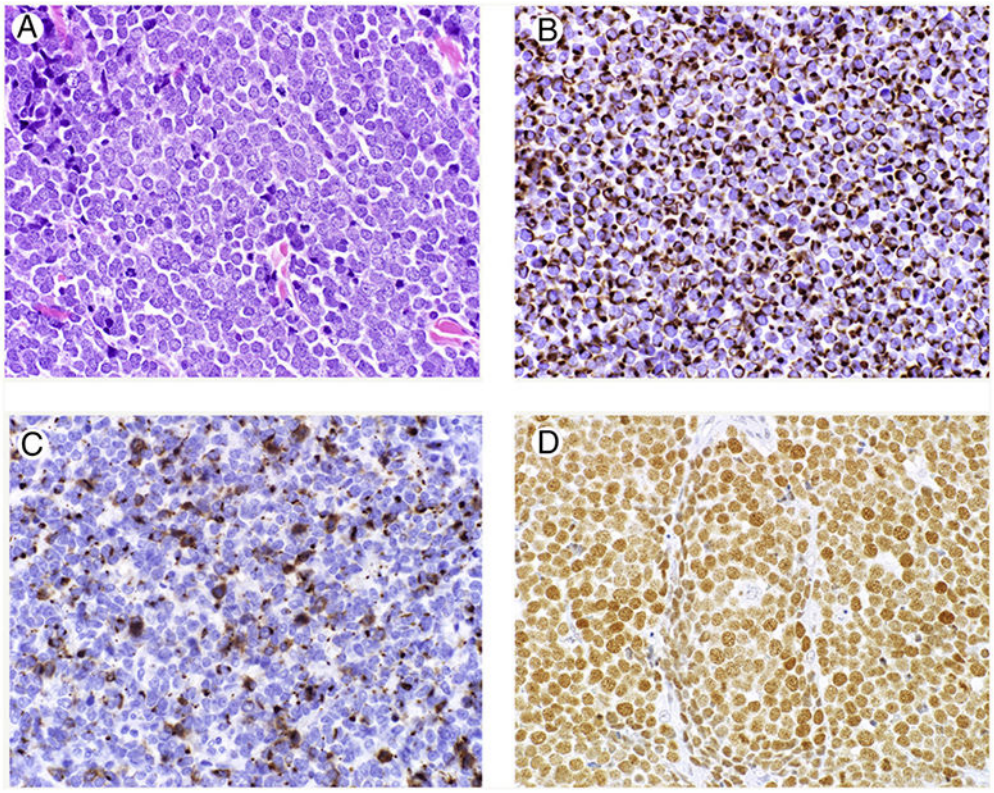

Figure 12. Immunohistochemistry for Merkel Cell Carcinoma:

(A) Monomorphous high-grade tumor with powdery chromatin demonstrates (B) dot-like CK20, (C) dot-like and diffuse neurofilament, and (D) nuclear Merkel cell polyomavirus large T antigen (clone CM2B4) expression. CK20 is expressed by 90% of Merkel cell carcinomas and is otherwise uncommonly expressed by neuroendocrine carcinomas (NEC) except for those arising in the parotid; neurofilament is expressed by 75% of tumors and appears reasonably specific. Up to 80% of Merkel cell carcinomas are polyomavirus-positive; the remainder are UV-light associated. Most CM2B4-positive Merkel cell carcinomas are CK20-positive, somewhat limiting this marker's diagnostic utility. Polyomavirus-driven Merkel cell carcinomas are remarkably monomorphous compared to all other NECs (i.e., you should be able to predict the result of the CM2B4 based on morphology!).

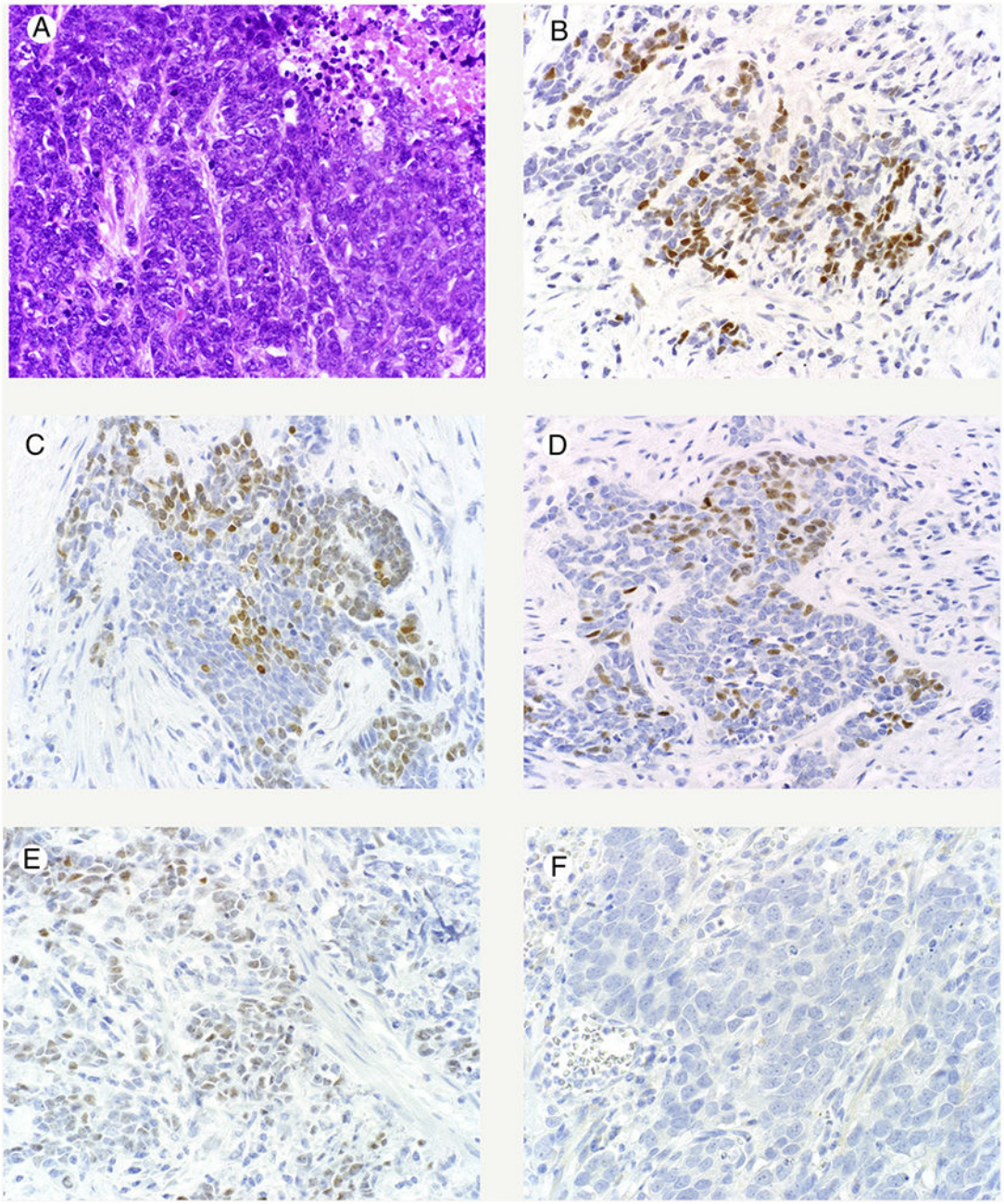

Figure 13. Poorly Differentiated Neuroendocrine Carcinomas (NEC) Demonstrate Marked Transcription Factor Lineage Infidelity:

(A) This endometrial NEC co-expresses (B) GATA-3, (C) SATB2, (D) p40, (E)MYC, and, naturally, does not express (F) PAX8. NECs tend to express multiple transcription factors independent of site of origin. Expression is typically patchy but can be rather extensive. The only transcription factors with a role in NEC site of origin assignment are TTF-1 and ASCL1 (for visceral origin) and strong SATB2 (for cutaneous origin).

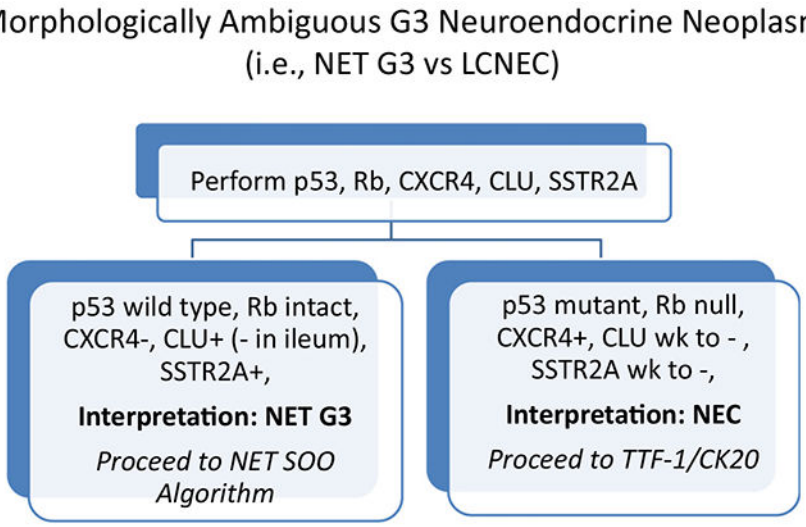

Figure 14. Immunohistochemical Algorithm for Morphologically Ambiguous G3 Neuroendocrine Epithelial Neoplasms.

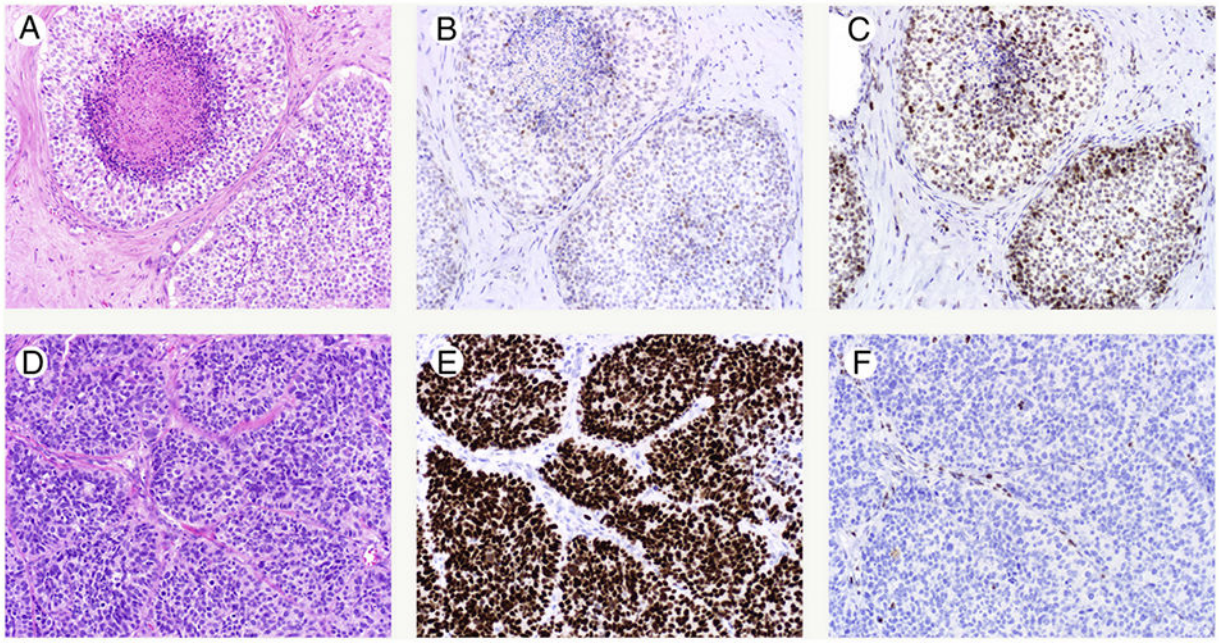

Figure 15. Immunohistochemistry to Distinguish Well-Differentiated Neuroendocrine Tumor (NET) G3 from Poorly Differentiated Neuroendocrine Carcinoma (NEC):

(A) This patient had a lung mass, and I favored a diagnosis of large cell NEC in this liver metastasis. (B) p53 demonstrates wild-type pattern staining and (C) Rb expression is intact, favoring NET. The tumor was found to express OTP (not depicted), further supporting the diagnosis. (D) This large cell NEC demonstrates (E) missense-mutation pattern p53 and (F) Rb loss, supporting the morphologic impression. The NET G3 versus NEC immunohistochemical classifier is predicated on NEC genetics.

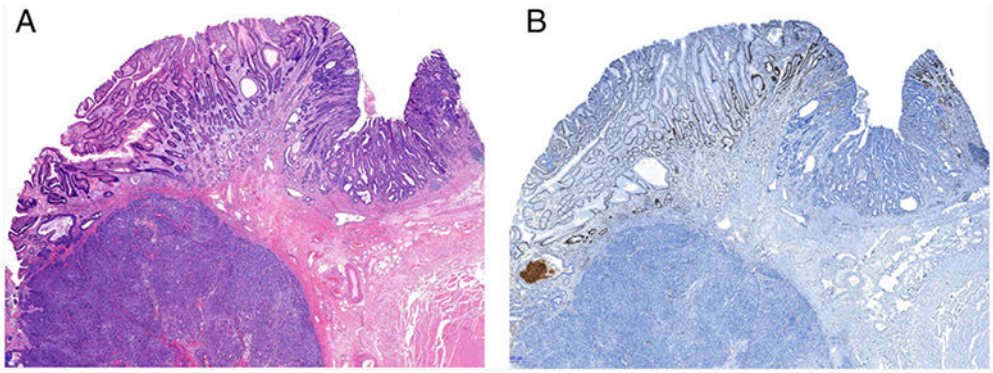

Figure 16. Rb Immunohistochemistry and the Relationship Between Non-Neuroendocrine and Neuroendocrine Carcinomas:

(A) This gastric large cell neuroendocrine carcinoma (NEC) arises in association with columnar dysplasia; (B) both components demonstrate Rb inactivation. In the GI tract, NECs arise from non-neuroendocrine precursors; many castration-resistant prostate cancers have a NEC phenotype; small cell lung cancer may arise from *EGFR*-mutant lung adenocarcinoma in the setting of anti-EGFR therapy. In these contexts, Rb inactivation, though insufficient on its own, appears to be permissive to the development of the NEC phenotype.

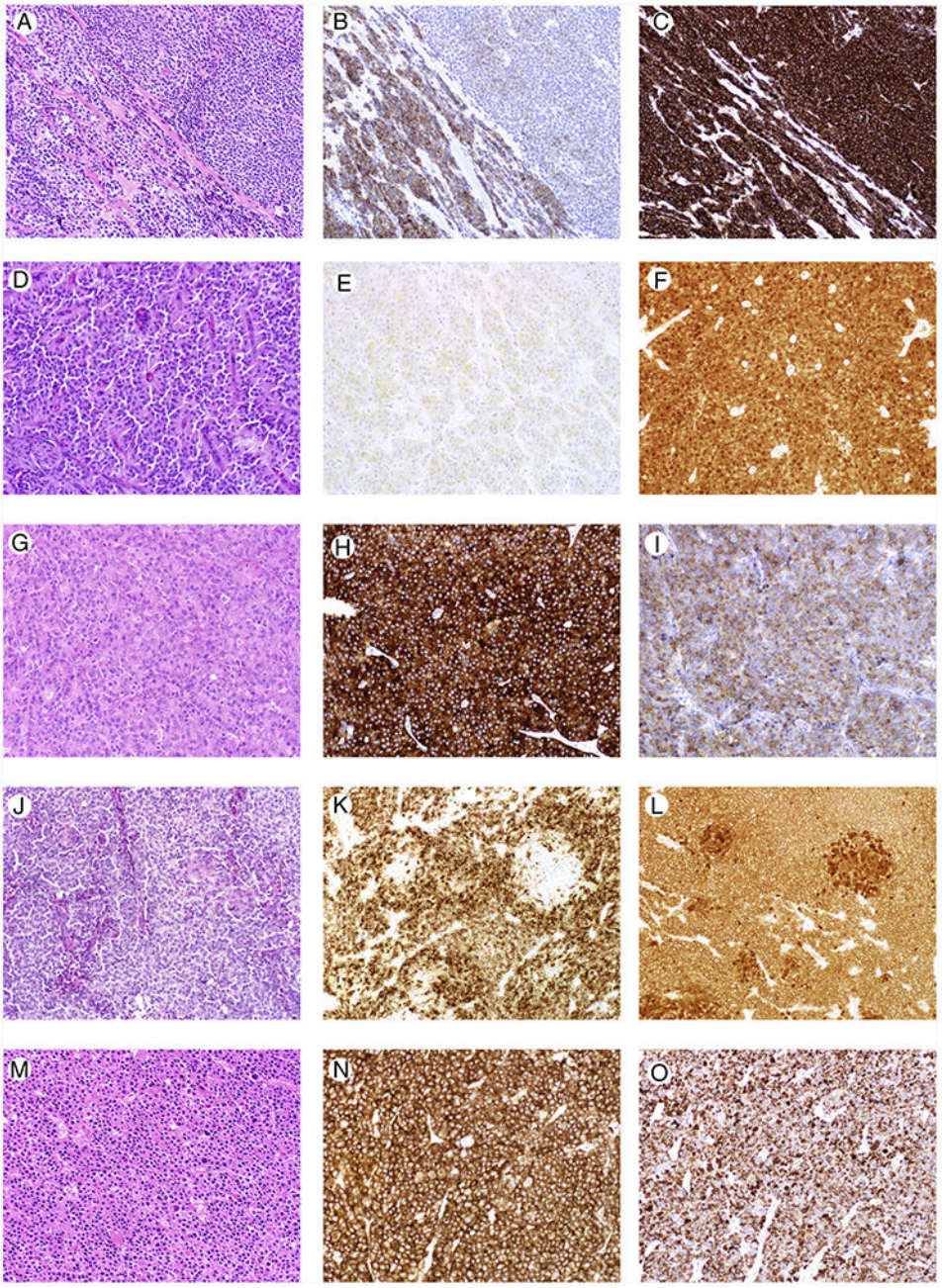

Figure 17. Lesions Apt to be Mistaken for Well-Differentiated Neuroendocrine Tumor (NET):
Each of these tumors demonstrates relatively monomorphous cytomorphology and general neuroendocrine marker-positivity (in many instances quite strong). (A) Gastric glomus tumor with (B) variable but overall rather extensive synaptophysin-positivity; (C) smooth muscle actin-positivity supports the diagnosis. (D) Solid pseudopapillary tumor with (E) modest synaptophysin-positivity; (F) nuclear beta-catenin-positivity supports the diagnosis in the appropriate morphologic context. (G) This mixed acinar-neuroendocrine carcinoma has the H&E-appearance of acinar cell carcinoma but (H) demonstrates diffuse, strong synaptophysin-positivity; (I) trypsin-positivity supports the morphologic impression. (J) This

pancreatoblastoma, with morules evident at the right center and upper left, was misdiagnosed as a NET based on (K) this strongly positive chromogranin A; (L) heterogeneous nuclear beta-catenin staining, which has a predilection for the morules, is typical; the tumor also demonstrated multifocal trypsin-positivity (not depicted). (M) Adrenal cortical carcinoma demonstrates (N) diffuse, strong synaptophysin-positivity; (O) melan A-positivity supports the diagnosis. I always consider adrenal cortical carcinoma in presumed NETs in which immunohistochemistry-based site of origin assignment is uncertain and in tumors described as “retroperitoneal” in location.

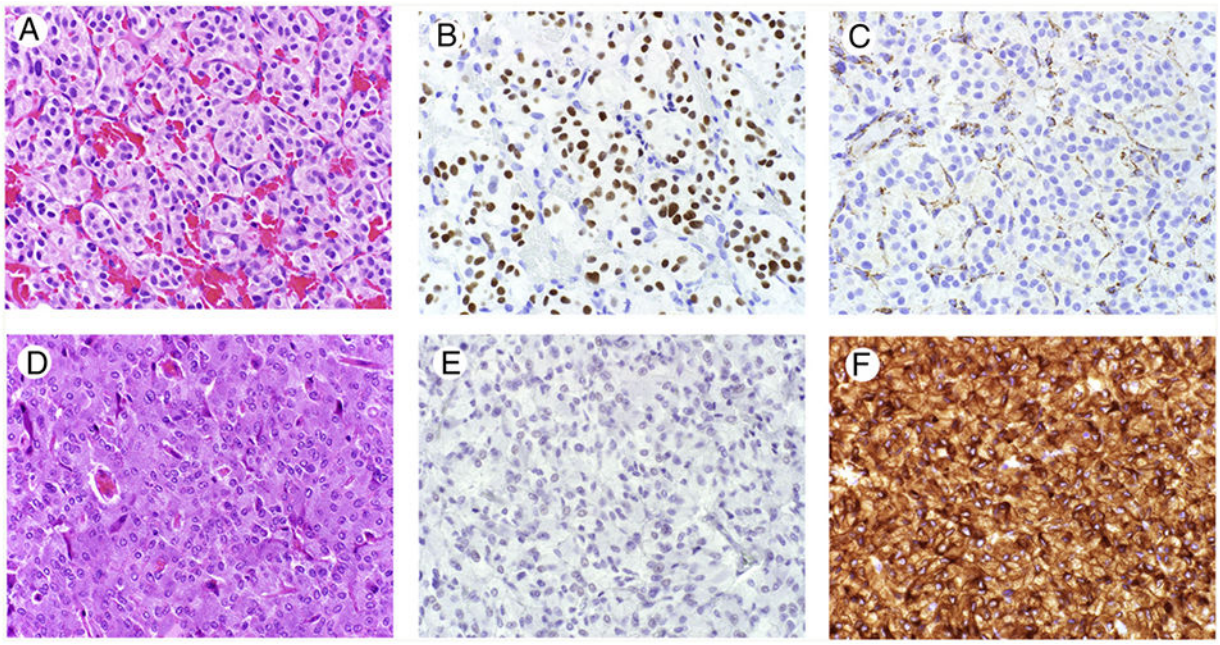

Figure 18. Immunohistochemistry for Paraganglioma/Pheochromocytoma:

(A) This paraganglioma demonstrates characteristic zellballen architecture; (B) strong GATA-3-positivity supports the diagnosis in this well-fixed biopsy of a metastatic tumor; (C) loss of SDHB expression, with intact staining in endothelium and sustentacular cells, also supports a diagnosis of paraganglioma or pheochromocytoma and suggests the possibility of hereditary paraganglioma-pheochromocytoma syndrome. (D) This pheochromocytoma is the “essence of amphophilic;” (E) GATA-3-positivity is barely discernable in this resected tumor; (F) diffuse, strong tyrosine hydroxylase expression is seen in 100% of pheochromocytomas and 40% of paragangliomas and staining is very robust.

Table 1

2019 WHO Classification of Gastroenteropancreatic Neuroendocrine Neoplasms

Classification/Grade	Ki-67 Proliferation Index	Mitotic Count*
(Well-Differentiated) Neuroendocrine Tumor:		
G1	<3%	<2 per 2 mm^2
G2	3–20%	2–20 per 2 mm^2
G3	>20%	>20 per 2 mm^2
(Poorly Differentiated) Neuroendocrine Carcinoma:		
G3	>20%	>20 per 2 mm^2
Mixed Neuroendocrine-Non-Neuroendocrine Neoplasm		

Note:

*2 mm^2 corresponds to 10 HPF for microscopes with a field diameter of 0.5 mm at 400x magnification. (My microscope, and many of yours, has a field diameter of 0.55 mm at 400x, in which case 2 mm^2 corresponds to 8.4 HPF.)

Table 2

2015 WHO Classification of Bronchopulmonary Neuroendocrine Neoplasms

Classification/Grade	Mitotic Count	Necrosis	Other Features
Carcinoid Tumor:			
Typical carcinoid	<2 per 2 mm^2	Absent	Tumor must be ≥0.5 cm
Atypical carcinoid	2–10 per 2 mm^2	Present	Diagnosis based on presence of either or both features
Poorly Differentiated Neuroendocrine Carcinoma:			
Small cell carcinoma	>10 per 2 mm^2	Frequent	Characteristic histology
Large cell neuroendocrine carcinoma	>10 per 2 mm^2	Frequent	Organoid morphology and expression of at least 1 general neuroendocrine marker

Table 3

Required and Recommended Reporting Elements for Biopsies and Resections of Neuroendocrine Epithelial Neoplasms

Required Data Element:	Associated Required or Recommended Immunohistochemistry
Diagnosis: well-differentiated neuroendocrine tumor (typical or atypical carcinoid tumor for lung) or poorly differentiated neuroendocrine carcinoma (small cell or large cell-type)	• Chromogranin A and synaptophysin (or INSM1) to establish neuroendocrine nature (required) • Broad-spectrum epithelial marker to confirm epithelial nature (highly recommended in primary and regional disease and required in distant metastasis) • p53 and Rb are recommended in the distinction of well-differentiated neuroendocrine tumor G3 from poorly differentiated neuroendocrine carcinoma; other immunostains may be useful in this differential
Ki-67 proliferation index (proliferation index >20% is implied for poorly differentiated neuroendocrine carcinoma and performance is not mandatory)	• Ki-67 on at least one block of tumor (required) • Ki-67 on additional blocks (e.g., matched primary and metastasis) (recommended)
Mitotic count per 2 mm^2 (assessed in at least 10mm^2 [50 HPF for microscopes with a field number of 20] and expressed as mitotic figures per 2 mm^2; in biopsies with fewer HPF it is reasonable to express the total number of mitotic figures per the total number of HPFs; for poorly differentiated neuroendocrine carcinoma a “G3 range”-mitotic count is implied and performance is not mandatory)	
Grade: G1, G2, or G3 (G3 is implied for poorly differentiated neuroendocrine carcinoma and need not be explicitly stated)	
Data elements in CAP Cancer Protocol: for resection specimens	
Recommended Data Element:	
Comment on site of origin (for metastasis of occult origin)	• Panel in a well-differentiated neuroendocrine tumor to include widely available markers of midgut (CDX2), pancreatic (polyclonal PAX8 and/or PR), lung (TTF-1), and rectal (SATB2) origin; if available, islet 1 and OTP are the best pancreatic and lung NET markers and serotonin may be useful to supplement the sensitivity of CDX2 for midgut • Panel in a poorly differentiated neuroendocrine carcinoma to include TTF-1 for visceral origin and CK20 for cutaneous origin; neurofilament, CM2B4, and strong SATB2-positivity may also be helpful to support a cutaneous origin