Circulating Tumor Cells and EpCAM Expression in Neuroendocrine Tumors

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Abstract

Purpose: Neuroendocrine tumors (NET) are heterogeneous tumors with widely variable survival. It is unknown whether they express EpCAM (epithelial cell adhesion molecule) and thus whether NET circulating tumor cells (CTC) are detectable. We systematically investigated EpCAM expression and CTC detection in patients with metastatic NETs and evaluated the potential of CTCs to predict radiological progression.

Experimental Design: EpCAM protein expression was evaluated in 74 samples of formalin-fixed, paraffin-embedded NET tissue by immunohistochemistry. Seventy-nine patients with metastatic NETs (42 midgut, 5 unknown primary, 19 pancreatic, 13 bronchopulmonary) had blood samples drawn for CTC isolation and enumeration utilizing the CellSearch platform. Patients were classified as having progressive or nonproliferative disease on the basis of serial imaging.

Results: Strong homogeneous, membranous EpCAM expression was observed in all ileal (n = 26) and pancreatic NETs (n = 16), whereas variable EpCAM expression was observed in bronchopulmonary NETs (n = 13). Forty-three percent of midgut and 21% of pancreatic NETs had CTCs detected with a range of 0–62 and 0–11, respectively. The absence of CTCs was strongly associated with stable disease (P < 0.001). There was a moderate correlation between CTC levels and urinary 5-hydroxyindole acetic acid (r = 0.5, P = 0.007) and between CTC levels and burden of liver metastases (B = 8.91, P < 0.001). There was no or low correlation between CTC levels and Ki-67 (r = 0.08, P = 0.59) and serum chromogranin A (r = 0.246, P = 0.03).

Conclusions: This is the first systematic analysis showing EpCAM expression and CTC detection in NETs. CTCs seem to be associated with progressive disease and may provide useful prognostic information given the variable survival rates in these tumors.

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Introduction

Neuroendocrine tumors (NET) are a rare, heterogeneous group of tumors arising most commonly from the gastrointestinal tract, pancreas, and lungs. Their natural history is variable, with median survival ranging from approximately 6 months in aggressive high-grade tumors to up to 20 years in more indolent disease (1). Current methods of monitoring progression in NETs and response to treatment include serial imaging over many years. Although there are general NET biomarkers such as plasma chromogranin A (CgA) and urinary 5-hydroxyindole acetic acid (5-HIAA), there is a lack of prospectively validated prognostic and predictive biomarkers.

Recent technological advances have enabled circulating tumor cell (CTC) enumeration and characterization by different methods. The CellSearch platform, an automated immunomagnetic enrichment and staining system, has been utilized to detect CTCs with high sensitivity, specificity, and reproducibility (2). Large studies have reported that the number of CTCs in patients with metastatic breast cancer before commencing a new therapy to be an independent predictor of progression-free and overall survival (3, 4), with similar results reported in metastatic colorectal and prostate cancer (5, 6). Therefore, it has been suggested that CTC detection can aid appropriate patient stratification and design of tailored treatments, which are the aims of ongoing studies (such as SWOG S0500).

The CellSearch platform requires EpCAM (epithelial cell adhesion molecule) expression to isolate CTCs. EpCAM is a 39- to 42-kDa transmembrane epithelial glycoprotein (7), overexpressed in human adenocarcinomas (8). Its exact function is yet to be fully elucidated, but its expression...
Translational Relevance

For the first time, we have systematically demonstrated EpCAM (epithelial cell adhesion molecule) expression in the majority of neuroendocrine tumors (NET), suggesting an epithelial origin to these rare tumors that were once thought to be derived from cells of the neural crest. EpCAM expression has allowed us to detect circulating tumor cells (CTC) in patients with NETs, and we show that rates of detection are particularly high in midgut tumors. We also report the association between absence of CTCs and radiologically stable disease, whereas the presence of CTCs seems to be associated with progressive disease. CTC enumeration may therefore be a useful means of stratifying patients for therapeutic intervention or clinical trials that is increasingly important as the number of active agents for treating NETs increases. Furthermore, the detection of CTCs in NETs opens the way for predictive and prognostic studies as currently applied to metastatic breast and prostate cancer.

enables the CellSearch platform to enrich CTCs via immunomagnetic separation with iron particles coupled to EpCAM antibodies. Although series have reported a small neuroendocrine subset of lung cancers (8) and insulomomas (9) to overexpress this epithelial marker, the systematic analysis of EpCAM expression in NETs has not been undertaken to our knowledge. Originally thought to be derived from cells of the neural crest sharing secretory and histologic properties with neural cells, it is debated whether NETs are epithelial in origin (10, 11) and thus it has been assumed that CTCs cannot be isolated in NETs as they should not express EpCAM (12, 13).

The aims of this study were to systematically investigate EpCAM expression in NETs, to identify and quantify CTCs in patients with NETs, and to evaluate their potential to predict radiological progression.

Materials and Methods

Immunohistochemistry

The study included 74 patients with a histopathologic diagnosis of NET in which blocks of formalin-fixed and paraffin-embedded tissue were available. The tumors were classified according to the site of origin and criteria of the World Health Organization and graded according to the European Neuroendocrine Tumour Society (ENETS) proposal for grading and staging of NETs (14, 15).

Briefly, 3-μm sections of tumor tissue were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase was blocked with 0.5% H₂O₂ in methanol for 10 minutes. Thereafter, sections were subjected to antigen retrieval for 10 minutes in 0.1% trypsin at 37°C. Immunohistochemical staining was performed with the NovoLink polymer detection system (Novoceastra). Sections were incubated with mouse anti-human EpCAM monoclonal antibody (ESA, clone VU-1D9; Novoceastra) at a dilution of 1:50 for 1 hour at room temperature, post–primary block for 30 minutes, followed by NovoLink polymer for 30 minutes. Reaction products were visualized with the application of diaminobenzidine substrate chromogen solution. Slides were counterstained in hematoxylin and mounted.

Normal small and large bowel epithelial cells, pancreatic acini, and islet cells and bile duct epithelial cells served as internal positive controls. Stromal cells were used as internal negative controls. All cases were evaluated independently by 2 examiners, including 1 pathologist, without knowledge of pathologic data. Any discordant results were reviewed together to reach agreement. Scoring was based on intensity of staining: 0, negative; 1, weakly positive; 2, moderate; and 3, strongly positive. Extent of tumor staining was scored, in which 10 random high-power fields were assessed and the average percentage of positive staining cells was estimated (1, <25%; 2, 25%–75%; and 3, >75%). The product of staining intensity and extent was used as the overall score giving final values of 0, 1, 2, 3, 4, 6, and 9. Scores of 0 were classified as negative, 1–2 as weak, 3–4 as moderate, and 6–9 as strong staining.

Cell culture and immunofluorescence

The bronchial NET cell line, NCI-H727, was maintained in RPMI 1640 (PAA Laboratories) supplemented with 10% FBS, 2 mmol/L of L-glutamine, penicillin, and streptomycin. A breast cancer cell line (as a negative control for CD56 expression), MCF-7, was maintained in MEM (PAA Laboratories) supplemented with 10% FBS, 2 mmol/L of L-glutamine, penicillin, and streptomycin at 37°C. Sterile 12-mm diameter coverslips were placed in wells of a 24-well plate (BD Falcon). Approximately 2 × 10⁵ cells were seeded into each well and incubated at 37°C for 24 hours at 5% CO₂. After media was removed, cells were washed, and then fixed in 1 mL of 1% formaldehyde for 10 minutes. After fixative was removed, 0.1 mol/L of glycine was added to each well to neutralize for 5 minutes. To permeabilize the cells, 0.1% Triton X-100 was added to each well for 10 minutes before further washing. To prevent nonspecific binding, cells were blocked with 0.1% BSA for 30 minutes. Cells were incubated with the FITC-conjugated mouse anti-human antibody synaptophysin (Acris Antibodies) at a dilution of 1:20 or the Alexa Fluor 488–conjugated mouse anti-human antibody, CD56 (BD PharMingen) at a dilution of 1:20 for the Alexa Fluor 488–conjugated mouse anti-human antibody, CD56 (BD PharMingen) at a dilution of 1:20 for 1 hour at room temperature. After further washing, coverslips were removed from each well and mounted onto slides with Vectashield (Vector Laboratories Ltd.) and sealed with clear nail varnish before visualization.

Patients

This study was approved by the Local Ethics Committee and all patients provided written informed consent. Eligible patients had pathologically confirmed NETs categorized according to their primary site of origin: midgut,
pancreas, bronchopulmonary, or of unknown primary; with metastatic disease measurable by Response Evaluation Criteria in Solid Tumors (RECIST). Although some patients had undergone resection of the primary, all patients had metastatic disease. Patients that had undergone chemotherapy, biological therapy, interferon, receptor-targeted radiolabeled therapy, or embolization within the previous 2 months were excluded. To assess whether a patient had progressive or nonprogressive disease, imaging with computed tomography (CT) or magnetic resonance imaging (MRI) within 6 weeks of sampling, was compared with the previous imaging. Comparisons were performed by an independent radiologist who recorded absolute changes in tumor dimension and classified them according to RECIST.

Plasma CgA and 24-hour urinary 5-HIAA, where applicable, were evaluated. Radiological burden was assessed by quantification of hepatic tumor load from 4 to 6 slices of a CT/MRI scan with the most amount of disease by a semiquantitative approach. Hepatic tumor burden was categorized as 25% or less, more than 25% but 50% or less, more than 50% but 75% or less, or more than 75%. Data were collected on primary site, duration of diagnoses, any previous treatment received, and whether the primary tumor had been resected.

CTC enumeration

Blood samples (7.5 mL) were drawn into CellSave tubes (Veridex LLC) and maintained at room temperature. The CellSearch (Veridex LLC) platform was used for isolation and enumeration of CTCs as previously described (3). In brief, the platform consists of a semiautomated system that enriches the sample for cells expressing EpCAM with antibody-coated ferroparticles. The identification of CTCs by immunofluorescence staining was performed with CellSearch Analyzer II (Veridex LLC), a semiautomated fluorescence-based microscopy system. The fluorescently labeled monoclonal antibodies, anti-CD45–allophycocyanin (APC) and anti–pan-cytokeratin (CK)-phycoerythrin (PE) are used to distinguish epithelial cells from leukocytes. CTCs were defined as 4,2-diamidino-2-phenylindole-dihydrochloride (DAPI)-stained cells lacking CD45 and expressing cytokeratin. NETs have been found to express low molecular weight cytokeratins in previous studies (16–18). All evaluations were performed by 2 independent operators without knowledge of clinical status. Technical details of the CellSearch platform including accuracy, precision, linearity, and reproducibility have been described elsewhere (2).

Analysis of synaptophysin and CD56 expression on CTCs was performed on the platform by using FITC-conjugated mouse anti-human synaptophysin antibody (Acris Antibodies) and Alexa Fluor 488–conjugated mouse anti-human CD56 antibody (BD PharMingen) as additional markers, respectively. A selected patient’s sample was processed alongside samples of healthy control blood spiked with $10^3$ NCI-H727 and $10^4$ MCF-7 cells as well as unspiked healthy blood. The antibodies were substituted for PBS for antibody omission controls. For the CD56 run, NCI-H727 was used as a positive control and MCF-7 as a negative control. For the synaptophysin run, NCI-H727 was used as a positive control and leukocytes as an internal negative control. The antibody concentrations were 100 µg/mL for the synaptophysin antibody and 12 µg/mL for the CD56 antibody. The positivity of synaptophysin and CD56 of neuroendocrine CTCs was evaluated by 2 independent operators by using the research mode of CellSearch Analyzer II with an integration time of 0.8 seconds.

Results

Immunohistochemical analysis of EpCAM expression

Expression of EpCAM in normal tissue. EpCAM was expressed mostly on the basal or basolateral cell membrane of small and large intestinal, appendiceal, and bile duct epithelia. Variable EpCAM expression, including some cytoplasmatic staining, was seen in pancreatic islets, pancreatic acini, and gastric oxyntic glands. EpCAM was negative in hepatocytes, mesothelium, and gastric foveolar epithelium. Variable staining, including some cytoplasmatic staining, was seen in pancreatic islets. These results are consistent with previous published work (8).

Expression of EpCAM in NET. All ileal ($n = 26$), pancreatic ($n = 16$), unknown primary ($n = 2$), and gastric ($n = 4$) NETs showed strong (score 6–9) homogeneous membranous staining for EpCAM (Fig. 1). Moderate to strong staining was seen in appendiceal ($n = 7$) NETs. EpCAM expression was not affected by grade. Bronchopulmonary NETs ($n = 13$) showed variable EpCAM expression from negative, weak to strong staining. EpCAM distribution was also variable in bronchopulmonary NETs: EpCAM was observed in cell membranes and in cytoplasm. EpCAM was not expressed in paranglioma ($n = 1$). Details can be found in Supplementary Table S1.

Patients for CTC evaluation

Eighty patients with metastatic NETs were recruited for CTC evaluation. One sample was excluded because of...
postsampling hemolysis. Clinical characteristics are shown in Table 1.

**CTCs in NETs**

Of patients with metastatic midgut tumors, 43% had CTCs detected with a mean ± SEM of 8.9 ± 2.8 CTCs per 7.5 mL of blood (range = 0–62; Fig. 2). Fewer patients with pancreatic (21%) and bronchopulmonary NETs (31%) had detectable CTCs, although the 2 patients with the greatest number of CTCs both had bronchopulmonary NETs (Fig. 2). In addition, 4 of 5 patients with NETs of unknown primary had CTCs present with a mean ± SEM of 4.2 ± 1.2 (range = 0–7). There was no significant difference in the presence ($P = 0.61$) or amount ($P = 0.21$) of CTCs between those on somatostatin analogues and those who were not.

**Figure 1.** Immunohistochemistry with EpCAM. A, gastric mucosa with absent expression, apart from, in oxyntic cells. B, ileal NET resection with intense membranous staining of NET and of normal mucosa (short arrow) with negative stroma (long arrow). C, EpCAM positivity in ileal NET at high power. D, EpCAM-positive appendiceal NET (short arrow) with positive normal appendix mucosa (long arrow). E, normal pancreas with positive acini, islet, and duct. F, pancreatic NET with membranous staining at high power. G, poorly differentiated gastric NET with membranous and cytoplasmic staining. H, bronchopulmonary NET with cytoplasmic staining (short arrow) and negative lung parenchyma/alveoli (long arrow).
To confirm the neuroendocrine origin of CTCs, the expression of synaptophysin and CD56 was evaluated on CTCs from a patient with metastatic midgut NET and on cell line controls. By immunofluorescence, synaptophysin was expressed on NCI-H727 and MCF-7, and CD56 on NCI-H727 but not on MCF-7. Spiked NCI-H727 processed on the CellSearch platform was positive for synaptophysin and CD56; MCF-7 was positive for synaptophysin but negative for CD56. Eighty-two percent of the patient’s CTCs were positive for synaptophysin and 21% for CD56 (Fig. 3).

Twenty-six of the 79 patients who had blood samples enumerated for CTCs had archival histopathologic tissue available for assessment of EpCAM expression (7 midgut, 2 liver metastases with unknown primary, 8 pancreatic, 6 bronchopulmonary resections, 3 bronchial biopsies). All 7 midgut NETs and 2 liver metastases from an unknown primary had strong staining for EpCAM. Five and 2 of these had CTCs present, respectively. All 8 pancreatic NETs (4 pancreatic resections, 4 biopsies) had strong EpCAM staining but only 1 had CTCs present. Four of the bronchopulmonary NET patients had moderate or strong EpCAM expression; 3 of these had CTCs present. Five bronchopulmonary NETs had weak or no EpCAM staining; none had CTCs. CTCs were detected only in those patients with bronchopulmonary NETs that had moderate to strong expression of EpCAM.

### CTC correlation with existing markers

In midgut and unknown NETs, there was a moderate correlation between CTC levels and urinary 5-HIAA, where available (r = 0.5, P = 0.007, n = 28). For all NETs, because of small category size, the liver burden categories 50%–75% and 75% were combined. A significant association between CTC levels and burden of liver metastases was found (B = 8.91, 95% CI = 4.3–13.5, P < 0.001; Supplementary Fig. S1). There was no or low correlation between CTC levels and Ki-67 (r = 0.08, P = 0.59; Supplementary Fig. S2), CgA (r = 0.246, P = 0.03; Supplementary Fig. S3), or resection of primary (U = 698.5, P = 0.68).

### CTCs and progressive disease

Of the 66 patients with metastatic disease from midgut, pancreatic, or unknown primary who had CTCs evaluated, 63 had imaging within 6 weeks and a previous scan for comparison. The 3 remaining patients had been diagnosed recently and only imaged once. Bronchopulmonary NET patients were excluded from this analysis because of variable EpCAM expression and thus CTC detection. Eighteen of 19 (95%) patients who had progressive disease according to RECIST had CTCs detected compared with 9 of 44 (20%) patients who had nonprogressive disease (Supplementary Fig. S4). This was a significant difference ($\chi^2 = 31.4, P < 0.001$), with no statistical difference in other factors between progressors and nonprogressors (Table 2).

### Table 1. Clinical characteristics of NET patient sample

<table>
<thead>
<tr>
<th></th>
<th>Pancreatic (n = 19)</th>
<th>Midgut (n = 42)</th>
<th>Bronchopulmonary (n = 13)</th>
<th>Unknown primary (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), y</td>
<td>60 (38–87)</td>
<td>61.5 (36–79)</td>
<td>57 (30–80)</td>
<td>62 (30–64)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td>Male 10 20 8 0</td>
<td>Female 9 22 5 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>Low 8 24 6 0</td>
<td>Intermediate 7 16 5 5</td>
<td>High 4 2 2 0</td>
<td></td>
</tr>
<tr>
<td>Burden of liver metastases, %</td>
<td>≤25 10 20 10 1</td>
<td>25 ≤ 50 7 15 2 4</td>
<td>50 ≤ 75 2 4 1 0</td>
<td>&gt;75 0 1 0 0</td>
</tr>
<tr>
<td>Unknown</td>
<td>Ongoing somatostatin analogue therapy</td>
<td>Yes 8 31 0 1</td>
<td>No 11 11 13 4</td>
<td></td>
</tr>
<tr>
<td>Duration of diagnosis, median (range), mo</td>
<td>58.5 (2–166)</td>
<td>60 (2–278)</td>
<td>22 (8–287)</td>
<td>16 (9–63)</td>
</tr>
<tr>
<td>Previous treatments</td>
<td>Resection of primary 4/19</td>
<td>23/42 6/13</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemotherapy 6 2 2 2</td>
<td>Embolization 1 5 0 1</td>
<td>Radionuclides 1 10 1 1</td>
<td>Liver resection 2 4 1 0</td>
</tr>
</tbody>
</table>
absolute changes in target lesions were assessed, 23 of 27 (85%) patients with detectable CTCs had growth of tumor lesions, and 31 of 36 (86%) patients without CTCs had no growth or spontaneous shrinkage (Fig. 4). There was no association between the number of CTCs and absolute increase in tumor size.

Discussion

To our knowledge, this is the first systematic analysis of EpCAM expression and CTCs in NETs. All gastroenteropancreatic NET tissue showed strong membranous EpCAM expression regardless of grade. The origin of NETs is still debated, but the expression of this carcinoma-associated antigen adds evidence to an epithelial origin rather than being derived from the neural crest as was once originally thought (10, 19). EpCAM upregulates c-myc and cyclins, promoting cell cycling and enhancing proliferation (20–24). Anti-EpCAM therapy has been trialed in metastatic breast cancer, colorectal cancer, and in malignant ascites (25–30) and EpCAM expression in NETs presents an opportunity for EpCAM directed therapy (9). However, caution should be taken as normal pancreatic and intestinal tissue express EpCAM. The CellSearch method of CTC enrichment has been validated previously and only 0.3% of healthy controls and benign cases have 2
or more CTCs per 7.5 mL of blood (2), although the rate of CTCs in healthy controls depends on criteria used for analysis of the images provided by the CellSearch system (31). We have shown that CTCs in our patient group are neuroendocrine in origin by using the NET unique immunohistochemical profile. In those with midgut and unknown primary NETs, 47% had CTCs detectable, 68% of whom had greater than 5 CTCs/7.5 mL. These levels are comparable with other tumor types, although not as high as in metastatic breast and prostate cancers, perhaps reflecting the indolent nature of most NETs (2). Although prognostic cutoff levels of CTCs have been determined in other cancers (3, 5, 6), further studies are required to determine these in NETs.

Table 2. Characteristics of cases with nonprogressive versus progressive disease in patients with metastatic midgut, pancreatic, or unknown primary NETs

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonprogressive disease (n = 44)</th>
<th>Progressive disease (n = 19)</th>
<th>Test/P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), y</td>
<td>59.5 (38–79)</td>
<td>56.1 (30–77)</td>
<td>U = 0.36/P = 0.37</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td>Fisher’s exact/P = 0.57</td>
</tr>
<tr>
<td>Low</td>
<td>22</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>19</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Burden of liver metastases, %</td>
<td></td>
<td></td>
<td>Fisher’s exact/P = 0.39</td>
</tr>
<tr>
<td>≤25</td>
<td>24</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>25 ≤ 50</td>
<td>16</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CgA, mean (range), pmol/L</td>
<td>297.8 (33–1,000)</td>
<td>359.4 (34–1,000)</td>
<td>t = 0.46/P = 0.65</td>
</tr>
<tr>
<td>Resection of primary</td>
<td></td>
<td></td>
<td>Fisher’s exact/P = 0.99</td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>28</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Duration of diagnosis, median (range), mo</td>
<td>58.5 (5–278)</td>
<td>55 (10–108)</td>
<td>U = 343/P = 0.99</td>
</tr>
<tr>
<td>Ongoing somatostatin analogue therapy</td>
<td></td>
<td></td>
<td>Fisher’s exact/P = 0.77</td>
</tr>
<tr>
<td>Yes</td>
<td>22</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>12</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Interval to last scan, median (range), mo</td>
<td>18 (12–56)</td>
<td>18 (10–30)</td>
<td>U = 347/P = 0.76</td>
</tr>
<tr>
<td>CTC</td>
<td>35</td>
<td>1</td>
<td>Fisher’s exact/P &lt; 0.001</td>
</tr>
<tr>
<td>≥1</td>
<td>9</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Percentage increase in lesions on imaging grouped by the presence of CTCs. Each bar represents an individual case. Cases without CTCs are grouped on the left-hand side of the chart (CTC = 0); cases with CTCs detected on the right-hand side (CTC ≥ 1).
Despite widespread EpCAM positivity in pancreatic NETs, only a small proportion had CTCs detected compared with midgut NETs, consistent with findings in pancreatic adenocarcinomas (2). Explanations include loss of EpCAM expression, slow shedding of CTCs from pancreatic NETs, or unidentified factors particular to this pancreatic sample. Absent EpCAM expression has been cited as a reason for the lack of CTCs in a substantial number of cases of metastatic breast cancer (32), but until now, there has been no study dealing with synchronous CTC detection and EpCAM expression in patients.

Lack of EpCAM expression may be relevant in bronchopulmonary NETs, as CTCs can only be detected on this platform when the primary tumor expresses EpCAM. Those bronchopulmonary NETs that were EpCAM positive had the highest CTC counts perhaps due to the absence of portal filtration that has recently been shown to decrease CTCs (33). Our sample of bronchopulmonary NETs was small, with only 13 cases, and the significance of EpCAM expression in bronchopulmonary NETs requires further investigation.

The absence of CTCs in NETs is associated with stable disease as defined by RECIST, whereas the presence of CTCs seems to be associated with progressive disease. Smaller increases in tumor size not attaining RECIST also seems to be associated with CTCs. Radiological methods of monitoring for progression in NETs and response to treatment may be confounded by interobserver variability and the fibrotic reaction often seen in NETs (34, 35). Given the varied survival with NETs compared with other tumors, this dependency on serial imaging is costly and exposes patients to radiation. In NETs, CTCs may be of prognostic value in discriminating progressing from stable tumors, which may assist stratification for aggressive therapy at time of diagnosis. Given the delayed response seen in NETs with chemotherapy and radionuclides (36, 37), CTCs may offer predictive information early during therapy and may be useful in monitoring response to therapy without repeated exposure to radiation. We recognize limitations in this analysis whereby tumors were classified as progressive making retrospective comparisons with previous scans in heterogeneous groups. However, the median interval between scans was similar between patients who had tumor progression and those with no tumor progression. In addition, the sample size was sufficient to detect this association between CTC presence and progressive disease. We also note the similar histologic grades between patients with and without tumor progression, which may partly reflect small sample size with few cases of high-grade NETs. The number of CTCs did not correlate with the existing prognostic markers Ki-67 proliferation index or plasma CgA. Ki-67 is evaluated in tumor specimens obtained at diagnosis, which may occur several years previously and may not reflect dynamic changes during the course of the disease. The association between CTCs and 24-hour urinary 5-HIAA in midgut NETs could be explained by tumors being more metabolically active when CTCs are present or by CTCs secreting metabolically active compounds into the circulation. The association between CTCs and burden conflicts with some studies in other cancers in which such an association was not found (38). The confirmation of CTCs in NETs presents an exciting opportunity to perform molecular characterization of NETs without invasive biopsies, and this may accelerate development of new therapies. Given the variable survival of NETs and increasing armory of treatment options (36, 37, 39–42), CTCs could be utilized as a prognostic marker in NETs to stratify therapy, and in real-time monitoring of tumor growth or treatment response. Because of the heterogeneity of our patient population with respect to therapy, we were not able to distinguish between the role of CTCs as a prognostic or predictive marker and large collaborative prospective studies are needed to address this important question.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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