A multianalyte algorithm PCR-based Blood Test outperforms Single Analyte ELISA-based blood tests for Neuroendocrine Tumor Detection

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ABSTRACT

A key issue in management of neuroendocrine tumors (NETs) is specific and sensitive biomarkers. Measurements of single analytes in blood are widely utilized but have significant limitations. We developed a 51 transcript blood NET signature and compared it with standard approaches [1, 2]. The multigene signature was evaluated in prospectively collected NETs (n=41, 61% small intestinal, 50% metastatic, 44% under treatment). These were age (NETs: mean 56.9 years, range: 31-76; controls: mean 56.4, range: 33-75) and sex-matched (M:F 10:31) with controls (1:1). Samples were analyzed by 2-step PCR protocol and ELISAs: (DAKO-CgA), pancreastatin (CusaBio-PST) and neurokinin A (RayBiotech-NKA). Sensitivity comparisons included chi-square, non-parametric measurements and ROC analyses. The NETest identified thirty eight of 41 NETs with equivalent performance metrics: sensitivity/specificity 93% and an AUC of 0.96. For the single analyte ELISA assays, metrics ranged from 31-93% and AUCs from 0.55-0.67. The multigene transcript NETest significantly outperformed single analyte tests (Z-statistic=4.85-6.58, p<0.0001). We conclude that a 51 panel multigene blood transcript analysis is significantly more sensitive and efficient (>93%) than any single analyte assay (CgA, PST or NKA) for NET detection. Our data indicate that a blood-based multigene analytic measurement will provide increased sensitivity and specificity in minimally invasive disease detection.

BACKGROUND

•Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are common (incidence: 2/100,000), occurring as frequently as testicular tumors, Hodgkin's disease, gliomas and multiple myeloma and are estimated to have a prevalence of 35/100,000 [3].

•They represent a heterogeneous group of cancers both in terms of tumor biology and the variety of bioactive products they synthesize and secrete, and exhibit a range of different behaviors (proliferation and/or metastasis) which reflects the diverse cells (and sites) of origin.

•There is a paucity of effective therapies as well as accurate tools to assess therapeutic efficacy.

•Strategies currently include detection of blood Chromogranin A (CgA) or measurements of other tumor-associated products including pancreastatin and neurokinin A [4] but none of these approaches are FDA-accepted as a supportable biomarkers.

•Single analyte approaches exhibit significant limitations including low sensitivities and specificities and measurements are affected by other diseases e.g., cancer as well as medications including acid inhibitory therapy.

•Identification of a peripherally accessible, molecular fingerprint using PCR-amplification of target genes, has successfully been undertaken in other cancers e.g., breast and colon, and is used in a variety of measures including prognosis, identification of metastasis and recurrence, prediction of therapy response and metastasis-free survival for node-negative, untreated primary cancers.

•The advantages of developing multianalyte assays with algorithmic analyses (MAAA) methodology to accurately assess a tumor group arising from many different cells and with numerous biological profiles therefore is self-evident.

•We have developed a peripheral blood PCR-based tool (*NETest*) that exhibited correct call rates of 91-97% with sensitivities and specificities of 85-98% and 93-97% for the identification of GEP-NENs [3].

•This methodology has now been recognized as more accurate than the currently used clinical standard CgA assay and could supplant it [5].

•We evaluated this test in a prospective setting against CgA as well as two other markers currently used in NET management – pancreastatin and neurokinin A.

Critical Issue

A key issue in management of neuroendocrine tumors (NETs) is specific and sensitive biomarkers. Measurements of single analytes in blood are widely utilized but have significant limitations.

Hypothesis

A multianlyte test will provide increased sensitivity and specificity for the detection of neuroendocrine tumors.

Key Findings

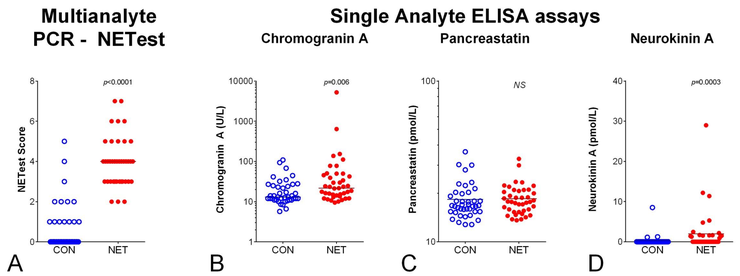

Figure 1. Differences in NETest score, chromogranin A levels, pancreastatin and neurokinin A in age-sex matched NETs and controls (n=41 each). The MAAA-NETest was significantly higher (p<0.0001) in NETs compared to controls (**1A**). CgA levels were also significantly elevated in NETs than in controls (p<0.01) (**1B**) but pancreastatin levels did not differentiate the two groups (**1C**). Neurokinin A levels were, however, elevated in NETs (p<0.001) (**1D**).
CON = control group, NET = neuroendocrine tumor group.

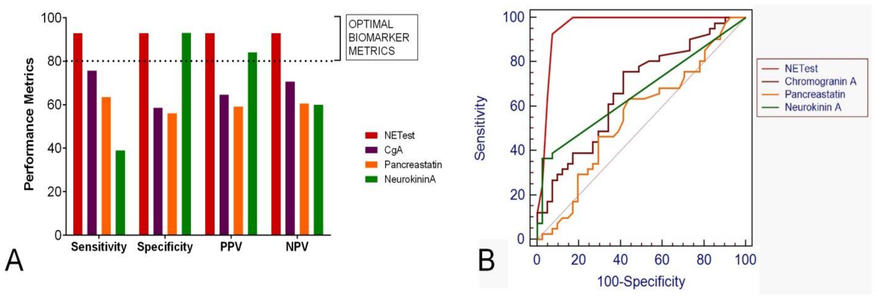

Figure 2. Performance metrics for the MAAA-NETest versus the single analyte ELISAs for CgA, pancreastatin and neurokinin A in the 41 matched NETs and controls. **2A**) The sensitivity, specificity, PPV and NPV for the MAAA-NETest were all >90%. The metrics for CgA ranged from 58.5-75.6%, for pancreastatin it was: 56.1-63.4% and for neurokinin A: 39-93%. **2B**) Receiver operating characteristic (ROC) curves for PCR gene analysis compared to CgA, pancreastatin and neurokinin A. The AUC for PCR gene analysis was 0.96 and for CgA 0.67. For pancreastatin it was 0.56 and for neurokinin A it was 0.66. The NETest significantly (p<0.0001) outperformed the single analytes.
NETest = multigene test, CgA = Chromogranin A, SENS = sensitivity, SPEC = specificity, PPV = positive predictive value, NPV = negative predictive value. The dotted line (**2A**) represents 80% (standard cut-off level for biomarkers) [8].

Conclusions

1. The multi-transcript molecular signature is both sensitive and specific (>90%) for the detection of neuroendocrine tumor disease.
2. The PCR test is robust and significantly more sensitive and specific (accurate) (p<0.0001) than currently used single analytes including Chromogranin A, pancreastatin and neurokinin A.

References

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Methods

All peripheral blood samples (5ml, K_2 EDTA tube) were collected and analyzed according to an IRB protocol (Yale University School of Medicine). The protocol was specifically approved for this study. Written consent was obtained from all study participants.
Matched cases and controls: We prospectively collected NET patients (Sept-Dec 2013) and controls, matching the 41 cases with a control (1:1) by sex and age to within 2 years. The ethnicity was exclusively Caucasian. There were no differences in sex distribution: M: F = 10:31, both groups) or age between the two groups (NETs: mean 56.9, range: 31-76; controls: mean 56.4, range: 33-75) confirming appropriateness of matching.
Multianalyte Assay (Whole blood samples)
Transcripts (mRNA) were isolated from whole blood using the mini blood kit (Qiagen: RNA quality >1.8 $A_{260:280}$ ratio, RIN>5.0) with cDNA produced using the High Capacity Reverse transcriptase kit (Applied Biosystems: cDNA production 2000-2500ng/ul) [1,2].
Real-time PCR analysis and NETest score: Real-time PCR was performed using Applied Biosystems products. PCR values were normalized to *ALG9* (DDC_T), using the control group as the population control (calibrator sample) [1,2]. A NET score (0-8) is derived from the PCR data; a value ≥2 is a positive tumor score.
Single Analyte Assays (Plasma samples)
Matching plasma samples (to whole blood) were used for single analyte ELISAs.
1. Chromogranin A : CgA was measured using the DAKO ELISA kit (K0025, DAKO North America, Inc., Carpinteria, CA) [3]. A cut-off of 14 Units/L (DAKO) was used as the upper limit of normal.
2. Pancreastatin: This was measured using the CUSABIO kit (#CSB-E09209h). The assay range is 31.25-2000pg/ml with a sensitivity of 7.8pg/ml.
3. Neurokinin A: NKA was measured using the RayBiotech kit (#EIA-NEA1). This has an assay range of 0.8-1000pg/ml with a published sensitivity of 0.8pg/ml.
Statistical analyses: Sensitivity comparisons using respectively χ-square, non-parametric measurements and ROC analysis were made between the MAAA-PCR test and single analyte plasma ELISAs for detection of NET. Predictive feature importance values for each test were derived using the mean decrease in Gini coefficient, following construction of a random forest model with 10-fold cross-validation. Prism 6.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) were utilized.

Results

Table 1: Performance Metrics

	AUC	SE*	95% CI**
NETest	0.957	0.0249	0.888—0.990
CgA	0.673	0.0593	0.561—0.773
Pancreastatin	0.555	0.0643	0.441—0.665
Neurokinin A	0.664	0.0607	0.551—0.765

* Hanley & McNeil, 1982 [6]
** Binomial exact
AUC = area under the curve, CI = confidence interval, SE = standard error

Table 2: Pairwise comparison of ROC curves

	Difference between AUCs	SE*	95% CI	Z-statistic	P-value
NETest vs CgA	0.284	0.0625	0.162—0.406	4.548	p<0.0001
NETest vs Pancreastatin	0.403	0.0682	0.269—0.536	5.902	p<0.0001
NETest vs Neurokinin A	0.294	0.0603	0.175—0.412	4.867	p<0.0001
CgA vs Pancreastatin	0.119	0.0845	-0.0470—0.284	1.404	p=0.1602
CgA vs Neurokinin A	0.00952	0.0819	-0.151—0.170	0.116	p=0.9075
Pancreastatin vs NeurokininA	0.109	0.0833	-0.0541—0.272	1.31	p=0.1901

* Hanley & McNeil, 1983 [7]
AUC = area under the curve, CI = confidence interval, SE = standard error.

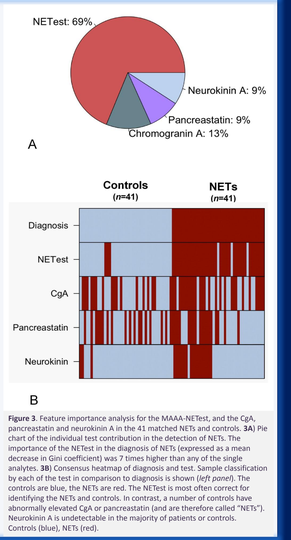

Figure 3. Feature importance analysis for the MAAA-NETest, and the CgA, pancreastatin and neurokinin A in the 41 matched NETs and controls. **3A**) Pie chart of the individual test contribution in the detection of NETs. The importance of the NETest in the diagnosis of NETs (expressed as a mean decrease in Gini coefficient) was 7 times higher than any of the single analytes. **3B**) Consensus heatmap of diagnosis and test. Sample classification by each of the test in comparison to diagnosis is shown (*left panel*). The controls are blue, the NETs are red. The NETest is most often correct for identifying the NETs and controls. In contrast, a number of controls have abnormally elevated CgA or pancreastatin (and are therefore called "NETs"). Neurokinin A is undetectable in the majority of patients or controls. Controls (blue), NETs (red).

NETest Performance Metrics

Sensitivity = 93% Specificity = 93%
PPV = 93% NPV = 93%

Acknowledgements

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