Validation of Neurokinin A Assays in the United States and Europe

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Objectives: International cooperative group trials require specific, sensitive biomarker assays that are validated between continents. Neurokinin A (NKA) has been shown to be a powerful independent predictor of a poor prognosis in well-differentiated midgut neuroendocrine tumors. We hypothesized that NKA concentrations of clinical specimens evaluated in NKA assays in the United States and the United Kingdom would be equivalent, even though assay techniques were significantly different.

Methods: Frozen clinical specimen aliquots were shipped from the United States to the United Kingdom (n = 67), and from United Kingdom to the United States (n = 50). In addition, spiked plasma standards and medium-spiked standards were exchanged. Samples from the United States were directly assayed in a radioimmunoassay, whereas the UK specimens were extracted, and the reconstituted specimens assayed in the radioimmunoassay. Neurokinin A values from the 2 studies were analyzed by regression analysis.

Results: The NKA values from the US and UK laboratories were essentially identical (United States to United Kingdom, $r^2 = 0.88$, $P < 0.0001$; and United Kingdom to United States, $r^2 = 0.96$, $P < 0.0001$).

Conclusions: Validation of biomarker assays across continents will ensure that laboratory observations made by researchers are equivalent and that prediction of clinical outcomes based on these assays is also reliable.

Key Words: biomarkers, prognosis, neuroendocrine tumors, carcinoid, clinical study, midgut

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Well-differentiated neuroendocrine tumors (NETs) of the midgut arise from Kulchitsky cells. Commonly, these tumors (often called "typical" midgut carcinoids) are initially treated with combinations of surgery and somatostatin analogs. Although less aggressive than their poorly differentiated counterparts, over time these lesions are responsible for significant morbidity and mortality. Despite surgery and somatostatin analog therapy, many patients ultimately die of these tumors. Several other treatment options have become available in recent years. Timing the use of these treatments optimally is crucial; indolent tumors can be watched, but those that become aggressive must be treated within the window of opportunity. Therefore, the progressive nature of metastatic well-differentiated NETs mandates that patients undergo interval restaging with radiographic imaging and radionucleotide scans. These individuals also require serial determination of plasma and urine biomarkers to detect postoperative tumor persistence or early tumor recurrence.[1–3]

To date, serial determinations of urinary 5-hydroxyindole acetic acid (5HIAA) and plasma chromogranin A (CGA) values are the most commonly utilized biomarkers for initial disease staging and the evaluation of tumor progression in well-differentiated midgut NETs. Neither of these laboratory tests, however, is without problems. Urinary 5HIAA measurement is cumbersome, and false-positive results are frequent because of diet. Chromogranin A is a general marker for NETs and is not specific for midgut tumors. A larger problem is that CGA measurement is also commonly associated with false-positive results because of high concentrations found with atrophic gastritis or proton-pump inhibitor therapy. Other biomarkers such as pancreastatin and neurokinin A (NKA) are rapidly gaining acceptance as significant contributors to the ongoing evaluation of these patients.[1–6] Neurokinin A is of particular importance because it is specific to this group of patients.[7–9]

Turner et al[7] have shown that high NKA concentrations (>50 pmol/L, >50 pg/mL) portend a poor prognosis in patients with midgut NETs. Serial determination of NKA concentration in patients with midgut NETs may offer a significant advantage over measurement of other markers in predicting a low 3- or 5-year survival rate. In a retrospective study (n = 150 patients), these authors demonstrated that circulating NKA was an excellent independent indicator of poor prognosis in patients with well-differentiated midgut NETs. Patients with midgut NETs whose NKA was greater than 50 pmol/L (50 pg/mL) had a 3-year survival rate of 10% in contrast to a 65% survival for those patients with an NKA concentration of less than 50 pmol/L. Data from this study also showed that the most recent NKA is the best prognostic indicator of survival.[7] Those patients who have critically elevated NKA values, but who undergo successful antitumor therapy that decreases their NKA concentration less than the critical 50 pmol/L (50 pg/mL) cutoff value, continue to have a good prognosis, despite their NKA concentration having once risen greater than the critical threshold value. An interim report from an ongoing prospective study corroborates these findings (Fig. 1).[8]

Based on the data from this study, patients with NKA values less than 50 pmol/L (50 pg/mL) should have an excellent 3-year survival rate. In these good prognosis patients, other indicators such as upward trends of CGA, pancreastatin, or 5HIAA may be used to determine if additional therapies should be undertaken or a change of therapy is warranted. In contrast, in those patients with NKA values greater than the critical value of 50 pmol/L, immediate changes in therapy should be undertaken in an attempt to lower the NKA value to less than 50 pmol/L. Therapy that decreases the NKA to less than this 50 pmol/L cutoff

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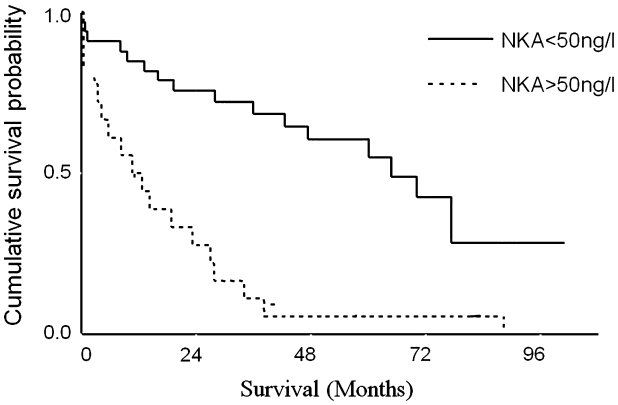

FIGURE 1. Kaplan-Meier survival curves for individuals with NKA values of less than 50 pg/mL and those patients with NKA values greater than 50 pg/mL as determined in Belfast. Corresponding US values would be less than or more than 57 pg/mL. These are preliminary data from an ongoing trial by Dr Ardill in Belfast. The n value is not available.

value is associated with a continuing improved survival, whereas failing to suppress NKA to less than 50 pmol/L predicts a short survival.[4]

Traditionally, most large multi-institutional clinical studies in patients with well-differentiated midgut NETs are conducted by the pharmaceutical industry as part of registration trials for drugs, devices, or other treatment modalities. As part of these trials, companies often collect specimens for biomarker determination and send them to a central reference laboratory. Clinicians, even those at academic centers, often do not have access to these highly sophisticated laboratories. Many well-respected, highly sophisticated commercial laboratories exist in the United States and in Europe, and several of these laboratories offer highly sensitive and specific assays for NET-associated biomarkers. Development of international cooperative group clinical protocols will require biomarker assays that are proven to be equivalent, and their results will have to be validated across continental borders. Equally important is the concept that general standardization of assays across reference laboratories so that multinational study centers deal with 1 set of normal values has a great deal of clinical relevance. With the advent of the European Neuroendocrine Tumor Society and the North American Neuroendocrine Tumor Society, it is likely that collaborative intercontinental studies will be designed that require serial biomarker determinations to gauge the response to therapy. These cooperative group trials will not be designed or carried out by the pharmaceutical industry and the physicians caring for patients on these studies may not have easy access to laboratories that offer validated biomarker assays that can be run on specimens from different continents. To date, there have been no comparative studies to validate biomarker assays in the United States and in other continents.

We hypothesized that the NKA assays that are offered commercially in the United States and the United Kingdom would yield essentially identical results, even though they use significantly different assay methodology. To test this hypothesis, clinical samples sent for commercial evaluation of their NKA concentrations were divided into 2 separate aliquots, frozen, exchanged between laboratories, and assayed for NKA by Inter Science Institute (ISI), Inglewood, Calif (G.M.), and the Royal Victoria Hospital in Belfast, Northern Ireland, United Kingdom (J.E.A.).

If we are able to show that the NKA assay results from the UK and from the US groups are equivalent, we may be able to use these 2 laboratories' NKA values interchangeably for clinical studies in American and European centers. Furthermore, if we can prove equivalence of results in these 2 assays, then the clinical implications of previous studies performed in Europe by the Belfast group can be extended to patients in the United States who have their NKA assayed by ISI.

METHODS

Cross-Validation of 2 NKA Radioimmunoassay Methodologies

Cross-validation of 2 methodologically different radioimmunoassays (RIAs) for NKA was achieved by comparison of test results generated by analysis of same samples by both assays ("split-tube" testing). The 2 NKA immunoassays were developed independently by the Regional Regulatory Peptide Laboratory (RRPL), Royal Victoria Hospital, and ISI. In these assays, different antisera, different methods of sample preparation, different methods of manufacturing of tracer, different incubation conditions, and different methods for separation of bound from free-labeled tracer were used by both these analytically and clinically validated immunoassay methods.

The comparison of assay methodologies involved the cross-validation of standards, quality-control samples, baseline and spiked normal plasma samples, comparisons of the linearity of high-sample-dilution studies, and the analysis of a wide range of clinical samples with low, intermediate, and high NKA concentrations. A total of approximately 100 coded samples analyzed previously in Belfast or in California by their respective assay procedures were shipped frozen on dry ice to the alternate laboratory to be analyzed by the recipient's unique NKA assay procedure. The intent of this project was to cross-validate the results of patient samples measured by 2 NKA RIAs, one in the United States and one in Europe.

Sample Preparation for the NKA RIAs Performed at ISI and Belfast

Samples were sent in August 2009 from the United States to the United Kingdom for a cross-validation study. One hundred coded samples were sent on dry ice from ISI to Belfast to be analyzed by the NKA RIA procedure developed by the RRPL, Royal Victoria Hospital. These samples contained (1) preformulated NKA standards prepared from same stock solution of the peptide as used at ISI for assay of same samples; (2) preformulated quality-control samples; (3) normal plasma samples (n = 10); (4) the same 10 normal plasma samples supplemented with either 80 (L1) or 160 pg/mL (L2) (n = 10 per group, n = 20 total); and (5) samples previously analyzed at ISI, NKA samples with either low, 0 to 20 pg/mL (16 samples); medium, 21 to 60 pg/mL (15 samples); or high, greater than 61 pg/mL of NKA (6 were undiluted, and 17 were diluted with assay matrix). All samples were supplied in a ready-to-use form and contained an optimized amount of the proprietary protease inhibitor cocktail.

In October 2009, specimens prepared in Belfast were packed in dry ice and shipped by express courier to ISI as part of this prospective, ongoing NKA assay cross-validation study. In this portion of the study, 103 coded samples were sent on dry ice from Belfast to be analyzed by ISI NKA RIA. These tubes consisted of (1) undiluted patient plasma specimens, (2) standards from the Belfast assay, (3) assay buffer spiked with analyte, (4) assay controls, and (5) serially diluted patient plasma.

Neurokinin A assay of ISI samples was done after approximately the same time of storage at −20°C at ISI and the RRPL, according to their own, optimized assay procedure. Each laboratory used its own NKA reference standard and its own antibody,

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tracer, and matrix in the established, direct, or extraction-based assay methodology. Test results were read from the dose-response curve constructed from in-house reference standards. Assay of the Belfast samples followed the same format as above assay of ISI samples: each laboratory used its own reference standard, its own antibody, its own tracer, and its own matrix in their direct or extraction-based assay methodology. All 103 Belfast samples were analyzed as received, ready-to-use samples. An optimized amount of protease inhibitor was added to each test tube to neutralize proteolytic enzymes.

The Belfast Assay

Specimen Collection and Preparation

Blood specimens were collected into EDTA tubes, placed on ice, and maintained at 4°C until the time of centrifugation at 4°C. Centrifugation had to take place within 2 hours of specimen collection. Plasma was decanted and stored at −20°C. Neurokinin A was extracted from plasma with acidified alcohol, dried down, and stored at 4°C up to 15 days until the peptide assay was performed.

RIA of NKA

Antiserum to the N-terminal region of NKA was raised in rabbits and was used at a final dilution of 1:160,000. ^{125}I-labeled NKA of high specific activity was prepared by a modification of the Chloramine T method and purified by reverse-phase high-performance liquid chromatography. More recently, radiolabeled NKA of high specificity was purchased from PerkinElmer (New England Nuclear [Waltham, Mass], product code NEX252010UC). Radiolabeled product was stored in acidified alcohol at −20°C.

Because no national or international standard is available for NKA, standards were prepared from peptide supplied by Bachem (Torrance, Calif; product code H3745). The stock standard was prepared at a concentration of 1 mg/L and stored and frozen at −20°C. This standard was stable for 6 months.

Because there is no quality-assurance material available for NKA, suitable quality-assurance samples were prepared in-house. Three controls, high, medium, and low concentrations, were prepared in the assay medium, and a second set of quality-assurance samples with similar peptide concentration was prepared in stripped human plasma. The target values for controls were 20, 50, and 100 ng/L and were run in over 10 consecutive assays. The analysis of the medium-based controls was performed directly without any further preparation. The human plasma–based controls were analyzed following the extraction procedure for clinical samples.

Assay specimens were reconstituted in phosphate buffer (0.05 M, pH 7.4) at the time of assay. Standards were prepared across the range 7.8 to 1000 ng/L. The assay was performed at 4°C. Unknowns, standards, and quality-assurance samples were incubated with diluted antiserum for 24 hours at 4°C. Antibody was diluted to give approximately 45% binding of a new preparation of radiolabeled NKA. Radiolabeled NKA was added to each tube at a concentration to give approximately 6000 counts per minute. The assay was incubated for a further 24 hours at 4°C. Antibody bound from free NKA was separated using a solid phase, a second antibody method with a 15-minute centrifugation step. The supernatant was decanted and discarded. The pellet was counted in a gamma counter, and a calibration curve constructed. Unknown concentrations were calculated using Multi Calc software (www.softwareandgames.com) (smooth spline curve fit).

Neurokinin A assay has been performed consistently in Belfast since 1985. Since 2002, new higher-affinity antisera have been used. From that time, the assay has been subjected to stringent regulation and is now a National Health Service–accredited assay in the United Kingdom and as such is suitable for measurement of NKA in human clinical specimens. It is the only such NKA assay in the United Kingdom and Ireland.

Analytical Sensitivity and Reproducibility of the Belfast Assay

Sensitivity of the Belfast assay was defined as that concentration of NKA standard, which shows 5% suppression in bound counts. This was established over 12 consecutive assays. The mean sensitivity was 7.4 ng/L (range, 5.9–8.7 ng/L).

All specimens with NKA concentrations greater than 100 ng/L were serially diluted in assay buffer. All were diluted parallel to the NKA standards.

Interassay and intra-assay variation was established by repeating high, medium, and low controls throughout all assays. In 12 consecutive assays, the coefficient of variation was 6.5, 4.7, and 4.6 (high, medium, and low controls), and for quality controls, which also had been subjected to the plasma extraction, 5.4, 8.1, and 10.7, respectively.

The ISI Assay

The NKA RIA developed at ISI was based on a direct, extraction-free assay of the analyte in the patient's plasma. In this assay, there was no need for liquid- or solid-phase extraction of NKA, and thus, there was no requirement to monitor the efficiency of analyte recovery for each analyzed sample. To eliminate the degradation of NKA and its tracer by plasma or serum proteases present in patient samples, a proprietary enzyme inhibitor mixture was added to each specimen and to each test tube used in the assay. This protease inhibitor step replaces the extraction step used in the Belfast assay and permits rapid preparation of an interference-free analytical sample.

Neurokinin A antiserum was developed using a polyclonal antibody raised in rabbit to the N-terminal region of NKA. It was diluted in the assay buffer to a working dilution of 1:50,000. This dilution bound 25% to 35% of a new lot of tracer. High-purity and high-immunopotency NKA peptide (Bachem) was used for the preparation of standards and quality-control samples in the assay matrices. A proprietary in-house manufactured, ^{125}I-labeled NKA peptide served as a tracer to monitor the immune reaction between the antigen and the anti-NKA antibody.

Quality-control samples were prepared by supplementation of assay matrix with the same high-purity NKA peptide as used

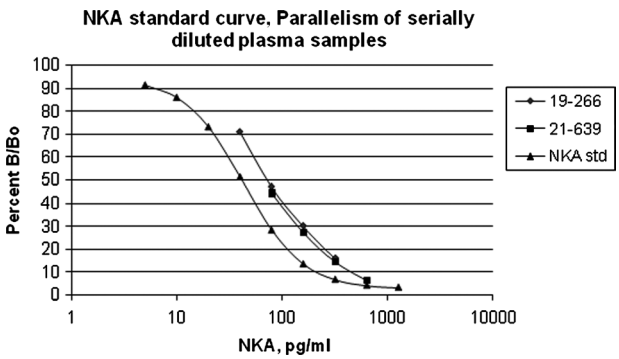

FIGURE 2. Graphic demonstration of the immunochemical identity between synthetic NKA standards and NKA peptide(s) in peripheral blood.

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for generation of standards. The target concentrations for the low, medium, and high control pools were 25, 75, and 200 pg/mL of matrix, respectively.

All steps of the assay were performed at 4°C using sodium phosphate-buffered saline supplemented with 0.2% bovine serum albumin as the assay buffer. A pool of human plasma devoid of the analyte (hypoplasma) served as assay matrix for generation of standards and quality-control samples. An optimized concentration of the proprietary protease inhibitor was added to each plasma specimen, each quality-control sample, or each NKA standard. Standard, patient plasma, or quality controls (100 μL) were preincubated in duplicate with 100 μL of working dilution of anti-NKA antiserum for 24 hours at 4°C, followed by addition of ^{125}I-labeled NKA tracer (100 μL) for an additional 24 hours at 4°C incubation.

Separation of antibody-bound tracer from free tracer was achieved by the addition of titered normal rabbit serum (200 μL) and goat anti–rabbit immunoglobulin G (200 μL).

Radioactivity in counts per minute of tracer bound to the antiserum in equilibrium with a specific dose of standard (B) (in picograms per milliliter) was expressed relative to the tracer bound (Bo) in the absence of a competing standard as a percent B/Bo. The percent B/Bo was then used to generate the calibration curve and the estimation of NKA in analyzed specimen. The effective range (ie, the lowest to the highest dose reliably measured by the optimized NKA RIA procedure) was given by the dose of standard yielding a 90% and a 10% B/Bo based on 12 assays performed over 8 months. The effective linear range of the NKA assay was determined to be 8.3 to 340 pg/mL.

Analytical Sensitivity of the ISI Method

The analytical sensitivity of the direct, extraction-free NKA RIA developed by ISI was defined by the concentration of the peptide that would reduce the binding of the tracer by 10% relative to the binding given by the analyte-free sample in Bo tubes. Twelve assays, performed over an 8-month time frame, with same

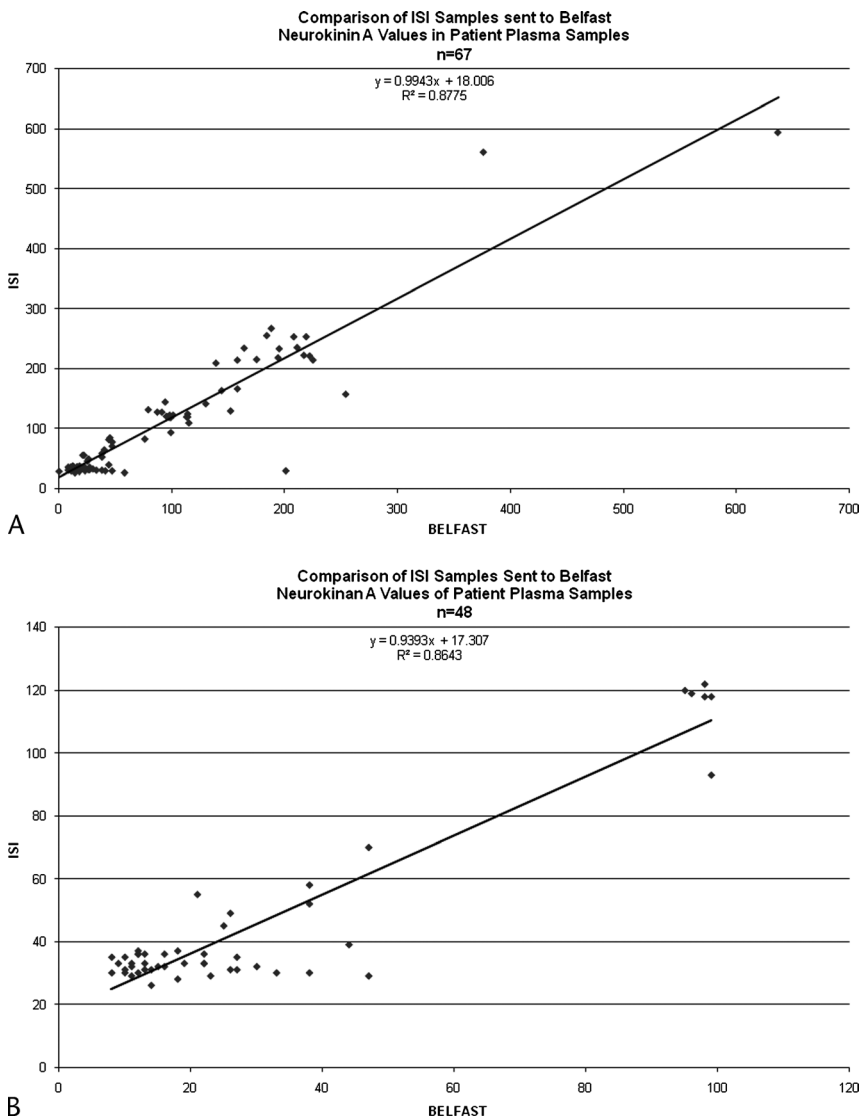

FIGURE 3. A, The regression analysis of NKA values (in pg/mL) reported from split-tube samples (n = 67) assayed in a US laboratory and Northern Ireland laboratory. These values are essentially identical (r^2 = 0.8775, $P < 0.0001$). B, The regression analysis of NKA values 0 to 100 from split-tube samples. The n value is 48, and r^2 = 0.8643.

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batch of antiserum, the same NKA peptide standard, and 4 different lots of in-house–manufactured tracer yielded a mean sensitivity of 12.5 pg/mL with a range of 8.3 to 16.7 pg/mL.

Parallelism

Samples containing high concentrations of native human NKA were serially diluted in the standard diluent. The antibody-bound radioactive NKA tracer of each dilution was plotted against the standard curve. Parallelism between the natural, immunoreactive NKA peptide found in peripheral circulation and the synthetic NKA peptide selected as the reference material is demonstrated in Figure 2. This identity of immune reaction indicates that the selected synthetic standard accurately reflects human NKA peptide(s) present in the analyzed specimen.

Reproducibility

Interassay reproducibility was determined from test results obtained for 3 quality-control pools in 12 consecutive assays. The coefficient of variation for the low pool (mean value, 42.9 pg/mL) was 8.4%; the medium pool (mean value, 101.7 pg/mL), 7.8%; and the high pool (mean value, 211.4 pg/mL), 6.4%.

Intra-assay reproducibility was determined from the analysis of 20 single test results generated for 3 quality-control pools in 1 assay. The coefficient of variation for the low pool (mean value, 38 pg/mL) was 4.6%; for the medium pool (mean value, 80.4 pg/mL), was 3.4%; and for the high pool (mean value, 146.7 pg/mL), 1.6%.

Statistical Analysis

Neurokinin A values from the 2 laboratory trials (United States to United Kingdom and United Kingdom to United States) were individually collated and placed into Excel spreadsheets. Neurokinin A values from each split-tube clinical specimen were paired and sorted in ascending order. In addition, the specimen results were segregated by the laboratory in which the sample originated. Neurokinin A values from clinical specimens from the United States were compared with NKA values obtained from the Northern Ireland laboratory by regression analysis.

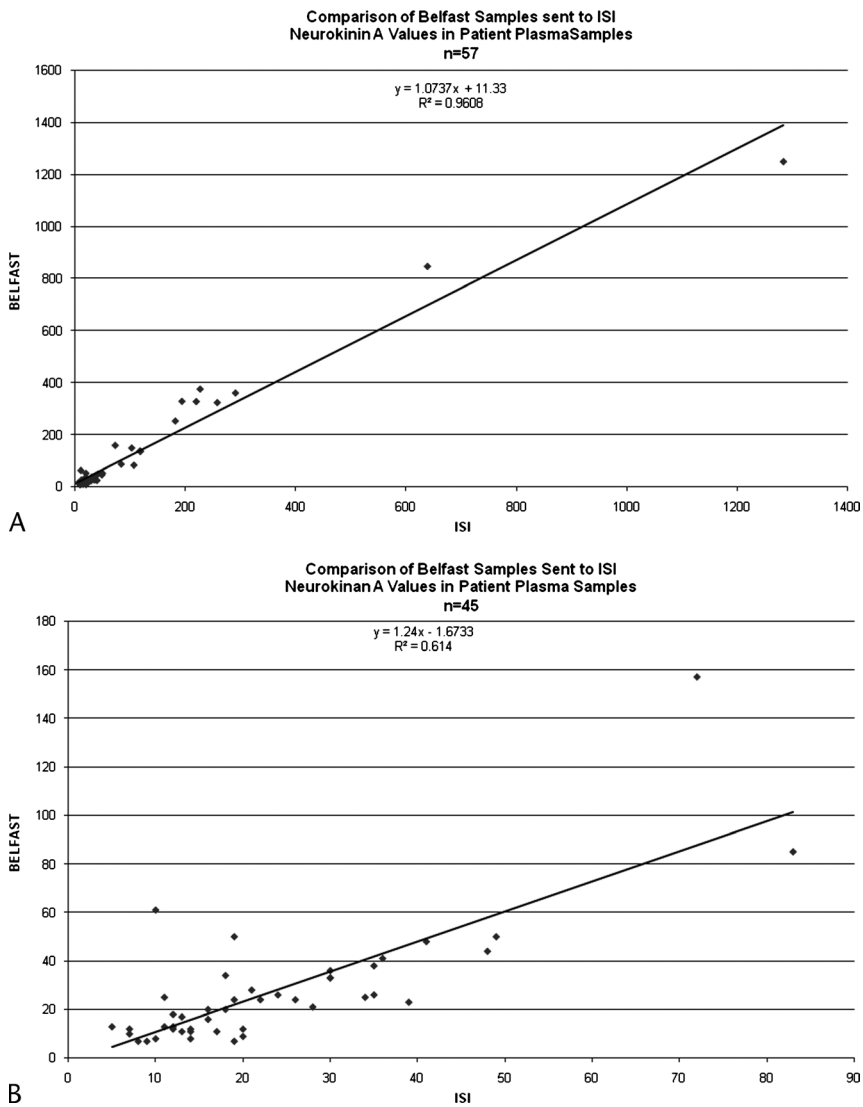

FIGURE 4. A, The result of regression analysis of NKA values (in pg/mL) from split-tube samples (n = 57) assayed in a US laboratory and Northern Ireland laboratory. These values are essentially identical ($r^2 = 0.9608$, $P < 0.0001$). B, The values from a subset of NKA 0 to 100 regraphed to show the linearity between the results from ISI and Belfast. The n value is 45, and $r^2 = 0.614$.

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RESULTS

Figures 3 and 4 summarize the comparisons of NKA data obtained from ISI samples analyzed in Belfast by an extraction-based assay and Belfast specimens assayed at ISI by a direct, extraction-free assay. The regression analysis of the NKA values from the United States sent to the United Kingdom study (n = 67 patient samples) showed a high degree of correlation ($r^2 = 0.89$), and this correlation was highly statistically significant ($P < 0.0001$) (Fig. 3). Similarly, the clinical specimens that were sent from the Belfast laboratory to the US laboratory (n = 50) also showed a high degree of correlation ($r^2 = 0.96$), and this correlation was statistically highly significant ($P < 0.0001$) (Fig. 4).

DISCUSSION

Based on the work of Ardill et al,[8] NKA values can provide highly significant prognostic data to the clinician caring for patients with well-differentiated midgut NETs. These tumors (previously called "typical" midgut carcinoids) often may have a relatively indolent clinical course. Conversely, in some patients, a high rate of tumor growth, bulky mesenteric nodal involvement, intraperitoneal metastasis, the development of liver or other distant metastasis may portend a more rapid downhill course and a decreased 5-year survival rate. The ability of the NKA assay to identify patients who have a poor prognosis and to identify a subset of patients who have the need for immediate changes in therapy may be invaluable. The role of NKA as a "prognosis marker" has been outlined by the group at the Royal Victoria Hospital in Belfast. This group is also responsible for the development of the NKA assay used to determine these prognostic indices.[4] No US laboratory, to date, has shown that its NKA assay values are comparable to the NKA assay results from the Belfast laboratory. Thus, until now, United States–based NKA assay values could not be used in conjunction with the Belfast's group survival data as a rationale for immediate and profound changes in clinical therapy. This article outlines 2 collaborative split-tube studies that tested the NKA assay results from clinical samples analyzed in both the ISI laboratory from the US and the Belfast laboratory in the United Kingdom.

This study demonstrates that samples assayed for NKA in both US and UK locations yield essentially identical "raw" results. The ability of a US and European centers to exchange NKA samples as part of a collegial and collaborative study indicates that the imagined barriers to developing international laboratory methods or assays for NET biomarkers are easily overcome. We found not only nearly identical trends between the 2 assays, but also nearly identical actual values. Given that NKA is a small molecule, it appears that the polyclonal NKA antisera increased independently in the ISI and Belfast laboratories recognized almost the same 3 or 4 epitopes of the NKA amino acid sequence. Similar studies need to be performed to cross-validate the assays for other critical NET biomarkers.[1–3] These split-tube comparison studies will be critical for future multi-institutional, multinational, and international collaborative group studies.

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