

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Hara K, Shiga A, Fukutake T, et al. Association of HTRA1 mutations and familial ischemic cerebral small-vessel disease. *N Engl J Med* 2009;360:1729-39.

Supplementary Methods

Primer Sequences for Originally Established Polymorphic Markers.

To narrow the candidate interval, we established five new microsatellite markers, namely, *M1236*, *M1238*, *M1241*, *M1260*, and *M1264*, based on simple repeat information obtained from the 2006 human reference sequence in the University of California Santa Cruz Genome Browser Database (<http://genome.ucsc.edu/index.html>).

The following table is a summary of amplification primers for the markers.

Marker	Forward Primer Reverse Primer	Start position on chromosome 10
<i>M1236</i>	ATTACAGGCATGAGCCACTG TTGTCTGCCATACATGCTGC	124,022,184
<i>M1238</i>	GGGAACTAAGAGATGCTGAG TGTTGCTACCTTTTGCATCTC	124,148,100
<i>M1241</i>	AAACTAGGCTTGCCACAAG AGGGTGCCACTTGCTATTTG	124,567,939
<i>M1260</i>	ACGAGACAAGACTTCTTTCAG CCACAGTAGTAACCTCTTTAG	126,435,697
<i>M1264</i>	AAAATTACCGGGCACATTAC CTCATGATACGTTAAGGGAAG	126,863,922

Reverse Transcription-Polymerase Chain Reaction Assay to Amplify *HTRA1* and

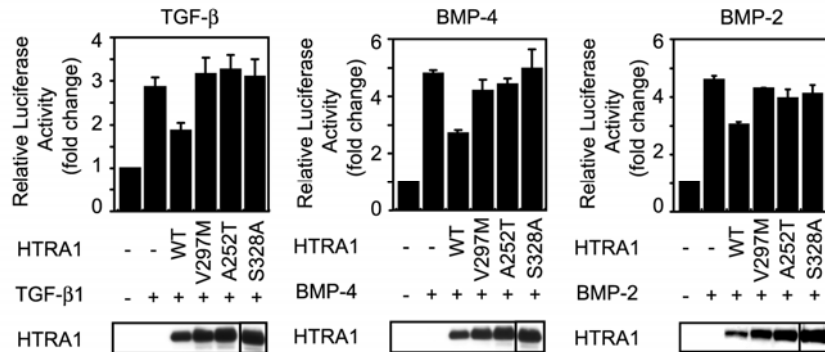
NOG mRNA

To determine whether R370X nonsense mutation in *HTRA1* resulted in loss of mRNA from a mutant allele, we analyzed mRNA from whole blood. Total RNA was

isolated with the PAX Gene Blood RNA kit (Pre-Analytix). cDNA was synthesized with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). PCR was performed with the following primer pair: forward primer, 5'-CGCCATCATCAACTATCG-3'; reverse primer, 5'-GTCAAAGTCTTGAGTGTCC-3'. RT-PCR products were analyzed on a 2% agarose gel and sequenced with the use of the same primers.

To quantify *HTRA1* mRNA levels in cultured skin fibroblasts, we performed real-time RT-PCR using TaqMan[®] Gene expression assays (Applied Biosystems) (Hs01016151_m1 for *HTRA1* and Hs99999905_m1 for glyceraldehyde 3-phosphate dehydrogenase as a control). Real-time RT-PCR amplification was carried out on an ABI Prism 7100 Sequence Detection System (Applied Biosystems). To quantify *NOG* mRNA levels in cultured skin fibroblasts in relation to the β -*actin* mRNA levels as control, the following primer pairs were designed: for *NOG* mRNA (forward primer, 5'-CCAGCACTATCTCCACATC; reverse primer, 5'-GCAGCGTCTCGTTCAGATC); for β -*actin* mRNA (forward primer, 5'-CTTCTACAATGAGCTGCGTG; reverse primer, 5'-GTCTCAAACATGATCTGGGTC).

Supplementary Figures

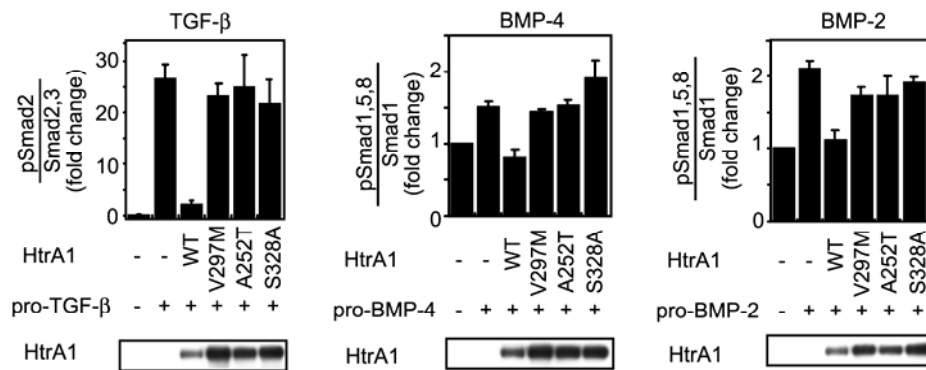


Supplementary Figure 1. Expression Level of Transfected HTRA1 Proteins in

TGF- β Family-Mediated Transcriptional Response Assay. C2C12 cells were

cotransfected with pRL-TK renilla luciferase expression plasmid, wild-type (WT) or mutated HTRA1 expression plasmid, and the following constructs: (left) (SBE)₄-firefly luciferase vector (TGF- β responsive reporter vector) and vectors containing *SMAD2*, *SMAD4*, and *TGF- β 1* (encoding pro-TGF- β 1 with two point mutations C223S/C225S); (middle) pGL3-Id985WT-firefly luciferase vector (BMP responsive reporter vector) and vectors containing *SMAD1*, *SMAD4*, and *BMP-4* (encoding pro-bone morphogenetic protein 4); (right) pGL3-Id985WT-firefly luciferase vector (BMP responsive reporter vector) and vectors containing *SMAD1*, *SMAD4*, and *BMP-2* (encoding pro-BMP-2).

Data represent the mean with standard error of normalized firefly luciferase/renilla luciferase activities from three independent experiments (top panel). The HTRA1 proteins were stained with anti-V5 antibody (bottom panel).



Supplementary Figure 2. Expression Level of Transfected HTRA1 Proteins in

Assay of Phosphorylation of Smad proteins. HEK293 cells were cotransfected with

WT or mutated HTRA1-V5 expression vectors, and the following constructs: (left)

vectors containing *SMAD2*, *SMAD4*, and *TGF-β1* (encoding pro-TGF-β1 with two point

mutations (C223S, C225S)); (middle) vectors containing *SMAD1*, *SMAD4*, and *BMP-4*

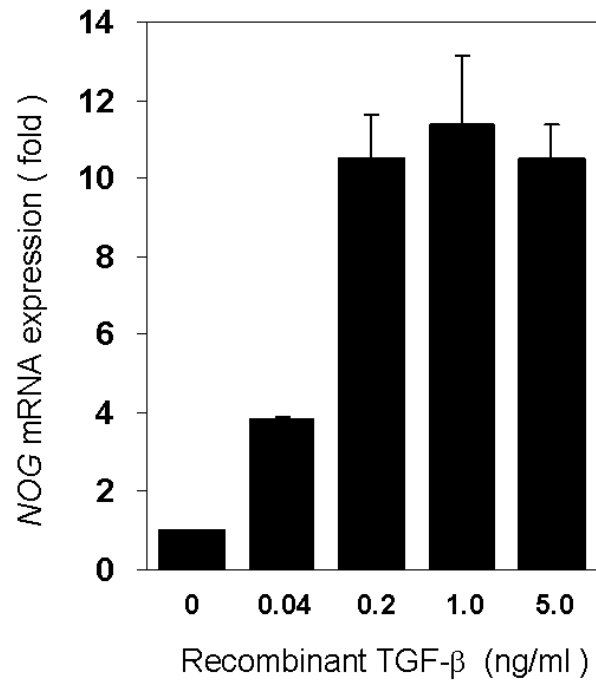
(encoding pro-bone morphogenetic protein 4); (right) vectors containing *SMAD1*,

SMAD4, and *BMP-2* (encoding pro-BMP-2). The ratio of phosphorylated Smad proteins

was examined by immunoblotting of whole cell lysates. Data represent the mean with

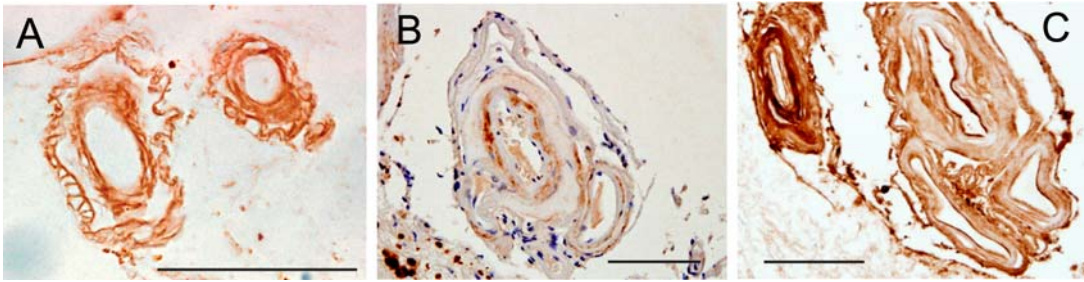
standard error of four independent experiments (top panel), and the HTRA1 proteins

were stained with anti-V5 antibody (bottom panel).



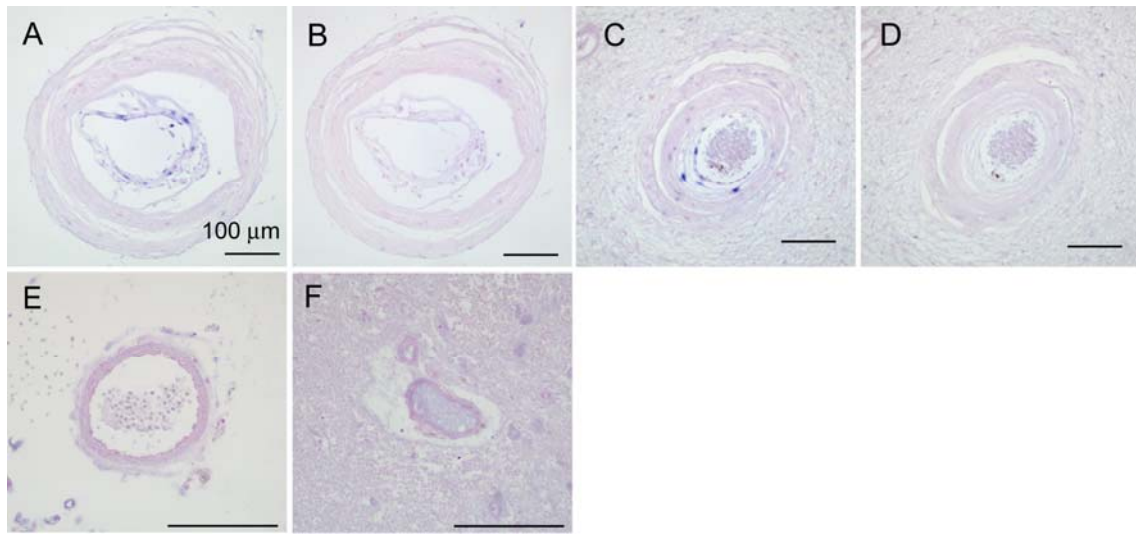
Supplementary Figure 3. Effects of TGF- β 1 on *NOG* mRNA Expression in

Cultured Skin Fibroblasts. Cultured skin fibroblasts from healthy Japanese subjects (n = 3) were treated with recombinant TGF- β 1 at 0.04-5.0 ng/ml for 2 hours. *NOG* mRNA levels as a fold change of levels in cells without TGF- β 1. The bars represent the standard errors.



Supplementary Figure 4: Immunohistochemical Analysis in Cerebral Small

Arteries. In panels A, B, and C, small cerebral arteries of autopsied subject II-3, family 5 (homozygous for the A252T mutation) show increased expression of an extra domain-A of fibronectin (panel A) and a versican in intima (panel B) and increased expression of TGF-β1 in media (panel C).



Supplementary Figure 5: mRNA Expression of Extra Domain-A of Fibronectin in Cerebral Small Arteries. In situ hybridization was carried out on the small cerebral arteries of autopsied subject II-1, family 6 (homozygous for the R302X mutation) with the use of antisense (panels A and C) and sense probes (panels B and D) derived from an extra domain-A of fibronectin. Panels E and F, in situ hybridization analysis of extra domain-A of fibronectin antisense probe in the cerebral small arteries of autopsied control subject (40-year-old female with amyotrophic lateral sclerosis).