Analytical and Bioanalytical Chemistry (2018) 410:1679–1688
https://doi.org/10.1007/s00216-017-0803-y

RESEARCH PAPER

An immunocapture-LC-MS-based assay for serum SPINK1 allows simultaneous quantification and detection of SPINK1 variants

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Received: 16 October 2017 / Revised: 27 November 2017 / Accepted: 1 December 2017 / Published online: 9 January 2018

Abstract

Pancreatic secretory trypsin inhibitor Kazal type 1 (SPINK1) is a 6420 Da peptide produced by the pancreas, but also by several other tissues and many tumors. Some mutations of the *SPINK1* gene, like the one causing amino acid change N34S, have been shown to confer susceptibility to recurrent or chronic pancreatitis. Detection of such variants are therefore of clinical utility. So far SPINK1 variants have been determined by DNA techniques. We have developed and validated an immunocapture-liquid chromatography-mass spectrometric (IC-LC-MS) assay for the detection and quantification of serum SPINK1, N34S-SPINK1, and P55S-SPINK1. We compared this method with a time-resolved immunofluorometric assay (TR-IFMA) for serum samples and primer extension analysis of DNA samples. We used serum and DNA samples from patients with acute pancreatitis, renal cell carcinoma, or benign urological conditions. With the help of a zygosity score calculated from the respective peak areas using the formula wild-type (wt) SPINK1/(variant SPINK1 + wt SPINK1), we were able to correctly characterize the heterozygotes and homozygotes from the samples with DNA information. The score was then used to characterize the apparent zygosity of the samples with no DNA characterization. The IC-LC-MS method for SPINK1 was linear over the concentration range 0.5–1000 μg/L. The limit of quantitation (LOQ) was 0.5 μg/L. The IC-LC-MS and the TR-IFMA assays showed good correlation. The median zygosity score was 1.00 (95% CI 0.98–1.01, $n = 11$), 0.55 (95% CI 0.43–0.61, $n = 14$), and 0.05 (range 0.04–0.07, $n = 3$) for individuals found to be wt, heterozygous, and homozygous, respectively, for the N34S-SPINK1 variant by DNA analysis. When DNA samples are not available, this assay facilitates identification of the N34S- and P55S-SPINK1 variants also in archival serum samples.

Keywords Immunocapture · TATI · SPINK1 · Quantitative LC-MS assay

Introduction

Serine peptidase inhibitor Kazal type 1 (SPINK1, also known as pancreatic secretory trypsin inhibitor or PSTI and tumor-associated trypsin inhibitor or TATI) is a 6240 Da peptide that occurs at high concentrations in the pancreas and pancreatic fluid, where it represents 0.1 to 0.8% of the total protein [1]. The main function of SPINK1 in the pancreas is to serve as the first line of defense against premature activation of trypsinogens [1, 2]. SPINK1 is also expressed in several other normal tissues, like the kidney [3], prostate [4], stomach, small intestine, and colon [5]. In these, SPINK1 may function as a growth factor, play a role in reproduction, or modulate apoptosis and normal tissue differentiation and repair [6]. SPINK1 is also expressed in several cancers [6, 7].

Pancreatitis may be induced by inadvertent activation of trypsinogens within the pancreas. It is characterized by

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inflammation and necrosis with swelling of pancreatic cells and organelles, and loss of plasma membrane integrity. This leads to increased leakage of pancreatic enzymes and SPINK1 into circulation. As a result, the concentrations of SPINK1 and trypsinogens in plasma and urine may increase more than 100-fold [8–10]. The sensitivities of serum SPINK1, amylase, and elastase I in acute pancreatitis (AP) are similar [11], but because SPINK1 also acts as an acute phase reactant [12], it has not replaced the amylase assay in routine diagnostics of AP. There are several known mutations in the SPINK1 gene which also alter the amino acid sequence [13, 14]. The incidence of the most common variants N34S (6214 Da, dbSNP:rs17107315) and P55S (6231 Da, dbSNP:rs111966833) is 0.5–2.5% [15, 16] and 0.5–3% [14–17], respectively. However, in chronic pancreatitis (CP) patients, the respective incidences are 9.1–28.6% [16, 18–22] and 0.9–7.3% [15–17, 21]. In a Finnish population, the N34S variant was found in 7.8% of AP patients, but there was no difference in the frequency of the P55S variant between the patients and controls [21]. The mechanism behind the association of the N34S variant with pancreatitis is not clear, because it is not known to alter expression, affinity for trypsin, or trypsin inhibitory activity [23–25].

Previously described enzyme-linked immunosorbent assays (ELISAs) [26–28], a time-resolved immunofluorometric assay (TR-IFMA) [28, 29], and radioimmunoassays (RIAs) [7, 10] for SPINK1 are based on antibodies, which are not known to distinguish between different SPINK1 variants. We describe here a novel assay based on immunocapture of SPINK1 from serum and detection and simultaneous quantification of wild-type (wt) SPINK1, N34S-, and P55S-SPINK1 variants by liquid chromatography-mass spectrometry (IC-LC-MS).

Materials and methods

Samples

We used serum samples from patients with benign urological conditions, with renal cell carcinoma, and suspicion of AP. In addition, we analyzed serum samples from patients ($n = 34$) with known *SPINK1* variations confirmed by PCR amplification and minisequencing [21]. AP was diagnosed as described [30] at the Department of Surgery of Helsinki University Central Hospital. Samples from AP patients were collected during 2010–2012, from renal cell carcinoma (RCC) patients and benign controls during 2004–2006 and AP patients with known *SPINK1* variations during 1998–2004, except for one sample that was collected in 2008. Patient characteristics are described in the Table 1. All samples were stored at − 20 °C until analysis. Serum from an apparently healthy individual was used as low level (11.2 μg/L) and, when spiked with SPINK1 (63.4 μg/L), as high level quality assurance (QA) sample. The QA samples were aliquoted and stored at − 80 °C. The study was approved by the ethical committee of Helsinki University Central Hospital, Finland. Informed consent was obtained from all individuals.

Production of calibrator and internal standard

Synthetic cDNAs encoding different SPINK1 forms and optimized for expression in *Escherichia coli* were from GenScript. For the production of SPINK1 calibrator, we used a cDNA encoding wt SPINK1 (Uniprot: P00995) and for an internal standard a cDNA encoding SPINK1 with an S25T-mutation (Fig. 1). Both cDNAs lacked the part encoding signal peptide (amino acids 1–23). The SUMOstar Expression System (LifeSensors) was used for expression and purification of both wt and S24T-mutated SPINK1 peptides in house. Synthetic cDNA sequences were cloned into pE-SUMOstar(Kn) vector and expressed in *E. coli* as fusion proteins with an N-terminal SUMO protein carrying a His-tag at the N-terminus. For peptide expression, *E. coli* BL21 (DE3) cells (Novagen) were induced overnight with 0.5 mM isopropylthio-β-galactoside (Calbiochem) after the optical density reading at 600 nm of cell suspension reached 0.6. The synthesized His-tagged peptides were purified using Ni-NTA agarose (QIAGEN) and SUMO protein was released from SPINK1 polypeptides by digestion with recombinant His-tag containing SUMOstar protease. The protease and SUMO protein were removed by incubation with Ni-NTA agarose. Finally, SPINK1 was purified by immunoaffinity chromatography and reversed-phase HPLC as previously described [31]. The concentrations of wt SPINK1 and S25T-SPINK1 were measured by the TR-IFMA for SPINK1 (see below) and absorbance at 280 nm. Typical yields were ~1 mg SPINK1 per liter of bacterial culture. The SPINK1 fractions were stored at − 20 °C. A SPINK1 stock solution (73.3 mg/L) was prepared by diluting purified wt SPINK1 with 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl (Tris-buffered saline, TBS) containing 50 g/L bovine serum albumin (BSA, essentially fatty acid and globulin free ≤ 99%, Sigma Aldrich), and stored at +4 °C. Calibrators (0.5–1000 μg/L) were diluted freshly from the stock solution with TBS containing 10 mg/L BSA. To prepare the internal standard (IS) stock solution (7.8 mg/L), purified S24T-SPINK1 was diluted with TBS containing 50 g/L BSA and stored at + 4 °C. IS working solution (150 μg/L) was diluted freshly from the stock solution with TBS containing 10 mg/L BSA.

Immunocapture of SPINK1

Antibody (MAb 6E8) recognizing SPINK1 [28] was conjugated to magnetic Protein G Mag Sepharose (called “beads”) using the crosslinking protocol provided by the manufacturer (GE Healthcare). With this protocol, the antibody is covalently bound to the beads and it is not eluted with the antigen.

Table 1 Patient data. Pancreatic amylase, amylase, and C reactive protein (CRP) were analyzed with the modular clinical chemistry analyzer (Hitachi Ltd.) using reagent kits from Roche Diagnostics BmbH

Patient group	Suspected AP	RCC	Benign urological conditions	Confirmed SPINK1 genotype
Men/Women, *n*	24/8	18/14	15/16	23/11
Median age, years (range)	51 (25–86)	62 (43–87)	55 (26–79)	52 (20–86)
Median CRP, mg/L (range)	137 (6–414)	–	–	24 (5–397)
Median pancreatic amylase, U/L (range)	342 (8–2746)	–	–	–
Median amylase, U/L (range)	366 (12–3618)	–	–	616 (59–5642)
AP/other patients	29/3	–	–	6/28[a]
Etiology, *n* biliary/alcoholic/unknown	9/20/3	–	–	–
Genotype, *n* Wt/N34S-heteroz./N34S-homoz./P55S-heteroz.				11/14/3/6
Clear cell/papillary RCC, *n*	–	22/14	–	–
Hematuria/other, *n*	–	–	19/12[b]	–

[a] Chronic pancreatitis 5; alcoholic pancreatitis 10; biliary pancreatitis 5; other 7; unknown 1

[b] Nephrolithiasis 5; ureterolithiasis 3; pollacisuria 2; cystitis recidivans 1; prostate hyperplasia 1

Magnetic beads were coupled in batches (500 μL of slurry at a time, 1 mg MAb/100 μL bead slurry) and the coupled beads were stored at + 4 °C.

To 100 μL of serum, QA samples and calibrators 20 μL of IS (150 μg/L) was added. The calibrators were forwarded for LC-MS analysis without capturing. SPINK1 in QA and serum samples was captured using 20 μL of magnetic anti-SPINK1 bead slurry, i.e., 4 μL of beads. The beads were first washed twice with TBS. Then, 100 μL of sample with IS was added to the beads and incubated for 15 min with gentle shaking at room temperature (Vortex-Genie 2 with a multiple sample head, Scientific Industries). The beads were washed twice with 2 M urea in TBS and once with TBS. Bound SPINK1 was eluted from the beads by adding 50 μL of 0.1% TFA and incubating for 20 min with shaking. The supernatant was removed and the elution was repeated. The supernatants were combined and the eluted sample was centrifuged for 1 min at 1100 g to remove possible residual magnetic particles.

LC-MS

The instrumentation consisted of an Agilent 1200 liquid chromatography (Agilent Technologies) and a 4000 QTRAP mass spectrometer (AB Sciex) equipped with a Turbo-V electrospray ion source. We used a Polaris C18-A column (3 μm particle size, 50 × 2 mm) with a Polaris C18-A guard column (Agilent Technologies). Elution buffers were 0.1% formic acid (FA) in water (A) and 0.1% FA in methanol (B). After injection of the sample (20 μL), the proportion of buffer B was ramped from 20 to 100% in 3 min and maintained at 100% for further 3 min. The proportion of B was then reduced to 20% in 0.5 min and the column was equilibrated for further 3.5 min. The flow rate was 300 μL/min. The ion source spray voltage was 5500 V and temperature 500 °C. The gas settings were curtain gas 55 psig, nebulizer gas 50 psig, heater gas 45 psig, and collision gas setting 10. For detection and quantification, we used pseudo-MRM with the following transitions (*m*/*z*) with 250 ms dwell time: 1041.4/1041.4 (SPINK1, $z = 6$, for quantification), 1249.4/1249.4 (SPINK1, $z = 5$, for confirmation), and 1043.5/1043.5 (IS, $z = 6$), and with dwell time 100 ms: 1036.7/1036.7 (N34S-SPINK1, $z = 6$) and 1039.5/1039.5 (P55S-SPINK1, $z = 6$).

Analytical validation of the assay

The limit of detection (LOD) was based on a signal-to-noise ratio (S/N) of 3 ($n = 10$). Linearity was determined by preparing eight calibrators with concentrations of 0.5–1000 μg/L in

A

MKVTGIFLLS ALALLSLSGN TGADSLGREA KCYNELNGCT KIYDPVCGTD GNTYPNECVL

CFENRKRQTS ILIQKSGPC

B

MKVTGIFLLS ALALLSLSGN TGADTLGREA KCYNELNGCT KIYDPVCGTD GNTYPNECVL

CFENRKRQTS ILIQKSGPC

Fig. 1 The protein sequence of SPINK1. Protein sequence of wild-type SPINK1 (**a**). The signal sequence is in gray. N34 and P55 are underlined. The sequence of mutated (S25T) SPINK1 used as internal standard (**b**). The mutated threonine is bolded

TBS containing 10 mg/L BSA. Evaluation of the resulting calibration curves was performed with 12 separately prepared dilutions. The stability of calibrator solutions was studied by preparing six calibrator solutions (1–500 μg/L), which were aliquotted and they were kept either at − 20 °C or at + 4 °C for 1 week to 3 months. The stability was estimated by calculating the coefficient of variation (CV%) of relative peak areas (calibrator peak area / IS peak area). The calibration curves were derived using $1/x^2$ weighted linear least-squares regression by the quantitation option of the Analyst 1.5 software (AB Sciex). The accuracy (mean relative error, RE%) and the precision (CV%) were calculated. The limit of quantitation (LOQ) and linear range were defined as the lowest concentration and range, respectively, which could be measured with an inaccuracy and imprecision < 20%. Intra- and inter-assay variation were calculated from 14 replicates in one and 15 consecutive assays, respectively. Recovery of added SPINK1 was calculated from three samples supplemented with 0 μg/L, 20 μg/L, or 100 μg/mL of recombinant SPINK1. The matrix effect was calculated from three immunopurified samples supplemented with 0, 10, or 300 μg/L of purified SPINK1 and 25 μg/L of IS, respectively. To avoid carry-over, we recommend sampler needle and injector valve washing procedures. The effect of potential interfering substances is described in the results.

Time-resolved immunofluometric assay

The time-resolved immunofluorometric assay (TR-IFMA) for SPINK1 was performed as previously described [28, 29]. Briefly, MAbs F62-6E8 and F62-11B3 were used as capture and tracer antibodies, respectively. F62-11B3 was labeled with europium (Eu) using isothiocyanatophenyldiethylenetriamine-N1,N2,N3,N4-tetraacetate chelated with Eu(III) [32]. Microtitration wells were coated with F62-6E8 and 25 μL of calibrators (from 0.5 to 90 μg/L) and samples together with 200 μL DELFIA® assay buffer (PerkinElmer) were added to the wells and incubated at room temperature for 1 h. All incubations were performed under constant shaking (DELFIA® Plateshake, PerkinElmer). The wells were washed twice with DELFIA® Wash solution (PerkinElmer) and 50 ng of Eu-labeled tracer in 200 μL of assay buffer was added per well and incubated for 30 min. After four washes, DELFIA® Enhancement solution (PerkinElmer) was added with a Delfia® plate dispenser (PerkinElmer) and the fluorescence measured for 1 s per well using a Victor2 1420 Multilabel HTS Counter (PerkinElmer).

Statistical methods

The coefficient of variation, Student's *t* test, and Bland-Altman plot were calculated with Analyse-it for Microsoft® Excel 2003 (version 2.04, http://www.analyse-it.com/). Other analyses were performed with R (version 2.13.0) package "psych" (version 1.2.8, http://personality-project.org/r/), "epicalc" (version 2.15.1.0, http://CRAN.R-project.org/), and with "MethComp" (version 1.22, http://bendixcarstensen.com/MethComp/).

Results

Validation of the LC-MS assay for SPINK1

SPINK1 and the IS eluted at a retention time of 3.3 min (Fig. 2). The LC-MS assay for SPINK1 was linear over the concentration range of 0.5–1000 μg/L, but as working calibrators we routinely used 1–500 μg/L (Fig. 3). Calibrators at concentrations of 2–500 and 5–500 μg/L were stable for 2 months at − 20 °C and at + 4 °C, respectively. The LOD was 0.15 μg/L and the LOQ was 0.5 μg/L. The intra-assay CVs were 6.4 and 4.2% and the inter-assay CVs 17.7 and 16.4% at concentrations of 11 and 75 μg/L, respectively. The recovery of added SPINK1 (20 and 100 μg/L) in serum samples containing 11.2, 50.3, and 273 μg/L endogenous SPINK1 was 87–100%. When spiked into immunopurified serum, we observed ionization suppression to 88 and 58% for 10 and 300 μg/L of SPINK1, respectively, and to 77% for the IS (25 μg/L). As calculated from the peak areas of samples spiked with SPINK1 (10 and 300 μg/L) or IS (25 μg/L) either pre- or post-immunocapture, the recovery of the immunopurification was 91 and 129% for SPINK1, respectively and 94% for the IS. When measured by TR-IFMA, the recovery for SPINK1 was 100%.

Detection of SPINK1 variants by LC-MS

The *m*/*z* difference of the sextuply charged ions of wt SPINK1 (6240 Da), N34S- (6214 Da), P55S- (6231 Da), and S25T-SPINK1 (6255 Da) used as IS allows mass spectrometric analysis of each SPINK1 form separately. The mean SPINK1 concentration in serum samples from AP patients with confirmed N34S-SPINK1 ($n = 17$) or P55S-SPINK1 ($n = 6$) variant was 143 μg/L (95% CI 49.8–164 μg/L) as measured by the TR-IFMA. The mean wt SPINK1 concentration by LC-MS assay was 67.2 μg/L (22.1–81.7 μg/L) and the mean total SPINK1 concentration, i.e., the sum of wt SPINK1, and N34S-SPINK1 or P55S-SPINK1 was 107 μg/L (49.8–163.4 μg/L).

To estimate the ability of our LC-MS assay to distinguish between homozygous and heterozygous SPINK1 variation carriers, we compared the peak areas of the samples from individuals with confirmed *SPINK1* genotype [21]. The peak area of wt SPINK1 and N34S-

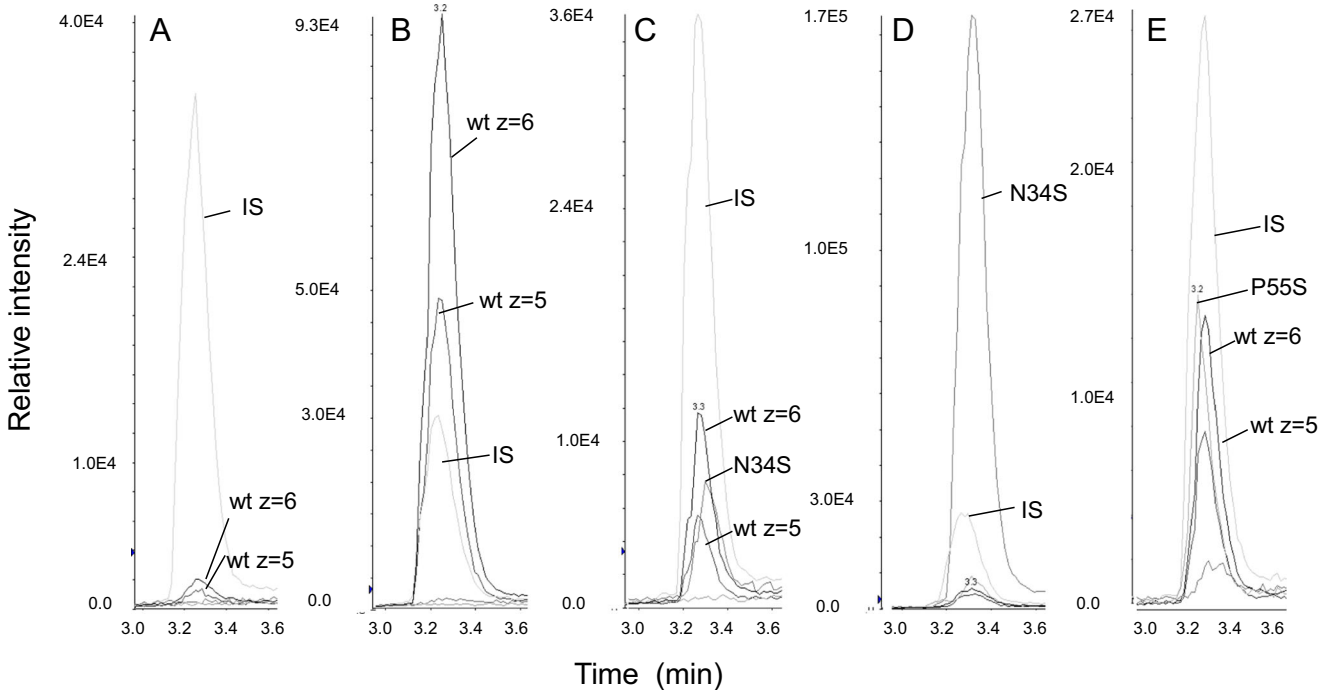

Fig. 2 LC-MS chromatograms. LC-MS chromatograms of a 5 μg/L recombinant SPINK1 calibrator (**a**) and samples from confirmed wild-type (**b**), heterozygous N34S- (**c**), homozygous N34S- (**d**), and heterozygous P55S-SPINK1 (**e**) individuals. The lines from black to the lightest gray represent wt SPINK1 *m/z* 1041.4 ($z = 6$) and *m/z* 1249.4 ($z = 5$), N34S-SPINK1 (*m/z* 1036.7, $z = 6$), P55S-SPINK1 (*m/z* 1039.5, $z = 6$), and IS (*m/z* 1043.5, $z = 6$), respectively. *Y*-axis shows the peak height (cps) and *x*-axis the chromatographic elution time (min)

SPINK1 is approximately equal in samples from heterozygous subjects (Fig. 2c). However, in the chromatogram of a homozygous N34S-SPINK1 carrier's sample (Fig. 2d), only a minor if any signal of wt SPINK1 is seen. In order to numerically represent the apparent genotype based on LC-MS, we calculated a zygosity score as the ratio of wt SPINK1 to total SPINK1, i.e., wt SPINK1/(mutated SPINK1 + wt SPINK1), from the respective peak areas of the samples. In samples from confirmed homozygous N34S-*SPINK1* carriers ($n = 3$), confirmed heterozygous N34S-*SPINK1* carriers ($n = 13$), and control subjects with confirmed wt *SPINK1* ($n = 15$), the median

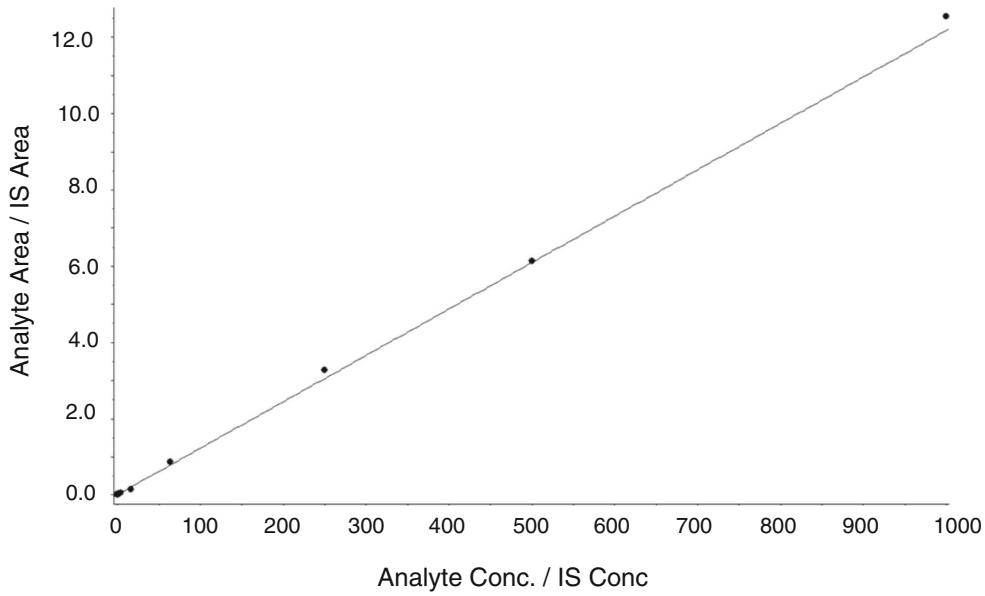

Fig. 3 Calibration curve of the IC-LC-MS analysis SPINK1 calibration curve showing the linear range of the assay

zygosity score was 0.05 (range 0.04–0.07), 0.55 (95% CI 0.43–0.61), and 1.00 (95% CI 0.98–1.01), respectively (Fig. 4). The result of one heterozygous N34S carrier (1.03) was excluded as an outlier after repeated DNA and LC-MS analyses. Patients with confirmed P55S-*SPINK1* (Fig. 2e) variant were all heterozygous ($n = 6$) with a median zygosity score of 0.56 (range 0.53–0.76). Variability of the zygosity score was studied with repeated assays ($n = 5$–7) of each different type of patients. The coefficient of variation in the zygosity score was the lowest (0.8–5.0%) for the wild-type samples (range 0.88–1.02), 4.9–21.5% for heterozygous N34S or P55S samples (range 0.31–0.76), and the highest (46.1–73.5%) for homozygous N34S samples (range 0.03–0.29).

Therefore, we suggest that a zygosity score > 0.8, 0.3–0.8, and < 0.3 indicates a wt, heterozygous, and homozygous genotype, respectively.

The effect of interfering substances

To study the effects of potential interfering factors to our assay, we selected among our patient samples (stripped of identifiers) those with elevated rheumatoid factor (449 and 986 IU/mL), lipemia (L-index 76 and 347 by Roche Modular clinical chemistry analyzer), monoclonal gammopathy with IgG6 (56 g/L), IgM1 (21 and 29 g/L), IgA2 (45 g/L) (by capillary electrophoresis and agarose gel electrophoresis followed by immunofixation), and one sample with interference in our THS assay (21.5 mU/L with Abbott Architect immunoanalyzer). After this sample was treated with heterophilic blocking tube (HBT from Scantibodies Laboratory, Inc., Santee, CA) and reanalyzed, the TSH result was 1.5 mU/L. Therefore, the interfering factor was likely to be heterophilic antibodies.

Serum samples from pancreatitis patients with confirmed wild-type or heterozygous N34S genotype (by DNA analysis) were diluted 1:2 with interfering serum samples. Interfering substances were found to cause decreased SPINK1 results (mean 74%, range 54–115% of expected concentration, Table 2). On the other hand, the interference was sample dependent, i.e., the change in expected concentration of two different patient samples by one interfering sample varied between 54–115% (rheumatoid factor) and 55–101% (monoclonal IgG6).

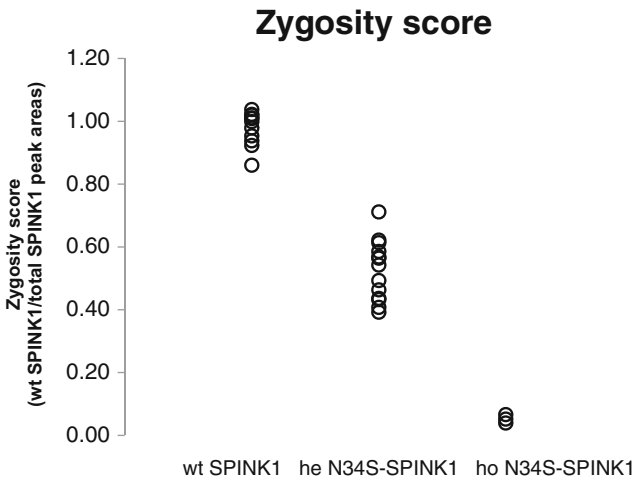

Fig. 4 Calculated zygosity scores according to confirmed genotype. Calculated zygosity scores (wt SPINK1/total SPINK1 peak areas) are plotted separately for samples from confirmed wt individuals (wt SPINK1), heterozygous N34S-SPINK1 carriers (he N34S-SPINK1), and homozygous N34S-SPINK1 carriers (ho N34S-SPINK1). The result (1.03) of one heterozygous individual is deleted as an outlier

Comparison of TR-IFMA and LC-MS assay for SPINK1

In addition to the patients with previously identified *SPINK1* variants, we analyzed 95 serum samples from patients with RCC, benign urological conditions, and suspected for AP. In AP patients, the mean serum concentration of SPINK1 was 243 μg/L (95% CI 178–307 μg/L) by the TR-IFMA and 274 μg/L (95% CI 181–368 μg/L) by the LC-MS assay. In RCC patients, the concentrations were 32.2 μg/L (95% CI 18.8–45.6 μg/L) and 45.4 μg/L (95% CI 16.9–73.9 μg/L) and in benign controls 11.3 μg/L (95% CI 9.7–12.9 μg/L) and 12.6 μg/L (95% CI 10.7–14.5 μg/L), respectively. There were no significant differences between SPINK1 concentrations as measured by the TR-IFMA and the LC-MS assay except in samples from benign controls ($p < 0.05$) (Fig. 5). The results by TR-IFMA (x) and LC-MS (y) correlated according to an equation $y = 1.17x - 13.6$ (Deming regression, $Sy|x = 0.498$, $R^2 = 0.94$, $n = 95$).

We observed by IC-LC-MS an apparent N34S-SPINK1 variant in 4 of 32 (12.5%) samples from suspected AP and in 1 of 32 (3.1%) samples from RCC patients. The genotype of these patients has not been confirmed by DNA analysis. The zygosity scores were 0.58, 0.55, 0.35, 0.54, and 0.05. This suggests that one of the patients is a homozygous and four are heterozygous N34S-*SPINK1* carriers. We did not see a P55S-SPINK1 variant in patient samples that were not previously genotyped.

Discussion

We have developed an immunocapture-LC-MS assay for simultaneous detection and quantification of serum wt SPINK1, and N34S-SPINK1 and P55S-SPINK1 variants. The assay employs a SPINK1 antibody coupled to magnetic beads, recombinant SPINK1 calibrator, and a

Table 2 The effect of interfering substances to SPINK1 concentration as measured by immunocapture-LC-MS. Serum samples were diluted 1:2 with samples containing the denoted amount of interfering substance. Theoretical SPINK1 concentration was calculated as the mean of SPINK1 concentration measured in the interfering sample and the patient sample

	Measured SPINK1 (μg/L)			Theoretical concentration	
Interfering factor	Interf. sample	Patient sample	Interf:Patient 1:1	μg/L	%
Rheumatoid factor 449 IU/mL	90	518	164	304	54
	100	45	83	72	115
Rheumatoid factor 986 IU/mL	27	518	156	272	57
	21	45	23	33	70
Lipemic index 76	80	518	224	299	75
	56	45	36	50	71
Lipemic index 347	36	518	197	277	71
	26	45	20	35	57
Monoclonal IgG6 56 g/L	53	518	158	285	55
	46	45	46	46	101
Monoclonal IgM1 21 g/L	137	518	230	328	70
Monoclonal IgM1 29 g/L	56	45	33	51	64
Monoclonal IgA2 45 g/L	70	518	202	294	69
	77	45	68	61	111
Heterophilic interference	14	256	93	135	69
	36	45	29	40	71
Mean					74
Min					54
Max					115

recombinant S25T-SPINK1 internal standard. The assay range is wide (0.5–1000 μg/L). The sensitivity (0.5 μg/L) is comparable to that of a previously described TR-IFMA (0.13 μg/L) and ELISA (0.8 μg/L) for SPINK1 [28] and clearly better than that of a former commercial radioimmunoassay (7 μg/L). The high sensitivity and the wide assay range of our new assay are advantageous both in clinical and research settings.

The present assay is based on pseudo-MRM employing low collision energy to reduce possible isobaric interferences.

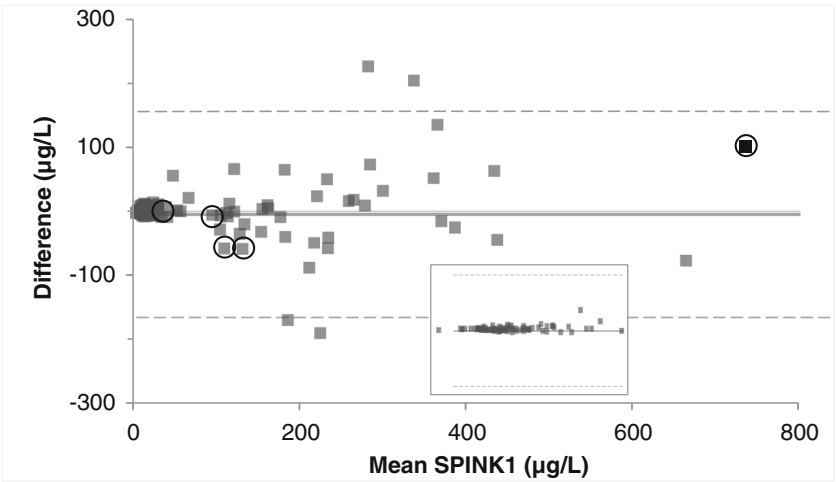

Fig. 5 Comparison of TR-IFMA and LC-MS results. Serum samples from patients suspected for AP ($n = 32$), with RCC ($n = 32$) and with benign urological conditions ($n = 31$) were measured by IC-LC-MS and TR-IFMA. In the Bland-Altman plot, the absolute difference between the two methods is plotted against their mean. Gray line shows identity and dotted line 95% limits of acceptance. The insert shows comparison of SPINK1 concentrations 4.7–96.6 μg/L with a log scale *x*-axis. One sample from an AP patient with a SPINK1 concentration 713 μg/L by the TR-IFMA and 1440 μg/L by the IC-LC-MS assay is not shown. Samples with newly found apparent heterozygous N34S-SPINK1 variants are circled

Especially at low concentrations, the mean SPINK1 concentrations were slightly higher by our IC-LC-MS than immunoassay suggesting a minor bias. In general, multiple reaction monitoring (MRM)-based assays are more specific and sensitive. However, an MRM assay of 6240 Da SPINK1 was not possible without an extra step of enzymatic digestion. We found that common known interfering factors may decrease the measured SPINK1 concentration. Thus, it should be kept in mind that immunocapture in non-diluted serum may be prone to interference, which is also the case with conventional immunoassays. The newly developed assay for total serum SPINK1 and the previously described TR-IFMA for SPINK1 [28] show relatively good correlation. Therefore, we consider the present straightforward pseudo-MRM assay suitable for quantification of SPINK1 and especially for detection of SPINK1 variants.

In LC-MS analysis, an ideal IS corrects for the recovery during sample preparation and chromatography and for the matrix effects during ionization. The most common practice is to employ a stable isotopically labeled (SIL) IS especially for small molecules. The use of an analog peptide is an alternative for SIL in peptide assays. A non-isotopic mutated IS peptide, in which leucine was replaced with alanine, has been compared to a SIL peptide for analysis of NAD(P)H:quinone oxidoreductase 1 (NQO1) [33]. NQO1 was quantified in extracts of cells and tumor tissues and there was no significant differences in the results between these two ISs. We used mutated recombinant S25T-SPINK1 as an internal standard. Such a mutation has not been described in endogenous SPINK1. Replacement of serine with threonine in SPINK1 was not expected to alter the chemical properties of the peptide. Indeed, the chromatographic and ionization properties of wt SPINK1 and S25T-SPINK1 were found to be similar. The acceptable correlation of our IC-LC-MS assay with TR-IFMA for SPINK1 suggests that S25T-SPINK1 is suitable for use as an IS.

Thus far, SPINK1 has been quantified by immunoassays often with high sensitivity. The selectivity depends on the antibodies employed, but immunoassays may lack specificity [34, 35]. A unique property of MS-based methods compared to immunoassays is the ability to detect different isoforms [36] and posttranslational modifications [31, 37]. For example, four different forms of Ras protein have been detected and quantified by LC-MS in tumor tissue, premalignant pancreatic cyst fluids, and cell lines [36]. However, only the relative abundance of these isoforms, not the absolute quantity, could be determined. Mass spectrometric detection of wt SPINK1, N34S-, and P55S-SPINK1 has been demonstrated earlier [31], but the method was not quantitative. We show that by using S25T-mutated SPINK1 as an IS, it is possible to quantify wt SPINK1, N34S-, and P55S-SPINK1 in serum samples. As expected, the concentration of wt SPINK1 by LC-MS was much lower than total SPINK1 concentration by TR-IFMA in serum samples from heterozygotes, but the total SPINK1 concentration (wt SPINK1 + mutated SPINK1) by LC-MS was in line with TR-IFMA results. This finding supports that the quantitative performances of S25T-, N34S-, P55S-SPINK1, and wt SPINK1 in our pseudo-MRM assay are similar.

We found that the IC-LC-MS analysis can be used to determine the apparent zygosity of the variants N34S- or P55S-*SPINK1* with the help of zygosity score. The score can be calculated based on the ratio of the peak areas of wt- and total SPINK1. By this means, the zygosity score of all confirmed wt subjects ($n = 11$), homozygous N34S carriers ($n = 3$), and 13 of 14 heterozygous subjects were in accordance with the DNA data. According to the zygosity score, one subject appeared to have repeatedly a wt phenotype (score 1.03) despite the heterozygous genotype. There are several possible explanations for this discrepancy. First, it is possible that both alleles are not always expressed equally at the protein level. The proportion of N34S-SPINK1 has been found to be higher than that of wt SPINK1 in urine ($n = 12$) and sera ($n = 2$) of heterozygous pancreatitis patients as shown earlier in a limited study [31]. On the other hand, this is not supported by our present data. Secondly, it is possible that yet another mutation results in the same m/z as that for wt SPINK1. Thirdly, a human mistake in sample tagging is possible. The number of patients ($n = 34$) with confirmed *SPINK1* variants in our study is limited. Therefore, further studies comparing the zygosity score and DNA analysis are warranted. The current results are, however, promising, and may provide a new DNA-free detection of known variants of SPINK1.

The presence of N34S-*SPINK1* variant, but not the zygosity, has been shown to associate with increased risk of developing CP after a sentinel AP incident [16, 21, 38–40]. However, the variant does not increase the risk of the first incident. The occurrence of the variant in Finnish general population is 2.5% [16, 21] and in patients with AP and CP, it is 7.8% [21] and 12% [16], respectively. Thus, the occurrence of N34S-SPINK1 in samples from AP (13%) and benign urological disease (1.6%) patients whose genotype has not been confirmed by DNA analysis is in line with earlier reports. Sentinel AP patients carrying the N34S-*SPINK1* variant have been shown to be about 19 times more prone to develop recurrent attacks [20]. Therefore, identification of N34S-*SPINK1* carriers with an initial attack of AP could be used to prevent recurrent attacks or progression of AP to CP. Furthermore, our new assay enables identification of SPINK1 variants also in archival samples when DNA is not available.

In conclusion, we have developed an immunocapture-LC-MS assay for serum SPINK1 and compared it with an immunoassay. The recombinant mutated SPINK1 used as an internal standard offers a cost-effective alternative to stable isotope-labeled peptides. In addition to wt SPINK1, our new assay facilitates detection and quantification of N34S- and

P55S-SPINK1 variants known to associate with pancreatitis. Moreover, by calculating a wt-to-total SPINK1 ratio, it is possible to determine the apparent zygosity of the variant without DNA analysis. Therefore, this assay could be used for variant screening and may help identifying patients with increased risk for CP due to *SPINK1* variants.

Acknowledgements The authors thank Ms. Maarit Leinimaa and Ms. Marianne Niemelä for expert technical assistance. This work was supported by the grants from the Academy of Finland, Finska läkaresällskapet, Sigrid Jusélius Foundation, Medical Faculty of the University of Helsinki, Biomedicum Helsinki Foundation, and Timo Lehtonen Urology Fund.

Compliance with ethical standards S. Ravela and L. Valmu work currently at ThermoFisher Scientific, but S. Ravela was a University of Helsinki employee and L. Valmu was a Finnish Red Cross employee at the time of the study. ThermoFisher Scientific has not participated nor supported this study. The authors have no other conflict of interests.

The study was approved by the ethical committee of Helsinki University Central Hospital, Finland. Informed consent was obtained from all individuals.

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