Blood mRNA Measurement (NETest) for Neuroendocrine Tumor Diagnosis of Image-Negative Liver Metastatic Disease

Anna Malczewska,[1] Lisa Bodei,[2] Mark Kidd,[3] and Irvin M. Modlin[4]

[1]Department of Endocrinology and Neuroendocrine Tumors, Medical University of Silesia, Katowice 40-514, Poland; [2]Memorial Sloan Kettering Cancer Center, New York, New York 10065; [3]Wren Laboratories, Branford, Connecticut 06405; and [4]Gastroenterological and Endoscopic Surgery, Yale University School of Medicine, New Haven, Connecticut 06520-8062

ORCiD numbers: 0000-0003-0321-4832 (A. Malczewska).

Context: Early cancer detection is critical to optimize treatment. This is particularly problematic in neuroendocrine tumors (NETs), which exhibit an ~5-year diagnostic delay due to covert symptoms, limitations in imaging, and circulating biomarkers. Despite development of continuous monitoring strategies utilizing advanced modalities [CT/MRI or ^{68}Gallium positron emission tomography (PET)/CT] or a repertoire of monoanalyte biomarkers [*e.g.,* chromogranin A (CgA), pancreastatin, serotonin], detection of minimal residual disease or microrecurrence remains elusive. Emerging molecular liquid biopsies (*e.g.,* NETest) provide a substantially improved threshold for disease detection.

Case Description: We describe the utility of a blood-based multigene PCR neuroendocrine measurement (NETest), which is representative of core molecular drivers of neuroendocrine tumorigenesis, to detect hepatic micrometastases in a patient with negative blood biomarkers and negative anatomical/functional imaging. The 52-year-old woman, who had undergone margin-negative resection for a NET of the ileocecal valve, developed persistently elevated NETest levels 8 months later. CT/MRI/^{68}Gallium PET and biomarkers remained negative. Blood multigene analysis identified disease, and peptide receptor radionuclide therapy (PRRT) was undertaken. Over 9 months, NETest levels increased (conventional biomarkers/imaging remained normal). Liver biopsy was undertaken, and foci of a 3-mm NET in segment VI were histologically documented. At 3.3 years after PRRT, the disease remained as a microscopic burden and stable biomarker/^{68}Gallium PET/MRI occult despite elevated blood levels of NET genes.

Conclusions: Blood measurement of NET transcripts can identify image- and CgA-negative disease. A NET liquid biopsy strategy has clinical utility in the early identification of residual or metastatic disease and optimizes consideration of adjuvant therapeutic intervention. ***(J Clin Endocrinol Metab* 104: 867–872, 2019)**

A critical limitation for successful management of small intestinal neuroendocrine tumors (SINETs) is early disease detection and identification of progression. Up to 50% of SINETs are metastatic at diagnosis, usually involving the liver (1). This reflects three issues. First, tumors are covert until hormonal (carcinoid) symptoms develop, which occurs after hepatic metastasis (in 30% to 40% of cases) (2). Second, biomarkers such as chromogranin A (CgA) have limited clinical utility, and third, imaging has identification limitations (3). CgA is compromised by numerous nonclinical conditions that elevate it (*e.g.*, renal failure, use of proton-pump inhibitors),

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Abbreviations: ^{68}Ga, ^{68}Gallium; CgA, chromogranin A; NET, neuroendocrine tumor; PET, positron emission tomography; PRRT, peptide receptor radionuclide therapy; R0, margin-negative; SINET, small intestinal neuroendocrine tumor; SSA, somatostatin analogue.

variability of assays, and false-negative results (3). There is also major disagreement regarding its use per National Comprehensive Cancer Network guidelines (category 3 biomarker) (2). Imaging and Response Evaluation Criteria in Solid Tumors likewise are well known to be limited (3). Up to ~70% of SINETs are estimated to be understaged by imaging (4). Histopathological examination reveals tumor deposits in >50% of resected specimens not detected by imaging (1, 5). Given this, standard strategies fail to detect progressive disease early, and appropriate therapy is delayed.

Liquid biopsy has considerable utility in other cancers and has recently been developed for neuroendocrine tumors (NETs; NETest) (6, 7). The strategy involves measuring circulating tumor RNA with high sensitivity and specificity. It can be repeated regularly (blood sample), and "omic" analyses yield real-time information regarding tumor behavior (6). It has been suggested that this will replace conventional mono-analyte biomarkers, which measure tumor secretory status and provide no information about the biological behavior of a cancer (3).

The NETest was derived from tumor transcriptome-based analysis and is an algorithmic evaluation of 51 neuroendocrine gene transcripts, representative of eight core drivers of neuroendocrine tumorigenesis: SSTRome, proliferome, metabolome, secretome, epigenome, growth factor signalome, plurome, and apoptome (6). PCR amplifications of genes isolated from peripheral blood are measured and scored to define disease activity (0% to 100%) (20%: upper limit of normal; 21% to 40%: low; 41% to 79%: moderate; ≥80%: high) (8). The test accurately detects NETs (>90%) and identifies progressive disease, completeness of surgical resection, and therapeutic responsiveness [*e.g.*, somatostatin analogues (SSAs)] (8).

We present a case in which blood molecular testing in the presence of normal blood CgA and negative ^{68}Gallium (^{68}Ga) positron emission tomography (PET) (Fig. 1; Table 1) identified biopsy-proven micrometastatic hepatic neuroendocrine disease. Informed consent was obtained from the patient.

Case Description

A 52-year-old woman presented in August 2013 with altered bowel movements and abdominal pain. CT enterography depicted wall thickening in the distal ileum/proximal cecum [Fig. 2(a)]. This was considered Crohn

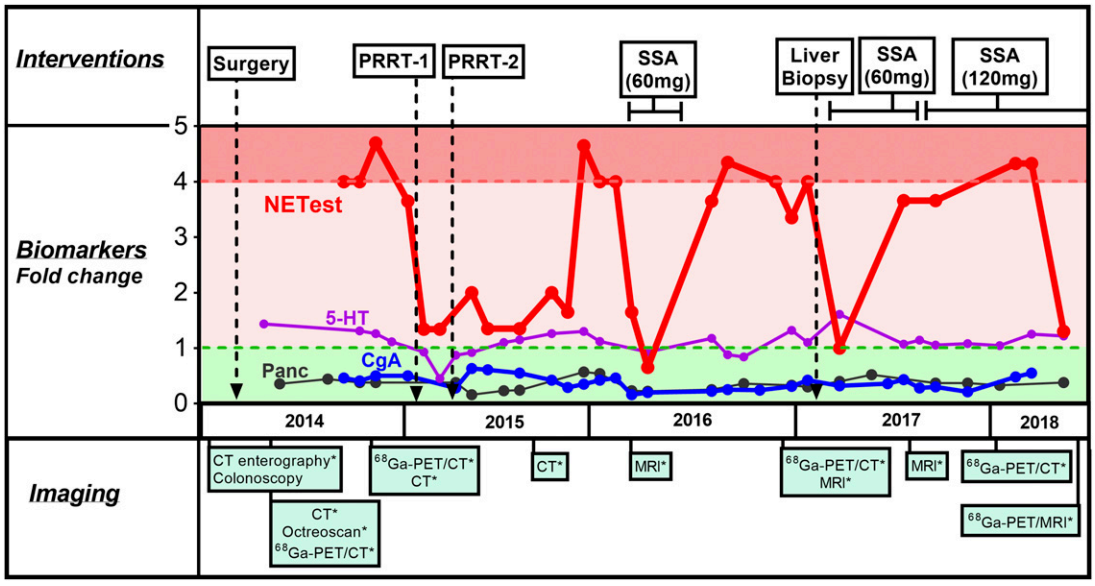

Figure 1. Timeline of patient's journey. The relationships of the NETest (red line) to interventions (upper panel) and imaging (lower panel) are shown against time. Biomarker values [CgA, pancreastatin (Panc), serotonin (5-HT)] and NETest scores are presented as fold-change (FC; above the upper limit of normal). Background color shows biomarker level ranges: green, normal range (≤1 FC, representing NETest scores from 0% to 20%); faded pink, elevated biomarker levels >1 to 4 FC (>1 to 4 FC range for the NETest represents the score range of 21% to 79% and low to moderate disease activity); red, ≥4 FC elevation of biomarker levels (NETest score ≥80%, corresponding with highly biologically active/progressive disease). Derivation of the NETest score: The test uses a two-step protocol (mRNA isolation, cDNA production, and PCR) from EDTA-collected whole blood. Blood gene expression of the 51 NET-specific markers (representative for all eight neuroendocrine tumorigenesis driver clusters) is normalized to a housekeeper gene and quantified vs a population control. Gene expression levels are related to an outcome based on four machine learning algorithms. The algorithmic output of these analyses enables the test to differentiate NET disease from controls and stable disease from progressive disease. To facilitate the clinical use of this information, the diagnostic score is represented as a correlate of clinical activity. Upper limit of normal is 20%; low disease activity (stable disease) is 21% to 40%; moderate activity is 41% to 79%, and high disease activity is ≥80%. Asterisk (*) indicates negative for NET. PRRT, peptide receptor radionuclide therapy; SSA, somatostatin analogue (lanreotide).

disease. At colonoscopy, a large ulcerated, friable mass obstructing the ileocecal valve was noted [Fig. 2(b)]. A laparoscopic right hemicolectomy with lymphadenectomy was undertaken (February 2014). No peritoneal or hepatic disease was evident. Histology confirmed a 5-cm well-differentiated NET [pT3N1 (9/22), margin-negative (R0), G2, Ki-67: 3%, lymphovascular invasion (+), perineural invasion (+)], 75% positive (3+) for somatostatin receptor (type 2). CT, octreoscan, and ^{68}Ga-DOTA-TOC PET/CT were all negative 3 months after surgery. The NETest was high (80%) and, when repeated, remained elevated (93%), whereas ^{68}Ga-DOTA-TOC PET/CT was negative. ^{177}Lutetium peptide receptor radionuclide therapy (PRRT) was undertaken (Rotterdam) with two cycles of ^{177}lutetium treatment (cumulative dose, 15 GBq). Following the first PRRT cycle, NETest measurements were low (27%). Subsequent to PRRT, NETest levels remained substantially elevated but decreased (May 2015: 40%; June 2015: 27%; October 2015: 40%; November 2015: 33%). This was interpreted as disease stabilization. A NETest in December 2015 was again elevated (93%) and remained high (80%), indicative of a progressive disease. In February 2016, on the basis of persistent NETest elevations, lanreotide depot 60 mg monthly was commenced. In March 2016, MRI was unremarkable. Thereafter, NETest levels decreased (33%). In July 2016, SSA was discontinued because of liver function test increases. Thereafter, the NETest increased (73%) and remained elevated (August 2016: 87%; January 2017: 80%). On December 2016, ^{68}Ga-DOTA-TATE PET/CT and MRI were negative.

On January 2017, symptomatic cholelithiasis prompted a cholecystectomy. The abdomen was normal, but random multiple peritoneal and core needle biopsies from segment VI were performed. The latter revealed foci of a metastatic tumor of ~3 mm (microscopic measurement; no mitotic figures were evident, and the tumor was too small for accurate Ki-67 assessment) [Fig. 2(c)]; peritoneal biopsies were negative. SSA was resumed (lanreotide depot 60 mg monthly), and the NETest in February 2017 was low (20%). In July 2017, the NETest was elevated (73%), but MRI was unremarkable. SSA was increased to 120 mg monthly, but NETest levels remained elevated throughout: September 2017 (73%) and March 2018 (87%). Follow-up ^{68}Ga-DOTA-TATE PET/CT (January 2018) and ^{68}Ga-DOTA-TATE PET/MRI (July 2018) [Fig. 2(d)] were both negative. The NETest in May 2018 remained elevated but low (27%).

The patient remains on SSA. Over this entire 5-year period, CgA and pancreastatin levels remained within the normal range. Blood serotonin levels fluctuated 1.1 ± 0.18 upper limit of normal (Fig. 1), whereas urinary 5-hydroxyindoleacetic acid measurement was undertaken once and was normal.

Discussion

A critical clinical problem after resection is the identification of minimal residual NET disease and characterization of its biological likelihood to progress. In this report, we demonstrated the sensitivity of the NETest for the identification of image-negative (CT, MRI, and ^{68}Ga PET) and biomarker-negative disease (CgA, pancreastatin, serotonin) in the presence of micrometastatic liver disease. Needle biopsy histology confirmed the blood molecular measurement. Repeated utilization of diverse imaging modalities (*e.g.*, CT, MRI, and ^{68}Ga PET/CT) failed to identify postoperative disease. Similarly, standard biomarkers failed to detect micrometastatic liver disease or disease progression.

Liver metastatic disease is an important prognostic factor in SINET; early detection facilitates and improves survival (1). For imaging, spatial resolution of 2 to 4 mm (CT/MRI) and 4 to 6 mm (^{68}Ga-SSA PET/CT or ^{18}F-fludeoxyglucose PET/CT) (9) infers delay in tumor detection. A 10-mm lesion is well known to exhibit at least an ~10^9 cell bulk, whereas circulating NET genes can be identified in as few as one tumor cell/mL (8). Micrometastatic disease is therefore far more likely to be identified by molecular amplification (gene expression–PCR) techniques than by imaging alone. Consistent with this observation are reports of the effectiveness of molecular techniques (PCR based) in SINET lymph nodes considered negative at immunohistochemistry (10). In 50% of histology-negative nodes, CgA transcripts (mRNA) were identifiable by PCR (10). Liver lesions have been identified in >50% of image-negative hepatic disease cases postoperatively (1, 5). In 54% of these cases, the size was ≤2 mm (5). The failure of anatomical or somatostatin receptor–based imaging to identify hepatic micrometastases (≤2 mm) is problematic in early detection (1). The real incidence of metastatic liver disease is likely much higher than reported. A molecular tool would therefore be of considerable utility in early identification of disease.

The presence of undetected hepatic micrometastases in SINETs would explain the clinical observation of frequent tumor recurrence following R0 primary resection or hepatic resection. In one study, 11 of 25 patients with an NET (44%) with intrahepatic recurrence after R0 hepatectomy exhibited hepatic micrometastases; no micrometastases were identified in seven who remained disease-free after surgical resection (1). This report demonstrates that hepatic micrometastases can be identified by the measurement of NET genes in blood.

Table 1. Summary of Imaging, Interventions, and NETest Measurements

Date	Interventions	Imaging	NETest Score (ULN 20)	Result Interpretation	NETest Effect on Management/ Treatment
1.4.2014	—	CT enterography	—	Crohn disease suspected	No NETest
1.16.2014	—	Colonoscopy	—	Ileocecal mass	No NETest
2.3.2014	Right hemicolectomy with lymphadenectomy	—	—	NET [pT3N1, R0, Ki-67: 3%, LVI (+), PNI (+); SSTR type 2: 75%, 3+]	No NETest
4.29.2014	-	CT/Octreoscan/^{68}Ga-DOTA-TOC PET/CT (−)	-	"Disease free"	No NETest
4.29.2014					
5.1.2014					
9.16.2014	—	—	**80%**	Imaging negative; "disease free" NETest high; recurrent/residual progressive disease	Need for adjuvant treatment [*e.g.,* PRRT/ based on tumor SSTR positivity (SSTR type 2: 75%, 3+)]
10.21.2014					
11.5.2014	—	—	**93%**		
11.6.2014	—	CT/^{68}Ga-DOTA-TOC PET/CT (−)	—		
1.5.2015	—	—	**73%**		
1.22.2015	**First PRRT cycle**	—	—	—	-
	—	—	27%	Good response to first PRRT cycle: NETest ↓ (low); disease stabilization	Monitor disease status (PRRT efficacy)
2.19.2015					
3.20.2015					
4.2.2015	**Second PRRT cycle**	—	—	—	-
5.28.2015	—	—	40%	Response to PRRT: NETest low; low disease activity; disease stabilization	Monitor disease status (PRRT efficacy)
6.25.2015	—	—	27%		
8.31.2015					
10.9.2015	—	CT (−)	—		
10.20.2015	—	—	40%		
11.17.2015	—	—	33%		
12.14.2015	—	—	**93%**	NETest high; disease biologically active/ progressive disease	Commence treatment (*e.g.,* SSA) and monitor its efficacy
1.6.2016	—	—	**80%**		
2.9.2016					
2.11.2016	**SSA (60 mg) start**	—	—	—	-
3.14.2016	On SSA (60 mg)	MRI (−)	—	Low NETest; stable disease SSA effective	Monitor disease status (SSA efficacy)
3.16.2016	On SSA (60 mg)	—	33%		
4.27.2016	On SSA (60 mg)	—	13%		
7.28.2016	**SSA (60 mg) last**	—	—	SSA withdrawn because of LFT increases	
8.17.2016	—	—	**73%**	No SSA; disease progression; high NETest; disease progressing, biologically active	Alter treatment to stabilize the disease?
8.24.2016	—	—	**87%**		
9.20.2016	—	—	**80%**		
12.13.2016	—	—	**67%**		
12.14.2016	—	^{68}Ga-DOTA-TATE PET/ CT, MRI (−)	—		
12.22.2016					
1.10.2017	—	—	**80%**		
1.23.2017	**Needle liver biopsies (n = 3), segment VI**	—	—	Liver micrometastasis positive on histology (hematoxylin and eosin); NETest accurate	Treat metastatic disease?
2.9.2017	**SSA resumed (60 mg)**	—	—	SSA reintroduced to treat metastatic disease	Treat metastatic disease
2.27.2017	On SSA (60 mg)	—	20%	SSA stabilized disease	Monitor disease status (SSA efficacy)
7.11.2017	On SSA (60 mg)	—	**73%**	NETest high despite SSA; disease progressive; alter treatment	Adjust treatment: SSA dose (↑)
7.17.2017	On SSA (60 mg)	MRI (−)	—		
8.28.2017	**SSA increased (120 mg)**	—	—	—	-
9.6.2017	On SSA (120 mg)	—	**73%**	NETest high; recent treatment alteration, monitor response to SSA dose change	Monitor response to ↑ SSA dose
1.19.2018	On SSA (120 mg)	^{68}Ga-DOTA-TATE PET/ CT (−)	—	Imaging negative despite known liver metastatic disease	Monitor disease status with liquid biopsy

(*Continued*)

Table 1. Summary of Imaging, Interventions, and NETest Measurements (Continued)

Date	Interventions	Imaging	NETest Score (ULN 20)	Result Interpretation	NETest Effect on Management/ Treatment
2.6.2018 3.5.2018	On SSA (120 mg)	—	**87%**	NETest high; recent treatment alteration, monitor response to SSA dose change	Monitor response to ↑ SSA dose
5.4.2018	On SSA (120 mg)	—	27%	NETest low; disease stabilization	Monitor disease status (SSA efficacy)
7.24.2018	On SSA (120 mg)	^{68}Ga-DOTA-TATE PET/ MRI (−)	—	Most advanced imaging technique negative for liver metastatic disease	Monitor disease status with disease-positive liquid biopsy

Information regarding interpretation of the results (imaging + NETest) and the effect of the NETest on treatment decision-making and management is included. Boldface indicates when substantial medical interventions were made.

Abbreviations: imaging (−), imaging modality negative for disease detection; LFT, liver function test; LVI, lymphovascular invasion; PNI, perineural invasion; PRRT, peptide receptor radionuclide therapy; R0, margin-negative; SSA, somatostatin analogue (lanreotide depot administered monthly); SSTR, somatostatin receptor; ULN, upper limit of normal.

Overall, these observations support the consideration of adjuvant treatment in NET disease.

In this patient, CgA was never elevated, irrespective of disease stage, alterations in disease activity, or various treatments (*e.g.*, SSA or PRRT). Similarly, pancreastatin (CgA derivative), an alternative biomarker, was of no value. Circulating serotonin and its urinary metabolite 5-hydroxyindoleacetic acid, indicators of tumor secretory status, were also not useful in defining disease. In contrast, a 51-gene NET-specific gene panel identified disease presence and progress despite repeated negative anatomical and functional imaging.

Conclusions

Use of a circulating gene expression biomarker is a novel strategy to optimize early disease detection. A liquid biopsy, requiring only venipuncture, is a viable strategy

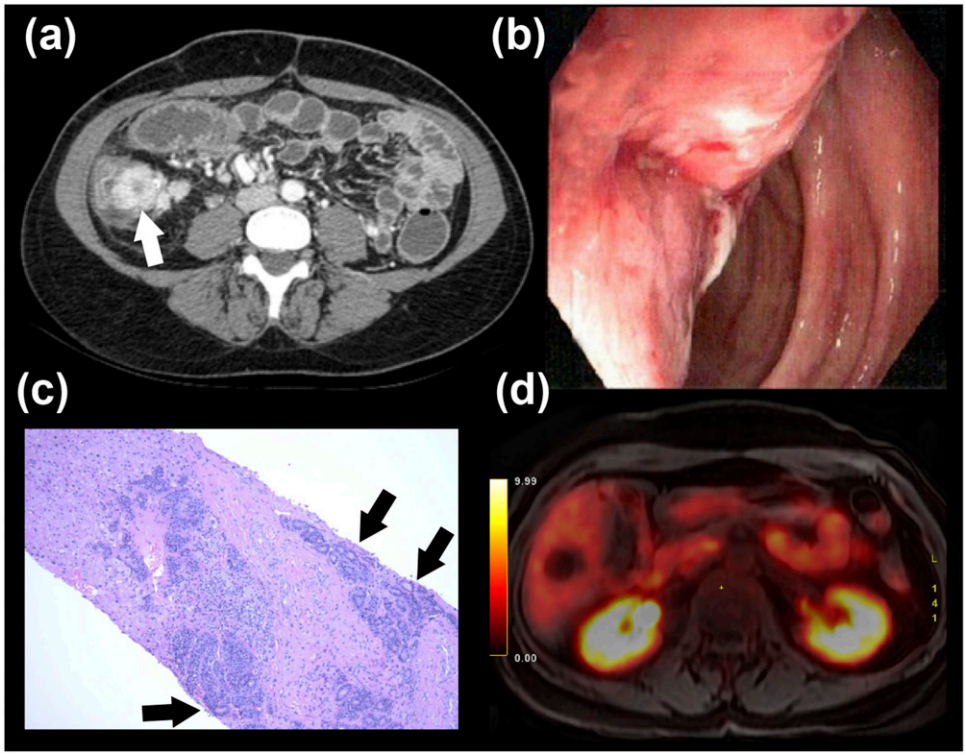

Figure 2. (a) CT enterography is suggestive of Crohn disease (arrow). (b) Colonoscopy shows an ulcerated tumor obstructing the ileocecal valve. (c) Liver biopsy with hematoxylin and eosin staining shows ~3 mm tumor aggregates (arrows). (d) ^{68}Ga-DOTA-TATE PET/MRI scan (July 2018) is disease-negative, with a large cyst in liver segment VI.

for monitoring the efficacy of treatment and progression of NET disease and provides a useful tool to minimize repetitive exposure to imaging. A prospective clinical trial that includes patients who are image-negative but NETest-positive would be of value to demonstrate the clinical utility of the liquid biopsy.

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Correspondence and Reprint Requests: Irvin M. Modlin, MD, Gastroenterological and Endoscopic Surgery, Yale University School of Medicine, 310 Cedar Street, New Haven, Connecticut 06520-8062. E-mail: imodlin@optonline.net.

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