tumor necrosis factor and IL-1 (13,14). IL-1 is normally expressed at low levels, but it is rapidly induced in response to local or peripheral insult (10,11). During inflammation, IL-1, a potent proinflammatory cytokine, is released with other cytokines, chemokines, prostaglandins, reactive oxygen species (ROI), NO and proteases (17). In addition to inducing genes in the iNOS system, IL-1 also leads to the expression of phospholipase A2 and cyclooxygenase-2 (Cox-2). Phospholipase A2 increases cellular levels of arachidonic acid, and Cox-2 catalyzes the first steps of prostaglandin synthesis (10-12). These lipid-derived inflammatory mediators can lead to cellular damage (18). In particular, Cox-2 generates ROI and contributes to tissue damage (10-12). IL-1 can also induce the expression of macrophage inflammatory proteins (MIPs), focal adhesion kinase (FAK), Mac-1 and CD4. Expression of cytokines, chemokines and inflammation-associated factors including IL-6, TNFa, MCP-1, MIPs, Cox-2 and iNOS are reduced in IL-1 receptorl null mice (10-12). These findings indicate that IL-1 is a key upstream regulator of the inflammatory and immune responses. Defective IL-1 signaling also appears to protect cells from inflammation associated cell death. Yamasaki et al. (19) reported that intracerebroventricular injection of neutralizing anti- IL-1β antibody to rats reduced ischemic brain damage. Mice lacking the IL-1 receptorl gene experience 50% less damage after cerebral hypoxia/ischemia (20).

There are two different IL-1 isoforms, IL-1α and IL-1β (10,11). Both are synthesized as precursor molecules that are then processed to their mature forms. However, pro-IL-1α is fully functional, whereas pro-IL-1β is biologically inactive. ICE, also known as caspase-1 is an intracellular cysteine protease that cleaves and activates pro-IL-1β. The 45-kD caspase-1 precursor requires two internal cleavages before becoming enzymatically active; the active form is a heterodimer comprised of a 10- and a 20-kD chain (12). Two caspase-1 heterodimers form a tetramer with two molecules of pro-IL-1β during the cleavage reaction. Macrophages from caspase-1-deficient mice do not release mature IL-1β upon stimulation with endotoxin (12). Mice lacking the gene for caspase-1 or

expressing a dominant negative form of caspase-1 also exhibit reduced ischemic brain damage (21,22). Interestingly, in the present study, increased caspase-1 expression was seen in the cerebral aneurysms of IL-1β deficient mice. It is possible that the absence of IL-1β perturbs the normal feedback regulation of caspase-1. Such a feedback system has not been described.

During apoptosis, nuclear fragmentation and the generation of ssDNA occur (9). From the ssDNA staining and TUNEL results, the apoptosis was mainly found in SMC.

We assessed the ssDNA expression and TUNEL in the study. We don't present the data about cytochrome-c release or caspase-9 expression. As for the pathway of cell death in aneurismal walls, additional experiments are necessary. As for cleaved casapase-3, we detected more obvious expression of that in wild-type mice than in IL-1β deficient mice, but this tendency didn't reach significance (P=0.058; n=5 per each group). As for wild-type mice, we used B10 mice. B10 mice are not complete wild-type. But IL-1β-/- mice was backcrossed for 3 generations to a B10. Thus it was the closest control that we could use. In our model, BAPN were used to induce aneurysms. It is possible that BAPN plays a role in apoptosis of vascular walls. The present data indicate that IL-1β plays an important role in the development and progression of cerebral aneurysms. Disruption of the IL-1β gene resulted in the reduced incidence of mature cerebral aneurysms.

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Figure legend

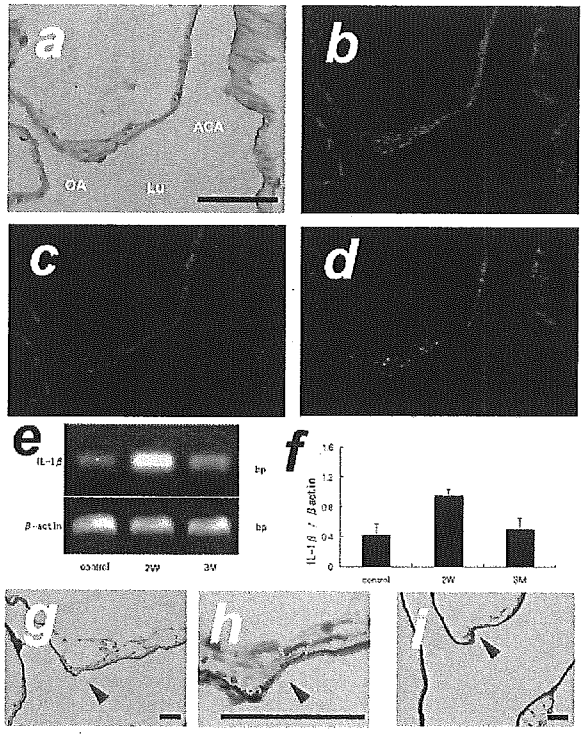
Figure 1. IL-1β expression in experimentally induced aneurysm in mice (a-f). a. hematoxilin and eosin staining of the arterial bifurcation of ACA(Anterior cerebral artery) and OA(Olfactory artery). b-d. Double staining for α-actin (red, b and c), and interleukin-1β (green, b and d) in ACA-OA bifurcation of this model. Merged cells in yellow signals (b) indicate that smooth muscle cells express interleukin-1β in induced mice aneurysms. e. RT-PCR analysis of IL-1β mRNA in control (n=4), 2 weeks after the operation (n=3) and 3 months after the operation (n=5). f. Densitometric analysis indicates that the expression of IL-1β mRNA is higher in 2 weeks group than control and 3 months group. These differences are statistically significant ($P < 0.05$). Classification of this study in Orcein staining (g-i). g and h. Early aneurysmal change sample. Fragmentation of internal elastic laminae is seen. (g. lower magnification, h. higher magnification). i. Advanced aneurysm sample. Vascular wall is protruded laterally at ACA just distal of juxta-apical groove. (lower magnification) ACA; Anterior cerebral artery, OA; Olfactory artery, Lu; Lumen side, * indicate $P< 0.05$ to the other groups, Bars; 50μm

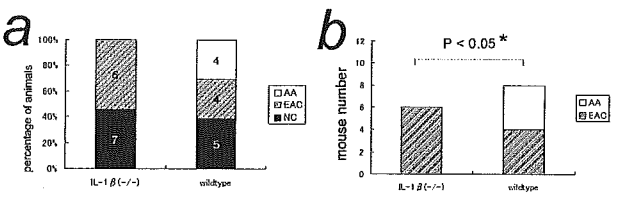
Figure 2. Characterization of experimentally induced aneurysms in IL-1β deficient and wild-type mice. a. Incidence of experimental cerebral aneurysmal changes in IL-1β -/- and wild-type mice. No significant differences are seen in the incidence of aneurysm development. b. Incidence of advanced aneurysmal changes among total aneurysmal changes. In IL-1β-/- mice, the incidence of advanced aneurysmal changes is significantly smaller than in wild-type mice (P=0.048).* indicate P<0.05.

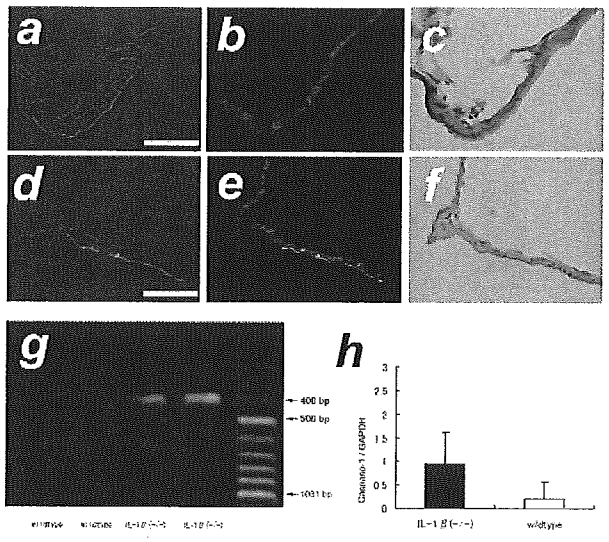
Figure3. Caspase-1 expression in experimentally induced aneurysms. a-c. In wild-type mice, little caspase-1 immunoreactivity was seen in vascular walls. (a, microscopic view; b, double staining for α-actin and caspase-1 (red, α-actin; green, caspase-1); c, hematoxylin and eosin staining). d-f. In IL-1β-/- mice, more caspase-1 immunoreactive cells were seen compared with wild-type mice. (d, microscopic view; e, double staining for α-actin and caspase-1 (red, α-actin; green, caspase-1); f, hematoxylin and eosin staining). Bars; 50μm. g, RT-PCR analysis of caspase-1 mRNA in wild-type and IL-1β-/- mice. h, Densitometric analysis showed that increased expression of caspase-1 mRNA in IL-1β-/- mice.

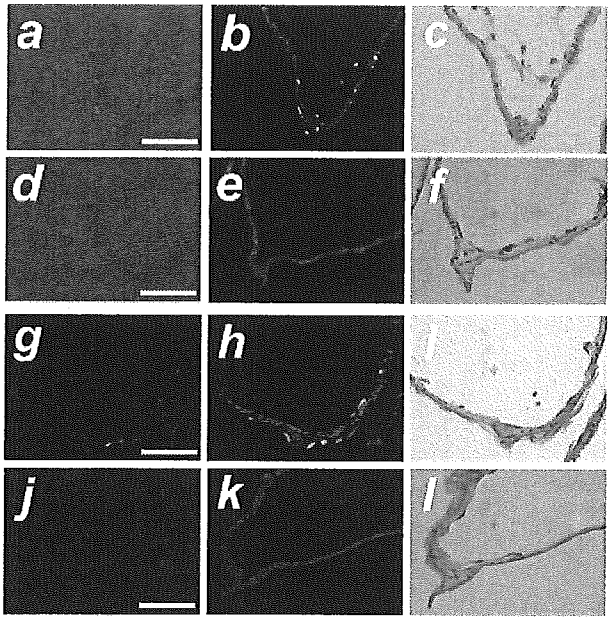
Figure 4. Single-stranded DNA expression (a-f) and TUNEL staining (g-l) in experimentally induced aneurysms in wild-type and IL-1β deficient mice. a-c. In wild-type mice, more ssDNA immunoreactive cells were seen compared to IL-1β-/- mice. (a, microscopic view (green, ssDNA); b, double staining for α-actin and ssDNA (red, α-actin; green, ssDNA); c, hematoxylin and eosin staining). d-f. In IL-1β-/- mice, little ssDNA immunoreactivity was seen in the media. (d, microscopic view (green, ssDNA); e, double staining for α-actin and ssDNA (red, α-actin; green, ssDNA); f, hematoxylin and eosin staining). g-i. More TUNEL-positive cells were seen in wild-type specimen. (g, microscopic view (green, TUNEL); h, double staining for α-actin and TUNEL (red, α-actin; green, TUNEL); i, hematoxylin and eosin staining). j-l. In IL-1β-/- mice, little TUNEL-positive cells was seen in the media. (j, microscopic view (green, TUNEL); k, double staining for α-actin and TUNEL (red, α-actin; green, TUNEL); l, hematoxylin and eosin staining). Bars; 50μm.

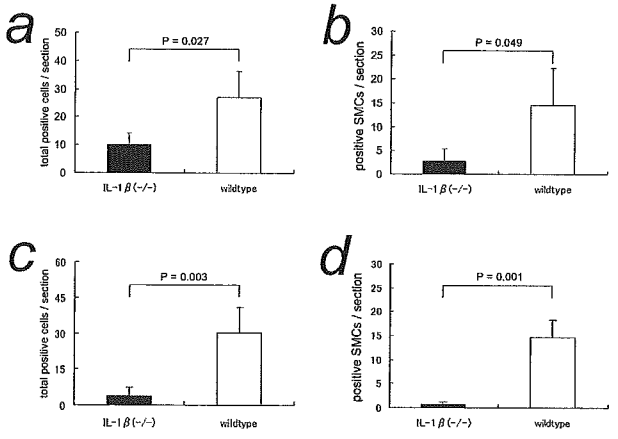
Figure 5. The number of ssDNA or TUNEL positive cells in the vascular walls of experimental aneurysms in wild-type and IL-1β deficient mice. The number of ssDNA immunoreactive cells per section in the media (a) and intima (b) was significantly smaller in IL-1β-/- mice (n=5) compared to wild-type animals (n=5). The number of TUNEL positive cells per section in the media (c) and intima (d) was significantly smaller in IL-1β-/- mice (n=5) compared to wild-type animals (n=5).











Author Information Page

Association Analysis of Common Variants of *ELN*, *NOS2A*, *APOE* and *ACE2* to Intracranial Aneurysm

Youhei Mineharu, MD; Kayoko Inoue, MD, PhD; Sumiko Inoue, PhD; Shigeki Yamada, MD, PhD; Kazuhiko Nozaki, MD, PhD; Katsunobu Takenaka, MD, PhD; Nobuo Hashimoto, MD, PhD; Akio Koizumi, MD, PhD

From the Department of Neurosurgery (Y.M., S.Y., K.N., N.H.); Department of Health and Environmental Science (K.I., S.I., A.K.), Kyoto University Graduate School of Medicine, Kyoto, Japan; and the Department of Neurosurgery, Takayama Red Cross Hospital (K.T.), Gifu, Japan.

Drs Y. Mineharu, K. Inoue, and S. Inoue contribute equally to this work.

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Correspondence to Akio Koizumi, MD, PhD, Professor, Department of Health and Environmental Sciences, Graduate School of Medicine Kyoto University, Konoe-cho, Yoshida, Sakyo-ku, Kyoto, 606-8501, Japan.

Fax: +81-75-753-4458, Phone: +81-75-753-4456

E-mail: koizumi@pbh.med.kyoto-u.ac.jp

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Title Page (R2)

Full title: Association Analysis of Common Variants of *ELN*, *NOS2A*, *APOE*, and *ACE2* to Intracranial Aneurysm

Cover title: Association of 4 Candidate Genes with IA

Number of tables: 4

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Key Words: intracranial aneurysm, genetics, SAH, single nucleotide polymorphism

Abstract Page

Background and Purpose— Previous studies have shown positive evidence of linkage of the intracranial aneurysm (IA) at chromosome 7q11, 17cen, 19q13, and Xp22. These regions contain *elastin (ELN)*, *nitric oxide synthetase2A (NOS2A)*, *apolipoprotein E (APOE)*, and *angiotensin-I converting enzyme 2 (ACE2)*, which are considered to be promising candidate genes for IA. We aimed to examine the association of single-nucleotide polymorphisms (SNPs) with IA in these candidate genes.

Methods— To identify polymorphisms in *NOS2A* and *ACE2*, all exons and exon-intron boundaries were screened by direct sequencing in 30 randomly selected controls. The program tagSNPs was used to select an optimal set of haplotype tagging SNPs (htSNPs). For *ELN* and *APOE*, SNPs were selected from previous reports. These selected SNPs were then genotyped in 362 cases with IA and 332 residential area matched controls. THESIAS software was used to investigate the association of alleles and haplotypes with IA by adjusting with covariates.

Results—We genotyped 8 SNPs in *ELN*, 8 SNPs in *NOS2A*, 3 ε alleles in *APOE* and 1 SNP in *ACE2*. No alleles or haplotypes of 4 candidate genes revealed any significant association with IA.

Conclusions— Investigated polymorphisms in this study were not associated with IA.