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PAD2 Dysregulation and Abnormal Protein Citrullination in ALS Disease Models

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Et al.

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Abstract

Amyotrophic lateral sclerosis (ALS) is a deadly neurodegenerative disease characterized by loss of motor neurons, paralysis and eventual death. The mechanism of ALS is still incompletely understood, and the disease is to date without an effective remedy. Protein arginine deiminase 2 (PAD2) converts peptidyl-Arginine into peptidyl-Citrulline, a post-translational modification referred to as citrullination. Aberrant expression of PAD2 and protein citrullination are increased in several neurodegenerative conditions. Whether this increase is involved in ALS is unknown. In this study, we investigated PAD2 and protein citrullination in two genetic mouse models of ALS expressing human mutant $SOD1^{G93A}$ and $PFN1^{C71G}$, and in human ALS spinal cord. We show that PAD2 gene expressions and protein citrullination are increased along ALS progression. These changes occur in areas with the most severe motor neuron degeneration including the spinal cord, and brainstem. We show that the increase in PAD2 and citrullinated proteins occur specifically in astrocytes, while decreasing in neurons. Citrullinated proteins also form non-astrocyte aggregate patterns; and are dominantly expressed in insoluble protein fractions. Furthermore, the ALS mice spinal cord shows altered citrullinome. Finally, knocking out PAD2 prevented protein citrullination in $SOD1^{G93A}$ mice, quicken disease onset, while slowing disease progression. These results demonstrate that aberration of PAD2 protein citrullination are key characteristics of reactive astrogliosis, and possibly drive some type of protein aggregation in the pathogenesis of ALS. Because protein citrullination alters protein functions, our results suggest that PAD2 and protein citrullination play a role in astrogliosis and astrocytic toxicity in ALS and other neurodegenerative conditions.

PAD2 is increase in $SOD1^{G93A}$ and $PFN1^{C71G}$ ALS mouse models

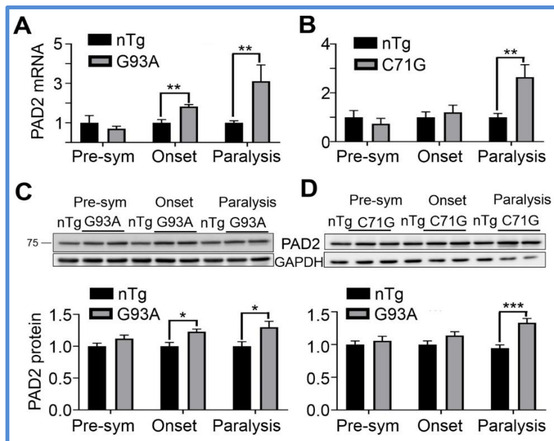


Fig. 1. A and B. Quantitative real-time PCR of PAD2 mRNA from the lumbar spinal cords of mice expressing mutant $SOD1^{G93A}$ and mutant $PFN1^{C71G}$, respectively. C and D. Western blot and Quantification of PAD2 protein from the lumbar spinal cords of mice expressing mutant $SOD1^{G93A}$ and mutant $PFN1^{C71G}$, respectively. P values are from student t-test comparing transgenic mice with their age matched controls. * = p<0.05, ** = p<0.01, *** = p<0.001.

PAD2 is progressively increased in astrocytes and decreased in neurons in $SOD1^{G93A}$ and $PFN1^{C71G}$ ALS mouse models

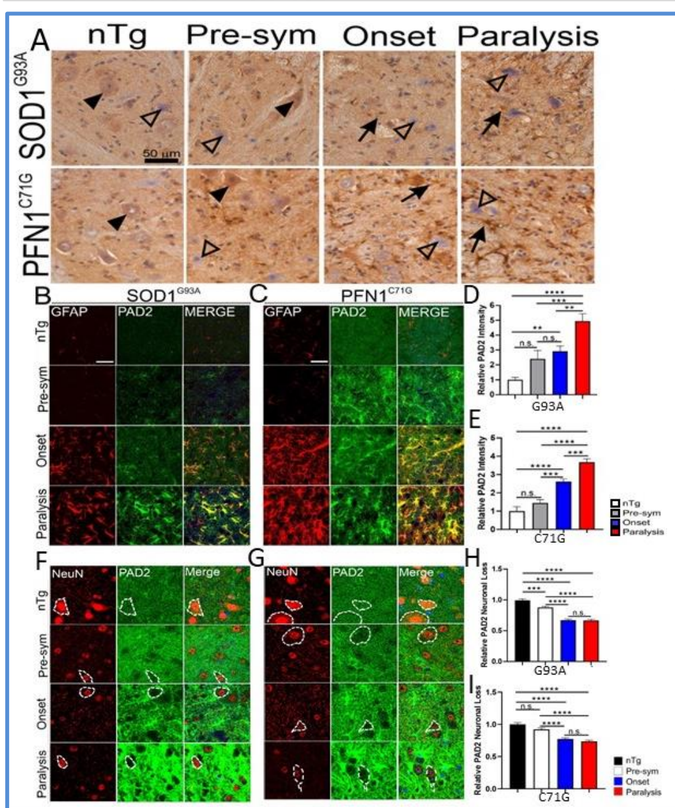


Fig. 2. A. Immunohistochemistry staining of PAD2 in ventral horn lumbar spinal cords of mutant $SOD1^{G93A}$ and $PFN1^{C71G}$ mouse models. B and C. Double immunofluorescence staining for PAD2 and GFAP in ventral horn lumbar spinal cords in mutant $SOD1^{G93A}$ and $PFN1^{C71G}$ mouse models respectively. D and E. Quantification of PAD2 fluorescent intensity in (B and C). F and G. Double immunofluorescence staining for PAD2 and NeuN in lumbar spinal cords of mutant $SOD1^{G93A}$ and $PFN1^{C71G}$ mouse models respectively. H and I. Quantification of PAD2 fluorescent intensity in NeuN positive neurons in (F and G).

Protein citrullination is increased in $SOD1^{G93A}$ and $PFN1^{C71G}$ ALS mouse models

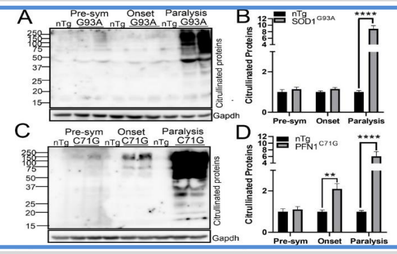


Fig. 3 A and B. Western blot and quantification of citrullinated proteins using the anti-modified citrulline (AMC) antibody detection method in $SOD1^{G93A}$ mouse model; and C-D in $PFN1^{C71G}$ mouse model. P values are from student t-test comparing transgenic mice with their age matched controls. **= p<0.01, **** = p<0.0001.

Protein citrullination increase in astrocytes in $SOD1^{G93A}$ and $PFN1^{C71G}$ ALS mouse models

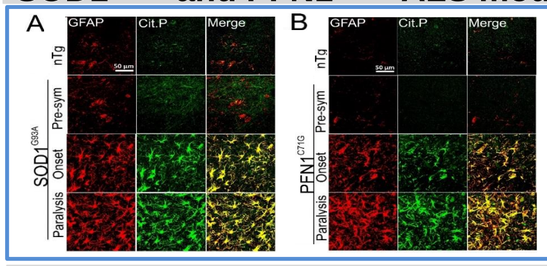


Fig. 4 A and B. Double immunofluorescence staining of spinal cord sections for GFAP and citrullinated proteins from different disease stages in $SOD1^{G93A}$ and $PFN1^{C71G}$, respectively.

The increase in PAD2 and protein citrullination correlates spatially with neurodegeneration in ALS mouse models

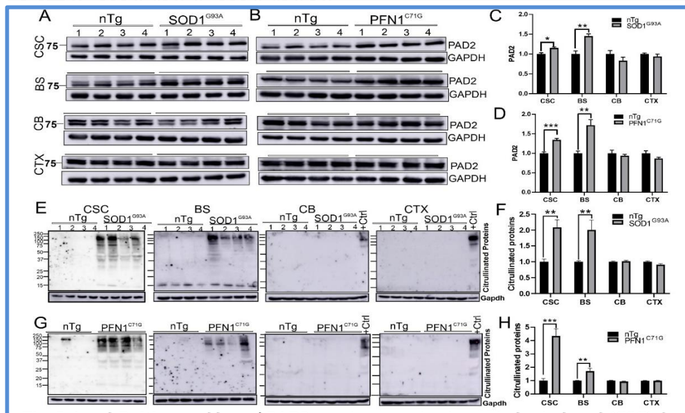
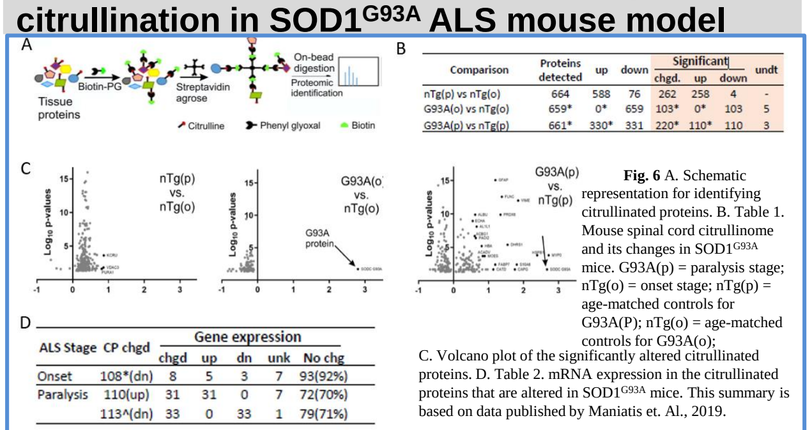


Fig. 5 A and B. Western blots of PAD2 protein expression in cervical spinal cord (CSC), brainstem (BS) cerebellum (CB) and cortex (CTX) in $SOD1^{G93A}$ and $PFN1^{C71G}$ mouse models respectively. C and D. Quantification of PAD2 protein band intensity in (A) and (B), respectively. E and F. Western blot and quantification of citrullinated proteins in $SOD1^{G93A}$ and; G and H. in $PFN1^{C71G}$ mouse model.

Proteomics demonstrate alteration of protein citrullination in $SOD1^{G93A}$ ALS mouse model

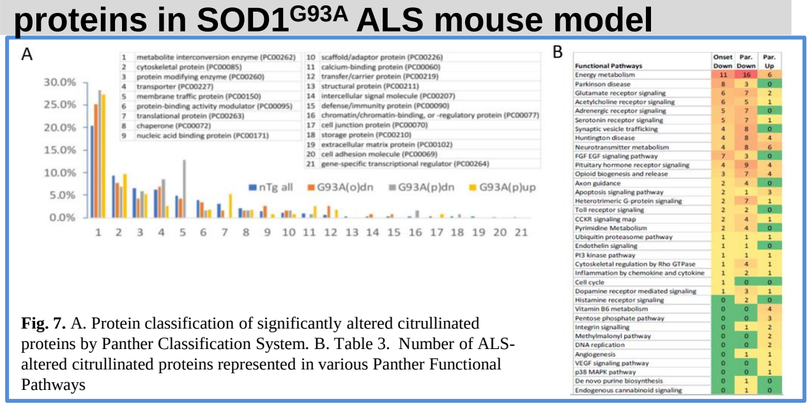


Comparison	Proteins detected	up	down	Significant chgd.	Significant up	Significant down	undt
nTg(p) vs nTg(o)	664	588	76	262	258	4	-
G93A(o) vs nTg(o)	659*	0*	659	103*	0*	103	5
G93A(p) vs nTg(p)	661*	330*	331	220*	110*	110	3

ALS Stage	CP chgd	Gene expression chgd	up	dn	unk	No chg
Onset	108*(dn)	8	5	3	7	93(92%)
Paralysis	110(up)	31	31	0	7	72(70%)
	113^(dn)	33	0	33	1	79(71%)

Fig. 6 A. Schematic representation for identifying citrullinated proteins. B. Table 1. Mouse spinal cord citrullinome and its changes in $SOD1^{G93A}$ mice. G93A(p) = paralysis stage; nTg(o) = onset stage; nTg(p) = age-matched controls for G93A(P); nTg(o) = age-matched controls for G93A(o); C. Volcano plot of the significantly altered citrullinated proteins. D. Table 2. mRNA expression in the citrullinated proteins that are altered in $SOD1^{G93A}$ mice. This summary is based on data published by Maniatis et. Al., 2019.

PANTHER classification of citrullinated proteins in $SOD1^{G93A}$ ALS mouse model



Functional Pathways	Onset Down	Par. Down	Par. Up
Energy metabolism	11	16	6
Parkinson disease	8	3	0
Glutamate receptor signaling	6	7	2
Acetylcholine receptor signaling	6	5	0
Adrenergic receptor signaling	5	5	1
Serotonin receptor signaling	5	7	1
Synaptic vesicle trafficking	4	8	0
Huntington disease	4	8	0
Neurotransmitter metabolism	4	8	0
FGF EGF signaling pathway	7	3	0
Pituitary hormone receptor signaling	4	9	4
Opioid biogenesis and release	3	7	4
Axon guidance	2	4	0
Apoptosis signaling pathway	2	1	3
Heterotrimeric G-protein signaling	2	7	1
Toll receptor signaling	2	2	0
CCKR signaling map	2	4	1
Pyrimidine Metabolism	2	4	0
Ubiquitin proteasome pathway	1	1	1
Endothelin signaling	1	1	0
PI3 kinase pathway	1	1	1
Cytoskeletal regulation by Rho GTPase	1	4	1
Inflammation by chemokine and cytokine	1	2	1
Cell cycle	1	0	0
Dopamine receptor mediated signaling	1	3	1
Histamine receptor signaling	0	2	0
Vitamin B6 metabolism	0	0	4
Pentose phosphate pathway	0	0	3
Integrin signalling	0	1	2
Methylmalonyl pathway	0	0	2
DNA replication	0	0	2
Angiogenesis	0	1	1
VEGF signaling pathway	0	1	1
p38 MAPK pathway	0	0	1
De novo purine biosynthesis	0	1	0
Endogenous cannabinoid signaling	0	1	0

Fig. 7. A. Protein classification of significantly altered citrullinated proteins by Panther Classification System. B. Table 3. Number of ALS-altered citrullinated proteins represented in various Panther Functional Pathways

Citrullinated proteins forms non-astrocyte aggregate patterns in $SOD1^{G93A}$ and $PFN1^{C71G}$ ALS mouse models

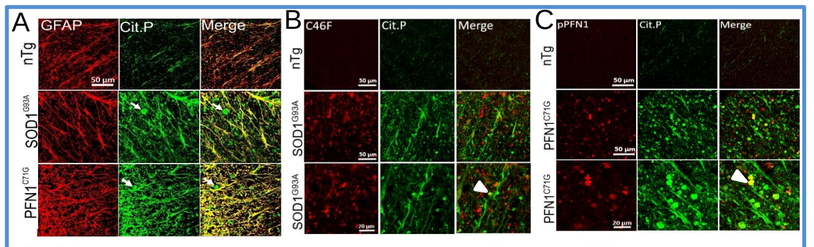


Fig. 8 Double immunofluorescence staining in the white matter of nTg and both ALS mouse models at paralysis stage for A. GFAP and citrullinated proteins. Arrow shows non-astrocyte aggregate patterns of citrullinated proteins B. C46F and citrullinated proteins. Arrowhead shows colocalization of SOD1 aggregate with citrullinated proteins. C. pPFN1 and citrullinated proteins. Arrowhead shows colocalization of pPFN1 with citrullinated proteins. NB. Lower panels are magnified images of middle panels

Citrullinated proteins increase in insoluble fractions in $SOD1^{G93A}$ and $PFN1^{C71G}$ ALS mouse models

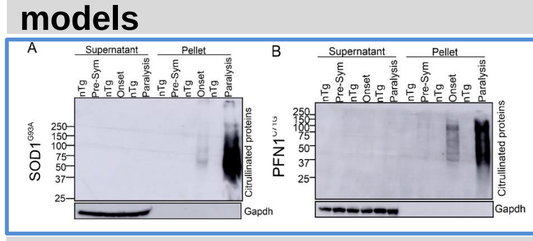


Fig. 9 A and B. Blots from sedimentation assay showing citrullinated proteins dominantly expressed in insoluble protein fraction from $SOD1^{G93A}$ and $PFN1^{C71G}$ mouse models, respectively, at different disease stages.

PAD2 knockout prevents protein citrullination in $SOD1^{G93A}$ mouse model.

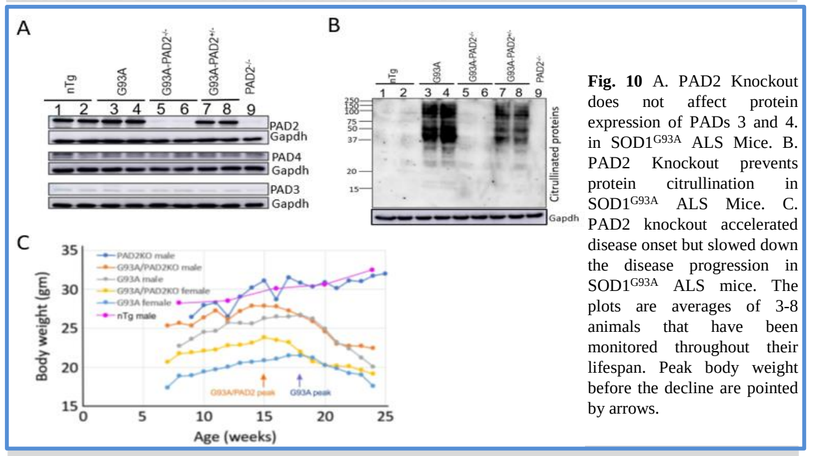


Fig. 10 A. PAD2 Knockout does not affect protein expression of PADs 3 and 4. in $SOD1^{G93A}$ ALS Mice. B. PAD2 Knockout prevents protein citrullination in $SOD1^{G93A}$ ALS Mice. C. PAD2 knockout accelerated disease onset but slowed down the disease progression in $SOD1^{G93A}$ ALS mice. The plots are averages of 3-8 animals that have been monitored throughout their lifespan. Peak body weight before the decline are pointed by arrows.

PAD2 and Protein citrullination increase in Human ALS spinal cord

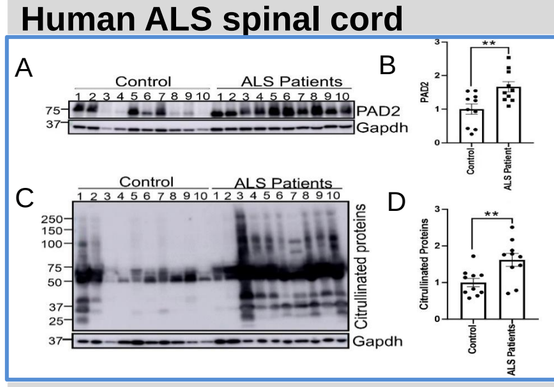


Fig. 11 A and B. Western blot and quantification of PAD2 protein in human ALS spinal cord, respectively. C and D. Western blot and quantification of citrullinated proteins in human ALS spinal cord, respectively P values are from comparing ALS patients and non-ALS controls. ** = p<0.01.

Summary

Our results show that PAD2 expression and protein citrullination increase as the disease progresses in two different genetic mouse models of ALS expressing mutant SOD1 and PFN1; and in ALS patients. This increase particularly occurs in the astrocytes, while decreasing in neurons. The alteration of PAD2 and protein citrullination seen in these mouse models coincides with areas showing motor neuron degeneration in ALS. The ALS mice spinal cord citrullinome shows a significant alteration in a wide range of protein classes and functional pathways. In addition, citrullinated proteins form non-astrocyte aggregate patterns; and are dominantly expressed in insoluble protein fractions, suggesting citrullination may possibly drive protein insolubility. Finally, PAD2 knockout prevented protein citrullination in ALS mice, quickens disease onset, while slowing disease progression. These results suggest that dysregulation of PAD2 and protein citrullination contribute in the pathogenesis of ALS.