Specific electron transport chain abnormalities in amyotrophic lateral sclerosis

Jerry Lin · Andrew Diamanduros ·
Soheli A. Chowdhury · Stephen Scelsa ·
Norman Latov · Saud A. Sadiq

Abstract In an amyotrophic lateral sclerosis (ALS) patient who also had an IgA gammopathy, autopsy studies identified the IgA in the surviving motor neurons. Further, the IgA bound the surface of isolated bovine motor neurons and inhibited neuronal proliferation in culture. To determine the pathologic basis of this IgA interaction with motor neurons, a neuroblastoma cDNA library was generated and screened with the IgA monoclonal antibody. Reactive clones were identified as flavin adenine dinucleotide (FAD) synthetase. To extend this finding to ALS in general, quantitative RT-PCRs were performed on blood samples from 26 ALS and 30 control blood samples to determine mRNA expression levels of FAD synthetase and other electron transport chain proteins, specifically riboflavin kinase (RFK), cytochrome C1 (CYC1), and succinate dehydrogenase complex subunit B (SDHB). All expression levels were measured against a control enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression levels for a non-respiratory chain protein (beta-actin) were also measured. We found that FAD synthetase expression levels were decreased in ALS samples compared to expression levels in controls ($P = 0.0151$). Expression levels for RFK, CYC1, and SDHB were also significantly decreased in the ALS group ($P = 0.0025$, $P = 0.0002$, and $P < 0.0001$, respectively). As control, expression levels for beta-actin did not show a significant difference between ALS and control groups ($P = 0.2118$). Our data show that a reduction in electron transport proteins, namely FAD synthetase, RFK, CYC1, and SDHB, is seen in patients with ALS. It is possible that this may have an effect on oxygen-dependent metabolic pathways. Human motor neurons may be particularly susceptible to injury if there is sub-optimal oxidative metabolism.

Keywords ALS · Mitochondrial disorders

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurological disease characterized pathologically by degeneration of upper and lower motor neurons. The clinical course is inexorably aggressive in the vast majority of cases and results in death within 4–5 years from disease onset [21]. Approximately 90% of all ALS cases arise sporadically, and the cause of sporadic ALS (SALS) remains unknown. Etiological considerations have included acquired superoxide dismutase 1 (SOD1) abnormalities, glutamate toxicity, oxidative stress, mitochondrial dysfunction, neurofilament abnormalities, genetic factors, viral infections, paraneoplastic disease, and autoimmune phenomena [8, 17, 19, 20, 26, 29].

In the past, autoimmune mechanisms in ALS were considered important because 5–10% of ALS cases are associated with paraproteins [22, 25, 30]. Further, an immune model of the disease was developed by

J. Lin · S. A. Chowdhury · S. A. Sadiq (✉)
Multiple Sclerosis Research Center of New York,
521 West 57th Street, 4th Fl., New York, NY 10019, USA
e-mail: ssadiq@msrcny.org

A. Diamanduros
Dept. of Biology, Georgia Southern University,
202 Georgia Ave, #8042, Statesboro, GA 30460, USA

S. Scelsa
Beth Israel Medical Center of New York, 10 Union Square East,
Suite 5D, New York, NY 10003, USA

N. Latov
Weill Medical College of Cornell University,
635 Madison Avenue, Suite 400, New York, NY 10022, USA

immunizing experimental animals with purified populations of spinal motor neurons [11]. However, the autoimmune hypothesis has not developed further, and immunosuppressive treatment has not had a therapeutic effect in patients with ALS [2, 4, 10].

Monoclonal gammopathies if associated with neurological diseases may provide important information about putative neural antigens [24]. Investigation of monoclonal antibodies in human disease is important because the monoclonality implies antigenic specificity. In diseases other than ALS, previous investigation of monoclonal antibodies has led to the discovery of anti-MAG antibody-associated neuropathy [15] and to the diseases associated with anti-GM1 antibodies [23]. Also, the target of the monoclonal gammopathy may help identify a key neuronal receptor or protein that is critical for motor neuron integrity. Disruption of its structure or function by any mechanism may lead to motor neuron death.

This study is based on the investigation of an IgA monoclonal antibody (MAb) associated with an autopsy-confirmed sporadic ALS patient. The clinical and immunopathological findings in our index patient were previously described [13, 24]. Briefly, the index patient was a 75-year-old female who presented with a 2-year history of muscle wasting, weakness, and bilateral Babinsky responses. Electromyographic findings were diagnostic of ALS, and the patient died of an unrelated cardiac arrhythmia. Autopsy confirmed the clinical diagnosis, and, in addition, the IgA MAb was present in surviving neurons and large neurites of the ventral horn. Our initial studies also found by immunocytochemistry that the IgA MAb bound the surface of neuroblastoma cells in culture and by Western blot reacted with a 65-kDa protein. The MAb also cross-reacted with the high molecular weight neurofilament protein (NFH). Our current investigations suggest that the 65-kDa protein is the electron transport chain enzyme, flavin adenine dinucleotide (FAD) synthetase. We extended these investigations to determine whether similar electron transport chain abnormalities occurred in other ALS patients.

Methods

Cellular Proliferation ELISA Assay

The Cell Proliferation ELISA Assay was purchased from Amersham Pharmacia Biotech (http://www.apbiotech.com). All procedures were performed as described in the manual. In summary, the assay is based on incorporation of the thymidine analogue BrdU into the DNA of proliferating cells. Cells are grown in microtiter plates at a density of 3.5×10^4 cells/ml with control or index patient sera for

53 h. BrdU was then added and incubated for an additional 22 h. Cells are grown in a 37°C humidified incubator with 5% CO_2 in DMEM supplemented with 10% FCS. Cells are then washed, fixed, DNA denatured, and permeablized. Incorporated BrdU is detected by peroxidase-labeled anti-BrdU antibodies. The substrate reaction is detected at 450 nm in microtiter plate spectrophotometer. Each experimental condition was done in triplicate. This assay was designed to determine the effects of the IgA MAb on neuronal proliferation and to compare the inhibition to that seen with control serum.

Generation and screening of cDNA library

Procedures for cDNA cloning and screening were performed as described in detail in Lambda ZAP cDNA Synthesis Kit (Stratagene, catalog no. 239616) and the picoBlue Immunoscreening Kit (Startagene, catalog no. 200372). In brief, first strand cDNA was synthesized from 5 μg of LAN-5 cell line mRNA (Micro-FastTrack Kit, Invitrogen, catalog no. K1520-02). The resulting phage titers were 1.4×10^7 pfu/ml for the initial library and 4.2×10^{10} pfu/ml for the amplified library. Twenty-four 150×20-mm plates were screened and yielded three positive clones. All clones were purified until all plaques were positive. Patient plasma was screened at a 1:1,000 dilution. Secondary affinity purified alkaline phosphatase conjugated goat anti-human IgA antibodies (Cappel, catalog no. 59286) were also used at 1:1,000. Plasmid purification was performed using Qiagen's Plasmid Mini Kit (catalog no. 12125).

Sequence analysis of purified reactive cDNA clone

All three positive clones were sequenced using the dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, catalog no. 403044) on the ABI Prism 310 Genetic Analyzer. All three clones were identical and revealed a sequence 833-bp long containing an open reading frame from base pair 2 through 737.

Protein expression and purification of reactive cDNA clone

New England Biolabs' Impact T7: One-Step Protein Purification System (catalog no. 6700) was selected for the expression and purification of our clone. All procedures were performed as described in the kit manual. The pTYB3 plasmid was used. Primers were designed to insert a NcoI site and a SapI site at the 5′ and 3′ of our reactive clone, respectively. In addition, 110 bases of the pBluescript phagemid, starting at the seventh base downstream from the ATG (Met) of pBluescript were included at the 5′ end

to facilitate protein expression. An extra glycine (GGT) amino acid was also included at the 3′ end to increase efficiency of the intein-mediated self cleavage. The resulting expressed protein is 32 kDa in mass. Protein concentration was determined using the Bradford method (Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 1996, p. 10.1.4). Protein purity was checked via Western blot, Coomassie Blue staining, and ELISA.

Epitope mapping

Sixty-four overlapping peptides of a truncated 737-bp sequence of the reactive insert were commercially synthesized and covalently bound to plastic pins (Mimotopes). The plastic pins are arranged in an ELISA plate array for use in a modified ELISA procedure. Each peptide is 15 amino acids in length with consecutive peptides having 12 identical amino acids with the previous and following peptides. The scale is 0.05 μmol. The peptides were screened against our index patient's serum at 1:1,000 dilution with the secondary anti-human IgA antibodies (Cappel, catalog no. 59286) also at 1:1,000.

Patient and control samples

Patient blood samples were collected by venous needle puncture according to guidelines approved by the Institutional Review Board. All patient samples were taken from clinically confirmed cases of ALS. Control samples were obtained from normal volunteers who had no neurological diseases. All samples were collected with informed consent.

Antibody ELISA screening

Sera were collected from 27 ALS patients and 30 controls. Then 0.2 μg of purified protein was used to coat each well. Sera and secondary antibody were used at 1:1,000. Each sample was screened in triplicate and optical densities (OD) averaged. Averaged ODs on each plate were calibrated (divided) to the average OD of the index patient at 1:25,600 on that plate. Plate-to-plate variations were accounted for using this method. The P value derived from the Mann–Whitney non-parametric t-test.

Quantitative real-time PCR

Total RNA was isolated from 26 ALS patients and 30 controls using Qiagen's PAXgene Blood RNA Kit (catalog no. 762164). cDNA was produced using 1 μg of total RNA (unless indicated otherwise) using Invitrogen's Superscript III First-Strand Synthesis System for RT-PCR (catalog no. 18080-051). Quantitative real-time PCR was performed on the Roche Lightcycler 2.0 using Lightcycler FastStart DNA MasterPlus SYBR Green I (catalog no. 03515885001) kits. The primers used are shown in Table 1.

Cycling conditions were as follows; 10 min at 95°C initial denaturing, 40 cycles of 10 s at 95°C denature, 10 s at 65°C annealing, and 20 s at 72°C extension. Standard curves were generated from pCR2.1 plasmids with the respective amplicon cloned in. Plasmid copy numbers were calculated using absorbance at 260 nm. Tenfold dilutions were made to generate the standard curve ranging from 10^7 to 10^1 fold copies. A relative quantitative experiment was performed on the samples. Expression levels were measured as a ratio to the GADPH gene. All samples were normalized to the same calibrator sample present on all runs.

Results

Index patient's IgA inhibits neuroblastoma LAN-5 proliferation in vitro

We previously reported that the MAb inhibited LAN-5 proliferation as measured via radioactive thymidine uptake assay [1]. To reaffirm this finding, we re-examined its effect on the proliferation of LAN-5 cells in vitro via the cell proliferation ELISA assay from Amersham Pharmacia Biotech. As seen in Fig. 1, the addition of control human sera increased LAN-5 proliferation in culture, while the

Table 1 Quantitative PCR primers

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Glyceraldehyde-3-phosphate dehydrogenase	GAAACTGTGGCGTGATG	GCTGTAGCCAAATTCGTTGTC
Beta-actin	CGGGAAATCGTGCGTGACA	ACGGAGTACTTGCGCTCAG
FAD synthetase 5′	GGGATTTGGGAACACGTTTAT	GGTGGCTACCTCATCAGGTACA
FAD synthetase 3′	GGAGGGGATGAAGGGACTATT	TCAGGTAATTTCCTCTGCACAG
Riboflavin kinase	GGCATCCCCACAGCTAATTTT	TGCTGAAATAAGTGACTCTAAAG
Succinate dehydrogenase complex subunit B	TTGCACCCGAAGGATTGAC	GATGGTGTGGCAGCGGTAT
Cytochrome C1	GGCATGGTGGTGAGGACTA	TTCTGGCAGGATGGCAAGC

Fig. 1 *Bar graph* of cell proliferation assay measuring BrdU incorporation. LAN-5 neuroblastoma cells were incubated with normal or index patient sera in the presence of the thymidine analogue BrdU. Cell proliferation is proportional to the amount of BrdU incorporated. Incorporated BrdU is detected by peroxidase labeled anti-BrdU antibodies. Each experimental condition was done in triplicate
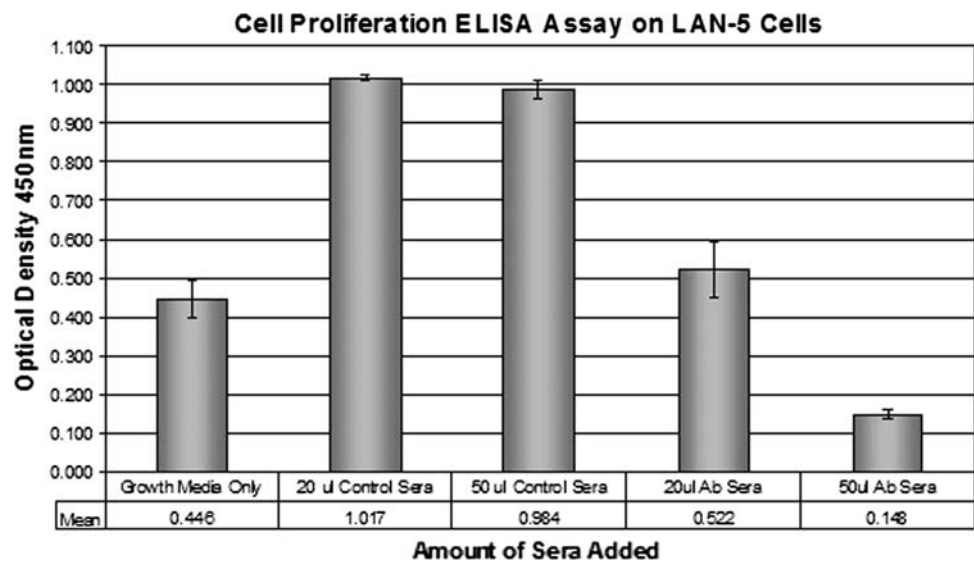
addition of the index patient's sera had a concentration-dependent inhibitory neuronal effect. This study suggests the index patient's IgA paraprotein has a pathogenic effect on neuronal proliferation.

Identification of IgA antigen as a 32-kDa FAD synthetase fragment

To further define the pathogenic role of our index patient's MAb, we identified the IgA's target antigen. A cDNA expression library was constructed from LAN-5 cells and screened with the MAb. Reactive clones were isolated, sequenced, and yielded a clone 833 bp in length. Bases 31-813 of our clone matched bases 1,410–2,192 of FAD synthetase isoform 1 (accession NM_025207) and bases 966–1,748 of FAD synthetase isoform 2 (accession NM_201398) as seen in Fig. 2a. Bases 1–30 appears to be a ubiquitous sequence found in the 5′ and 3′ end of many mRNA clones spanning across species. The bulk of our clone corresponds to the 3′ end of both isoforms of FAD synthetase.

To confirm the reactivity to the MAb, we expressed and purified our FAD synthetase fragment. Our recombinant protein yielded a 32-kDa protein. Western blotting (Fig. 2b right) and ELISA (Fig. 2c) both confirmed the reactivity of the MAb, with ELISA showing reactivity even at dilutions greater than 1:100,000. A Coomassie stain showed the purity of our purification (Fig. 2b left). The purified protein was used in our subsequent antibody screening experiment.

Identification of target epitope

Epitope mapping was performed to confirm reactivity in the FAD synthetase region of our reactive clone. Of the 64 overlapping peptides of 15 amino acids in length, 3 peptides showed reactivity to the MAb (Fig. 2d). Peptides 5, 6, and 7 correspond to bases 62–124 in our reactive clone and within the region matching FAD synthetase (Fig. 2a).

ALS patients' reactivity toward FAD synthetase

To investigate whether other ALS patients have reactivity toward FAD synthetase, we collected sera from 27 ALS patients and 30 controls and screened them against our recombinant protein. Sera were screened at three dilutions, 1:800, 1:1,600, and 1:3,200. Mann–Whitney tests were performed on the optical densities to determine if statistical significant differences exist between the two groups. A significant difference was found at 1:800 dilution ($P = 0.0316$); however at dilutions of 1:1,600 and 1:3,200, the differences were not significant ($P = 0.1118$ and $P = 0.1192$, respectively) (data not shown).

Relative quantitative polymerase chain reaction

In our index patient, we show that the binding of the IgA MAb may have a pathological role. Its binding may be compromising the action of FAD synthetase by decreasing the levels of functional FAD synthetase. To investigate whether ALS patients have abnormal expression levels of FAD synthetase, we performed relative quantitative PCRs on 26 ALS and 30 control blood samples (Table 2). We found that in comparison to control subjects, ALS patients had significantly lower levels of the oxidative enzyme. FAD synthetase is a mitochondrial enzyme that catalyzes the adenylation of flavin mononucleotide (FMN) to form FAD, a critical component in the electron transport chain. Two isoforms of FAD synthetase are known (Fig. 2a). Two sets of primers were designed; FAD 5′ amplified exclusively FAD synthetase isoform 1, whereas FAD 3′ amplified both
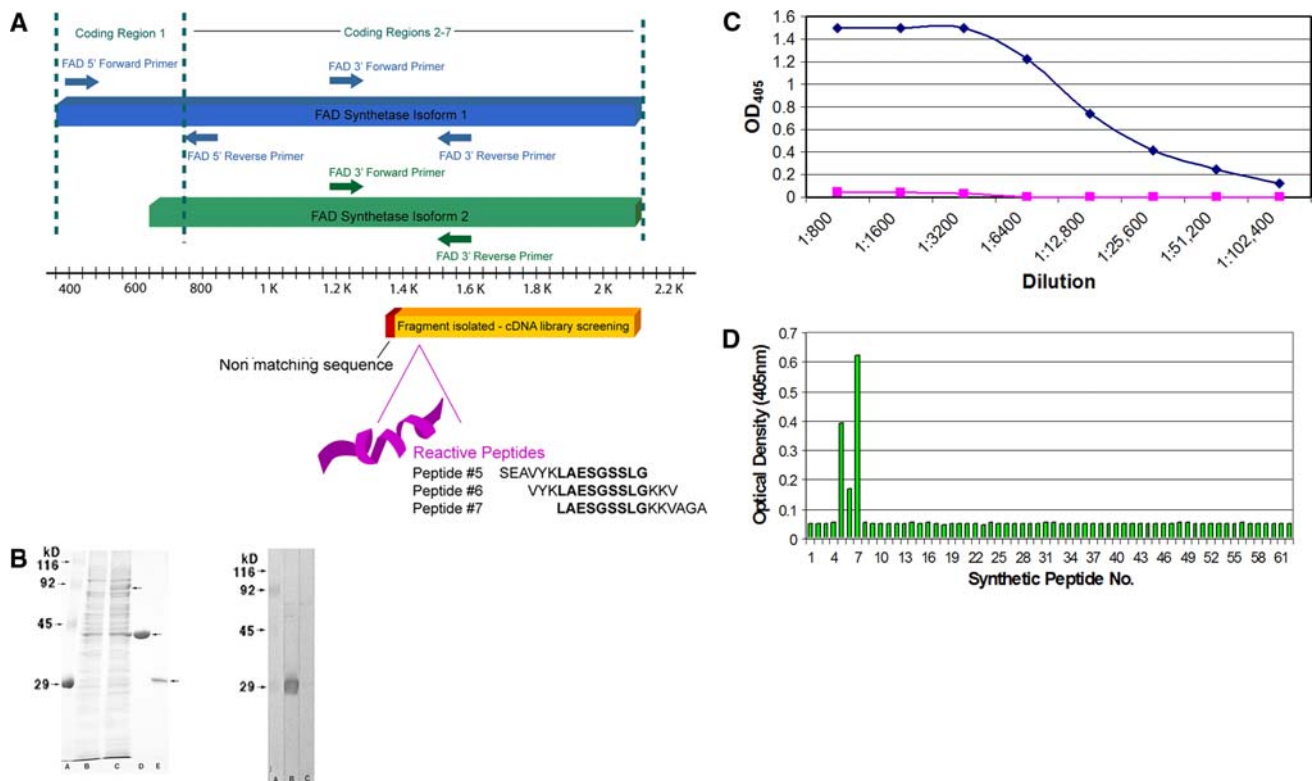
Fig. 2 a Graphical representation of the two known FAD synthetase isoforms and the isolated fragment from the cDNA library screening aligned. Isoform 1 codes for a 65-kDa protein and isoform 2 varies from isoform 1 by using a downstream ATP start site in coding region 1. Also shown are the locations of the reactive peptides from the peptide mapping and the two primer sets used for QPCR. FAD 5′ primer set only amplifies isoform 1, while FAD 3′ primer set amplifies both isoforms. **b** *Left* Coomassie stain of SDS-PAGE showing expression of our protein fragment. *Lane A* MW markers, *lane B* uninduced cell lysate, *lane C* induced cell lysate with arrow pointing to expressed protein fusion, *lane D* purified kit control, maltose-binding protein, *lane E* purified FAD synthetase fragment (32 kDa). *Right* Western blot showing reactivity to 32-kDa expressed fragment. *Lane A* MW markers, *lane B* Purified FAD Synthetase 32-kDa fragment with index patient plasma, *lane C* secondary antibody control. **c** Graph of ELISA showing reactivity of the MAb against our recombinant FAD Synthetase fragment. Index patient serum in *blue* and control serum in *pink*. **d** Epitope identification using peptide mapping of FAD synthetase fragment sequence with index patient plasma. *Peptides 5*, *6*, and *7* show increased reactivity

isoforms. We found a significant decrease in mRNA expression levels between the ALS group and the normal control group with both the FAD 5′ primer sets and the FAD 3′ primer set (Fig. 3, $P = 0.0201$ and $P = 0.0151$, respectively). We further investigated other mitochondrial components related to the electron transport chain and found significant reductions in mRNA expression levels of riboflavin kinase (RFK) ($P = 0.0025$), succinate dehydrogenase complex subunit B (SDHB) ($P < 0.0001$), and CYC1 ($P = 0.0002$) in ALS patients, but not in control subjects (Fig. 3). The control beta-actin gene was not significantly different between the two groups (Fig. 3). Our data as summarized in Table 3 indicate that mitochondrial function may be suboptimal in patients with ALS.

Discussion

The cause of SALS remains elusive. Our investigations are based on a patient with ALS who also had an IgA mAb. A number of observations in this patient suggested that the IgA mAb may be pathogenically related to the motor neuron degeneration. At autopsy, the IgA mAb was found to be present in the surviving motor neurons, and by immunofluorescence the antibody bound specifically to motor neurons in spinal cord cross sections [13]. Further, it bound to human neuroblastoma cells in culture and isolated bovine motor neurons. By Western blot it reacted with a 65-kDa protein and cross-reacted with the high molecular weight NFH and bound to axonal processes [24]. Finally, the mAb inhibited neuronal proliferation in culture [1].

Our investigations in the index patient identified the putative 65-kDa antigenic target of the IgA mAb as an enzyme involved in the cellular oxidative phosphorylation pathway, FAD synthetase. We further identified the reactive epitope of FAD synthetase by peptide mapping. The reactive epitope on FAD synthetase did not reveal any homology to the peptide sequence of NFH, and it may be that the IgA MAb cross-reactivity is based on a conformational epitope. Irrespective of the nature of cross-

Table 2 Relative expression ratios of ETC proteins

Sample	FAD 5′	FAD 3′	RFK	ACTB	CYC1	SDHB
ALS001	3.64	1.89	1.65	1.05	0.99	1.18
ALS002	1.69	1.16	1.47	0.97	0.99	1.14
ALS003	1.72	1.52	0.90	0.99	1.06	1.36
ALS004	4.18	2.27	1.53	0.99	1.56	1.30
ALS005	3.51	1.84	1.84	0.82	1.19	1.01
ALS006	4.75	2.01	2.69	0.65	1.25	1.38
ALS007	1.64	1.39	1.67	0.97	1.24	1.13
ALS008	1.19	1.29	1.56	0.82	0.96	1.04
ALS009	1.59	1.64	1.33	1.09	1.22	0.89
ALS010	0.95	1.02	0.79	0.82	0.98	0.81
ALS011	1.67	1.48	1.35	0.80	1.30	0.99
ALS012	1.08	1.18	1.21	0.76	0.99	1.08
ALS013	1.65	1.88	1.04	0.74	1.49	0.86
ALS014	1.09	1.13	0.95	0.74	0.99	1.04
ALS015	1.19	1.61	1.01	1.12	1.29	0.99
ALS016	3.03	1.77	2.32	0.63	1.37	1.24
ALS017	3.31	2.23	2.68	0.70	1.38	1.32
ALS018	1.36	1.58	1.17	1.03	1.19	1.01
ALS019	2.79	2.10	1.89	1.00	1.32	1.52
ALS020	1.60	1.57	1.01	1.05	1.27	1.19
ALS021	4.11	2.26	2.42	0.76	1.43	1.42
ALS022	2.07	1.62	1.17	0.73	1.24	1.21
ALS023	2.22	1.66	0.79	0.92	1.31	1.13
ALS024	1.76	1.69	0.97	0.94	1.29	1.05
ALS025	1.90	1.23	0.84	0.88	1.31	0.81
ALS026	2.43	2.06	1.93	0.67	1.29	1.19
CN001	2.05	1.74	2.35	0.97	1.37	1.35
CN002	3.74	2.20	2.62	1.10	1.45	1.47
CN003	3.45	1.84	2.08	1.24	1.46	1.36
CN004	2.42	1.48	2.28	1.11	1.07	1.61
CN005	2.61	1.52	2.06	1.04	1.33	1.33
CN006	5.86	2.61	4.23	0.81	2.02	2.28
CN007	2.51	1.42	1.28	1.22	1.27	1.15
CN008	2.51	1.88	1.99	0.98	1.68	1.44
CN009	1.60	1.96	1.42	0.98	1.25	1.06
CN010	3.31	1.80	2.07	0.89	1.44	1.22
CN011	2.39	1.75	1.41	1.23	1.32	1.31
CN012	2.37	1.69	2.05	1.28	1.20	1.63
CN013	2.95	2.19	3.10	1.23	1.52	1.47
CN014	2.65	1.70	2.35	1.00	1.45	1.51
CN015	2.01	1.57	1.96	0.85	1.28	1.31
CN016	3.53	2.38	1.93	0.81	1.77	1.50
CN017	2.37	2.21	2.01	1.13	1.37	1.43
CN018	1.84	1.92	1.35	0.93	1.33	1.24
CN019	2.61	2.08	1.91	0.86	1.32	1.45
CN020	1.79	1.91	1.18	0.87	1.44	1.15
CN021	2.61	2.56	1.61	1.02	1.54	1.19
CN022	3.75	2.59	2.00	0.76	1.92	1.54

Table 2 continued

Sample	FAD 5′	FAD 3′	RFK	ACTB	CYC1	SDHB
CN023	3.85	2.63	1.97	0.93	1.97	1.86
CN024	3.19	2.31	3.14	0.88	1.68	1.70
CN025	2.14	1.54	2.28	0.70	1.24	1.21
CN026	2.86	1.89	2.38	0.49	1.69	1.35
CN027	3.04	1.91	2.07	0.46	1.68	1.65
CN028	1.85	1.28	0.90	0.49	1.07	0.97
CN029	1.55	1.53	0.89	0.68	1.28	1.10
CN030	2.74	1.92	1.11	0.83	1.61	1.26

reactivity in our index patient, NFH dysfunction coupled with mitochnondrial abnormalities likely contributed to neuronal demise.

Our identification of the 65-kDa neuronal surface protein as FAD synthetase is enigmatic. This is because FAD synthetase is a critical member of the electron transport chain (ETC) and is thought to be subcellular in location [3, 9] and not at the cell surface. However, even the subcellular location (cytosolic or mitochondrial) of eukaryotic FAD synthetase is controversial and has not been fully investigated [6]. Whether FAD synthetase is also present on the neuronal cell membrane is unknown.

Because ALS is not an autoimmune disease, we did not expect to find anti-FAD synthetase antibodies in other patients with SALS. This was confirmed by ELISA that showed a lack of significant autoantibody reactivity to FAD synthetase in ALS patients compared to normal controls (data not shown). However, our finding of high titer (>1:100,000) anti-FAD synthetase antibodies in our index patient was intriguing as it suggested a plausible explanation for neuronal degeneration based on a disruption of oxidative metabolism. We thus investigated whether ETC. abnormalities other than those caused by autoantibodies were present in SALS patients.

By quantitative mRNA analysis, we found significant reductions in FAD synthetase as well as other key enzymes or proteins of oxidative phosphorylation, specifically RFK, SDHB, and CYC1. These findings suggest that ECT enzyme function was sub-optimal in our index ALS patient as a result of high titer autoantibody formation and in other ALS patients as result of down regulation of mRNA expression of these enzymes.

Our data support a dysfunction of oxidative metabolism in ALS. FAD synthetase is an enzyme that catalyzes the adenylation of FMN to form FAD coenzyme, an integral part of complex II of the electron transport chain. RFK is an enzyme that catalyzes the formation of FMN from riboflavin. FMN is reduced to $FMNH_2$ in complex I of the ETC by NADH. $FMNH_2$ then passes on the electrons to a Fe–S cluster. Succinate dehydrogenase complex subunit B,

Fig. 3 Relative quantitative PCR of FAD synthetase expression in the blood of ALS patients compared to normal controls. All oxidative phosphorylation enzymes or proteins examined were significantly decreased in the ALS group compared with the controls (FAD synthetase 5′ $P = 0.0201$, FAD synthetase 3′ $P = 0.0151$, RFK $P = 0.0025$, SDHB $P < 0.0001$, and CYC1 $P = 0.0002$). There was no significant difference in beta-actin levels, $P = 0.2118$. All relative quantitation is expressed as a ratio of gene of interest to GAPDH, and all samples were normalized to the same calibrator sample present on all runs
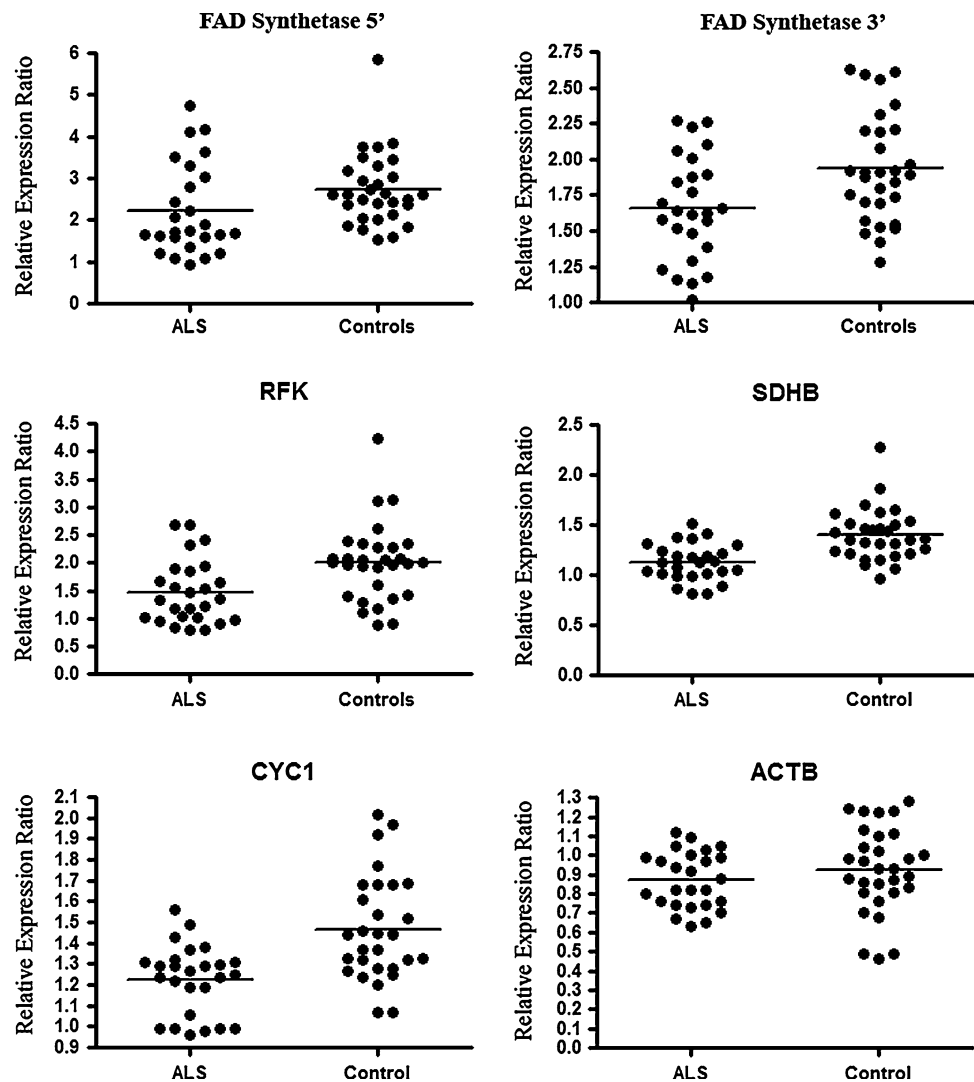

Table 3 Summary of electron transport chain proteins examined

Enzyme or protein	ETC. complex involved	P value (Mann–Whitney)
FAD synthetase	Complex II	$P = 0.0201$ and $P = 0.0151$
Riboflavin kinase	Complex I	$P = 0.0025$
Succinate dehydrogenase subunit B	Complex II	$P < 0.0001$
Cytochrome C1	Complex III	$P = 0.0002$
Beta-actin	N/A	$P = 0.2118$

iron sulfur, is part of complex II of the ETC. Fe–S subunit accepts electrons from $FADH_2$ and reduces ubiquinone. Cytochrome C1 (CYC1) is one of the subunits of the cytochrome bc1 complex of complex III of the

mitochondrial electron-transfer chain. It mediates the transfer of an electron from Rieske iron-sulfur protein to cytochrome c. Other electron transport chain enzymes or proteins were not investigated, and it is possible that in addition to complex I, II, and III, complex IV and V abnormalities also may be present in ALS. However, in this study our goal was to determine whether our index patient's abnormalities were an isolated pathogenic phenomenon or not. The data suggest that not only are electron transport chain abnormalities present in ALS, but further investigation is needed to fully map out the extent of mitochondrial involvement.

The mitochondrial abnormalities found in our patients with SALS may be a secondary phenomenon as the findings are based on peripheral blood analysis. Thus, oxidative pathways may be down regulated in ALS as a result of motor inactivity associated with the disease.

However, there may be a pathogenic basis for these findings. Thus, mitochondrial dysfunction may cause motor neuron death by predisposing them to calcium-mediated excitotoxicity, by increasing generation of reactive oxygen species and by initiating the intrinsic apoptotic pathway [16]. Whatever the underlying mechanisms are, it is possible that motor neurons are more vulnerable to injury at minimum levels of oxidative stress at which other cells may be unaffected [2].

Although the pathogenesis of SALS remains to be elucidated, others have postulated that mitochondrial abnormalities may have a pathogenic role. This subject was recently reviewed by Martin [17] and by Manfredi [16]. In SALS, mitochondrial morphological abnormalities have been described in several tissues, and decreased complex IV activity has been reported in spinal cord ventral horn and muscle tissue [5, 27]. Further, a cytochrome *c* oxidase subunit I microdeletion was described in a patient who developed motor neuron degeneration [7]. Further evidence for mitochondrial dysfunction as a precursor for motor neuron death comes from mutant mouse models of SOD1 [14, 18, 28]. Our study is compatible with these findings and is the first to show such abnormalities in the peripheral blood of ALS patients. It is also the first study to show a decrease in multiple enzymes or proteins of oxidative phosphorylation.

If our findings on mitochondrial dysfunction in ALS are validated, it could have therapeutic implications. Medications that up regulate oxidative enzymes, such as FAD synthetase [12] or specifically targeted anti-oxidants, may have clinical application in patients with ALS.

In conclusion, this is the first report of specific oxidative phosphorylation enzymes or proteins abnormalities in the peripheral blood of patients with ALS. It may be that any number of divergent factors, such as suboptimal enzyme activity, mitochondrial mutations, environmental toxins, or autoantibodies that affect mitochondrial function, may make motor neurons susceptible to degeneration. Further work is needed to determine the significance of these findings and their applicability to ALS patients.

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