**A NOVEL SUBSTITUTION OF PROLINE (P32L) DESTABILIZES**

**β2-MICROGLOBULIN INDUCING HEREDITARY SYSTEMIC AMYLOIDOSIS**

Tatiana Prokaeva,1 Tracy Joshi,1 Elena S. Klimtchuk,1 Victoria M. Gibson,1 Brian Spencer,1 Omar Siddiqi,1 \* Dobrin Nedelkov,2 Yueming Hu,2 John L. Berk,1 Sarah A.M. Cuddy,3 Surendra Dasari,4 April Chiu,5 Lauren A. Choate,5 Ellen D. McPhail,5 Haili Cui,1,6 Hui Chen,6 Eric J. Burks,6 Vaishali Sanchorawala,1 and Lawreen H. Connors1,6

*1Amyloidosis Center, Boston University School of Medicine, Boston, MA, USA*

*2Isoformix Inc., Phoenix, AZ, USA*

*3Amyloidosis Program, Department of Medicine, Brigham and Women’s Hospital, Boston, MA, USA*

*4Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA*

*5Department of Laboratory of Medicine and Pathology, Mayo Clinic, Rochester, MN, USA*

*6Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA, USA*

*\* Currently enrolled in the Cellular, Molecular and Biomedical Science PhD Program at University of Vermont, VT, USA*

***Corresponding Author:*** *Tatiana Prokaeva, MD, PhD*

*Amyloidosis Center, Boston University School of Medicine. 72 East Concord Street, K-510, Boston, MA 02118-2526, USA; e-mail address:* [*prokaeva@bu.edu*](mailto:prokaeva@bu.edu)*; phone: 617-358-4749; fax: 617-358-4735*

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**METHODS**

**Clinical data and samples collection**

Institutional Review Boards at Boston Medical Center, Brigham and Women's Hospital and Mayo Clinic approved informed consents for data and samples collection.

Demographic, clinical, laboratory, cardiac imaging, and histological data were collected from either medical records or databases maintained at participating institutions. Blood and plasma samples from the proband (II-3) were obtained at initial evaluation and stored at -20 °C until analysis. The following reference ranges were used:

*Boston University Amyloidosis Center*

NT-proBNP: <300 pg/mL normal, heart failure unlikely; ≥ 900 pg/mL high probability of heart failure

BNP: 0 - 53.2 pg/mL

Troponin-I: <0.013 ng/mL  
β2M: 1.3-2.4 mg/L.

*Brigham and Women's Hospital*

NT-proBNP: <900 pg/mL

Troponin T: 0-14 ng/L

**Immunoelectron microscopy**

Approximately 2 mm of formalin-fixed paraffin-embedded tissue samples were excised by hand from Congo red positive areas observed by light microscopy, oven heated at 62 °C for 5 minutes on filter paper, and transferred to a glass specimen vial containing 100% xylene for overnight deparaffinization. Deparaffinized specimens were rehydrated in serial ethanol concentrations and embedded in Lowicryl K4M (Electron Microscopy Sciences). Sections were cut at 2 μm and stained with toluidine blue for light microscopic examination to identify areas likely corresponding to amyloid deposits. Ultra-thin sections were cut at approximately 72 nm (silver sections) and mounted on Formvar film 150 mesh nickel grids. Grids were incubated overnight with primary polyclonal rabbit anti-human antibodies against kappa or lambda light chains (Agilent Teck; Cat. # A019102-2 and Cat. # A019302-2, respectively) or prealbumin (Agilent Teck; Cat. # A000202-2), or monoclonal mouse anti-human antibody against amyloid A (Agilent Teck; Cat. # M0759). Grids were washed and incubated for 1 hour with secondary goat anti-rabbit IgG antibody conjugated to 15 nm gold particles (Ted Pella; Cat. # 15727) at 1:20 dilution or with goat anti-mouse IgG antibody conjugated to 20 nm gold particles(Ted Pella; Cat. # 15753) at 1:10 dilution. Upon secondary antibody incubation, grids were washed and stained with uranyl acetate and lead citrate. Stained grids were examined and photomicrographs collected using a JEOL JEM1020 transmission electron microscope.

**Genetic analyses**

Genomic DNA was extracted from buffy coat cells stored in RNA later (Thermo Fisher) using a QiaAmp DNA Mini Kit (Qiagen) and served as the template for amplifications of all *B2M* gene coding regions (exons 1-3). The following primers were used for *B2M* gene amplification:

exon 1 – F: GGG TTT CCG TTT TCT CGA AT; R: CTT GGA GAA GGG AAG TGA CG;

exon 2 – F: CCA AGT TAG CCC CAA GTG AA; R: CCC TGA CAA TCC CAA TAT GC; and

exon 3 – F: TGG GTA GGA ACA GCA GCC TA; R: TTC TCC ACA TAG TGA GGG TTA TCA.

Each PCR was conducted in a 50 uL volume containing 2X Qiagen PCR Master Mix, 2uL of genomic DNA, and 0.2 uM of both forward and reverse primers. Thermal cycler conditions consisted of an initial denaturation step at 95 °C for 3 minutes followed by 35 cycles of denaturing at 95 °C for 30 seconds, annealing at 56 °C for 30 seconds, and extension at 72 °C for 45 seconds. PCR products were sequenced in both forward and reverse directions on an ABI s3730xL automated DNA sequencer (Applied Biosystems) at GENEWIZ (South Plainfield). The PCR product for exon 2 was cloned into a pCR 2.1 vector using a TA Cloning Kit (Invitrogen) to ascertain the heterozygous nature of the mutation.

Mutation nomenclature was based on the *B2M* transcript (NCBI reference sequence cDNA accession number, NM\_004048.3). Nucleotides were numbered according to the cDNA sequence with the first nucleotide corresponding to A of the ATG translation initiation codon as specified by the Human Genome Variation Society guidelines (<http://varnomen.hgvs.org/>). The β2M variant with 20-residue signal peptide and mature variant protein were designated as p.P52L and P32L, respectively. The Single Nucleotide Polymorphism Database (dbSNP; <https://www.ncbi.nlm.nih.gov/snp/>), Genome Aggregation Database (gnomAD; <https://gnomad.broadinstitute.org/>), [NHLBI GO Exome Sequencing Project (ESP;](https://esp.gs.washington.edu/drupal/)<https://evs.gs.washington.edu/EVS/>**), and** ClinVar archive (<https://www.ncbi.nlm.nih.gov/clinvar/>) were used to search for identified genetic variants and their association with clinical phenotype.

**Proteomic analysis of plasma β2-microglobulin by mass spectrometry immunoassay**

Prior to the mass spectrometric immunoassay (MSIA) assay, 5 μL of the samples were mixed with 145 μL PBS, 0.1% Tween. Activation of the MSIA-Tips (Thermo Fisher; Cat. # 991CUS01) with polyclonal goat anti-human β2M antibody (DAKO; Cat. # A0072) and capture of β2M from the patient and control (obtained from ProMedDx) plasma samples were performed as previously described [1]. To elute captured plasma β2M, 5 μL of Matrix-Assisted Laser Desorption Ionization (MALDI) matrix (20 g/L sinapic acid in 33% (v/v) acetonitrile and 0.4% (v/v) trifluoroacetic acid) were aspirated into each tip, pushed up and down through the tip five times, and then dispensed directly onto a 96-well formatted MALDI target. MALDI time-of-flight (MALDI-TOF) mass spectrometry was performed on a Bruker’s Ultraflex III MALDI-TOF instrument (Bruker) in a linear positive ion mode, with 150 ns delay, 20.00 kV and 18.45 kV ion source voltages, 5 kV lens voltage, and signal suppression of up to 4,500 Da. Three mass spectra (each consisting of 1000 summed laser-shots) were acquired for each sample. The mass spectra were externally calibrated using human hemoglobin peaks ([M+2H]2+, [M+H]+. The spectra were baseline subtracted (Convex Hull algorithm, 0.8 flatness), and smoothed (Savizky Golay algorithm, 5 m/z width and 1 cycle) using Flex Analysis software (Bruker Daltonics). The ratio of circulating β2M species was estimated using the peak height data of variant to wild-type β2M measured from the mass spectra profiles.

**Proteomic analysis of cardiac tissue-derived amyloid deposits by mass spectrometry**

Previously published proteomics methods were used for typing the amyloid deposits present in the formalin-fixed paraffin-embedded endomyocardial tissue biopsy [2]. Briefly, a 10-uM thick cardiac tissue section was cut and mounted on a special Director (OncoplexDX) slide. The section was stained with Congo red and mounted on a laser microdissection apparatus. Amyloid deposits were visualized under a fluorescent light and Congo red positive tissue fragments from an area of 60,000uM2 were collected. Proteins in the collected samples were extracted using heat, and digested into peptides by overnight incubation with trypsin. The peptide fragments were analyzed using a QExactive mass spectrometer (Thermo Fisher).

The MS/MS data were processed using a previously published informatics pipeline [3]. In brief, MS/MS data were searched against the SwissProt human reference database supplemented with reversed sequence entries to estimate protein and peptide identification probabilities. Two different search engines (Mascot and X!Tandem) were used to perform the peptide-spectrum matching and the results were processed using Scaffold software (Proteome Software). Proteins with at least one confident identification probability ≥0.9 were utilized for interpretation. An amyloid pathologist inspected the detected proteins for the presence of “amyloid signature” and amyloid type-specific proteins [4]. The data were re-interrogated for known and unknown mutations in amyloid type-specific proteins using a previously published pipeline [5].

**Site-directed mutagenesis, expression and purification of recombinant β2**-**microglobulin proteins**

A synthetic human β*2M* encoding DNA fragment with the sequence optimized for expression in *E. coli* was inserted into the pQE-1 vector to allow for expression of an N-terminal 6×His-tagged recombinant β2M (rβ2M) protein (GenScript). The QuickChange II Site-Directed Mutagenesis Kit (Invitrogen) was used to produce pQE-1 plasmids encoding the following rβ2M proteins for comparative analyses: rP32L, the current study variant; rP32G, a synthetic β2M extensively investigated *in vitro* [6]; rD76N, a previously reported amyloidogenic protein [7]; and wild-type rβ2M (rβ2Mwt).

After verification by DNA sequencing, the β2M/pQE-1 expression plasmids were transformed into *E. coli* Rosetta-gami 2 cells (Novagen) by heat shock. All four proteins were expressed and purified under non-denaturing conditions as previously described for immunoglobulin light chain proteins [8]**.** His-tags were removed from purified proteins using DAPase enzyme (Qiagen). Prior to use, the protein solutions were dialyzed against 5 mM sodium phosphate buffer, pH 7.4, 15 mM NaCl, a standard buffer used throughout the study. Protein purity was accessed by MALDI-TOF mass spectrometry. Two μL of MALDI matrix (20 g/L sinapic acid in 33% (v/v) acetonitrile and 0.4% (v/v) trifluoroacetic acid) was spotted and mixed with 2 μL of sample directly on the MALDI target, and allowed to dry at room temperature. MALDI-TOF MS was performed on a Bruker’s Ultraflex III MALDI-TOF instrument (Bruker) as described previously (see Proteomic analysis of plasma β2-microglobulin section).

**Biophysical and biochemical characterization of recombinant β2**-**microglobulin proteins**

Circular dichroism (CD) spectra and melting data were recorded in separate experiments for all β2M proteins by using a Jasco J-815 (Jasco Inc.) spectropolarimeter equipped with a thermoelectric temperature controller. Secondary structure was assessed by far-UV CD spectra recorded at 195-250 nm on samples containing 0.25 mg/mL (22 µM) rβ2M placed in 1 mm quartz cells. The data were normalized to protein concentration and expressed in units of molar residue ellipticity (MRE); secondary structure of was estimated from the far-UV CD spectra using the CDpro program. Thermal stability was assessed in melting experiments performed at 205 nm to monitor protein unfolding during sample heating from 25°C to 75°C at a constant rate of 1°C/min. The apparent melting temperature (Tm app) was determined by calculating the maximum of the first derivative from the melting data. For equilibrium chemical denaturation, 0.02 mg/mL of each rβ2M proteinwas pre-incubated with guanidine hydrochloride (0 to 4.6 M) at room temperature for 2 hours. The fluorescence intensity ratio, I355/I335, was recorded as a function of guanidine hydrochloride concentration to monitor changes in tryptophan exposure during protein unfolding. Data were fitted with a two-state equilibrium denaturation model from which the unfolding midpoint for guanidine hydrochloride concentration (*Cm*) was determined and expressed as the mean SD. For limited proteolysis, samples containing 0.4 mg/mL of protein in standard buffer were digested at 37 °C with trypsin, at a substrate to enzyme weight ratio of 200:1, for 5 to 120 minutes. The reaction was quenched at various time points using 1 mM phenylmethylsulfonyl fluoride. The proteolytic products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The formation of amyloid-like structures upon limited proteolysis of each β2M protein was monitored by thioflavin T (ThT) binding fluorescence using a TECAN microplate reader(Fisher Scientific). Individual samples containing 0.4 mg/mL protein, 0.05% NaN3, and 10 μM ThT were subjected to double-orbital shaking at 37 °C; ThT emission (λex=450 nm, λem=482 nm) was recorded at 10 minute intervals for 100 hours. The emission of ThT in buffer alone was subtracted from the data. All experiments were performed in triplicates.

For negative stain transmission electron microscopy**,** a 4 µL drop containing approximately 0.4 mg/mL of rP32L protein was incubated with trypsin for 5 days at 37 °C with double-orbital shaking at 168 rpm in a TECAN fluorescence microplate reader. Samples were deposited onto an EM grid, stained with 1% uranyl acetate, and blotted as previously described [9]**.** Electron micrographs were collected at 45,000x magnification using a CM12 transmission electron microscope (Philips Electron Optics) equipped with a Teitz 2Kx2K CCD camera (TVIPS).

**Analysis of amyloid-forming peptides of recombinant β2M P32L by MALDI-TOF**

Amyloid-forming peptides collected after incubation of rP32L with trypsin for 100 hours were re-suspended in loading buffer and separated using non-reducing SDS-PAGE. The area of the gel showing a peptide band of ~10 kD was selected, excised, and rinsed in a solution of 1:1 acetonitrile:water (v/v) on an orbital shaker for 15 minutes at room temperature. After supernatant removal, gel pieces were diced, dehydrated in acetonitrile, and further dried in a vacuum centrifuge. A volume of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate, sufficient to cover the gel pieces, was added and the proteins were reduced for 1 hour at 56 °C. After cooling to room temperature, the DTT solution was replaced with approximately the same volume of 55mM iodoacetamide in 100 mM ammonium bicarbonate. After a 45-minute incubation at ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 50-100 uL of 100 mM ammonium bicarbonate for 10 minutes, dehydrated by addition of acetonitrile upon supernatant removal, rehydrated in 100 mM ammonium bicarbonate, and dehydrated again with addition of the same volume of acetonitrile. The liquid phase was removed and the gel pieces were completely dried in a vacuum centrifuge.

The gel pieces were rehydrated in 100 uL of 50mM ammonium bicarbonate, and 5 uL of endoproteinase Lys-C (0.1ug/ul; NEB) was added in an ice bath. After incubation for 45 min, an additional 50 uL of 50mM ammonium bicarbonate was added to completely cover the gel slices during enzymatic cleavage (37 °C, overnight). Peptides were extracted by one change of 20 mM ammonium bicarbonate and one change of 0.5% trifluoroacetic acid in 50% acetonitrile at room temperature and dried.

Peptides were reconstituted in 20 uL of 0.1% trifluoroacetic acid and 10 uL was removed for desalting using UC18 zip tips (Millipore) according to procedure recommended by the manufacturer. Peptides were eluted from the tip using a solution of 10 mg/mL alpha-cyano-4-hyrdoxycinnamic acid in 0.1% trifluoroacetic acid (Sigma). Proteomic analysis was performed using an AB-Sciex 4800 MALDI-TOF/TOF instrument in positive reflectron mode and LC-MS/MS of selected peptides was obtained using a 2-kV positive mode method. A list of generated peptide masses was submitted to the ExPASy FindPept program (<https://web.expasy.org/findpept/>) for identification of peptides and determination of peptide coverages.

**SUPPLEMENTARY FIGURES**

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**Figure S1. Histological analysis of fat tissue in proband II-3**

**A.** Congo red-positive amyloid deposits viewed by light microscopy.

**B.** Polarized light view demonstrates apple-green birefringence of Congo red-positive amyloid deposits. Original magnification: panels A and B, x100

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**Figure S2. Proteomic detection of β2M variant P32L in the endomyocardial amyloid deposits in family members II-4 and II-2.**

**A-B.** Scaffold readouts of the top 20 proteins identified in Congo red positive areas of endomyocardial tissue biopsy by laser microdissection-assisted mass spectrometry-based proteomics method. The number in the box denotes total number of detected MS/MS spectra obtained for each protein in each independent dissection. The green box color denotes >95% probability that the spectra represent the identified protein. Amyloid-related proteins are starred and shown at the top of the list. The blue star marks β2M protein; data show abundances of the β2M in each of two tested samples (**A**. 60 and 65 spectra, respectively; **B**. 33 and 40 spectra, respectively) and the >95% probability suggests strong confidence in the accuracy of the data. Yellow stars mark universal amyloid-associated proteins (apolipoprotein E, serum amyloid P, and apolipoprotein IV). There was no evidence of other known amyloidogenic precursor proteins associated with cardiac amyloidosis.

**C-D**. MS/MS spectra showing the mutated peptide HLSDIEVDLLK in family members **C**. II-4 and **D**. II-2. Red asterisk highlights mutated leucine residue at position 32 in the y-ion series of the peptide.

**E-F**. Combined amino acid sequence coverage of the β2M protein (shown with underlined 20 residue signal peptide) was obtained from two dissections of congophilic amyloid deposits. Peptides identified by mass spectrometry-based proteomics method accounted for **E**. 41.2% and **F**. 48.7% of β2M sequence in family members II-4 and II-2, respectively (shown in bold letters). Mutated leucine residue is shown in red font. β2Mwt peptide was not detected in the amyloid deposits.

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**Figure S3. MALDI-TOF MS characterization of amyloid-forming peptides of rP32L variant**. The mass spectrum of Lys-C digested peptides derived from the amyloid-forming tryptic peptide of the rP32L variant protein is shown. Peptide ions are labeled with observed *m/z* values and the corresponding residue intervals. The ∆N6/∆C8 truncation was identified in the mass spectral profile. Insert shows SDS-PAGE of amyloid-forming tryptic peptides of rP32L that were present at ~100 h. Black arrow indicates the ~10 kDa protein band that was excised and subjected to in-gel digestion with Lys-C followed by MALDI-TOF analysis.

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**Figure S4. Effect of P32 mutation on stability and aggregation of recombinant P32L and P32G β2M variants.**

Recombinant β2M variant proteins, rP32L and rP32G, were generated and analyzed as described in Methods and Supplementary Appendix.

**A.** Melting data were recorded by CD at 205 nm to monitor unfolding of the proteins (0.25 mg/mL in standard buffer) during heating from 25 to 75°C at a rate of 1°C/min. The apparent melting temperature (Tm app) was determined from the first derivative maximum in the melting data. The vertical dashed lines indicate the Tm app for the rβ2M variants (rP32L: ~58°C; rP32G ~55.5°C), suggesting slightly less destabilizing effect of rP32L on the β2M structure.

**B.** Equilibrium denaturation by guanidine hydrochloride tracked by the ratio of fluorescence intensity at 355 nm and 335 nm (I355/I335) to monitor changes in solvent exposure of tryptophan during protein unfolding. rβ2M proteins (0.02 mg/mL) were pre-incubated with guanidine hydrochloride (0 - 4.6 M) at a room temperature for 2 hours. Data were fitted with a two-state equilibrium denaturation model. Equilibrium unfolding of rP32L and rP32G variants occurred at 2.2+0.2 M and 1.8 +0.2 M of guanidine hydrochloride, respectively, indicating decreased slightly less destabilizing effect of rP32L on protein structure.

**C.** SDS-PAGE of rP32G β2M variant at various stages of limited proteolysis; results for rP32L variant are shown in **Figure 3D**. The sample containing 0.4 mg/mL of protein was digested with trypsin at 37°C at substrate to enzyme weight ratio of 200:1 for 5 to 120 min as indicated on the top line; 0 indicates intact protein prior to incubation with trypsin. St indicates molecular weight standards. Both variants showed enhanced susceptibility to proteolysis by trypsin with rP32L proteolyzed slightly slower than rP32G.

**D.** Aggregation of rP32L and rP32G variants upon limited proteolysis with trypsin was monitored by ThT fluorescence. Proteins (0.4 mg/mL) were incubated at 37°C with trypsin at substrate to enzyme weight ratio of 200:1. Only rP32L variant demonstrated ThT binding to amyloid-like structures. Full-length proteins showed no evidence of aggregation.

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