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NOTE

Determining ProGRP and isoforms in lung and thyroid cancer patient samples: comparing an MS method with a routine clinical immunoassay

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Abstract This paper compares two methods to determine the tumor marker progastrin-releasing peptide (ProGRP): as routine assay, the automated time-resolved immunofluorometric assay (TR-IFMA), which allows total ProGRP determination; and the immunocapture liquid chromatography selected reaction monitoring mass spectrometry (LC–SRM-MS) method, which additionally allows isoform differentiation. The investigation included 60 serum samples from patients suffering from various cancer diseases which may cause elevated ProGRP levels (small cell lung carcinoma; SCLC, non-small cell lung carcinoma; NCLC; and medullary thyroid cancer; MTC, as well as some with unspecific diagnosis). The two methods showed good correlation (R^2=0.887). However, the MS method determines the total ProGRP concentration systematically approximately 30 % lower than the TR-IFMA, implying that the absolute values generated by the methods are not interchangeable. The MS method gives additional information about isoform levels in the samples, providing novel insight into isoform expression on the protein level.

Keywords Biomarker · Progastrin-releasing peptide · LC–SRM-MS · Signature peptide · Immunocapture · Immunoassay · Targeted protein analysis · Bottom-up

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Introduction

Reliable determination of tumor markers is essential for exploration of their potential as diagnostic-based effective molecular cancer indicators. Progastrin-releasing peptide (ProGRP) is an acknowledged marker for small cell lung carcinoma (SCLC) [1], and other neuroendocrine tumors such as medullary thyroid cancer (MTC). In addition, slightly elevated ProGRP levels are also found in non-small cell lung carcinomas (NSCLC) with histological subtypes such as squamous cell carcinoma (SCC) and adenocarcinoma [2]. At present, immunoassays are used for measuring ProGRP. The first commercially available immunoassay for ProGRP was the Advanced Life Science Institute's (ALSI's) ProGRP ELISA [3]. Recently, a new automated time-resolved immunofluorometric assay (TR-IFMA) was developed for measuring ProGRP, which appears to provide additional prognostic information [4, 5]. ProGRP occurs in concentrations less than 58.9 pg/mL in healthy subjects (97.5-percentile reference limit) [6] and increase in case of certain pathologies.

ProGRP exists as three isoforms at mRNA level, where types 1 and 3 are more abundant than the type 2. Recently, the presence of ProGRP isoforms 1 and 3 on the protein level in patient samples as well as the ability to quantify them with LC–MS was published [2]. The method is complementary to the immunometric assays also using immunobased sample preparation [2], but utilizing selected reaction monitoring (SRM) MS as a tool for detection. SRM MS allows isoform differentiation via the bottom-up approach, carried out by detecting unique peptides that unequivocally represents its parent protein. The signature peptides are NLLGLIEAK (for all known ProGRP isoforms) [2], LSAPGSQR (ProGRP isoform 1) [2], and DLVDSLLQVLNVK (ProGRP isoform 3) [2].

This paper had two aims: (1) to compare the performance of the automated TR-IFMA and MS methods in measuring

total ProGRP in serum samples, and (2) to investigate the pattern of quantitative isoform expression on the protein level. For this purpose, 60 patient samples were collected and analyzed with both methods.

Materials and methods

Reagents and standards

All chemicals used were of analytical grade. TPCK-treated lyophilized trypsin from bovine pancreas was of sequencing grade (Sigma-Aldrich, MO, USA). Aqueous reagents were prepared with water purified by a Milli-Q system (Millipore Corporation, MA, USA)

Recombinant ProGRP products, namely short ProGRP (aa 31–98), ProGRP isoform 1 (aa 1–125+8), and ProGRP isoform 3 (aa 1–115+8), were cloned from human cDNA (OriGene® Technologies), expressed in *Escherichia coli* (Promega) using pGEX-6P-3 constructs (GE Healthcare) and purified as described elsewhere [7]. The concentration of the short ProGRP (aa 31–98) was determined by the ALSI ELISA. The concentrations of ProGRP isoform solutions were determined by TR-IFMA against the short ProGRP (aa 31–98). Stock solutions of ProGRP and internal standard (IS), NLLGLIEA[K_$^{13}C_6{}^{15}N_2$] (purity>95 %, Sigma-Aldrich) were prepared as described elsewhere [2].

Serum samples

Human serum from healthy subjects was obtained from Ullevål Hospital, OUS (Oslo, Norway), and serum samples from cancer patients were supplied by Radiumhospitalet, OUS (Oslo, Norway). The patient samples used in this study were collected when the patients first presented at OUS, at a point when most patients had already started treatment at their local hospital. Thus, the samples were collected at different time points during treatment as well as with various staging of the cancer. All serum samples were stored at −30 °C. The application of antibody approaches to study ProGRP expression in patient samples was approved by the Norwegian Regional Committee for Medical Research Ethics (REK, http://helseforskning.etikkom.no).

The TR-IFMA method

Determination of total ProGRP was performed by the automated TR-IFMA. This is an immunofluorometric method comprising mAb E146 [7], as catcher, and mAb M16 [4], as tracer, with binding sites of aa 48–52 and aa 57–61, respectively. Calibrators were prepared using purified recombinant ProGRP (aa 31–98). Because the molecular weight of the calibrators used in the two methods were different, the concentrations were considered in molarity (M) for easy comparison of the results.

Analysis of samples

The samples were treated as briefly described here (for complete description, see [4]): 100 μL of calibrator or patient serum sample and 100 μL ProGRP buffer were added to duplicate wells, followed by shaking for 1 h. After six washes, 0.2 μg/well of Eu^{3+}-labeled M16 in 200 μL ProGRP buffer was added, and the plates were incubated for another hour under continuous shaking. After six washes, 200 μL/well of enhancement solution was added, followed by incubation with shaking at room temperature for 5 min, and subsequently fluorescence was measured in a time-resolved fluorometer.

The immunocapture MS method

Quantitative measurements of total ProGRP, in addition to isoform 1 and isoform 3 was performed by the immunocapture LC–MS/MS method previously developed and described by Torsetnes et al. [2]. The validated quantification ranges were 13–7,631 pM for total ProGRP, 35–3,469 pM for isoform 1, and 20–2,047 pM for isoform 3 [2]. In the construction of standard curves (n=2 for each calibration point), the serum was depleted for endogenous ProGRP before use. Calibrators used were ProGRP isoform 1 (aa 1–125+8) and isoform 3 (aa 1–115+8). Preparation of anti-ProGRP-coated (mAb146) magnetic beads was carried out as described elsewhere [8]. This yielded an anti-ProGRP concentration of 20 μg/μL mAb E146-coated beads in a 10 mg/mL beads solution. Total ProGRP was detected by means of NLLGLIEAK, ProGRP isoform 1 by means of LSAPGSQR, and ProGRP isoform 3 by means of DLVDSLLQVLNVK.

Preparation of matrix for calibration samples

To achieve ProGRP-free serum, healthy donor serum needed to be depleted for endogenous ProGRP. This was achieved by the following procedure: (1) Add 15 μL prewashed E146-coated magnetic beads to 1 mL serum sample, (2) rotate and shake for 1 h, and (3) remove beads with endogenous ProGRP from the serum, resulting in ProGRP-free serum.

Analysis of samples

The samples were treated as briefly described here (for complete description, see [2]):

> *Immunocapture of ProGRP*: 20 μL of prewashed E146-coated magnetic beads and 1 mL serum sample were added to each vial. These were rotated and shaken for 1 h before the beads were washed with the following series

of solutions: (1) 200 μL of PBS containing 0.05 % Tween 20, (2) 200 μL of PBS, (3) 200 μL of 10 mM Tris-HCl (pH 7.4), and (4) 200 μL of 50 mM ABC buffer.

Digestion of captured ProGRP: 80 μL 50 mM freshly prepared ABC buffer containing 6 μg/mL trypsin and IS yielding a concentration of 1,250 pM was added to the magnetic beads obtained from the immunoextraction, and the solution was incubated overnight at 800 rpm and 37 °C. The vials were then briefly centrifuged before the beads were withheld by a magnet and the solution of tryptic peptides transferred to new vials. The new vials were centrifuged at 5,000 rpm for 2 min to sediment remaining trace particles from the sample, and 50 μL supernatant was subsequently transferred to vials for LC–MS/MS analysis, and 40 μL was injected and analyzed.

Results and discussion

Quantifying ProGRP variations in patient samples

Since the MS method was able to simultaneously quantify both total ProGRP and the two ProGRP isoforms, all information on ProGRP variations was acquired from a single analysis of each patient sample. The isoforms were quantified, and for the current experiment, the lower limits of quantification (LLOQs) were set to 25 pM for isoform 1 (represented by LSAPGSQR), 20 pM for isoform 3 (represented by DLVDSLLQVLNVK) and 10 pM for total ProGRP (represented by NLLGLIEAK). These levels were lower for isoform 1 and total ProGRP than those described earlier (being 13 pM for total ProGRP and 35 pM for isoform 1) [2], but were here considered as quantifiable levels after analyzing the precision (RSD<20 %) and accuracy (bias<20 %) at these lower levels (data not shown) and were found to be acceptable on the basis of the European Medicines Agency (EMA) guidelines [9]. A typical chromatogram of a patient sample as well as a blank sample is shown in Fig. 1.

Comparing the MS method with the automated TR-IFMA

Correlation between the methods

The estimated concentrations of total ProGRP in 60 patient samples obtained by the TR-IFMA and the MS methods were compared as shown in the scatter plot in Fig. 2a. It indicates a linear relationship, although the *a* term (a=0.643) of the generated linear regression calculated from the results reveals a tendency of TR-IFMA to generate higher estimations of ProGRP levels than the MS method. This implies that the

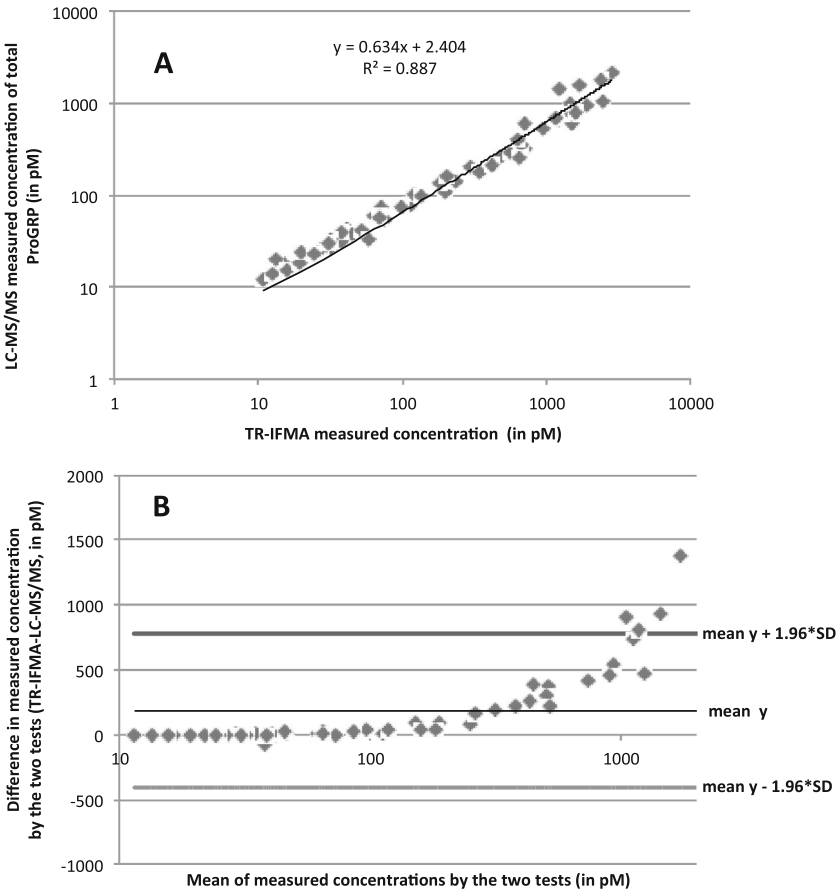

Fig. 1 A typical chromatogram of a patient sample and a ProGRP-depleted healthy serum sample. The patient sample is from a subject diagnosed with SCLC, and the measured concentrations were 127 pg/mL total ProGRP (signature peptide NLLGLIEAK), 24 pg/mL ProGRP isoform 1 (signature peptide LSAPGSQR), and 64 pg/mL ProGRP isoform 3 (signature peptide DLVDSLLQVLNVK)

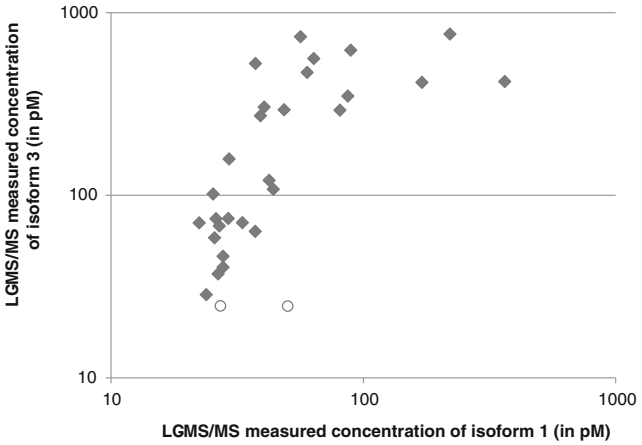

Fig. 2 Comparing measured concentrations of total ProGRP obtained by the two methods. **a** Correlation plot for total ProGRP measured with the automated TR-IFMA and the MS method. ProGRP is determined in 60 serum samples obtained from cancer patients. **b** Bland–Altman plot for total ProGRP measured with the automated TR-IFMA and the MS method. The results of the 60 serum samples are depicted through $S(x,y) = \frac{S1+S2}{2}, (S1-S2)$, where the S_1 is the result from the TR-IFMA method, and the S_2 is the corresponding result for the analysis of the same sample using the MS method. The mean of *y* values are given by the *thinnest added line*, and the *two thicker lines* give the 95 % CI of the *y* values given by −1.96×SD and +1.96×SD. These *thick lines* define the limits of agreement as bias±1.96×SD

methods may not be used interchangeably as they generate different absolute values.

Agreement of the methods

Because a good *correlation* does not necessarily imply good *agreement* between methods, a Bland–Altman plot was constructed (Fig. 2b). This plot allows one to check for systematic errors and outliers. Figure 2b shows an increase in deviating results in the samples with higher concentration levels of ProGRP, which concurs with the *a* term of 0.643 in the correlation plot. In other words, a high concentration of ProGRP gave a higher result in the TR-IFMA method compared to the LC-MS method. Owing to the very large concentration span (highest concentration more than 250 times higher than the lowest concentration), larger absolute deviations at higher concentrations are expected when methods systematically generate such different values. Nevertheless, only four of 60 patient samples (<7 %), all with high concentrations, fall outside the 95 % CI. To summarize: there is a good correlation between the methods; however, the results between the methods are not interchangeable.

Differences of the methods

Although the identical antibody (mAb E146) is utilized, there are several differences between the method design of the automated TR-IFMA and the MS method:

Firstly, the use of calibrators; the TR-IFMA uses short recombinant ProGRP (aa 31–98), whereas the MS method uses full-length recombinant ProGRP isoform 1 (aa 1–125+8) and isoform 3 (aa 1–115+8). In the absence of a defined international standard for ProGRP concentration, the TR-IFMA response is calibrated against the ALSI ELISA response to determine the concentrations of the three aforementioned calibrators. Consequently, all calibrators are standardized utilizing the one method and should under the same conditions exhibit similar results.

Secondly, the calibrator matrixes; ProGRP-depleted serum is used for the MS method and 5 % heat-treated buffered BSA for the automated TR-IFMA. Both stability and capture affinity of the different calibrators, as well as endogenous ProGRP, might be different in the two matrixes. It is also known for endogenous ProGRP that it might be unstable in serum. In addition and perhaps most likely, an additional freeze–thaw step of all patient samples before analysis by the MS method may have caused a degree of degradation, resulting in lower results for the MS method compared to the TR-IFMA, as tested previously.

The third and last aspect to be considered is the differing principles of detection. The TR-IFMA is based on epitope specificity of two ProGRP antibodies, mAb E146 and mAb M16; whereas, in the MS method, protein-specific tryptic peptides are produced after immunocapture by mAb E146 and then front-end purified by LC, before being detected by their mass-to-charge ratio by SRM MS. Though the epitope for mAb M16 and the site for signature peptide NLLGLIEAK for the MS method are somewhat overlapping, stability and/or conformation change may in different degrees alter the accessibility for mAb M16 and/or trypsin for the ProGRP variants.

All in all, the methods show good correlation and are in accordance for the determination of total ProGRP in the lower concentration ranges (<1,000 pM, see Fig. 2b). As for the systematic differences shown, these are not uncommon when comparing methods, as earlier demonstrated for the comparison of the two ProGRP immunometric assays, namely the ELISA and the TR-IFMA [5], and must also be expected between such different methods as presented here. The causes for these differences may derive from one or several of the reasons addressed above, but do not imply a difference in clinical specificity, and both methods deliver reliable, comparable results.

Exploring isoform expression

The isoform expression was explored in serum from patients with different forms of cancer where ProGRP protein expression is upregulated. All the 60 patient samples had quantifiable total ProGRP levels (levels above LLOQ), 53 of the 60 samples measured contained isoform 3 above LLOQ, and 29 of the 60 samples contained isoform 1 above LLOQ. All the

Table 1 The listed ratios on protein level are based on the quantifiable isoform levels found by the MS analysis of the 60 patient samples. The top row for protein level shows the results from all the quantifiable samples, and the bottom three rows are values subgrouped to some of the respective pathologies

Serum samples	Protein isoform 1 to total ratio ± SD	Protein isoform 3 to total ratio ± SD	Protein isoform 1 to isoform 3±SD
All patient samples	0.24±0.24 (n=29)	0.65±0.29 (n=53)	0.44±0.40 (n=29)
SCLC	0.21±0.14 (n=10)	0.56±0.15 (n=15)	0.38±0.23 (n=10)
Adenocarcinoma	0.47±0.61 (n=3)	0.53±0.43 (n=4)	0.79±0.35 (n=3)
MTC	0.16±0.09 (n=8)	0.74±0.31 (n=19)	0.25±0.10 (n=8)

29 samples containing quantifiable levels of isoform 1 also contained quantifiable levels of isoform 3; thus, a total number of 29 samples had quantifiable levels of both isoform 1 and 3. The protein isoform expression results are shown in Table 1 and Fig. 3.

As referred to in [2], three ProGRP isoforms are described to be expressed on the mRNA level. Besides the fact that mainly ProGRP mRNA types 1 and 3 (encoding ProGRP isoforms 1 and 3, respectively) are expressed, ProGRP mRNA type 1 has also been shown to be predominantly expressed over type 3 [10]. In the study presented here, the presence of mainly ProGRP isoforms 1 and 3 on the protein level seems to be in accordance with these earlier mRNA studies; however, it was seen that protein isoform 3 was in excess of isoform 1 in serum (average ratio isoform 3 to total ProGRP was 0.65± 0.29 vs. average ratio isoform 1 to total ProGRP was 0.29± 0.24, and average isoform 1 to isoform 3 ratio was 0.44±0.40, see Table 1). Figure 3 confirms this dominating expression of protein isoform 3 in the individual samples, by the 27 cases where isoform 3 is expressed in a higher concentration than isoform 1 against only two where the opposite is the case.

The isoform ratios were also grouped according to pathology for those cases where the diagnosis was available, to investigate ratios and deviations between and within the diagnosed conditions (Table 1). The table shows that the ratios between isoforms in serum samples from patients with SCLC, adenocarcinoma and MTC were somewhat different. A larger study should be performed to evaluate the diagnostic value of the isoform distribution.

Conclusion

A recently developed MS method [2] has been compared with an established immunoassay for total ProGRP to evaluate its conformity of and clinical applicability to ProGRP quantification. The two methods with different formats have been compared by analyzing 60 patient serum samples and

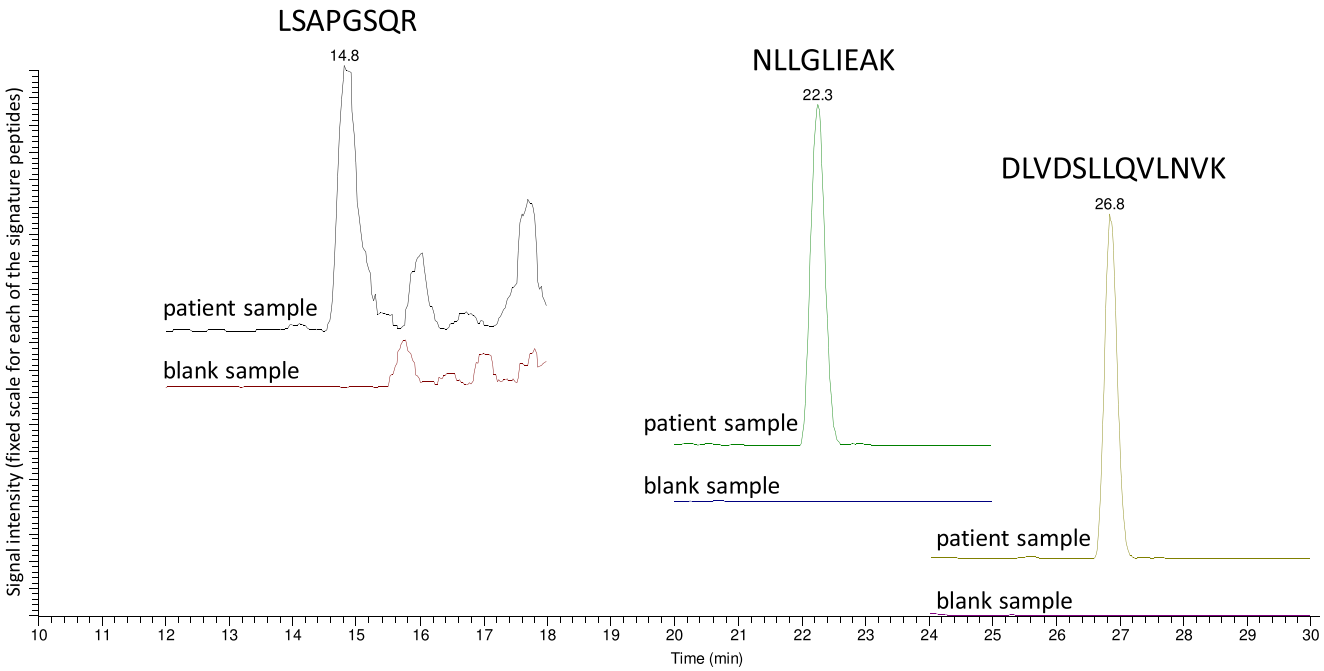

Fig. 3 The measured concentrations of isoforms 1 and 3 plotted against each other obtained with the MS method. For the cases where the isoform 3 concentration is higher than isoform 1, the measurement is marked with a *diamond symbol*. For the two samples where the isoform 1 concentration exceeded that of isoform 3 an *open circle* is assigned for the measurements

demonstrated to correlate. Interchangeability of the absolute concentration measurements is not possible because of the relative systematically lower levels in the MS method.

As a supplement, the MS method simultaneously provided quantitative determinations of isoform expression of two out of three known ProGRP isoforms in these samples. To our knowledge, this is the first paper presenting information on the endogenous isoform expression of ProGRP on the protein level. The protein isoform expression showed the predominance of isoform 3 expression relative to isoform 1, as well as great heterogeneity in the expression of various isoforms.

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