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Clinical Research Article

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Prognostic Threshold for Circulating Tumor Cells in Patients With Pancreatic and Midgut Neuroendocrine Tumors

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Abbreviations: AUROC, area under receiver operating characteristic curve; CgA, chromogranin A; CTCs, circulating tumor cells; ENETS, European Neuroendocrine Tumour Society; EpCAM, epithelial cell adhesion molecule; G, grade; GEP, gastroenteropancreatic; HR, hazard ratio; miRNA, microRNA; midgut NET, midgut neuroendocrine tumor; NET, neuroendocrine tumor; OR, odds ratio; OS, overall survival; PanNET, pancreatic neuroendocrine tumor; PFS, progression-free survival; ULN, upper limit of normal.

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Abstract

Background: Circulating tumor cells (CTCs) are detectable in patients with neuroendocrine tumors (NETs) and are accurate prognostic markers although the optimum threshold has not been defined.

Objective: This work aims to define optimal prognostic CTC thresholds in PanNET and midgut NETs.

Patients and Methods: CellSearch was used to enumerate CTCs in 199 patients with metastatic pancreatic (PanNET) (90) or midgut NETs (109). Patients were followed for progression-free survival (PFS) and overall survival (OS) for a minimum of 3 years or until death.

Results: The area under the receiver operating characteristic curve (AUROC) for progression at 12 months in PanNETs and midgut NETs identified the optimal CTC threshold as 1 or greater and 2 or greater, respectively. In multivariate logistic regression analysis, these thresholds were predictive for 12-month progression with an odds ratio (OR) of 6.69 ($P < .01$) for PanNETs and 5.88 ($P < .003$) for midgut NETs. The same thresholds were found to be optimal for predicting death at 36 months, with an OR of 2.87 ($P < .03$) and 5.09 ($P < .005$) for PanNETs and midgut NETs, respectively. In multivariate Cox hazard regression analysis for PFS in PanNETs, 1 or greater CTC had a hazard ratio (HR)

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of 2.6 ($P < .01$), whereas 2 or greater CTCs had an HR of 2.25 ($P < .01$) in midgut NETs. In multivariate analysis OS in PanNETs, 1 or greater CTCs had an HR of 3.16 ($P < .01$) and in midgut NETs, 2 or greater CTCs had an HR of 1.73 ($P < .06$).
Conclusions: The optimal CTC threshold to predict PFS and OS in metastatic PanNETs and midgut NETs is 1 and 2, respectively. These thresholds can be used to stratify patients in clinical practice and clinical trials.

Freeform/Key Words: circulating tumor cells, neuroendocrine tumor, PanNET, midgut NET

Neuroendocrine tumors (NETs) are a heterogeneous group of tumors that arise in diverse anatomic locations but most commonly the gastrointestinal tract and pancreas. According to SEER, the US Surveillance Epidemiology and End Research program, NETs make up 0.9% of all tumors, of which 60.5% are gastroenteropancreatic (GEP) and 27% are bronchial in origin (1). The annual incidence is between 2 and 5 per 100 000 population (2), but prevalence is higher because of prolonged survival. NETs vary greatly in terms of prognosis and response to treatment, but currently the only circulating biomarker recommended by the European Neuroendocrine Tumour Society (ENETS) is chromogranin A (CgA) (3, 4). The sensitivity of CgA in the diagnosis of GEP NETs varies between 62% and 75%, with specificity reported between 68% and 100% (5-7). In retrospective studies, high baseline levels are associated with worse progression-free (PFS) and overall survival (OS) (8). RADIANT 3, a phase 3, randomized, prospective study evaluating everolimus in pancreatic NETs, found that high levels of CgA at baseline were associated with worse outcome (hazard ratio [HR] 0.42 with $P < .001$) (9). However, CgA can be elevated in many other common conditions or by the concomitant use of certain drugs, and this decreases the sensitivity to between 10% and 35% (10). In up to 40% of GEP NETs, CgA is normal even in the presence of radiological progression and large-volume disease (11).

Circulating tumor cells (CTCs) have been evaluated as biomarkers in a wide range of tumors. The CellSearch platform, which allows immunomagnetic separation of CTCs expressing the epithelial cell adhesion molecule (EpCAM), has been approved by the US Food and Drug Administration for use in breast, prostate, and colorectal cancer following prospective trials demonstrating the prognostic value of CTCs at defined thresholds (12-14). Pancreatic (PanNETs) and midgut NETs have been shown to express EpCAM in tissue, and CTCs are detectable using CellSearch in a high proportion of patients (15). In a prospective study of 176 NET patients, the presence of one or more CTCs was shown to be an independent prognostic factor associated with a significantly increased risk of death. However, the patient population studied was heterogeneous with respect to the primary tumor, and the optimal prognostic CTC according to primary site was not defined. Additionally, the study was limited by short follow-up, with a median of 12.6 months in a patient group that has a 73% survival at 2 years (16). Here we have extended the study to allow the prognostic threshold of CTCs to be determined in separate, large cohorts of pancreatic and midgut NETs with a minimum follow-up of 3 years.

Materials and Methods

Patients

Patients older than 18 years with histologically proven PanNET or midgut NET and radiological evidence of metastases measurable by Response Evaluation Criteria in Solid Tumours, version 1.1 (RECIST 1.1) (17) were recruited. Functioning and nonfunctioning tumors were both included. Patients were excluded if they were participating in other clinical trials or had commenced treatment other than somatostatin analogues within 3 months prior to sample collection. Ethical approval was obtained for the study from the National Research Ethics Service (reference No. 13/LO/0376) and all patients provided written informed consent. Tumor grade was determined according to ENETS and World Health Organization 2010 guidelines (18). The presence of metastases was determined using cross-sectional imaging with magnetic resonance imaging and computed tomography, and also with somatostatin receptor scintigraphy using Octreoscan, Mallinckrodt Nuclear Medicine LLC (Covidien) or Gallium-68 DOTATATE positron emission tomography. The volume of liver metastases was determined from computed tomography and magnetic resonance images.

Enumeration of circulating tumor cells

Blood samples (7.5 mL) were collected from patients into CellSave preservative tubes (Menarini Silicon Biosystems), stored at room temperature, and processed within 96 hours of collection as previously described by Khan et al (15). Two operators independently reviewed each sample and both were blinded to the clinical details. When there was disagreement on whether a cell met the criteria for CTC, a third independent operator was required to arbitrate.

Statistics

Statistical analyses were performed using GraphPad Prism, version 6; Microsoft Excel; and Stata 14. In the validation study of CellSearch by Allard et al (19) and the aforementioned study by Khan and colleagues (16), a training set of 90 patients was used to define a prognostic threshold and a target of 90 was also used for each of the midgut and pancreatic cohorts in this series. To determine optimum CTC threshold, receiver operating characteristic (ROC) curves were plotted for progression at 12 months and death at 36 months for each primary. The optimal threshold was then applied in logistic regression analysis with other clinically significant variables in a univariate and multivariate model for those time points. Kaplan-Meier survival curves were plotted and Cox hazards regression analysis was also performed for OS and PFS using the optimum CTC threshold for each primary. OS was defined as the time from CTC sample to death, and PFS from the time of CTC sample to death or progression as defined by RECIST 1.1.

Results

Patient characteristics

Overall, 90 patients with PanNET, and 109 patients with midgut NET were recruited between September 2009 and July 2014. Patients were followed up until November 2017, with a median follow-up of 64 months (range, 1-98 months). All living patients had a minimum follow-up of 3 years. The demographics and clinical characteristics of the patients are shown in Table 1. A greater proportion of grade 1 (G1) tumors were seen in the midgut group (69%) than in the PanNET group (28%). There were 16 G3 tumors in the PanNET group compared to 3 in the midgut group. A higher proportion of midgut patients (27%) had CgA elevated beyond 10 times the upper limit of normal (ULN) compared to patients in the PanNET group (10%). All patients had liver metastases, although a greater number of extrahepatic metastatic sites were seen in the midgut group. CTCs were detected in 30 (33%) PanNET and 56 (51%) of midgut NET patients, which is consistent with previously published studies.

Defining prognostic circulating tumor cell threshold

In total, 46 patients with PanNET and 41 patients with midgut NET had progressed at 12 months. The area under the ROC (AUROC) curve for 12-month PFS in PanNET was 0.69 (95% CI, 0.6-0.78) and the optimum CTC threshold was 1 or greater CTC, with a sensitivity of 50% and specificity of 84%. For midgut NET the AUROC was 0.78 (95% CI, 0.69-0.87), and the optimal CTC threshold of 2 or greater CTCs was associated with a sensitivity of 70% and specificity of 83% (20). These thresholds were used to determine the predictive utility of CTCs. Univariate and multivariate logistic regression analysis was applied to predict progression at 12 months (Table 2). For PanNET, G3 and CTCs of 1 or greater both were predictive of significantly increased risk of progression at 12 months in multivariate analysis, whereas for midgut NET, G2 tumors, CgA greater than 10 times the ULN, and CTCs of 2 or greater were predictive. The odds ratio (OR) for G3 midgut NET was 5.47 (95% CI, 0.13-222.5), but this was not significant likely because only 3 of 109 cases were G3.

The optimal CTC threshold was also determined for 36-month survival using ROC analysis (20). At 36 months, there were 40 deaths in the PanNET and 43 deaths in the midgut NET cohorts. The AUROC for PanNET was 0.69 (95% CI, 0.59-0.78), with a sensitivity of 50% and specificity 80% with an optimum CTC threshold of 1 or greater. In midgut NETs, the AUROC was 0.75 (95% CI, 0.65-0.86) with the optimum threshold of 2 or greater CTCs giving a sensitivity of 70% and specificity of 72%. Logistic regression analysis was performed using these thresholds (Table 3). For PanNETs, liver volume between 50% and 75% and the presence of a CTC of 1 or greater were predictive of death in multivariate analysis. For midgut NETs, a CgA of 5 to 10 times the ULN and greater than 10 times the ULN was predictive, as was a CTC of 2 or greater.

The prognostic performance of CTCs to predict PFS and OS was also estimated using Kaplan-Meier survival curves comparing patients above and below the defined thresholds using the log-rank test (Fig. 1A-1D). The CTC thresholds were used in Cox hazards regression analysis along with the other clinical variables. The median PFS for PanNET with less than 1 CTC was 17.6 months and 6 months in patients with 1 or more CTC (HR 2.92; 95% CI, 1.79-4.78, $P < .001$) (Fig. 1A). For midgut NETs, the median PFS was 44.4 months in patients with fewer than 2 CTCs, whereas for those with 2 or more it was 7.3 months (HR 3.8; 95% CI, 2.4-6.01, $P < .001$) (Fig. 1B). The univariate and multivariate Cox hazards ratios are summarized for PanNETs and midgut NETs in Table 4. In the multivariate analysis for PanNET, G3 and a CTC of 1 or greater was associated with a significantly worse PFS consistent with the findings for 12-month PFS. Similarly, for midgut NETs, CgA elevated beyond 5 times the ULN and the presence of 2 or more CTCs were associated with significantly worse PFS. The univariate and multivariate Cox hazards ratios for OS are summarized in Table 5. The median OS for PanNETs with less than 1 CTC was not reached, compared to 19.2 months for patients with 1 or more CTCs (HR 3.31; 95% CI, 1.87-5.8, $P < .001$) (see Fig. 1C). G3 and a CTC

Table 1. Demographics and clinicopathological characteristics of all pancreatic and midgut neuroendocrine tumors

	PanNET, n = 90	Midgut, NET n = 109	*P*
Age at diagnosis median (range), y	54 (23-78)	51 (30-83)	.67
Age at time of sample, y	62 (23-89)	63 (40-85)	.52
< 55	34	31	
55-65	27	30	
> 65	29	48	
Sex, male/female	46/44	59/40	.41
Grade 1	28	68	< .05
Grade 2	46	38	.36
Grade 3	16	3	< .05
Liver disease, % < 25	37	47	.88
25-50	21	42	< .05
50-75	20	15	.14
> 75	12	5	< .05
No. of extrahepatic sites			
1	13	5	< .05
2	29	34	.88
≥ 3	48	70	< .05
Bone metastases	19	23	.98
No bone metastases	71	86	
CgA < 3 × ULN	59	47	< .05
> 3 × ULN - < 5× ULN	12	14	.99
> 5 × ULN-< 10 × ULN	10	19	.23
> 10 × ULN	9	29	< .05
CTC = 0	60	53	< .05
CTC ≥ 1	30	56	< .05
CTC ≥ 2	18	48	< .05
Median length of follow-up mos (range)	84 (1-198)	72 (4-324)	.18
Previous treatment			
SSTs	42	78	< .05
Chemotherapy	46	15	< .01
PRRT	10	45	< .01
TAE	5	10	.31
IFN	6	15	< .05
Sunitinib	3	0	
Everolimus	1	0	
Liver resection	7	6	.51
Resection of primary	28/90 (31%)	55/109 (50%)	.12

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; IFN, interferon; PanNET, pancreatic neuroendocrine tumor; PRRT, peptide receptor radiotargeted therapy; SSTs, somatostatin analogues; TAE, transarterial embolization; ULN, upper limit of normal.

of 1 or greater were significant in multivariate analysis. In midgut NETs, the median OS for patients with 2 or more CTCs was 24.5 months, as opposed to 77.7 months for those with fewer than 2 (HR 3.08; 95% CI, 1.9-5, $P < .001$) (see Fig. 1D). In multivariate analysis a CgA greater than 5 times the ULN and CTCs of 2 or greater were significant.

Discussion

The value of CTCs as a prognostic marker in NETs was initially demonstrated in a mixed population of primary tumors (16). However, a consensus paper on biomarkers in NETs by a panel of international experts concluded that further studies were needed to confirm whether CTCs correlated with prognosis (21). To our knowledge, this study represents the largest prospective biomarker study published to date in PanNETs and midgut NETs. It also benefits from longer follow-up compared to previously published studies, providing robust survival data.

In this study, we have defined the optimal prognostic threshold for PanNET and midgut NET as 1 or greater and 2 or greater CTCs, respectively. Reassuringly, the same thresholds were derived using both 12-month PFS and 36-month OS. Applying these thresholds both in logistic regression analysis and Cox hazards regression analysis, we have demonstrated a consistent relationship between

Table 2. Summary of univariate and multivariate logistic regression analysis for progression at 12 months from time of sampling for pancreatic neuroendocrine tumors and midgut neuroendocrine tumors

	PanNET			Midgut NET		
Univariate analysis	OR	CI	*P*	OR	CI	*P*
Age y, < 55	1.00			1.00		
55-65	1.04	0.38-2.87	.933	2.08	0.65-6.72	.219
> 65	1.59	0.59-4.33	.361	4.53	1.58-13	.005
Male	1.00			1.00		
Female	0.59	0.25-1.35	.210	1.93	0.87-4.31	.107
Grade 1	1.00			1.00		
Grade 2	1.12	0.43-2.89	.815	2.84	1.22-6.62	.016
Grade 3	5.78	1.34-24.92	.019	2.36	0.14-39.5	.549
Liver disease, % < 25	1.00			1.00		
25-50	1.49	0.51-4.41	.468	3.84	1.49-9.88	.005
50-75	4.93	1.47-16.54	.010	4.83	1.39-16.8	.013
> 75	2.30	0.61-8.66	.218	16.89	1.68-169	.016
Extrahepatic sites						
1	1.00			1.00		
2	1.13	0.3-4.31	.859	2.31	0.98-5.45	.057
≥ 3	2.44	0.69-8.59	.164	2.89	0.71-8.2	.14
No bone metastases	1.00			1.00		
Bone metastases	3.41	1.11-10.49	.032	2.14	0.84-5.45	.109
CgA < 3× ULN	1.00			1.00		
3-5 × ULN	1.66	0.47-5.83	.430	1.69	0.43-6.64	.453
5-10 × ULN	2.77	0.65-11.75	.168	7.24	2.22-23.6	.001
10 × ULN	1.48	0.36-6.07	.585	5.20	1.85-14.5	.002
No primary resection	1.00			1.00		
Resection	0.35	0.14-0.9	.030	0.48	0.22-1.05	.066
CTC < threshold	1.00			1.00		
CTC ≥ 1	5.29	1.96-14.27	.001	-	–	–
CTC ≥ 2	–	–	–	9.30	3.78-22.9	< .0001
Multivariate analysis						
Grade 1	1.00			1.00		
Grade 2	1.22	0.34–4.46	0.759	4.20	1.14–15.52	0.031
Grade 3	10.16	1.33-77.83	.026	5.47	0.13-225.8	.371
Liver, % < 25	1.00			1.00		
25-50	0.69	0.11-4.24	.688	1.86	0.51-6.78	.348
50-75	3.30	0.52-20.89	.204	1.65	0.3-9.2	.567
> 75	0.97	0.14-6.66	.976	4.86	0.3-78.77	.266
No bone metastases	1.00			1.00		
Bone metastases	3.82	0.69-21.07	.124	2.31	0.55-9.77	.254
CgA < 3× ULN	1.00			1.00		
3-5 × ULN	2.12	0.36-12.54	.407	0.96	0.14-6.34	.964
5-10 × ULN	2.09	0.34-12.71	.424	3.79	0.78-18.38	.098
10 × ULN	0.34	0.04-2.64	.304	5.72	1.36-24.08	.017
No primary resection	1.00			1.00		
Resection	0.24	0.05-1.08	.064	0.79	0.27-2.3	.665
CTC < threshold	1.00			1.00		
CTC ≥ 1	6.69	1.56-28.69		–	–	–
CTC ≥ 2	–	–	.01	5.88	1.82-19.01	.003

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; OR, odds ratio; PanNET, pancreatic neuroendocrine tumor; ULN, upper limit of normal.

CTCs and both PFS and OS. For PanNETs, the presence of 1 or more CTCs is associated with an OR of 6.69 and 2.87 for 12-month PFS and 36-month OS respectively, whereas for midgut NETs the presence of 2 or more CTCs is associated with an OR of 5.88 and 5.09, respectively. The Cox HRs for PFS and OS were also significant in univariate

Table 3. Summary of univariate and multivariate logistic regression analysis for prediction of death at 36 months from time of sampling for pancreatic neuroendocrine tumors and midgut neuroendocrine tumors

	PanNET			Midgut NET		
Univariate analysis	OR	CI	*P*	OR	CI	*P*
Age, y < 55	1.00			1.00		
55-65	0.63	0.22-1.81	.393	3.43	1.14-10.35	.029
> 65	1.56	0.58-4.22	.383	2.67	0.96–7.37	.059
Male	1.00			1.00		
Female	0.47	0.2-1.1	.083	1.22	0.55-2.7	.620
Grade 1	1.00			1.00		
Grade 2	0.82	0.31-2.18	.696	1.74	0.76-4	.194
Grade 3	6.70	1.54-29.03	.011	1.85	0.11-30.74	.669
Liver, % < 25	1.00			1.00		
25-50	3.99	1.25-12.72	.019	2.98	1.2-7.37	.018
59-75	10.87	3.03-39.09	.0003	3.74	1.11-12.65	.034
> 75	3.62	0.92-14.35	.067	13.09	1.32-129.6	.028
1 extrahepatic site	1.00			1.00		
2	2.04	0.46-9.06	.350	1.91	0.19-19.2	.581
3	3.94	0.96-16.13	.057	3.18	0.34-29.91	.312
No bone metastases	1.00			1.00		
Bone metastases	1.99	0.71-5.56	.188	0.98	0.38-2.52	.972
CgA < 3 × ULN	1.00			1.00		
3-5 × ULN	2.19	0.62-7.74	.223	3.17	0.88-11.43	.078
5-10 × ULN	1.57	0.41-6.01	.514	7.24	2.22-23.6	.001
10 × ULN	1.96	0.48-8.05	.353	5.20	1.85-14.57	.002
No primary resection	1.00			1.00		
Resection	0.41	0.16-1.07	.068	0.48	0.22-1.05	.067
CTC < threshold	1.00			1.00		
CTC ≥ 1	4.00	1.58-10.13	.003	–	–	–
CTC ≥ 2	–	–	–	6.15	2.64-14.35	< .001
Multivariate analysis						
Grade 1	1.00			1.00		
Grade 2	0.53	0.13-2.11	.365	0.78	0.25-2.4	.663
Grade 3	5.10	0.79–32.74	0.086	2.26	0.07–73.5	0.646
Liver, % < 25	1.00			1.00		
25-50	3.77	0.61–23.24	0.152	1.15	0.36–3.66	0.810
59-75	14.30	1.98–103.17	0.008	1.27	0.25–6.36	0.771
> 75	3.08	0.37–25.58	0.298	3.45	0.16–76.56	0.433
No bone metastases	1.00			1.00		
Bone metastases	2.18	0.45–10.63	0.335	0.46	0.12–1.71	0.244
CgA < 3× ULN	1.00			1.00		
3-5× ULN	1.78	0.32–10.03	0.513	2.35	0.47–11.7	0.298
5-10× ULN	1.10	0.16–7.47	0.923	6.50	1.57–26.9	0.010
10× ULN	0.43	0.06–3.25	0.410	6.26	1.67–23.4	0.006
No primary resection	1.00			1.00		
Resection	0.80	0.17–3.81	0.776	0.63	0.24–1.64	0.343
CTC < threshold	1.00			1.00		
CTC ≥ 1	2.87	1.74–11.1	0.026	-	-	-
CTC ≥ 2	-	-	-	5.09	1.65–15.7	0.005

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; OR, odds ratio; PanNET, pancreatic neuroendocrine tumor; ULN, upper limit of normal.

analysis both for PanNETs and midgut NETs. Significance was maintained in multivariate analysis with the exception of OS for midgut NET, which narrowly missed significance ($P = .06$). Grade was also an independent prognostic factor as has been widely reported but was more consistent for G3 tumors in PanNET. By contrast, CgA levels were not prognostic for PanNET but were informative in midgut NET when elevated 5 times the ULN or more.

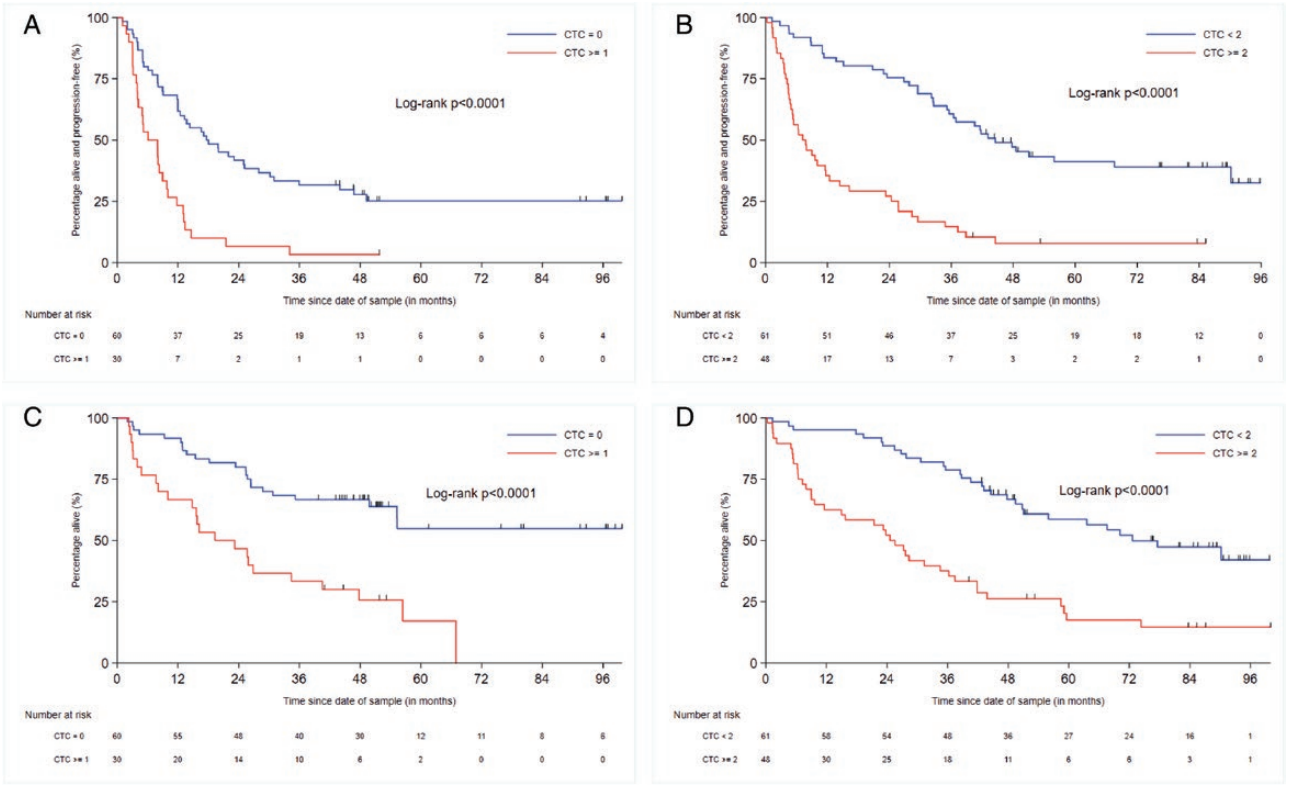

Figure 1. A-D, Kaplan-Meier survival curves. A, Progression-free survival (PFS) for pancreatic neuroendocrine tumors (PanNETs) above and below circulating tumor cell (CTC) threshold. B, PFS for midgut NETs. C, Overall survival (OS) for PanNETs. D, OS for midgut NETs.

Since the original study by Khan et al (16), a few smaller, prospective studies have evaluated CTCs as prognostic markers in NETs. A phase 2 study evaluating the effect of pazopanib, a multitargeted vascular endothelial growth factor and platelet-derived growth factor receptor antagonist, in GEP NETS included measurement of CTCs at baseline. The authors demonstrated that patients with no CTCs at baseline had a better response and longer PFS compared to those with one CTC or more (22). However, the study did not meet statistical significance, perhaps because of the small and heterogeneous patient population. A further prospective study by Khan and colleagues sought to investigate whether changes in CTC count in response to therapy could predict response and overall survival. It was found that patients with no CTCs at baseline or at first follow-up sample had the best OS followed by those that had a more than 50% reduction CTC. Patients that had less than a 50% decrease in CTC count, or an increase, had the worst survival (23). Molecular characterization of CTCs in NETs has also been explored and the expression of somatostatin receptors 2 and 5 has been demonstrated on CTCs enriched by CellSearch (24). The expression of CXCR4 on NET CTCs has also been reported, along with observation that bone metastases were strongly associated with the presence of CTCs (25).

The majority of studies published on the use of CTCs as a prognostic marker in cancer have used the CellSearch platform, and it remains the only US Food and Drug Administration–approved technology for CTC enumeration. There are many other technologies that have been used for isolation and detection of CTCs, but these studies are limited by the lack of reproducibility, poor correlation with clinical outcome, and cost.

Our study also demonstrated that CgA is an independent prognostic biomarker in midgut NETs. However, this is the case only for levels at least 5 times the ULN, which was the case in around 44% of midgut patients. For PanNETs there was no evidence that CgA had prognostic utility. More recently, the Neuroendocrine Neoplasms Test (NETest; Wren Laboratories) has been developed and evaluated in a number of studies. NETest measures the expression of 51 genes associated with development of neoplasia using reverse transcriptase polymerase chain reaction to develop a multigene signature from peripheral blood (26). By matching circulating transcripts with tissue transcripts, 30 of the 51 genes were classified into 9 clusters, and by determining expression across these 9 clusters a score between 0% and 100% has been derived that can differentiate between stable disease and progressive disease (27). A meta-analysis of heterogeneous studies suggests that the NETest may

Table 4. Summary of univariate and multivariate Cox hazard ratios for progression-free survival for pancreatic neuroendocrine tumors and midgut neuroendocrine tumors

	PanNET			Midgut NET		
Univariate analysis	HR	CI	*P*	HR	CI	*P*
Age, y < 55	1.00			1.00		
55-65	1.17	0.67-2.04	.574	1.39	0.76-2.53	.285
> 65	0.96	0.55-1.67	.873	1.79	1.04-3.09	.037
Male	1.00			1.00		
Female	0.51	0.32-0.82	.006	1.29	0.83-2.02	.264
Grade 1	1.00			1.00		
Grade 2	1.10	0.64-1.88	.737	2.17	1.35-3.48	.001
Grade 3	3.22	1.67-6.2	< .001	2.50	0.6-10.35	.207
Liver, % < 25	1.00			1.00		
25-50	1.73	0.94-3.17	.079	2.61	1.57-4.33	< .001
59-75	2.75	1.48-5.11	.001	2.62	1.32-5.2	.006
> 75	3.00	1.48-6.09	.002	5.71	2.15-15.19	< .001
1 extrahepatic site	1.00			1.00		
2	1.22	0.59-2.51	.586	1.47	0.44-4.95	.530
3	1.40	0.72-2.75	.321	2.53	0.79-8.11	.118
No bone metastases	1.00			1.00		
Bone metastases	1.67	0.96-2.89	.067	1.35	0.81-2.26	.253
CgA < 3 × ULN	1.00			1.00		
3-5 × ULN	1.48	0.74-2.94	.270	1.68	0.84-3.39	.145
5-10 × ULN	2.00	1-4.03	.051	2.92	1.57-5.41	.001
10 × ULN	2.49	1.2-5.19	.014	2.83	1.63-4.9	< .001
No primary resection	1.00			1.00		
Resection	0.47	0.27-0.82	.007	0.64	0.41-0.99	.046
CTC < threshold	1.00			1.00		
CTC ≥ 1	2.92	1.79-4.78	< .001	–	–	–
CTC ≥ 2	–	–	–	3.80	2.4-6.01	< .001
Multivariate analysis						
Grade 1	1.00			1.00		
Grade 2	1.26	0.66-2.4	.490	2.18	1.26-3.76	.005
Grade 3	3.02	1.41-6.46	.004	5.44	1.11-26.76	.037
Liver, % < 25	1.00			1.00		
25-50	1.22	0.58-2.57	.591	1.78	0.98-3.23	.060
59-75	1.82	0.87-3.8	.112	1.84	0.85-3.96	.121
> 75	1.58	0.64-3.9	.319	2.74	0.86-8.67	.087
No bone metastases	1.00			1.00		
Bone metastases	1.04	0.5-2.14	.919	1.19	0.64-2.2	.586
CgA < 3 × ULN	1.00			1.00		
3-5 × ULN	2.05	0.95-4.42	.068	1.33	0.6-2.97	.482
5-10 × ULN	1.49	0.65-3.41	.350	2.13	1.03-4.4	.041
10 × ULN	2.02	0.85-4.82	.112	2.49	1.27-4.91	.008
No primary resection	1.00			1.00		
Resection	0.56	0.27-1.15	.114	0.89	0.56-1.43	.642
CTC < threshold	1.00			1.00		
CTC ≥ 1	2.60	1.37-4.9	.003	–	–	–
CTC ≥ 2	–	–	–	2.25	1.32-3.84	.003

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; HR, hazard ratio; PanNET, pancreatic neuroendocrine tumor; ULN, upper limit of normal.

differentiate stable from progressive disease (28), but larger prospective studies in well-defined populations are required to define the role of the NETest as an independent prognostic marker.

Circulating microRNAs (miRNAs) have been investigated as potential markers of tumor behavior that can be measured in patient blood samples. In NETs, 31 candidate miRNAs were found to be similarly expressed in tissue

Table 5. Summary of univariate and multivariate Cox hazards ratios for overall survival for pancreatic neuroendocrine tumors and midgut neuroendocrine tumors

	PanNET			Midgut NET		
Univariate analysis	HR	CI	*P*	HR	CI	*P*
Age, y < 55	1.00			1.00		
55-65	0.57	0.27-1.19	.136	1.90	0.99-3.66	.054
> 65	1.00	0.53-1.92	.989	1.89	1.03-3.46	.039
Male	1.00			1.00		
Female	0.51	0.28-0.93	.027	1.19	0.73-1.92	.484
Grade 1	1.00			1.00		
Grade 2	1.08	0.54-2.17	.832	1.52	0.92-2.5	.101
Grade 3	4.90	2.24-10.7	< .001	0.80	0.11-5.87	.830
Liver, % < 25	1.00			1.00		
25-50	2.66	1.19-5.93	.017	2.15	1.25-3.7	.006
50-75	4.09	1.88-8.92	< .001	2.29	1.09-4.83	.029
> 75	3.22	1.35-7.67	.008	6.40	2.37-17.3	< .001
1 extrahepatic site	1.00			1.00		
2	1.44	0.55-3.78	.457	0.80	0.23-2.79	.727
3	2.41	0.97-5.99	.059	1.96	0.61-6.3	.258
No bone metastases	1.00			1.00		
Bone metastases	1.59	0.84-3	.158	1.48	0.85-2.57	.162
CgA < 3× ULN	1.00			1.00		
3-5 × ULN	2.24	1-5.03	.049	1.55	0.72-3.36	.263
5-10 × ULN	1.71	0.74-3.93	.206	3.24	1.67–6.28	< .001
10 × ULN	2.17	0.89-5.29	.087	2.78	1.53-5.05	.001
No primary resection	1.00			1.00		
Resection	0.48	0.24-0.96	.038	0.60	0.37-0.97	.037
CTC < threshold	1.00			1.00		
CTC ≥ 1	3.31	1.87-5.85	< .001	–	–	–
CTC ≥ 2	–	–	–	3.08	1.9-5	< .001
Multivariate analysis						
Grade 1	1.00			1.00		
Grade 2	0.63	0.25-1.58	.325	1.23	0.7-2.16	.478
Grade 3	4.88	1.89-12.6	.001	1.08	0.13-8.75	.939
Liver, % < 25	1.00			1.00		
25-50	1.25	0.43-3.67	.685	1.43	0.77-2.68	.261
50-75	3.30	1.19-9.11	.022	1.51	0.65-3.49	.336
> 75	2.46	0.71-8.59	.157	2.92	0.95-9.23	.06
No bone metastases	1.00			1.00		
Bone metastases	0.87	0.38-2	.751	1.10	0.57-2.13	.783
CgA < 3× ULN	1.00			1.00		
3-5 × ULN	4.17	1.44-12.1	.008	1.25	0.53-2.95	.616
5-10 × ULN	1.20	0.4-3.59	.740	3.03	1.43-6.44	.004
10 × ULN	1.21	0.41-3.62	.729	2.02	0.97-4.24	.062
No primary resection	1.00			1.00		
Resection	0.91	0.35-2.36	.850	0.84	0.5-1.41	.502
CTC < threshold	1.00			1.00		
CTC ≥ 1	3.16	1.46-6.81	.003	–	–	–
CTC ≥ 2	–	–	–	1.73	0.96-3.13	.06

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; HR, hazard ratio; PanNET, pancreatic neuroendocrine tumor; ULN, upper limit of normal.

and serum from patents with midgut NETs. High circulating levels of miR-22-3p and miR-21-5p and low levels of miR-150-5p when combined predicted worse OS (HR 0.47, $P < .002$) (29). The majority of studies to date have evaluated miRNAs in tissue samples and although there has been some correlation with tumor response, expression has been reported to be often only weakly associated with circulating levels (21). Currently, based on the published data,

circulating miRNA has not been validated as a biomarker in NETS. In conclusion, CTCs enumerated by CellSearch represent a robust biomarker that can be used to predict outcome in PanNET and midgut NET. Here we have defined the prognostic threshold for CTCs in PanNETs and midgut NETs, which allows clinicians to stratify patients in clinical practice or in the context of clinical trials. The technological advances in single-cell sequencing is now being applied to CTCs to extend their utility as liquid biopsies. This is expected to shed new light on tumor evolution and the biology of metastasis (30).

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