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Cancer Diagnostics

LC-MS/MS Quantification of Zn-α2 Glycoprotein: A Potential Serum Biomarker for Prostate Cancer

Olga P. Bondar, David R. Barnidge, Eric W. Klee, Brian J. Davis, and George G. Klee*

Background: **Zn-α2 glycoprotein (ZAG) is a relatively abundant glycoprotein that has potential as a biomarker for prostate cancer. We present a high-flow liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for measuring serum ZAG concentrations by proteolytic cleavage of the protein and quantification of a unique peptide.**

Methods: **We selected the ZAG tryptic peptide ^{147}EIPAWVPEDPAAQITK^{162} as the intact protein for quantification and used a stable isotope-labeled synthetic peptide with this sequence as an internal standard. Standards using recombinant ZAG in bovine serum albumin, 50 g/L, and a pilot series of patient sera were denatured, reduced, alkylated, and digested with trypsin. The concentration of ZAG was calculated from a dose–response curve of the ratio of the relative abundance of the ZAG tryptic peptide to internal standard.**

Results: **The limit of detection for ZAG in serum was 0.08 mg/L, and the limit of quantification was 0.32 mg/L with a linear dynamic range of 0.32 to 10.2 mg/L. Replicate digests from pooled sera run during a period of 3 consecutive days showed intraassay imprecision (CV) of 5.0% to 6.3% and interassay imprecision of 4.4% to 5.9%. Mean (SD) ZAG was higher in 25 men with prostate cancer [7.59 (2.45) mg/L] than in 20 men with nonmalignant prostate disease [6.21 (1.65) mg/L, $P = 0.037$] and 6 healthy men [3.65 (0.71) mg/L, $P = 0.0007$].**

Conclusions: **This LC-MS/MS assay is reproducible and can be used to evaluate the clinical utility of ZAG as a cancer biomarker.**

Zn-α2-glycoprotein (ZAG)[1] is a glycoprotein with a molecular mass of ~41 000 Da and a crystal structure similar to that of a class I major histocompatibility complex *(1, 2)*. Biochemically, ZAG stimulates lipid degeneration in adipocytes and appears to be involved in cachexia, a wasting syndrome that can affect people with cancer, AIDS, and other terminal illnesses *(3, 4)*. ZAG appears naturally in most body fluids, such as blood *(5)*, sweat *(6)*, seminal fluid *(7)*, breast cyst fluid *(8)*, cerebrospinal fluid *(9)*, and urine *(10)* and is also found in secretory epithelial cells of the liver and the gastrointestinal tract *(11)*.

Previous studies employing techniques such as immunohistology and 2-dimensional electrophoresis have reported that ZAG is overexpressed in certain malignant tumors and thus may serve as a potential cancer biomarker *(12, 13)*. ZAG quantification in serum by immunoassay found circulating concentrations ranging from 40 mg/L in healthy individuals to 120 mg/L in some diseased persons *(14)*. In this study we used liquid chromatography–tandem mass spectrometry (LC-MS/MS), at a high LC flow rate commonly used in the clinical laboratory (250 μL/min), combined with proteolysis, to quantify ZAG in serum. We also determined whether ZAG could be used as a specific biomarker for prostate cancer (PCa).

Materials and Methods

RECOMBINANT ZAG PROTEIN STANDARD

The recombinant ZAG protein used in this study was a gift from Dr. Bjorkman (Division of Biology, Howard Hughes Medical Institute, California Institute of Technology). The protein was generated in CHO cells by transfection with a ZAG vector using Lipofectamine 2000 (Invitrogen) *(2)*. The recombinant ZAG stock concentration was found to be 1.2 g/L as determined by BCA total protein analysis (Pierce). The purity of the protein was

Department of Laboratory Medicine and Pathology, Mayo Clinic and Mayo Foundation, Rochester, MN.

* Address correspondence to this author at: Mayo Clinic and Mayo Foundation, 200 First St. SW, Rochester, MN 55905. Fax 507-284-4542; e-mail klee.george@mayo.edu.

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[1] Nonstandard abbreviations: ZAG, zinc-α2 glycoprotein; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PCa, prostate cancer; BSA, bovine serum albumin; LOQ, limit of quantification; LOD, limit of detection.

estimated to be >95% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 1A, inset). Standard curves were generated by diluting the recombinant ZAG into 5% bovine serum albumin (BSA) in phosphate-buffered saline (10 mmol sodium phosphate, 150 mmol sodium chloride, pH 7.2).

SERUM DIGESTION

All digests were performed on 125 μL aliquots of serum. No prior purification or removal of high-abundance proteins such as serum albumin was performed. Samples were first denatured with 6 mol/L urea and followed by reduction in 15 mmol/L dithiothreitol for 60 min at 40 °C. Samples were then alkylated with 50 mmol/L iodoacetamide for 60 min in the dark at room temperature. After reduction and alkylation we decreased the urea concentration by diluting the samples to 490 μL with 50 mmol/L hydroxymethylaminoethane, pH 8, containing 10 mmol/L $CaCl_2$. We then added 1 mg of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (Sigma-Aldrich) and digested the sample overnight at 37 °C.

LC-MS/MS

A Waters Q-TOF Premier quadrupole time-of-flight mass spectrometer was used to identify and acquire the relative abundances of all the tryptic peptides generated from the digest of recombinant ZAG. A more thorough explanation of the LC-MS/MS conditions used can be found elsewhere *(15)*. The tryptic peptide from ZAG found to have the greatest response by this method was ^{147}EIPAWVPFDPAAQITK^{162} (hereafter referred to as tpZAG147–162). The sequence EIPAWVPFDPAAQITK was found to be specific to Chain D, Human Zinc-A-2-Glycoprotein (accession no. 1ZAG_D, gi:7246026) via a BLAST search, suggesting that this sequence could be used to specifically quantify ZAG. This same tryptic peptide for ZAG was recently described by Anderson and Hunter *(16)*.

LC-MS/MS CONDITIONS

Absolute quantification was performed using a CTC Analytics HTC PAL autosampler (LEAP Technologies), a Shimadzu 10-AD binary pumping system, and an API 5000 triple quadrupole mass spectrometer (Applied Biosystems). For each run we injected a total of 10 μL of sample onto a 50 × 2.1 mm TARGA C_{18} column (Higgins Analytical) at a flow rate of 250 μL/min, with a total run time of 30 min. The gradient used consisted of solvent A (water, 4 mmol/L ammonium acetate, 1 g/L formic acid) and solvent B (methanol, 4 mmol/L ammonium acetate, 1 g/L formic acid) starting at 5% B for 2 minutes, ramping to 95% B over 24 min, holding at 95% B for 2 min, back to 5% B in 1 min, and then holding to 30 min. The API 5000 instrument source parameters were CAD: 10, CUR: 30,

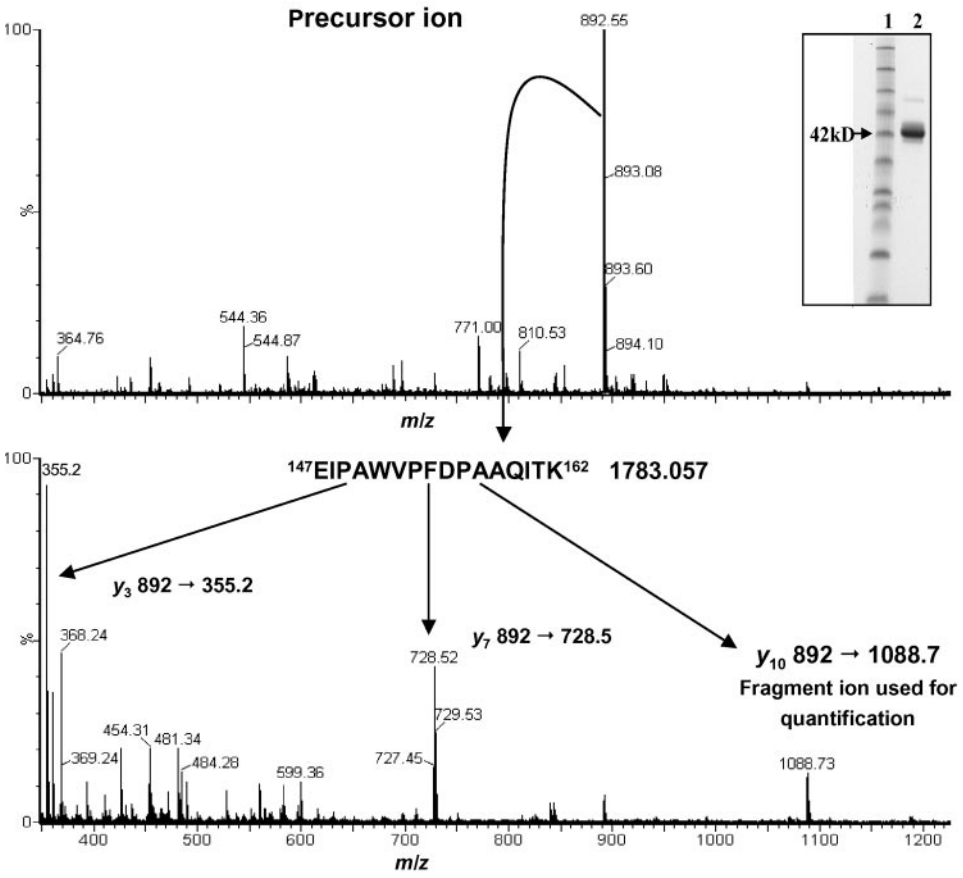

Fig. 1. (*A*), Precursor ion spectra of ZAG trypsin digest.

Inset in (*A*) shows 1-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel of recombinant ZAG used for optimizing protein cleavage and as a standard for the absolute quantification of ZAG from human serum (*lane 1*, protein molecular mass standards; *lane 2*, 5 μg recombinant ZAG loaded and stained with Coomassie Blue). (*B*), MS/MS spectrum of tpZAG147–162. This tryptic relatively high molecular weight 1088.7 Da. The primary transition y^*_{10} 892.5→1088.7 and the secondary transition Y_7 892.55→728.5 have been monitored.

GS1: 40, GS2: 40, IS: 5500, TEM: 600, DP: 225, and EP: 5. Using a 200 ms dwell time the doubly charged precursor ion for the ZAG tryptic peptide tpZAG147–162 at *m/z* 892 was selected in Q1 and 3 singly charged transitions were monitored in Q3: the *y*-ion PFDPAAQITK at *m/z* 1088 (CE: 40, CXP: 40), the y-ion PAAQITK at *m/z* 728.4 (CE: 40, CXP: 40), and the internal fragment ion PAW at *m/z* 355.2 (CE: 50, CXP: 26).

stable isotope-labeled peptide internal standard

The stable isotope peptide ^{147}EIPAWVPEDPAAQITK^{162} was synthesized in the Mayo Proteomics Research Center on an ACT 396 Multiple Peptide Synthesizer (Advanced ChemTech), using recommended procedures for 1,3-di-isopropylcarbodiimide activation and coupling. Stable isotope-labeled proline (5 ^{13}C, 1 ^{15}N-Fmoc-Proline, Isotech) was coupled in the peptide sequence at positions 7 and 9 to give a total molecular mass shift of +12 Da from the nonlabeled peptide and a monoisotopic molecular mass of 1775.92 Da. We added 2 μL of the internal standard in concentration of 2 nmol/mL (3.6 μg/mL) to 10 μL of sample before injection into the LC-MS/MS.

location of selected peptide in 3-dimensional structure of zag

To coordinate the MS/MS measurements with potential immunoassay measurement systems, we specifically looked for a peptide sequence that was on the exterior of the 3-dimensional structure of ZAG. This exterior location would more likely be an immunologic binding site for appropriately targeted antisera, rather than an internal site. The IT7V Zn-α-2-glycoprotein:baculo-ZAG PEG 200 structure was downloaded from the RCSB Protein Data Bank *(1)*. The structure was determined using x-ray diffraction with a 1.95 Å resolution. The DeepView/Swiss-PdbViewer 3.7 program *(2)* was used to visualize and highlight the epitope site ^{147}EIPAWVPEDPAAQITK^{162} *(17, 18)*

study subjects

We used serum samples from 3 pilot groups of men in this study. The 1st group included healthy men (n = 6, ages 46 to 58 years), the 2nd group, men with nonmalignant prostate biopsy results (n = 20, ages 59 to 83 years), and the 3rd group, men with PCa (n = 26, ages 56 to 84 years). The men with nonmalignant prostate biopsy results had pathology reports of normal (n = 8), inflammation (n = 4), prostate intraepithelial neoplasia (n = 6), and atypical acinar proliferation (n = 2). Median follow-up of the 29 men with PCa was 26.5 months (range 3.8–43.6 months). The prostate-specific antigen values were ≤4 μg/L (2 patients); 4.1–10 μg/L (6 patients); 10.1–20 μg/L (7 patients); and >20 μg/L (11 patients). Gleason scores were available for 25 patients at the time of initial diagnosis; 36% had a total Gleason score of 6, 32% a score of 7, 16% a score of 8, and 16% a score of 9 or 10. We obtained the specimens from the Mayo Foundation Prostate Specialized Program in Research Excellence (SPORE). These specimens were collected in 2002–2006, according to a protocol approved by Mayo Clinic Institutional Review Board (#1937-00).

calculations and statistics

We defined the limit of quantification (LOQ) for ZAG as the response for digested recombinant ZAG added to 50 g/L BSA matrix that gave a signal-to-noise value of 10. The limit of detection (LOD) for ZAG was defined as the concentration of standard that gave a signal-to-noise value of 3. We used 2-tailed Student *t*-tests (SAS software) to compare the ZAG concentrations in the 3 pilot study groups.

Results

The most critical step in the protein cleavage approach to quantification of a protein is the selection of a cleaved peptide that will provide adequate analytical specificity and sensitivity. Selection of the tryptic peptide tpZAG147–162 for ZAG quantification was determined empirically by performing LC-MS/MS on tryptic digests of recombinant ZAG. The most abundant tryptic peptide observed was tpZAG147–162 (Fig. 1A). Fortuitously, this tryptic peptide has 3 proline residues that produce strong fragment ions with *m/z* values of 355.2, 728.5, and 1088.7 Da (Fig. 1B). All ZAG quantification was performed using the y_{10} =1088.7 fragment ion response since this transition had the best signal-to-noise and LOQ for ZAG from human serum. Also fortuitous was the location of this peptide on the external part of the molecule (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4).

Quantification was performed using a standard curve prepared by serial dilutions of recombinant ZAG added to 50 g/L BSA in phosphate-buffered saline as a surrogate serum matrix. Fig. 2 shows 3 ion chromatograms: (*a*) the digest of recombinant ZAG added to 50 g/L of BSA at a concentration of 1.3 mg/L, (*b*) ZAG tryptic peptide produced after the digest of patient serum, and (*c*) the stable isotope-labeled tpZAG147–162 added to the ZAG standard after digestion. We added 2 μL of the internal standard in concentration of 2 μmol/L (3.6 mg/L) to 10 μL of sample. A comparison of the response for equimolar amounts of ZAG vs the internal standard peptide was performed by generating a standard curve, with each point in the curve having equimolar amounts of ZAG and internal standard. Linear regression analysis of this curve resulted in $y = 1.054x - 0.283$ with an $R^2 = 0.9965$. These results demonstrated that ZAG was completely digested in the 50 g/L BSA matrix, as the regression line slope was ~1.0 and the intercept reflected minimal background interference. After standard curves were found to be linear and reproducible, patient and normal donor serum samples were digested and analyzed.

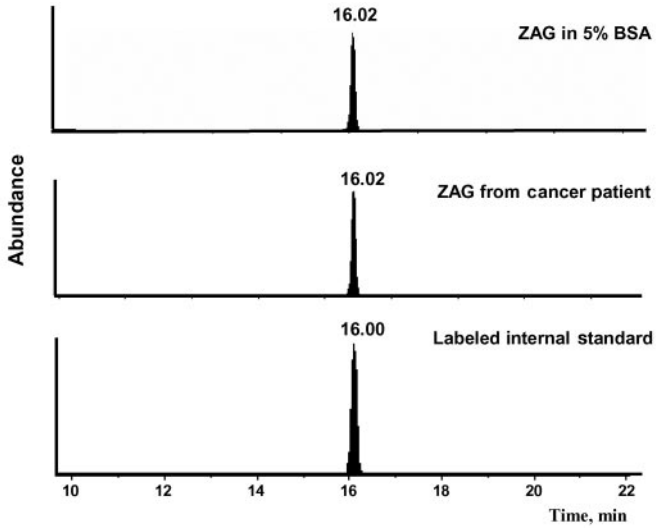

Fig. 2. Chromatograms showing the response from the LC-MS/MS analysis of the ZAG tryptic peptide tpZAG147–162 produced after the digest of 1.3 mg/L of recombinant ZAG added to a 50 g/L BSA matrix (*A*), ZAG tryptic peptide tpZAG147–162 produced after the digest of patient serum (*B*), along with the stable isotope-labeled internal standard (*C*) 2 μL of the internal standard in concentration of 2 nmol/mL (3.6 mg/L) was added to 10 μL of ZAG tryptic digest before injection to LC-MS/MS (*C*).

The trace for the PCa patient (Fig. 2B) shows the superior signal-to-noise afforded by the y_{10} transition for the tpZAG147–162 peptide. Controls run without supplementing ZAG or stable isotope-labeled tpZAG147–162 into 50 g/L BSA matrix showed low background response for the y_{10} transition, as did controls for the internal standard transition in human serum digests where no internal standard was added. Since ZAG free human serum was not available, standard addition of recombinant ZAG into pooled normal male sera was performed to further confirm the origin of the peak observed in serum digests. Recoveries were also determined by adding recombinant ZAG to pooled normal male sera. Two different supplemented concentrations were evaluated, 3 mg/L (within normal range) and 6 mg/L (mean value for nonmalignant prostate disease). The results are shown in Table 1. In addition, a 5-point dilution series was made from a nonmalignant prostate serum sample by adding 50 g/L BSA matrix. Linear regression analysis of the dilution series, plotted as the known concentration vs the calculated concentration, was found to be $y = 0.8112x + 2.063$ with an $R^2 = 0.9695$. Fig. 3 shows a typical standard curve used for quantification, demonstrating the linearity associated with recombinant ZAG added to the BSA matrix over a range of 0.33 to 10.4 mg/L. The LOQ was 0.32 mg/L and the LOD was 0.08 mg/L. This LOD translates into ~20 fmol of tpZAG147–162 loaded on column for a 10 μL injection.

To evaluate intra- and interassay imprecision, we prepared 2 serum pools from normal male and normal female sera. Aliquots of these pools were reduced, alkylated, and digested separately and then analyzed using our LC-MS/MS method over a period of 3 consecutive days. The results of this analysis (Table 1) demonstrated the reproducibility of our method; 10 separate digests performed and run over 3 consecutive days showed CVs <7%. The ZAG concentration from normal male sera was between 2.6 and 4.7 mg/L with an average concentration of 3.65 mg/L. The average concentration of ZAG in men with nonmalignant prostate disease was 6.21 mg/L, and the average ZAG concentration in men with PCa was 7.59 mg/L. Student *t*-tests performed on the 3 sample sets comparing samples of normal, nonmalignant disease, and PCa samples showed statistically significant increases in the concentration of ZAG across these groups (Table 2) when analyzed by our LC-MS/MS method.

Table 1. Assay performance characteristics.

Recovery in normal male pool

Added, μg	Measured, mg/L	% Recovery
0	2.98	
3	5.76	93%
6	9.8	114%

Intra- and interassay precision

	Intraassay		Interassay	
	Male pool	Female pool	Male pool	Female pool
Replicates	5	5	15	15
Mean, mg/L	3.27	3.42	3.26	3.38
SD, mg/L	0.16	0.22	0.14	0.20
CV, %	4.99	6.30	4.36	5.92

Discussion

The method presented here for the quantification of ZAG in serum by LC-MS/MS is based on the technique first

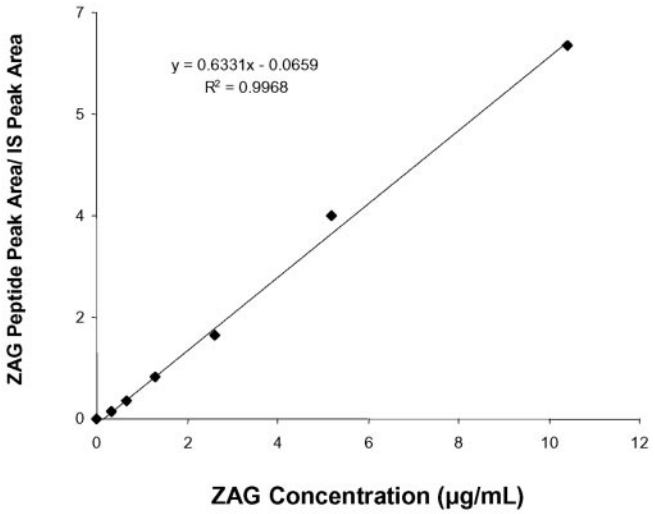

Fig. 3. Linear regression analysis of a standard curve composed of recombinant ZAG added to a 50 mg/L BSA matrix over a range of 0.33 to 10.4 mg/L.
(Figure represents 1 typical run.)

Table 2. Comparison of serum ZAG values in normal individuals, patients with nonmalignant prostate disease, and patients with PCa.

	Serum ZAG concentration, mg/L				
Group	Number	Average	SD	*P* values	
Normal	6	3.65	0.71	0.0013	
Nonmalignant prostate disease	20	6.21	1.65	0.0007	0.037
PCa	25	7.59	2.45		

described by Barr et al. *(19)* and modified by our group and others for use in quantifying proteins from serum *(16, 20)*. Recently, Anderson and Hunter described the same tryptic fragment of ZAG protein (tpZAG147–162) that can be used for identification of ZAG among 53 plasma proteins ranging in abundance from albumin (~70 g/L) down to fibronectin (~1 mg/L) *(16)*. We performed absolute quantification of ZAG by using pure recombinant protein for our standards, and a stable isotope-labeled synthetic peptide with the same sequence as tryptic fragment tpZAG147–162 as an internal standard. The concentration of ZAG in serum is relatively high (our findings showed ~3–8 mg/L), making it possible to quantify the protein by LC-MS/MS directly from digests of serum, without purification or depletion of high-abundance proteins. We were also able to use a high LC flow rate of 250 μL/min and an electrospray source, both of which are routinely used in clinical laboratories. We anticipate that the method will be a valuable resource for determining the concentration of ZAG in larger cohorts of patients to determine the utility of ZAG as a cancer biomarker.

If follow-up LC-MS/MS cohort studies demonstrate the ZAG protein has clinical utility as a cancer biomarker, development of an immunoassay may be desirable. The position of the tpZAG147–162 fragment on the periphery of the protein's tertiary structure suggests that this peptide may also provide an accessible epitope for antibody binding and facilitate future assay development. This LC-MS/MS assay, based on an external peptide, could potentially be used as a reference method for immunoassays, especially if the immunoassays also target the same external region of the ZAG molecule illustrated in Fig. 1 in the online Data Supplement.

ZAG has been identified as a biomarker associated with multiple diseases, including prostate, bladder, and breast cancer *(3, 6, 7)*. Therefore, ZAG concentrations by themselves are unlikely to be a specific screening test for any targeted disease, such as PCa. Nonetheless, ZAG may be a valuable biomarker to increase sensitivity for early disease detection if it is combined with other more tissue specific biomarker markers, such as prostate-specific antigen. ZAG also may have utility for differentiating more aggressive forms of cancer. However, a large number of measurements will be needed to further define the screening or prognostic value of ZAG, and the LC-MS/MS assay described in this paper should be a valuable tool for conducting these studies.

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