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A novel *ENG* mutation causing impaired co-translational processing of endoglin associated with hereditary hemorrhagic telangiectasia

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ABSTRACT

Hereditary hemorrhagic telangiectasia (HHT) is an inherited autosomal dominant vascular dysplasia caused by mutations in mainly the endoglin gene (*ENG*) or activin-like kinase receptor 1 (ALK1) gene (*ACVRL1*). We investigated the molecular basis of HHT in a Japanese patient, and identified a novel missense mutation in *ENG* (c.38 T>A, p.Leu13Gln) located in the signal peptide's hydrophobic core, but not in *ACVRL1*. In experiments in COS-1 cells, the Leu13Gln (L13Q) mutant endoglin appeared to be expressed as a precursor form, probably due to impaired protein processing. Flow cytometry analyses of the COS-1 cells transiently expressing recombinant endoglins revealed that the wild-type endoglin was detected on the cell surface, but the L13Q mutant was not. We also analyzed expression patterns of the recombinant endoglins by immunofluorescent staining, and found that the wild-type co-localized with the endoplasmic reticulum (ER), but the L13Q mutant did not. These results implied that the L13Q mutant endoglin fails to insert into the ER, probably due to destruction of the hydrophobic core structure in the signal peptide to be recognized by signal recognition particles. Thus, the Leu13 in the signal peptide of endoglin might be essential for correct protein processing through the ER and cell-surface expression. Taken together, the novel c.38 T>A mutation in *ENG* would impair co-translational processing of the endoglin, and could be responsible for HHT in this patient.

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Rendu-Weber syndrome, is an inherited autosomal dominant vascular dysplasia with a frequency of 1 in 10,000, and exhibits age-related penetrance with variable expressivity [1-3]. The most common clinical manifestations involve the development of vascular abnormalities seen as telangiectases on skin and lesions in nasal mucosa that readily bleed. Further clinical manifestations are pulmonary, cerebral, hepatic and, in rare cases, spinal cord arteriovenous malformations. These may cause serious complications such as stroke, brain abscess, hemorrhage, or venous thromboembolism [4-6].

HHT type 1 (HHT1) is caused by a mutation in the gene encoding endoglin (*ENG*) located on the long arm of chromosome 9 (9q34) [7-9]. HHT type 2 (HHT2) is caused by a mutation in the activin-like kinase receptor 1 (ALK1) gene (*ACVRL1*) located on the long arm of chromosome 12 (12q13) [10-12]. Many mutations have been identified in *ENG* and *ACVRL1* genes and support the haploinsufficiency model for HHT, that is that the remaining wild-type allele is unable to contribute sufficient protein for normal function [13]. Endoglin is a homodimeric integral membrane glycoprotein that interacts with signaling receptor complexes for several members of the transforming growth factor-β (TGF-β) superfamily, and composed of disulfide-linked 90-kDa subunits [14-16]. It is expressed primarily in the vascular endothelial cells of capillaries, arterioles, and venules, as well as in activated monocytes, syncytiotrophoblasts, and some leukemic cells. Two alternatively spliced variants, long- and short-form endoglins, are encoded by *ENG*. It is suggested that short-form endoglin is induced during endothelial senescence and plays opposite roles with respect to the predominant long-form endoglin, contributing to age-dependent vascular pathology [13].

Abbreviations: ALK1, activin receptor-like kinase-1; HHT, hereditary hemorrhagic telangiectasia; HHT1, HHT type 1; TGF-β, transforming growth factor-βSRP,, signal recognition particle; RFLP, restriction fragment-length polymorphism; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; RT, room temperature; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; DAPI, 4'6-diamidino-2-phenylindole-2HCl.

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ALK1 is also expressed on endothelial cells, and is a type I receptor of the TGF-β superfamily [17].

TGF-β family cytokines are multifunctional proteins that regulate proliferation, differentiation, migration, adhesion and apoptosis of various cell types, and mediate their cellular effects through a heteromeric complexes of type I and type II transmembrane serine-threonine kinase receptors [5]. Ligand binding induces association of the type I and type II receptors, leading to a unidirectional phosphorylation event in which the type II receptor phosphorylates the type I receptor, thereby activating its kinase domain. The activated type I receptor phosphorylates receptor regulated Smads (R-Smads) and these activated R-Smads bind subsequently to the common mediator (Co-Smad), Smad4. The R-Smad/Co-Smad complexes accumulate in the nucleus where it regulates transcription by interacting with many specific DNA-binding proteins. Although the core of the TGF-β receptor complex is formed by the association of type I and type II receptors, it may also contain auxiliary receptors such as endoglin. In endothelial cells (ECs), the TGF-β type II receptor and two distinct TGF-β type I receptors, the EC-restricted ALK1 and the broadly expressed ALK5, are expressed [18,19]. Endoglin is necessary for efficient TGF-β/ALK1 signaling and indirectly inhibits TGF-β/ALK5 signaling, thereby promoting the activation state of EC during angiogenesis [20].

To date, 282 different mutations have been reported in the *ENG* gene and 246 distinct mutations in the *ACVRL1* gene (human gene mutation database: HGMD, http://www.hgmd.cf.ac.uk/ac/all.php). Some missense mutations in the signal peptide region of endoglin have been reported, but their molecular basis has yet to be investigated in detail. In this study, we analyzed the *ENG* gene in a Japanese patient with HHT, and found a novel missense mutation in the signal peptide region of endoglin. We further investigated the molecular basis of HHT in the patient through expression analyses of the mutant endoglin in COS-1 cells.

1. Materials and Methods

1.1. Sample preparation

Ethical approval for this study was obtained from the Ethics Committee of the Nagoya University School of Medicine. Citrated blood samples were obtained from the patient and his sister with informed consent. Genomic DNA was isolated from the peripheral leukocytes by phenol extraction.

1.2. Polymerase Chain Reaction (PCR) and DNA sequencing

The protein-coding exons and exon-intron boundaries of *ENG* and *ACVRL1* were amplified by the polymerase chain reaction (PCR) using gene-specific primers (Suppl. Table 1). The PCR products were electrophoresed on agarose gels, and purified with a QIAEX II Gel Extraction Kit (QIAGEN, GmbH, Germany). We performed direct sequencing of the purified PCR products with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI Prism 310 Genetic Analyzer (Applied Biosystems).

1.3. PCR-Restriction Fragment Length Polymorphism (RFLP) analysis

PCR-mediated restriction fragment length polymorphism (PCR-RFLP) was performed to detect the missense mutation (c.38 T>A) identified in the *ENG* gene of the patient. Briefly, we amplified a part of the *ENG* gene containing the c.38 T>A mutation by PCR using an exon 1 sense primer and a partially mismatched antisense primer (5'-GACACCTACTTGTGGGGCTGAGGGTGC-3': the mutated nucleotide is underlined), which was designed to destroy the *Pst*I site near the mutation. The PCR products were digested with *Pst*I and analyzed by electrophoresis on 3% NuSieve 3:1 agarose (LONZA, Walkersville, MD).

1.4. Cell culture and reagents

African green monkey kidney COS-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The EAhy926 cells, which are human umbilical vein endothelial cell (HUVEC)-like cells, were generously donated by Dr. Cora-Jean S. Edgell (University of North Carolina, Chapel Hill, NC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; CCB from Nichirei Biosciences, Tokyo, Japan) and 100× antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan). Tunicamycin was purchased from Sigma-Aldrich (St Louis, MO), and dissolved in Hybri-Max® DMSO (Sigma-Aldrich).

1.5. Construction of the endoglin expression vector and transient transfection

Full-length human endoglin cDNAs, encoding the 658-amino acid long form (L-ENG) and 625-amino acid short form (S-ENG), were prepared by reverse transcription (RT)-PCR from normal peripheral mononuclear cell mRNAs. These cDNAs were inserted into the pCI Mammalian Expression Vector (Promega, Madison, WI) between *Eco*RI and *Sal*I sites (pL-ENG^{WT} and pS-ENG^{WT}).

To introduce a c.38 T>A transversion, we used a Quik Change Lightning Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, with the following primers; 5'-GTTGCCCTGCAGCTGGCCAGCTGCAGCC-3' (sense: the mutated nucleotide is underlined), 5'-GGCTGCAGCTGGCCAGCTG CAGGGCAAC-3' (antisense), and prepared L13Q mutant endoglin expression vectors (pL-ENG^{L13Q} and pS-ENG^{L13Q}).

For transient transfection, COS-1 cells were seeded at a concentration of 5×10^5 cells in 60 mm dishes or 2×10^5 cells in 35 mm dishes. After 18 hr, the respective endoglin expression vector or a mock vector (pCI) (0.5 μg or 5 μg in 60 mm dish, or 1 μg in 35 mm dish) was transfected using Lipofectoamine2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Following experiments were performed at 48 hr after transfection.

1.6. Western Blot analysis

The transfected cells were lysed in SDS sample buffer with or without β-mercaptoethanol (β-ME), boiled, and subjected to 10% SDS-PAGE followed by Western blotting. The Western blot was performed as described previously [21] with minor modifications. Primary antibody against endoglin (H-300; Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Cytoskeleton, Denver, CO), Smad3 or phospho-Smad3 (Cell Signaling Technology, Danvers, MA) was probed in a dilution of 1:1000, and after being washed, horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) was probed. Signals were visualized with a chemiluminescent HRP substrate on Immobilon™-Western (Millipore Corp., Billerica, MA). The intensities of the bands were quantified by ATTO CS analyzer (ATTO, Tokyo, Japan) and represented as relative values of endoglin against β-actin, respectively.

1.7. Flow cytometry analysis and Cell-surface biotinylation-immunoprecipitation (IP) analysis

COS-1 cells were transiently transfected with 1 μg of pL-ENG^{WT} or pL-ENG^{L13Q} in 35 mm dish, and harvested using 1 mM EDTA in PBS after 48 hr. The cells were washed with PBS, and resuspended in flow cytometry buffer (1% FBS, 0.1% NaN_3 in PBS). The cells in flow cytometry buffer were divided into aliquots, and treated with blocking buffer (1% BSA in flow cytometry buffer) for 15 min at room temperature (RT). After centrifugation, the cells were incubated with anti-endoglin antibody (H-300; a rabbit polyclonal antibody raised against amino acids 27–326 of human endoglin, Santa Cruz Biotechnology) or isotype-matched rabbit IgG (10 μg/mL) in blocking buffer for 15 min at RT. Anti-rabbit IgG-Alexa488 antibody (Invitrogen) was