Case Report

CLINICAL CASE REPORT: LECT2-ASSOCIATED ADRENAL AMYLOIDOSIS

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ABSTRACT

Objective: Leukocyte chemotactic factor-2 (LECT2)-associated amyloidosis is a newly discovered cause of amyloid and has thus far been reported to involve only the kidneys. We report a case of amyloidosis of the adrenal gland, along with involvement of the kidney, in a patient with primary aldosteronism.

Methods: Tissue samples from the patient were analyzed using tandem mass spectrometry to identify the amyloid protein, followed by Western blot. DNA sequencing was performed to evaluate genetic polymorphisms.

Results: Tandem mass spectrometry identified the amyloid protein as LECT2, and its identity was confirmed by Western blot. Genetic analysis revealed the patient to be homozygous for a genetic polymorphism of the *LECT2* gene. Serum levels of LECT2 were undetectable, and no mutations were found in the *CTNNB1*, *KCNJ5*, *ATP1A1*, or *ATP2B3* genes.

Conclusion: This case appears to be the first published report to describe LECT 2-associated amyloidosis both outside of the kidney and within the adrenal gland. **(AACE Clinical Case Rep. 2015;1:e59-e67)**

Abbreviations:
CT = computed tomography; **CTNNB1** = catenin (cadherin-associated protein), beta 1; **LDL** = low-density lipoprotein; **LECT2** = leukocyte chemotactic factor-2; **LECT2 AA** = LECT2-associated amyloid A amyloidosis; **PA** = primary aldosteronism; **SDS** = sodium dodecyl sulfate

INTRODUCTION

Amyloidosis is a generic set of diseases with multiple specific etiologies that share the common feature of pathogenic fibrillary deposits in various tissues. The amyloid deposits arise either from normal plasma proteins produced in excess (secondary amyloidosis) or from a growing list of abnormal proteins arising from mutant genes (multiple subsets of primary amyloidosis). The dominant pattern of tissue deposition is determined mostly by the specific form of amyloidogenic protein. For example, secondary amyloid A (AA) amyloidosis characteristically affects the kidneys, liver, gut, vasculature, and adrenal glands (though clinically significant adrenal dysfunction is rare). The primary amyloidoses have different patterns of tissue deposition determined by the unique tissue affinities for each particular type of amyloid protein. In 2008, a new form of amyloid, designated leukocyte chemotactic factor-2 (LECT2)-associated amyloidosis (LECT2 AA) was described; the mutant LECT2 has thus far been reported to involve only the kidneys (1,2).

We report a case of a patient referred to the National Institutes of Health for primary aldosteronism (PA). He was found to have LECT2 amyloidosis of the adrenal gland, along with involvement of the kidney. Genetic analysis revealed that he was homozygous for a polymorphism of the *LECT2* gene. Extensive infiltration of the adrenal gland by LECT2 amyloid was associated with excess production of aldosterone by the gland, resulting in PA.

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METHODS

Tissue Homogenization

A frozen tissue section (~2 mm^3) of the patient's adrenal gland and tissue from a nonaffected control (female, 52 years of age, diagnosis: mild nodular cortical hyperplasia) was serially homogenized on ice. First, 300 μL of 50 mM Tris-HCl buffer (pH 8) containing 5 mM dithiothreitol (DTT) was added and the tissue was disrupted. The sample was centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the supernatant was removed. Next, 300 μL of Tris-HCl buffer containing 5 mM DTT and 0.1% (w/v) sodium dodecyl sulfate (SDS) was added to the remaining pellet and homogenized on ice. The resulting sample was centrifuged again and the supernatant was collected. This step was repeated with 200 μL of buffer. The pellet samples were solubilized with 50 μL of Liquid Tissue® (Expression Pathology, Gaithersburg, MD) solubilization buffer, and the sample was heated at 95°C for 90 minutes. The sample was then placed on ice and centrifuged at 10,000 × *g* for 15 minutes at 4°C, and the supernatant was prepared for SDS-polyacrylamide gel electrophoresis (PAGE). The resulting gels were stained with Coomassie blue or processed for Western blot analysis.

Protein Digestion and Identification

Coomassie-stained gel bands were rinsed twice with 200 μL of 100 mM ammonium bicarbonate. Protein digestion, peptide extraction, and mass spectrometry analysis were carried out as previously described (3). Protein identification was obtained using the SwissProt database (downloaded March 21, 2012; taxonomy, human; 20,329 sequences) via the Mascot search engine. Additional details are provided in the Supplemental Materials.

LECT2 Western Blotting

A 10-μL aliquot of the third solubilization step (Tris/SDS) and 2-μL aliquot of the Liquid Tissue® extracted solution from both normal and amyloid samples was mixed with sample buffer and reducing agent as noted above (final concentration, 1× each). SDS-PAGE was carried out as noted above, and Western blot analysis was performed as previously described using a 1:1,000 diluted mouse anti-human LECT2 antibody (clone 102717; R&D Systems, Minneapolis, MN) (3).

DNA Extraction and Sequencing

DNA was extracted from peripheral blood and fresh frozen adrenal sample according to standard protocols. The *LECT2*, catenin (cadherin-associated protein), beta 1 (*CTNNB1*) (exons 3 and 5), potassium inwardly rectifying channel, subfamily J, member 5 (*KCNJ5*), ATPase, Na^+/K^+ transporting, alpha 1 polypeptide (*ATP1A1*), and ATPase, Ca^{2+} transporting, plasma membrane 3 (*ATP2B3*) genes were amplified and directly sequenced as described in detail in the Supplemental Materials.

Immunohistochemical Staining

All formalin-fixed paraffin-embedded slides were prepared and stained using a standard protocol (Supplemental Material). The slides were incubated overnight at 4°C with anti-β-catenin mouse monoclonal antibody (1:200 dilution; catalog number, 610154; BD Transduction Laboratories™, San Jose, CA). All antibodies were titrated with negative and positive controls to obtain optimal staining. Antibodies used for CYP11B1 and CYP11B2 staining were from Abgent Inc (San Diego CA; CYP11B1, catalog number, AP8723a; CYP11B2, catalog number, AP11213c).

CASE REPORT

A 60-year-old obese (body mass index, 35.7 kg/m^2) Mexican-American male with a 10-year history of insulin-requiring type 2 diabetes mellitus, nondialysis-dependent renal failure (estimated glomerular filtration rate, 18 mL/min/1.73 m^2), with rapid-onset nephrotic-level proteinuria and PA was referred for optimal management of PA. Prior work-up at an outside institution for other secondary causes of hypertension in the setting of an adrenal adenoma included an evaluation for Cushing disease, with a low-dose dexamethasone suppression test, and urinary metanephrine levels, both of which were within normal limits. Oral salt loading, also done at an outside institution, was consistent with the diagnosis of PA, with an elevated urinary aldosterone in the setting of an elevated urinary sodium level. Testing done at the National Institutes of Health as per Endocrine Society guidelines (4) revealed: baseline aldosterone (35 ng/dL; normal, <21 ng/dL) and suppressed plasma renin (<0.6 ng/mL/hour; ratio, 58) on presentation. Saline suppression confirmatory testing showed a 4-hour aldosterone value of 22 ng/dL, with a suppressed plasma renin level of <0.6 ng/mL/hour, along with the presence of hypokalemia (Table 1). A computed tomography (CT) scan revealed a 2.8-cm mass in the right adrenal and a 1-cm mass in the left adrenal gland, both with low attenuation suggestive of bilateral lesions. However, adrenal venous sampling revealed unilateral disease of the left gland (Table 2). The patient was managed on terazosin, verapamil, and hydralazine for his hypertension, and during initial testing (including saline suppression testing), the patient was also managed on clonidine, which was tapered off prior to adrenal venous sampling. The patient opted for left unilateral adrenalectomy, as previous management with antihypertensives, including aldosterone antagonists (due to hyperkalemia), was unsuccessful.

Other testing revealed a low-density lipoprotein (LDL) level of 18 mg/dL, total cholesterol of 91 mg/dL, triglyceride of 202 mg/dL, high-density lipoprotein (HDL) of 33

Table 1
Clinical and Laboratory Evaluation Pre- and Postadrenalectomy

	Presentation (February 14, 2011)	Preoperative (March 18, 2011)	Postoperative (March 30, 2011)
Aldosterone (ng/dL)	35	35	<4.0
Plasma renin activity (ng/mL/hour)	<0.6	<0.6	2.7
Potassium (mmol/L)	3.4	3.1	4.1
Antihypertensive medications (number)	3	3	2, reduced dosages
Serum creatinine (mg/dL)	2.5	4.6	3.2

mg/dL, apolipoprotein A1 of 127 mg/dL (normal, 110 to 205 mg/dL), and apolipoprotein B of 47 mg/dL (normal, 55 to 140 mg/dL) while on simvastatin 40 mg daily. An earlier lipid panel obtained while the patient was not on cholesterol-lowering medication showed: LDL of 56 mg/dL, total cholesterol of 151 mg/dL, triglyceride level of 326 mg/dL, and HDL level of 29 mg/dL. Prior cardiac catheterization was notable for the absence of atherosclerosis. Cardiac CT scan obtained during the current hospitalization revealed minimal coronary artery calcification only in the left anterior descending region. The dyslipidemia was suggestive of hypobetalipoproteinemia (5).

Renal ultrasound evaluation was limited by body habitus and the left kidney could not be visualized. However, there was no evidence of enlargement or hydronephrosis in the right kidney.

Family history was significant for hypertension, with 6 of his 7 siblings diagnosed with hypertension in their 30s. No information on secondary causes of hypertension in the siblings was available. Two of his 3 children, also in their 30s, had been recently diagnosed with hypertension. All 3 children were tested and ruled out for PA at our center. Imaging of the heart, kidneys, and adrenal glands did not reveal any evidence of amyloidosis, and none of the children had proteinuria to suggest incipient LECT2 amyloid of the kidneys. Sequencing of the *LECT2* gene revealed they were homozygous for the polymorphism.

Table 2
Adrenal Venous Sampling Results

Adrenal vein sampling time[a]	Aldosterone (ng/dL)	Cortisol (μg/dL)	Aldosterone to cortisol ratio
−5 Minutes			
Right	49	26.7	1.83
Left	430	23.5	18.29
Peripheral	20	15.7	1.27
0 Minutes			
Right	4	14.4	0.28
Left	670	23.7	28.20
Peripheral	18	16.2	1.13
+10 Minutes			
Right	310	731.0	0.42
Left	1,100	350.0	3.14
Peripheral	27	22.0	1.22
+15 Minutes			
Right	300	616.0	0.48
Left	2,000	301.0	6.60
Peripheral	29	22.7	1.27

[a] Time before (−) and after (+) adrenocorticotropic hormone stimulation.

In this patient, kidney biopsy was performed at the time of complete laparoscopic left adrenalectomy to determine the etiology of rapid-onset renal failure with nephrotic syndrome. Postoperatively, blood pressure improved: two antihypertensives were reduced in dosage, while the third was discontinued. Hypokalemia resolved. On postoperative day 1, plasma renin was detectable at 2.7 ng/mL/hour, and aldosterone was suppressed, at <4.0 ng/dL. One week postoperatively, the patient reported that he was feeling much better than prior to surgery, and we found decreased variability in blood pressure readings and improvement in blood sugar control.

Adrenal pathology revealed the presence of abundant amyloid without evidence of a nodule or hyperplasia. Kidney biopsy also showed amyloid infiltration.

RESULTS

On gross examination, the left adrenal gland, which measured 10 × 4 × 3 cm, appeared focally nodular, with a thickened adrenal cortex. Microscopic examination of the adrenal gland and kidney biopsy revealed the presence of abundant eosinophilic material. The glomeruli of the kidney were globally sclerosed, and Congo red staining of both tissues revealed the presence of green birefringence on polarization (Fig. 1 *A*, *B*). Examination under electron microscopy demonstrated abundant, randomly oriented, filaments of approximately 10 nm in diameter. CYP11B2 staining was positive but not as strong as that seen for CYP11B1 (Fig. 1 *C*, *D*).

Tandem mass spectrometry was used to identify the amyloid protein as LECT2 (Mascot score, 99). Western blotting confirmed the identity of LECT2, with a strong band observed at approximately 15 kDa, which was consistent with the predicted molecular weight. Two additional strong bands were observed at approximately 28 and 45 kDa, which may have represented dimer and trimer forms of the LECT2 protein. Western blot analysis of adrenal tissue processed identically from an unaffected individual did not reveal LECT2 levels that were detectable (Fig. 2). Serum levels of LECT2 were below the level of detection in our patient (lower limit, 0.25 ng/mL). Two age- and gender-matched control patients also had undetectable serum enzyme-linked immunosorbent assay levels (data not shown).

The *LECT2* gene was sequenced using genomic DNA extracted from peripheral blood leukocytes from the patient's children and from DNA extracted from the

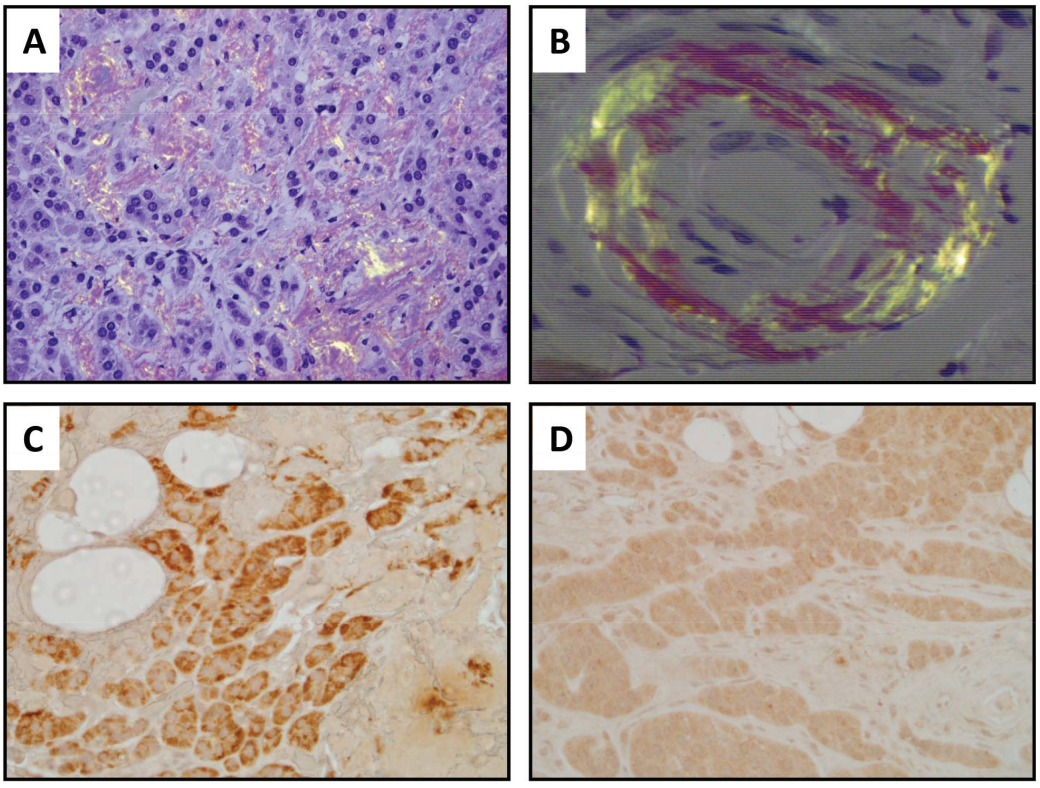

Fig. 1. Adrenal and kidney tissue histopathology. Congo red–stained images of adrenal tissue with polarization at 40× magnification (*A*); Medium-sized interlobular artery of kidney. The intimal layer thickening is typical of hypertensive injury; the medial thickening includes deposition of Congophilic material, which shows typical apple-green birefringence of amyloid deposits under polarized light. Congo red stain, 100× magnification (*B*); CYP11B1 staining, adrenal (*C*); and CYP11B2 staining, adrenal (*D*).

patient's adrenal tissue. The patient was found to be homozygous for the G allele, a known polymorphism of the LECT2 gene usually present in the heterozygote state, at nucleotide 172 of the coding cDNA, which results in the substitution of a valine for isoleucine at amino acid 40 of the mature LECT2 protein.

To determine if there was an association between a mutation in *CTTNB1* and LECT2 amyloidosis, exons 3 and 5 of *CTTNB1*were sequenced from DNA extracted from the adrenal tissue. No mutations were observed in either exon. Sequencing of the *KCNJ5*, *ATP1A1*, and *ATP2B3* genes (which have been implicated in hyperaldosteronism) was also negative for any pathogenic mutations.

The staining pattern for β-catenin of the patient's adrenal lesion was membranous and cytoplasmic (Fig. 3). Analogous β-catenin staining was found in a patient with hyperaldosteronism due to adrenal hyperplasia also negative for *CTTNB1* mutations (Fig. 3).

DISCUSSION

LECT2 AA was first reported by Benson et al (6) in 2008 in a kidney biopsy specimen from a patient with nephrotic syndrome secondary to unexplained renal amyloidosis. In total, LECT2 AA has been reported in 11 patients.

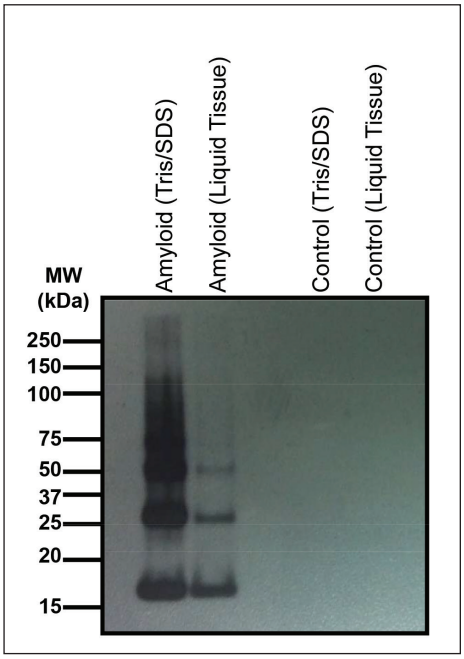

Fig. 2. LECT2 Western blot analysis of adrenal tissue homogenates. Aliquots from the second Tris/sodium dodecyl sulfate (SDS) solubilization step and Liquid Tissue buffer solubilization were analyzed for the presence of LECT2 via Western blot. Strong bands were observed for the amyloidosis sample (left lanes), whereas bands were not observed for the control tissue homogenate (right lanes). *MW* = molecular weight markers.

All reports have only shown renal involvement, although this may be due to selection bias and biopsies limited to the kidney (2,7). This case appears to be the first published report to describe LECT2 AA both outside of the kidney and within the adrenal gland, suggesting that LECT2 AA is not strictly a renal disease. Furthermore, in this case, it was associated with an adrenal disorder, PA. This finding is particularly noteworthy, as previous cases of adrenal amyloidosis showed some evidence of adrenal insufficiency.

In patients with nephrotic syndrome, aldosterone and renin levels have been found to be similar to control subjects. However, compared with control subjects, subjects with nephrotic syndrome exhibit a greater response with regards to urinary sodium excretion with the administration of spironolactone (8). Therefore, this patient's aldosterone excess was not likely the result of nephrotic syndrome. More likely, the nephrotic syndrome was the result of infiltration of the kidneys by LECT2 AA. It is not clear if the LECT2 AA and PA were related in this patient, and if so, how.

The amino acid structure and function of LECT2 was first described by Yamagoe et al (9) in 1996 in relation to its role in the inflammatory response. LECT2 was isolated

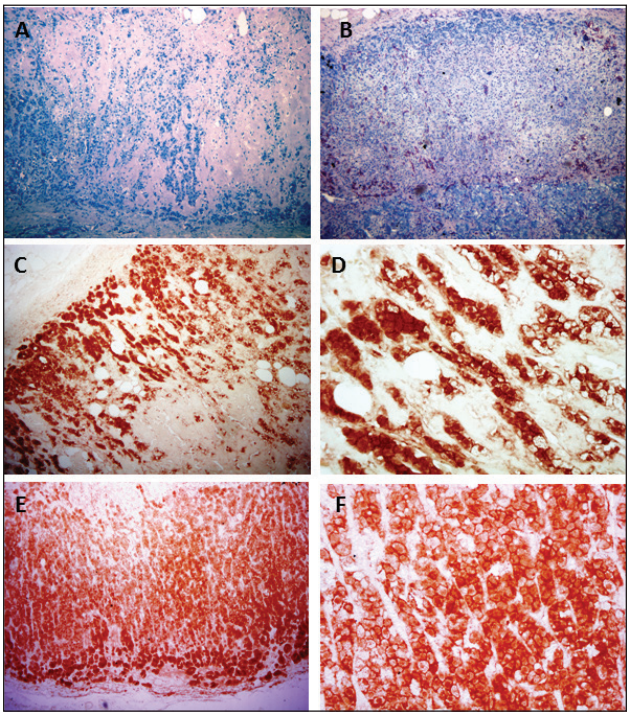

Fig. 3. Expression of β-catenin in patient's left adrenal gland and in a patient with aldosterone-producing adrenal hypeplasia and no *CTNNB1* mutations or other abnormalities of β-catenin signaling. Hematoxylin and eosin–stained section of (*A*) the patient's adrenal, showing pink, homogeneous masses of amyloid; (*B*) a patient without *CTNNB1* mutations; (*C*, ×5; *D*, ×10) β-catenin staining showing a membranous and cytoplasmic pattern; (*E*, ×5; *F*, ×10) adrenocortical hyperplasia without amyloid. β-Catenin staining shows a membranous and cytoplasmic pattern, as in the patient with adrenal amyloidosis and hyperaldosteronism.

as a neutrophil-chemotactic factor produced by T cells (9). More recently, *LECT2* has been described as a target gene of β-catenin. The β-catenin pathway plays an essential role in Wnt signaling, and mutations in β-catenin have been linked to tumorigenesis (10-12). Studies on liver carcinogenesis due to β-catenin mutations demonstrated induction of *LECT2* expression (13). This upregulation of *LECT2* was specific to tumors induced by β-catenin oncogenesis, in contrast to β-catenin-independent tumors, in which there was no increased expression of *LECT2*. Furthermore, studies in mice with β-catenin-induced liver tumors demonstrated that those mice with *LECT2* gene knockouts develop more aggressive metastatic tumors (14). Activation of *LECT2* is reported to be related to a proinflammatory response, leading to development of tumors with a better prognosis. This suggests that LECT2 and its chemotactic properties play a protective role in tumorigenesis. Activating mutations in the Wnt signaling pathway have also been linked to adrenocortical tumors, and β-catenin mutations have been reported as the most frequent mutation in adrenocortical adenomas (15). Recent data by Berthon et al (16) showed that Wnt/β-catenin activation is a frequent alteration, found in 70% of aldosterone-producing adenomas; however, it is not clear what role, if any, LECT2 plays in adrenal tumorigenesis or the development of PA, as in our patient (16).

Characterization of patients with LECT2 amyloidosis mostly comes from a small case series by Murphy and colleagues (2), in which 7 of 10 patients with LECT2 AA were Mexican American. Serum LECT2 levels were obtained in 2 of the 10 patients, one of which had undetectable levels, as in our patient, whereas the other patient had a level in the normal range. LECT2 levels in serum have been shown to be elevated during acute liver injury, and serum LECT2 levels have been found to be a prognostic indicator in acute liver failure (17). It is unknown, however, what role serum LECT2 levels play in the evaluation of LECT2 amyloidosis.

Of the 4 out of 10 patients tested for genetic polymorphisms in the case series, all 4 patients were found to be homozygous for the G allele at position 172 of the cDNA, as in our patient. It has been hypothesized that the substitution of valine (G allele) for isoleucine (A allele) results in a destabilized protein with a higher propensity to form amyloid fibrils (2).

Although PA has been linked to cardiovascular disease, and diabetes is a known cardiovascular risk equivalent, this patient had no evidence of cardiovascular disease. Cardiac catheterization performed prior to his hospitalization showed minimal atherosclerosis. Additionally, a cardiac CT scan performed during his hospitalization revealed a lower Agatston coronary calcium score than would be expected, given his multiple risk factors. One possible explanation for the apparent lack of significant cardiovascular disease could be his unusual lipid profile. The LDL level was extremely low, suggestive of hypobetalipoproteinemia, although the elevated triglyceride level argues against this diagnosis. Genetic deletions or mutations in ApoA-1 have been associated with systemic amyloidosis, although they are associated with low HDL, and, with complete gene deletion, premature coronary artery disease (18-20). It is not clear if there was a link between the patient's unusual lipid profile and amyloidosis. It is likely, however, that his low LDL level was cardioprotective.

LECT2 AA was found in both renal and adrenal tissue in this patient, although it is possible that other organs were involved. The patient died at home unexpectedly a few weeks following surgery, after his discharge from the hospital. No clinical information was obtained by the family regarding the cause of death. The possible etiologies of his sudden death include a conduction abnormality due to cardiac amyloidosis, pulmonary embolism, or other causes. As no postmortem examination was performed, it is impossible to know if LECT2 AA played a role. Further review of imaging, including cardiac CT and echocardiogram obtained prior to the patient's death, did not reveal any evidence of cardiac amyloidosis.

This is an unusual clinical case in that the patient had PA, LECT2 AA, and a lipid panel suggestive of a hypobetalipoproteinemia. It also represents the first published case of LECT2 AA in the adrenal glands. It is not clear whether LECT2 AA and PA were related in this patient. In addition, it is unclear why no adrenal nodule was found on pathology despite imaging that would suggest the contrary. A recent report by Nanba et al (21) describes distinct subtypes of PA with differing anatomic and microscopic features, which may account for this as well as the low expression of CYP11B2.

CONCLUSION

The absence of a *CTTNB1* mutation and the noncharacteristic pattern of β-catenin staining are not supportive of a role for β-catenin in the hyperaldosteronism of this patient. In contrast, patients with *CTTNB11* mutations and/or *CTTNB1* involvement in their disease have mostly nuclear β-catenin staining (16). Although many questions remain unanswered, this case report invites further investigation into LECT2 AA and its role in the adrenal glands.

ACKNOWLEDGMENT

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DISCLOSURE

The authors have no multiplicity of interest to disclose.

SUPPLEMENTAL MATERIALS

Protein Identification via Mass Spectrometry

Mass spectrometry was carried out on a model 4800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems). Mass spectra (MS) were acquired in the positive ion mode with internal calibration. Tandem mass spectra (MS/MS) were acquired with 1-kV acceleration energy in the 'gas-off' mode (unimolecular decay). MS and MS/MS data were searched against the SwissProt database (downloaded, March 21, 2012; taxonomy, human; 20,329 sequences) via the Mascot search engine with the following search parameters: MS tolerance, 0.15 Da; MS/MS tolerance, 0.06 Da; fixed modifications, carbamidomethyl (C), for the second analysis only. Significant hits resulting in identification were considered, with 2 peptide identifications and individual ion scores >28 (P<.05) indicating identity or extensive homology.

DNA Extraction and Sequencing of the *LECT2*, *CTTNB1*, *KCNJ5*, *ATP1A1*, and *ATP2B3* Genes

DNA was extracted from blood and fresh, frozen adrenal tumor samples of the patient using a Gentra Purgene kit (Qiagen) according to the manufacturer's protocol. Purified DNA was quantified using a NanoDrop 2000 (Thermo Scientific) or Qubit 2.0 fluorometer (Life Technologies). Mutation analyses for exons, exon-intron boundaries, and flanking intronic regions were performed for the *LECT2*, *CTTNB1*, *KCNJ5*, *ATP1A1*, and *ATP2B3* genes. The sequences of primers used are shown in Supplemental Table 1. Polymerase chain reaction (PCR) conditions are available on request. The PCR product (~50 μL) was mixed with 10 μL of Orange G loading dye and the solution was loaded onto a 1.5% (w/v) agarose gel and electrophoresed at 120 V. The bands were then imaged and each band was excised from the gel. DNA was purified from the excised gel bands using a Mini Elute kit (Qiagen) according to the manufacturer's protocol.

The sequencing reaction was carried out using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). A total of 10 to 20 ng of the purified exon was prepared according to the manufacturer's protocol. The resulting dye reaction was purified using the ethanol purification method. Briefly, 2.5 μL of 125 mM ethylenediaminetetraacetic acid was added to the reaction solution and mixed. Then, 50 μL of cold ethanol was added to the mixture. The samples were incubated on ice for 15 minutes and then centrifuged at 4°C for 15 minutes at 14,000 rpm. The supernatant was removed and 200 μL of cold 75% ethanol was added to the tube. The solution was centrifuged at 4°C for 15 minutes at 14,000 rpm. The supernatant was discarded and the resulting product was dried via centrifugal vacuum. The purified product was resuspended in 11 μL of Hi-Di solution. Sequencing was performed on an Applied Biosystems 3130XL Genetic Sequencer.

Enzyme-linked Immunosorbent Assay (ELISA)

Whole blood from patients was collected in a red top tube (~10 mL). Samples were centrifuged at 2,200 rpm for 5 minutes. The supernatant (serum) was transferred to fresh tubes (200-μL aliquots) and frozen at −80°C until use. Serum LECT2 concentrations were measured using the human LECT2 ELISA from USCN Life Science (catalog number E95541Hu) according to the manufacturer's protocol.

Immunohistochemistry

Paraffin sections (4-μm thickness) were mounted on a glass slide, deparaffinised in xylene, rehydrated in a decreasing series of alcohol solutions, and washed in phosphate-buffered saline (PBS; pH 7.3). Antigen retrieval was achieved by boiling the slides for 30 minutes in a rice cooker in a 1:100 dilution of a citrate-based antigen-unmasking solution (H-3300, Vector Laboratories), cooling to room temperature, and washing with PBS. After endogenous peroxidase had been quenched with an aqueous solution of 3% hydrogen peroxide/H_2O for 30 minutes and the sample was washed with PBS, the sections were incubated at 4°C overnight with a β-catenin monoclonal mouse antibody (BD Transduction Laboratories™, San Jose, CA; catalog number 610154) at a 1:200 dilution in antibody diluent solution and then washed with PBS. Next, the sections were incubated with secondary antibody (anti-mouse Ig, ImmPRESS Reagent kit; Burlingame, CA) for 30 minutes at room temperature. Color development was performed with 3,3'-diaminobenzidine.

Supplemental Table 1
Sequences of Primers Used in the Present Study

Gene	Exon	Forward primer	Reverse primer
LECT2	1	TCGATATGCACCTAGCCTTATC	GCCAAATACACAAGTCTTTGTTG
	2	CTGGAAGGACGATCTTCCC	CATGCTGTCAGAACTGTGAGC
	3	GTTCTGGCAGCTTCAAGC	CATCAGCTAGTTTGGGTACACTC
	4	CCTCTCCTGAGTTCATTCAAC	TCAAGCAGAAAGGTTGCTAG
KCNJ5	2.1	GAGGATTTCACGCCCTGAC	CCCGGATATAAGCAATGAGC
	2.2	CGCTTCAATTGCTCGTCTT	ATGAACTCCCCCTCTTTGGT
	2.3	TCCAACAACGCAGTCATCTC	CCAGTACCCCTCAAACCACA
	3	ATGGATGGATGAACAGATGAC	GTTTATGGTTCCTCCCCAC
CTTNB1	3	TCTTGGCTGTCTTTCAGATT	TCACTATCCACAGTTCAGCA
	5	TCAAGGGGAGTAGTTTCAGA	TGGTTGCCATAAGCTAAAAT
ATP1A1	1	GACGCTGGGGCTTAGCTT	GGGTCTGCCTATCCTCCTTC
	2	ATTCATGGCCTCACTTTTCC	TGGGGGATTAAAAATCATGG
	3	TGCACTGAAGAAAACATCTGC	TCAAAAAGTTTCAGGGAAAAGAA
	4	ATCCTTATTGCAACCGTCCA	GAGGAAATGGAAACGAAGGA
	5	TAGAGCCACGGGCCCTAA	GGGCTGTCAAGTCATCCACT
	6	GGACACTACCTTCTCTTTGTTGG	GCCCGAACAGCAGAGCTA
	7	GGCTGGTGTATTCACATGACA	CCAACACCCATGCCAGAC
	8	GTGTAAAATCCGTGGCTTCC	ATCAAACATCCAGAATAAAACATAAG
	9	CAAACTTCAGAAGAAGGTTGGA	TCCCATACCCTTGACTCTTCA
	10	AACACAGCCTTTGAAGTTAATGC	TGCACAGACCACTCCAGGT
	11	CCCATCCTCTGCTACCTCGT	TCAAGGGCAAAATTTAAAGC
	12	TTGTTTTCCACCATGGACTG	GGAGGATTGCCCCTAAAAGA
	13	AAGGTAGGCGGGCTGAATAG	TCTGGACCTCAAAAGGCAAC
	14	GGCAGTTGCATTCAACACAT	CAGTGCAGGTACATTTCCTACTCA
	15	TGCATAAGAATATCCCCTCTTGA	CCCTCTCTATGCCTATGAGCTT
	16	TGCATCGCACTATTTCCATC	GAGACAAGCACACTGGAAACTG
	17	CTGCCCATTAGCATCCATTT	GGATTGGGACACACACTGG
	18	CAAAAGGTTCACAATATTAGCTTCC	TGAAAGTCGCCTGGATTTG
	19	TTATCAACCAGGGGGAAACA	TGCAGAAGACAGAAGATGAGC
	20	ATGCAGGTGAAGAGCCAGAG	TTGAAAACTTGGAGGTCAGATG
	21	TCTGCCATTTTAATGCATAGC	GCCTCAGGATTTGTTAGTCTGA
	22	TTTCTTTATTTGAACTGTGTTCGT	TGACTATGGTCAGTTTGCTGGT
	23	TGAGGTCCCATGTTTGGATT	ATAAAAACCTTCCCCGCTGT
ATP2B3	1	CTTAGCAGCTTTCTCACCGC	AGGGAAATGGCTGAGAGGAG
	2	TTCTGCACTTCGAGGGTCAT	TCTCCCCCTCAAGGGAAAT
	3	CAGTCAGTCAGGGCCACATA	CAGTGGAGGAGACATCACCA
	4	CCCTCATCATCCTGGAGGT	GCTGTGGGGACAGTTGATTC
	5	GGATGCAAATGTCCTCGTTC	CAACCTGAAGGTTATGCTGGA
	6	AAGAAGGCCACCAGCAATC	TGTGCTCTCTCCTCCCTCTG
	7	AGGAGGCGAGAGAAGGGAAG	CACACATCACCTGCCACATC
	8	CGAGACCGTGTCCATACCTC	CCCAGTTTATACTGCCACCAA
	9	ACTCCTCCACCCCAACAAG	ATGAGCACCTTGCAACAGC
	10	CAGCATGGAGGGTGGTCT	GGCCTCTTTTACCACGACAA
	11	GACTAAGCCCCCAATCCAGT	CTACCAGCTCGGACAAGCTC
	12	TGATGGAGGAAGGGGTACTG	AAGGACGCTGCAGAGCTAAG
	13	CTTAGCTCTGCAGCGTCCTT	CAAATGGACCCAGCTTCCTA
	14	CAGGGTTGTGGTGACAGATG	CCCACTTCCTGGTACAAAGC
	15	GGGGTGGGATGTTATTCAGA	TCTCACAATCTGGCCCTCA
	16	TGAAATCCTGGTGATGTGGA	ACAGAATCTGAGCCCGAGGT
	17	TCCTGAGTAATGCGTCATGC	CCAGTACTAGGGTGTGACGA
	18	GACAAGGGTGGCTCAGGA	GAAGCAGGGTTAGGGCAAGT
	19	ACTACGCCAGTTCTCAAGG	CAGCAAGACCAAAGGGTTTC
	20	AGGGCCCTGTCCAAACAT	GAGAGACACCGTCTGG
	21	TACCTTCCTTCCACGTTTCC	GTCCCCATATGTGTGTGTGC

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