2-way ANOVA. E, F: There were significant main effects of day ($F(1, 20) = 196.9$ and 418.0, $p<0.001$ in Aβ40 and Aβ42, respectively) and dose ($F(4, 20) = 4.16$, $p = 0.013$ and $F(4, 20) = 91.9$, $p<0.001$ in Aβ40 and Aβ42, respectively), and significant interaction between day and dose ($F(4, 20) = 25.4$, $p<0.001$ in Aβ42) by 2-way ANOVA. *, $^{\#}p<0.05$, **, $^{\#\#}p<0.01$, ***, $^{\#\#\#}p<0.001$, significantly different from respective vehicle-treated groups by Dunnett's test.
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containing 0.2% Triton X-100 for 10 min (permeabilization). After blocking with 2% BSA in PBS, cells were incubated with primary antibody diluted with blocking buffer and then washed with PBS. Finally the cells were incubated with secondary antibodies and mounted using ProLong Gold antifade reagent with DAPI (Invitrogen). The immunoreactive cells were visualized using an LSM 700 Laser Scanning Microscope (Carl Zeiss, Jena, Germany) and a Biorevo BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Quantitative real-time RT-PCR

Total RNA was isolated from cells using TRIZOL reagent (Invitrogen). Contaminating DNA was removed using the TURBO DNA-free kit (Ambion, Austin, TX), and cDNA was synthesized using ReverTra Ace-α (Toyobo, Osaka, Japan), according to the manufacturers' protocols. Real-time PCR was performed using the StepOnePlus system (Applied Biosystems) and SYBR green reagent (TAKARA, Shiga, Japan). The primers used are listed in Table S2 in the supporting information.

HiPS cell culture and differentiation into neuronal cells

HiPS cells, 253G4 [14] (passage 20–30) or hES cells, H9 were cultured on mitomycin C-treated mouse embryonic fibroblasts in primate ES medium (ReproCELL, Kanagawa, Japan) supplemented with bFGF (Wako Pure Chemicals, Osaka, Japan). To obtain cortical neurons derived from iPS cells, we partially modified a previous method [12,15]. For neural induction, partially dissociated iPS cell colonies, 40–100 μm in diameter, were selected with Cell Strainer (BD Falcon, BD Bioscience, Bedford, MA) and plated on poly-L-lysine (Sigma-Aldrich)/Laminin (BD Biosciences) (PLL/LM)-coated dishes (P1) in N2B27 neuronal differentiation medium [DMEM/F12 (Invitrogen), Neurobasal (Invitrogen), N2 (Invitrogen), B27 minus vitamin A (Invitrogen), L-Gln (Invitrogen)], supplemented with 100 ng/ml human recombinant Noggin (R&D Systems, Minneapolis, MN) and 1 μM SB431542 (Sigma-Aldrich) for 17 days. At day 10, primary colonies were split into small clumps using 200 U/ml collagenase with $CaCl_2$ and plated into PLL/Entactin-Collagen IV-Laminin (Millipore) (ECL)-coated dishes (P2). At day 17, P2 cells were dissociated using Accutase (Innovative Cell Technologies, San Diego, CA) and cultured on PLL/ECL-coated dishes (P3). Finally, at day 24, cells dissociated with Accutase were passed through a 40-μm cell strainer (BD Biosciences), counted, and cultured on PLL/LM/Fibronectin (Millipore)-coated 24-well plates at 2.5×10^4 cells/well in N2B27 medium supplemented with 10 ng/ml BDNF, GDNF, and NT-3 (R&D Systems). Medium changes for cell culture were carried out once every two or three days until day 52.

Aβ sandwich ELISA

At days 38, 45, and 52, two-day incubated conditioned media were collected from cultured neuronal cells and centrifuged at 4,000 *g* for 10 min. The resultant clear supernatants were subjected to sandwich ELISA (Wako) with a combination of monoclonal antibodies specific to the midportion of Aβ and specific to the C-terminal of Aβ40 or Aβ42, to determine the amounts of secreted Aβ, as described previously [20,28,30]. We also examined the inhibitory effect of each drug on Aβ production. All media were replaced with new media containing each drug and two-day conditioned media were analyzed as mentioned above.

Western blot analysis

Western blot analysis was performed as previously described with minor modification. In addition to conditioned media, cell lysates were also collected, extensively washed with PBS, and lysed

Table 1. Panel of Aβ monitoring systems.

Human sample	**Aβ40**	**Aβ42**	**Ref.**
Brain tissue [AD]	↑ (AD/NC)	↑ (AD/NC)	[39]
CSF [AD]	-	↓ (AD/NC)	[37]
	→ (AD/NC)	↓ (AD/NC)	[29]
Plasma [AD]	↑ (AD/NC)	→ (AD/NC)	[29]
iPS cell-derived neuronal cells	Measurable	Measurable	This report
Mouse model	**Aβ40**	**Aβ42**	**Ref.**
Brain [PDAPP]	↑ (Aging)	↑ (Aging)	[36]
Brain [APP23]	↑ (Tg/non-Tg)	↑ (Tg/non-Tg)	[27]
Brain [Tg2576]	↑ (Aging)	↑ (Aging)	[28]
	↑ (Tg/non-Tg)	↑ (Tg/non-Tg)	
CSF [Tg2576]	↓ (Aging)	↓ (Aging)	[28]
Plasma [Tg2576]	↓ (Aging)	↓ (Aging)	[28]
Cell line	**Aβ40**	**Aβ42**	**Ref.**
[APP_{NL}-H4]	Measurable	Measurable	[30]
[CHO-APP_{NL}/SH-SY5Y-APP]	Measurable	Measurable	[4]

AD, Alzheimer's disease; NC, normal control; Tg, transgenic mouse model.
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directly with 1× sample buffer (EzApply; ATTO, Tokyo, Japan). The media or cell lysates were separated by 5–20% gradient or 7.5% [FL-APP] or 10% [β-actin] sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). The blots were probed with an appropriate primary antibody, followed by HRP-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare). The protein bands were visualized using an enhanced chemiluminescence (ECL) detection method (GE Healthcare), and band intensity was analyzed with a densitometer (LAS-4000; GE Healthcare), using the Science Laboratory 2001 Image Gauge software (Fujifilm, Tokyo, Japan). Immunoreactive protein content in each sample was calculated based on a standard curve constructed with each recombinant protein or one of the samples. Each set of experiments was repeated at least two times to confirm the results. The level of β-actin protein, measured by quantitative western blotting using β-actin antibody, was used as an extraction and loading control.

LDH assay

Cell toxicity assays were performed using a cytotoxicity detection kit (LDH, Roche, Mannheim, Germany) according to the manufacturer's protocol.

Statistical analysis

All data were expressed as mean ± SD. Comparisons of mean among more than three groups were done by one-way or two-way ANOVA, followed by *post-hoc* test (PRISM, GraphPad software). *P* values ≤0.05 indicated significant differences.

Supporting Information

Figure S1 Cholinergic neuronal marker-positive cells were observed in hiPS cell-derived neuronal cells. Expression levels of ChAT (A) and VAChT (B) were quantified by qPCR (n = 3) and normalized by that of GAPDH. "Fold expression" represents the ratio of expression on the given day compared to day 38. ChAT- (C) and VAChT (D)-positive cells were observed a little at day 52.
(TIF)

Figure S2 Percentages of the three isoforms of APP (APP770, APP751, and APP695) at 38, 45, and 52 days. Each column represents mean ± SD of 8 assays. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Tukey's test.
(TIF)

Figure S3 New hsAPPα and sAPPβ antibodies specifically detect human sAPPα and sAPPβ by western blots, respectively. Human neuroglioma H4 cells overexpressing wild-type APP (APP_{WT}-H4 cells) were treated with α-secretase activator (12-*O*-tetradecanoylphorbol 13-acetate (TPA)), α-secretase inhibitor (TNF-α protease inhibitor-2 (TAPI-2)), or β-secretase inhibitor (see Protocol S1). Brain lysates of APP-knockout mice (APP-KO) were used as negative control. Immunoblots of conditioned media and supernatants of brain lysates were probed by anti-hsAPPα or anti-sAPPβ antibody. sAPPα or sAPPβ derived from both exogenous APP695 and endogenous APP770/751 are detected by each antibody. The increase in sAPPα by α-secretase activator and the reduction in sAPPα by α-secretase inhibitor effectively reached 434% and 50% of control (DMSO), respectively (upper panel). The decrease in sAPPβ by β-secretase inhibitor effectively reached 11% of control (lower panel). Neither sAPPα nor sAPPβ in the APP-KO brain was detected by anti-hsAPPα or anti-sAPPβ antibody, respectively. An asterisk indicates a non-specific band.
(TIF)

Figure S4 Immunocytochemical characterization of human ES cell (H9)-derived neuronal cells. (A) Time-dependent morphological changes of cells reseeded in a 24-well plate. Neuronal and glial cells were stained by anti-Tuj1 (left; red), anti-synapsin I (left; green), anti-MAP2 (right; red), and anti-GFAP (right; green) antibodies and DAPI (right; blue) at 38, 45, and 52 days. Scale bar, left; 20 μm, right; 50 μm. (B) ICC staining of Tbr1-, Ctip2-, Cux1- and Satb2-positive cells at day 52. (C–E) Neurotransmitter phenotypes at day 52. PAG (red)- and GAD (green)-positive (C), Glut1 (green)- and Tuj1 (red)-positive (D), and GABA (green)- and Tuj1 (red)-positive cells (E). Blue, DAPI. Scale bar, 50 μm.
(TIF)

Figure S5 Aβ production was modulated by several drugs in human ES cell-derived neuronal cells. β-Secretase inhibitor (BSI) (A, B), γ-secretase inhibitor (GSI) (C, D), and NSAID (E, F) were added into hES cell-derived neuronal cell cultures at day 36 (dotted line) and 50 (bold line), and two days later amounts of Aβ40 and Aβ42 secreted into the conditioned media were measured. The ratios Aβ40/FL-APP and Aβ42/FL-APP are expressed as percentages of the vehicle-treated group at day 52 and represent mean ± SD of 3 assays. *, $^{\#}p<0.05$, **, $^{\#\#}p<0.01$, ***, $^{\#\#\#}p<0.001$, significantly different from respective vehicle-treated groups by Dunnett's test.
(TIF)

Figure S6 Expression levels of reprogramming factors of iPS cells in neural differentiation. Total and transgene (Tg) expression levels of Sox2, Oct3/4 and Klf4 were measured by qPCR. Bold and dotted lines represent total and transgene expressions, respectively. "Fold expression" represents the ratio of the expression level compared to the total expression level at day 0 (iPS cells).
(TIF)

Figure S7 Aβ production was modulated by GSI in human iPS cell (201B7)-derived neuronal cells. γ-Secretase inhibitor (GSI) was added into the hiPS cell line, 201B7-derived neuronal cell cultures at day 36 (dotted line) and 50 (bold line), and two days later amounts of Aβ40 (A) and Aβ42 (B) secreted into the conditioned media were measured. The ratios Aβ40/FL-APP and Aβ42/FL-APP are expressed as percentages of the vehicle-treated group at day 52 and represent mean ± SD of 3 assays.
(TIF)

Protocol S1 Sampling method for checking antibody specificity.
(PDF)

Table S1 Effects of secretion inhibitors on cell viability measured by LDH assay at day 52.
(DOCX)

Table S2 qPCR primers.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NI HI NY MA. Performed the experiments: NY MA NI. Analyzed the data: NY MA NI HI. Contributed reagents/materials/analysis tools: SK KT IA HH TK KM TCS TN TA SY. Wrote the paper: NY MA SY NI HI.

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High Incidence of *NLRP3* Somatic Mosaicism in Patients With Chronic Infantile Neurologic, Cutaneous, Articular Syndrome

Results of an International Multicenter Collaborative Study

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Objective. **Chronic infantile neurologic, cutaneous, articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease (NOMID), is a dominantly inherited systemic autoinflammatory disease. Although heterozygous germline gain-of-function *NLRP3* mutations are a known cause of this disease, conventional genetic analyses fail to detect disease-causing mutations in ~40% of patients. Since somatic *NLRP3* mosaicism has been detected in several mutation-negative NOMID/CINCA syndrome patients, we undertook this study to determine the precise contribution of somatic *NLRP3* mosaicism to the etiology of NOMID/CINCA syndrome.**

Methods. **An international case–control study was performed to detect somatic *NLRP3* mosaicism in NOMID/CINCA syndrome patients who had shown no mutation during conventional sequencing. Subcloning and sequencing of *NLRP3* was performed in these mutation-negative NOMID/CINCA syndrome patients and their healthy relatives. Clinical features were analyzed to identify potential genotype–phenotype associations.**

Results. **Somatic *NLRP3* mosaicism was identified in 18 of the 26 patients (69.2%). Estimates of the level of mosaicism ranged from 4.2% to 35.8% (mean ± SD 12.1 ± 7.9%). Mosaicism was not detected in any of the 19 healthy relatives (18 of 26 patients versus 0 of 19**

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relatives; $P < 0.0001$). In vitro functional assays indicated that the detected somatic *NLRP3* mutations had disease-causing functional effects. No differences in *NLRP3* mosaicism were detected between different cell lineages. Among nondescript clinical features, a lower incidence of mental retardation was noted in patients with somatic mosaicism. Genotype-matched comparison confirmed that patients with somatic *NLRP3* mosaicism presented with milder neurologic symptoms.

***Conclusion.* Somatic *NLRP3* mutations were identified in 69.2% of patients with mutation-negative NOMID/CINCA syndrome. This indicates that somatic *NLRP3* mosaicism is a major cause of NOMID/CINCA syndrome.**

Chronic infantile neurologic, cutaneous, articular (CINCA) syndrome (MIM no. #607715), also known as neonatal-onset multisystem inflammatory disease (NOMID), is a dominantly-inherited autoinflammatory disease that is characterized by neonatal onset and the triad of urticarial-like skin rash, neurologic manifestations, and arthritis/arthropathy. Patients often experience recurrent fever and systemic inflammation. NOMID/CINCA syndrome is the most severe clinical phenotype of the cryopyrin-associated periodic syndromes (CAPS) that also include the 2 less severe but phenotypically similar syndromes familial cold autoinflammatory syndrome (FCAS; MIM no. #120100) and Muckle-Wells syndrome (MIM no. #191900). CAPS are caused by mutations in the *NLRP3* gene, which is a member of the nucleotide-binding oligomerization domain–like receptor (NLR) family of the innate immune system (1,2).

NLRP3 is an intracellular "sensor" of danger signals arising from cellular insults, such as infection, tissue damage, and metabolic deregulation, and it has been highly conserved throughout evolution. NLRP3 associates with ASC and procaspase 1 to constitute a large multiprotein complex termed the NLRP3 inflammasome. When activated, the NLRP3 inflammasome converts the biologically inactive procaspase 1 into active caspase 1. Caspase 1 produces the cytokines interleukin-1β (IL-1β) and IL-18, which are mainly involved in the inflammatory response (3). Available research suggests that mutated *NLRP3* induces autoactivation of the NLRP3 inflammasome in CAPS patients, resulting in an uncontrolled overproduction of IL-1β.

Most CAPS patients carry heterozygous germline missense mutations in the *NLRP3* coding region ("mutation-positive" patients) (4,5). More than 80 different disease-causing mutations have been reported to date (6). However, ~40% of clinically diagnosed NOMID/CINCA syndrome patients show no heterozygous germline *NLRP3* mutation during conventional Sanger-sequencing-based genetic analyses ("mutation-negative" patients). Comparisons of NOMID/CINCA syndrome patients with and without heterozygous germline *NLRP3* mutations have revealed no differences in clinical features or response to treatment (4,7).

In a previous study, we identified a high incidence of somatic *NLRP3* mosaicism in "mutation-negative" NOMID/CINCA syndrome patients in Japan (8). We therefore hypothesized that somatic *NLRP3* mosaicism may be implicated in the etiology of the disorder, although its precise contribution remains unclear. The aim of the present study was to evaluate both the frequency of *NLRP3* somatic mosaicism in NOMID/CINCA syndrome patients and the association between somatic mosaicism and clinical phenotype using an international cohort of mutation-negative NOMID/CINCA syndrome patients.

PATIENTS AND METHODS

Study design and participants. International collaborators were contacted to identify mutation-negative NOMID/CINCA syndrome cases. A total of 20 DNA samples were received from 4 centers: France (n = 6), The Netherlands (n = 4), Spain (n = 3), and the US (n = 7). DNA samples had been extracted from peripheral blood mononuclear cells or whole blood. All 20 samples had been subjected to conventional sequencing, and no *NLRP3* mutations had been identified. In each case, the accuracy of the clinical diagnosis had been confirmed according to the diagnostic criteria (7). The 6 previously reported Japanese cases and 1 Spanish case with *NLRP3* somatic mosaicism were also included (8,9). DNA samples were also collected from 19 healthy relatives of 8 patients (8 from France, 5 from Japan, 2 from Spain, and 4 from the US) to evaluate the causality of somatic *NLRP3* mosaicism in a case–control manner, since the clinical features may be modified by genetic and environmental factors.

Written informed consent for *NLRP3* gene analysis was obtained from all patients and controls. The study was approved by the Institutional Review Board of the Kyoto University Graduate School of Medicine and was conducted in accordance with the Declaration of Helsinki.

Data collection. *Demographic and clinical data.* The clinicians responsible for each mutation-negative NOMID/CINCA syndrome patient completed a questionnaire to document characteristics such as age, sex, race, symptoms, clinical findings, clinical course, and prognosis. No clinical data were obtained from the healthy controls.

Investigation of NLRP3 gene mosaicism. Disease-causing mutations in NOMID/CINCA syndrome patients have

only been reported in exons 3, 4, and 6 of *NLRP3* (6). Thus, the present sequencing was focused on a search for somatic mosaicism of these 3 exons and their flanking intronic regions. After amplifying these genomic regions with the proofreading polymerase chain reaction (PCR) enzyme KOD-Plus polymerase (Toyobo) and dA addition with an LA *Taq* polymerase (Takara Bio), the amplicons were subcloned into pCR2.1-TOPO vector (Invitrogen). Ninety-six clones were selected at random for each amplicon. The subcloned amplicons were retrieved by PCR with LA *Taq* polymerase. They were then treated with ExoSAP-IT (USB) and proteinase K (Promega) prior to direct sequencing. The cloned exons were sequenced at the Kazusa DNA Research Institute using a BigDye Terminator kit (version 3.1) and an ABI 3730 DNA sequencer (Life Technologies). Mosaicism was indicated by the detection of >2 subclones carrying the same base variation at the same position in 96 clones.

To purify leukocyte subpopulations, freshly drawn whole blood was separated using sequential dextran and Ficoll-Hypaque density-gradient centrifugation methods. Cell sorting to select T cells, B cells, and monocytes was performed with an AutoMACS Pro Separator (Miltenyi Biotec) or a FACSVantage System (BD Biosciences), as described elsewhere (8,9). The purity of each cell lineage was >90%. The level of mosaicism was determined by sequencing each source of genomic DNA from 80 clones.

Plasmids and cell lines. To determine whether the identified NLRP3 mutants cause disease, experiments for assessing 2 pathologic functions were performed as described elsewhere (8). Briefly, ASC-dependent NF-κB activation was performed by a dual-luciferase reporter assay in HEK 293FT cells transfected with NLRP3 mutants. Transfection-induced cell death in the human monocytic cell line THP-1 was performed by transfecting green fluorescent protein–fused mutant NLRP3 into THP-1 cells and then measuring the dead cells with 7-aminoactinomycin D.

Statistical analysis. The study was designed to detect mosaicism at a 5% allele frequency with >95% possibility. To satisfy this condition, it was necessary to sequence at least 93 clones per patient. The following calculation was used to estimate the number of clones that had to be sequenced: $P = 1 - (1 - 0.05)^n - n(0.05)(1 - 0.05)^{n-1}$ ($n = 93$, $P = 0.956$). The study was designed to analyze 96 PCR-fragment clones from each patient. The error rate of the PCR reactions was estimated using a proofreading KOD-Plus enzyme. We analyzed a plasmid vector carrying a normal *NLRP3* exon 3, in which 2 distinct errors were detected by sequencing 91 clones. The calculated error rate for this result was 1/87,451 (2/[1,922 bp × 91 clones]). Thus, the probability was negligible that the same errors would be detected more than twice in 96 clones from 1 patient.

To calculate the sample size, we calculated the prevalence of somatic mosaicism among mutation-negative NOMID/CINCA syndrome patients. Eight cases of somatic mosaicism were identified among 15 mutation-negative NOMID/CINCA syndrome patients who were subsequently analyzed by the subcloning method described above. It was assumed that the maximum number of possible somatic mosaicism cases among family controls was 1. On the basis of these data and this assumption, it was calculated that 19 controls were required to ensure a 2-sided alpha level of 0.05 and a power of 0.8.

Continuous variables are presented as the mean ± SD or as the median and interquartile range. Categorical variables are presented as numbers and ratios (with percentages). To compare clinical data between patients with and patients without mosaicism, the Wilcoxon rank sum test was used for continuous variables and Fisher's exact test was used for categorical variables. Fisher's exact test was used to compare the difference in mosaicism ratio between cases and controls. The chi-square test was used to compare the difference in the level of mosaicism between different sources of genomic DNA from each patient.

Table 1. Somatic mosaicism among mutation-negative NOMID/CINCA syndrome patients*

Country, patient	Sequence variant	Protein variant	Mosaicism, %
France			
F1	1298C>T	T433I	5.2
F2	907G>C	D303H	4.2
F3	1315G>C	A439P	21.9
F4	1216A>G	M406V	9.2
F5	1698C>A	F566L	11.5
F6	None	–	–
Japan			
J1	1709A>G	Y570C	12.2
J2	790C>T	L264F	4.3
J3	919G>A	G307S	10.7
J4	1699G>A	E567K	6.5
J5	907G>C	D303H	11.9
J6	None	–	–
Spain			
S1	920G>T	G307V	9.6
S2	907G>C	D303H	19.1
S3	None	–	–
S4	None	–	–
US			
A1	1065A>T	K355N	18.8
A2	1698C>A	F566L	14.6
A3	1704G>C	K568N	9.4
A4	2263G>A	G755R	35.8
A5	None	–	–
A6	None	–	–
The Netherlands			
N1	1699G>A	E567K	6.3
N2	2263G>A	G755R	6.3
N3	None	–	–
N4	None	–	–

* *NLRP3* mosaicism was detected in 18 of 26 patients (69.2%) with neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurologic, cutaneous, articular syndrome (CINCA syndrome). When samples from 19 healthy relatives of these patients were investigated, no somatic mosaicism was detected. The *P* value from the comparison of the cases and the controls (18 of 26 versus 0 of 19) was statistically significant ($P < 0.0001$).

RESULTS

Somatic *NLRP3* mosaicism in mutation-negative NOMID/CINCA syndrome patients. A heterozygous germline *NLRP3* mutation was detected in 1 of the 27 samples, and this was therefore excluded from the analyses. For each patient, 96 clones were selected at random for each amplicon. These were then sequenced. *NLRP3* mosaicism was detected in 18 of 26 patients (69.2%), and the level of allelic mosaicism ranged from 4.2% to 35.8% (mean ± SD 12.1 ± 7.9%; median 10.2%) (Table 1). Seven of the detected *NLRP3* mutations were novel (p.G307S, p.K355N, p.M406V, p.T433I, p.F566L, p.E567K, and p.K568N). The remaining mutations have been reported previously in NOMID/CINCA syndrome patients as disease-causing heterozygous germline mutations (p.L264F, p.D303H, p.G307V, p.A439P, p.Y570C, and p.G755R). Each of the 3 *NLRP3* mutations, p.F566L, p.E567K, and p.G755R, was detected in 2 unrelated patients. *NLRP3* mutation p.D303H was detected in 3 unrelated patients.

Analyses in family controls. To validate the clinical relevance of the *NLRP3* mosaicism identified in mutation-negative NOMID/CINCA syndrome patients, samples from 19 healthy relatives were investigated. No somatic mosaicism was detected in any of these samples. The *P* value from the comparison of cases and controls (18 of 26 versus 0 of 19) was statistically significant ($P < 0.0001$).

Functional effects of the identified somatic *NLRP3* mutations. Since disease-causing heterozygous germline mutations in *NLRP3* have been implicated in necrosis-like programmed cell death and ASC-dependent NF-κB activation (8), experiments were performed to determine whether the mutations identified in patients with somatic mosaicism showed the same effects. All of the identified mutations induced both THP-1 cell death (Figure 1A) and ASC-dependent NF-κB activation (Figure 1B). The in vitro effects of these novel mutations were similar to or even more pronounced than those of previously reported *NLRP3* mutations. This strongly suggests that all mutations showing somatic mosaicism have pathogenic effects, including the novel mutations identified in the present study.

Mutation frequency of *NLRP3* among various cell lineages and 1 tissue type. To explore the origin of the *NLRP3* mosaicism, mutational frequency was evaluated in various cell lineages and 1 tissue type from 4 Japanese patients with *NLRP3* somatic mosaicism. In each patient, the same mutations were found in all of the cell lineages investigated (neutrophils, monocytes, T cells, B cells) and in the buccal mucosa tissue, and no significant difference in mutation frequency was observed between these sources (Table 2).

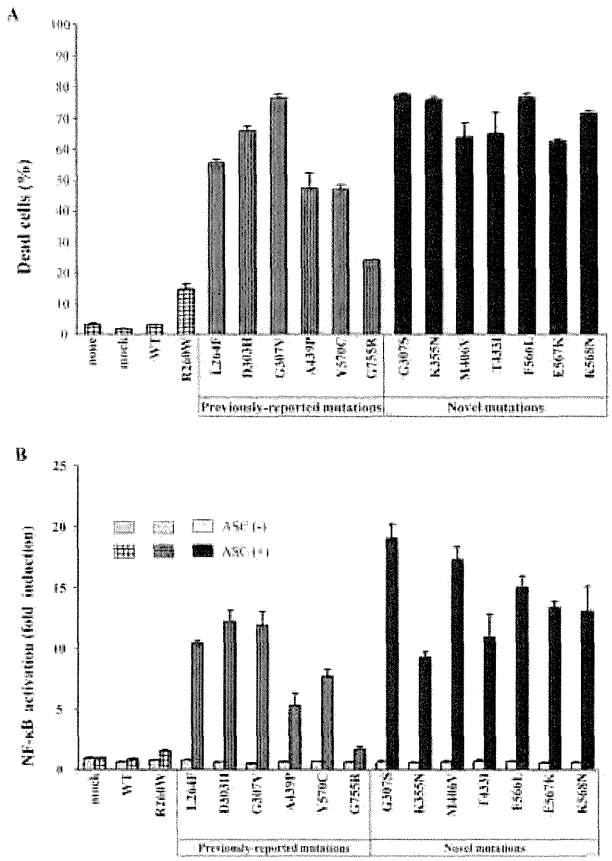

Figure 1. In vitro functional assessment of the identified *NLRP3* mosaicism mutations. **A,** Necrotic cell death of THP-1 cells induced by the identified somatic *NLRP3* mosaicism mutations. Green fluorescent protein (GFP)–fused mutant NLRP3 was transfected into THP-1 cells. The percentage of dead cells (7-aminoactinomycin D positive) among GFP-positive cells is shown. Values are the mean ± SD of triplicate experiments, and data are representative of 2 independent experiments. None = nothing transfected; mock = vector without NLRP3; WT = wild-type NLRP3; R260W = NLRP3 with p.R260W (frequent mutations in patients with cryopyrin-associated periodic syndromes). **B,** ASC-dependent NF-κB activation induced by the identified somatic *NLRP3* mosaicism mutations. HEK 293FT cells were cotransfected with WT or mutant NLRP3 in the presence or absence of ASC. The induction of NF-κB is shown as the fold change compared with cells that were transfected with a control vector without ASC (set at 1). Values are the mean ± SD of triplicate experiments, and data are representative of 2 independent experiments.

Table 2. Distribution and quantification of *NLRP3* mutations among sources of genomic DNA (4 cell lineages and 1 tissue type)*

Patient	Sequence variant	Protein variant	Mosaicism, %				
			Neutrophils	Monocytes	T cells	B cells	Buccal mucosa
J1	1709A>G	Y570C	12.6	9.8	8.0	9.5	8.3
J3	919G>A	G307S	9.1	10.8	6.9	10.6	9.0
J4	1699G>A	E567K	3.5	2.3	3.7	3.4	2.2
J5	907G>C	D303H	14.4	8.7	7.7	8.5	13.5

* No significant differences in the level of mosaicism were observed among the sources of genomic DNA.

Phenotype–genotype analysis. Given the previously reported genotype–phenotype association between the *NLRP3* gene and CAPS, the clinical characteristics of NOMID/CINCA syndrome patients with somatic *NLRP3* mutations were compared with those of patients from previous reports who had the same *NLRP3* mutations but with heterozygous germline status (1,4,10–13) (Figure 2) (further information is available at http://web16.kazusa.or.jp/download/). All of the patients in these 2 groups had an early onset of the disease, fever, and urticarial rash. The presence of arthritis, bony overgrowth, contractures, hearing loss, and seizure varied in each group of patients, and no significant difference was detected. However, whereas most patients with heterozygous germline *NLRP3* mutations presented with mental retardation, this was not the case for patients with somatic *NLRP3* mosaicism. A comparison was also made between the clinical data from patients with somatic *NLRP3* mosaicism and the data from patients with neither germline nor somatic *NLRP3* mutations. Again, a lower incidence of mental retardation was observed in patients with somatic *NLRP3* mosaicism

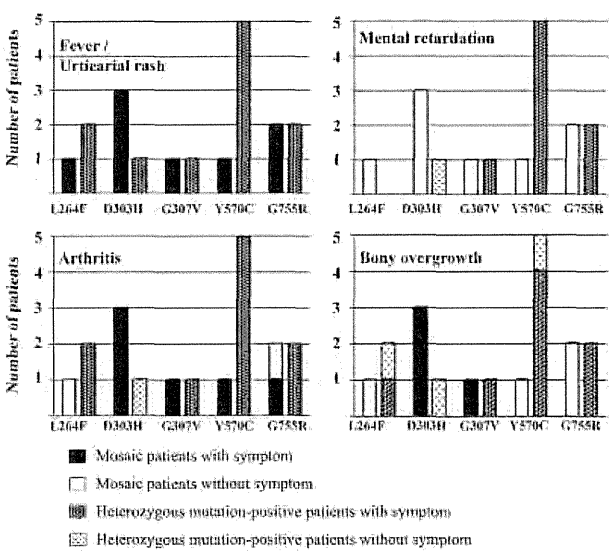

Figure 2. Comparison of the clinical profiles of patients carrying somatic *NLRP3* mutations and patients carrying the same mutation, but with germline status. Clinical profiles of patients with mosaicism and those of patients with heterozygous germline mutations are compared for each protein variant (L264F, D303H, G307V, Y570C, and G755R). The data on 4 typical clinical symptoms are shown. Total numbers of patients with mosaicism and total numbers of patients with heterozygous mutation examined are shown as a bar for each protein variant. Each bar is stratified according to the presence or absence of the symptom. For the protein variant L264F, no data on mental retardation were available for the patient with a heterozygous germline mutation.

Table 3. Clinical profiles of patients with somatic *NLRP3* mosaicism and patients with neither germline nor somatic *NLRP3* mutations*

	Patients with somatic *NLRP3* mosaicism (n = 18)	Patients with neither germline nor somatic *NLRP3* mutations (n = 8)
Age, median (IQR) years	12 (1–30)	10 (3–21)
No. of men/women	10/8	3/5
Age at onset, median (IQR) months	0 (0–24)	0.5 (0–33)
Fever	17/17	7/7
Urticarial rash	14/14	8/8
Mental retardation	4/17	6/8
Meningitis	13/17	5/8
Seizures	2/18	1/7
Hearing loss	10/18	2/7
Arthritis	14/17	7/8
Bony overgrowth	12/17	6/7
Contractures	7/17	4/7
Walking disability	8/18	3/7
Biologic therapy	10/15	3/8

* Except where indicated otherwise, values are the number with the feature/the total number of patients assessed. A lower incidence of mental retardation was observed in patients with somatic *NLRP3* mosaicism ($P = 0.03$). No other significant differences were detected between the groups. IQR = interquartile range.

($P = 0.03$). No other significant differences were detected (Table 3) (further information is available at http://web16.kazusa.or.jp/download/).

DISCUSSION

The present international multicenter study investigated 26 NOMID/CINCA syndrome patients who were mutation negative according to conventional sequencing along with 19 family controls to determine whether low-level mosaicism is a disease-causing genetic mechanism. Following our first report of low-level somatic mosaicism in a NOMID/CINCA syndrome patient (14), we reported a new method of detecting low-level *NLRP3* mosaicism, in which lipopolysaccharide (LPS) induced cell death specifically in *NLRP3* mutation–positive monocytes (8). However, this method requires fresh live monocytes, special equipment such as a cell sorter, and experience in its use due to the rapid time course of LPS-induced necrotic monocytic death. For these reasons, application of this method was not feasible in an international collaborative study. We therefore opted to use genomic DNA, since it is easier to handle and can be stored and shipped. Based on our previous study in Japanese patients showing that the frequency of mutant alleles could be <5%, we designed a subcloning and Sanger-sequencing strategy that could detect this very low allelic mutation frequency.

Presuming that the present cohort is representative of the 40% of NOMID/CINCA syndrome patients who are mutation negative according to conventional sequencing, the results suggest that ~28% of all NOMID/CINCA syndrome patients may carry somatic *NLRP3* mosaicism. CAPS patients present with a continuous spectrum of symptoms, and a degree of genotypic overlap is observed between disease subtypes. Although the present study focused on the most severe NOMID/CINCA syndrome phenotype, it is possible that somatic *NLRP3* mosaicism may also occur in milder forms of CAPS. The presence of somatic mosaicism should also be investigated in patients with other dominantly inherited autoinflammatory diseases caused by gain-of-function mutations and who are mutation negative according to conventional sequencing.

Among the 18 patients with somatic *NLRP3* mosaicism, we found 6 mutations that have previously been identified in NOMID/CINCA syndrome patients as heterozygous germline mutations. We also identified 7 novel mutations, which were confirmed as being functionally active and presumably pathogenic. Functional in vitro assays showed that these novel mutations had greater disease-causing capacity than the previously described mutations. This suggests that the novel mutations may be deleterious and unrecognized if inherited as heterozygous germline mutations.

The present study also addressed the important question of how somatic *NLRP3* mosaicism modifies clinical presentation. Although no statistically significant differences in age at disease onset, skin symptoms, joint involvement, or response to IL-1 blockade were detected, milder neurologic involvement was observed in patients with somatic mosaicism. Comparisons with NOMID/CINCA syndrome patients carrying the same *NLRP3* mutations but with heterozygous germline status made this tendency more prominent. Although the level of somatic mosaicism in blood leukocytes was relatively low, it remains unclear how these low-level mutations influence clinical presentation, including disease severity. One interesting hypothesis is that the difference in the severity of neurologic manifestations is a function of the level of mosaicism. For ethical and technical reasons, it was not possible to evaluate the level of mosaicism in central nervous system (CNS) cells or glial cells in the present study, and this therefore awaits investigation in future studies.

The mechanism through which *NLRP3* somatic mosaicism occurs also requires elucidation. The present study demonstrated that similar proportions of neutrophils, T cells, B cells, monocytes, and buccal cells carried the mutated allele. Therefore, the mutation leading to mosaicism must have arisen before the pluripotent stem cells committed to hematopoietic progenitor stem cells or ectoderm-derived nonhematopoietic cells. Several mechanisms for mosaicism have been proposed, including chimerism due to cell fusion with an aborted dizygotic twin and a mutational event during early embryogenesis (15). The latter mechanism is more likely in the present cohort, since mosaicism at similar frequency was detected in several cell types. To verify the hypothesis of a mutational event during embryogenesis, and to determine the point at which this occurred, it would be helpful to analyze other tissues. However, obtaining such tissues from patients may be ethically problematic.

Approximately 12% of the patients in the present cohort carried neither germline nor somatic *NLRP3* mutations and may therefore be considered to be genuinely mutation negative. However, it is possible that these patients have *NLRP3* mutations that have been overlooked. A recent report described a mutation in the 5′-untranslated region of *NLRP3* in a patient with FCAS

(16), although it remains unclear how this noncoding mutation causes disease. Another possibility is that an extremely low frequency of NLRP3 mosaicism may have been missed. The subcloning and Sanger-sequencing strategy used in this study set the detection limit of mosaicism at 5%. Considering the range of *NLRP3* mosaicism detected (4.2–35.8%), the median (10.2%), and the identification of 2 patients with <5% mosaicism, it is indeed likely that patients with an even lower level of *NLRP3* mosaicism may have been overlooked. Recent advances in next-generation DNA sequencing technology may resolve this technical problem, although the associated error rate could be problematic. Another possibility is that *NLRP3* mutations were present in uninvestigated cell lineages, such as those from CNS tissue, bone tissue, or skin. Future studies of NOMID/CINCA syndrome should investigate these tissues while searching for mutations in other genes.

In conclusion, the present study has clearly demonstrated that a significant proportion of NOMID/CINCA syndrome patients who were mutation negative according to conventional sequencing carried somatic *NLRP3* mutations with a variable degree of mosaicism. Clinicians should therefore consider somatic mosaicism as a possible cause of disease in mutation-negative NOMID/CINCA syndrome patients and implement appropriate therapy. The early diagnosis of NOMID/CINCA syndrome and prompt initiation of therapy would improve clinical outcome. Further goals in this research field are the refinement of genetic screening and the verification of the functional consequences of all detected somatic mutations. Systematic screening for somatic mosaicism will provide new insights into the etiology of human disease.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Ohara and Nishikomori had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data. Tanaka, Izawa, Saito, Oshima, Ohara, Nishikomori, Goldbach-Mansky, Aksentijevich, de Saint Basile, Neven, van Gijn, Frenkel, Aróstegui, Yagüe, Merino, Ibañez, Pontillo, Takada, Imagawa.

Analysis and interpretation of data. Sakuma, Morimoto, Kawai, Yasumi, Nakahata, Heike.

ROLE OF THE STUDY SPONSOR

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２０１１年1月２７日プレスリリース

東京大学
THE UNIVERSITY OF TOKYO

平成 24 年 1 月 27 日
国立大学法人 東京大学 医科学研究所

報道関係者各位

厚労科研費「再生医療臨床実現化ハイウェイ研究事業」

～事業開始のお知らせと報道機関説明会の開催案内～

このほど、東京大学医科学研究所(所長:清野　宏)は、平成23年度厚生労働科学研究費補助金「難病・がん等の疾患分野の医療の実用化研究事業(再生医療関係研究分野)」(再生医療臨床実現化ハイウェイ研究事業)を実施することとなりましたので、お知らせします。本研究事業について、2月7日(火)に当研究所教授で研究代表者の中井謙太が報道機関説明会を開催します。

本研究事業では、情報通信技術を活用して研究機関間の連携体制を構築することにより、高い安全性、有効性及び品質を有するヒト幹細胞の研究開発とその細胞を用いた再生医療の早期実現化を目指すとともに、研究機関が継続的に革新的技術を創出できるように、オープン・イノベーション環境の礎を築きます。また、別紙1のとおり、本研究事業の運営に関する指導、助言等を行う研究評価委員会を設置します。

報道機関説明会へのご参加を希望される方は、別紙2をご参照の上、期日までにお申し込みください。

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本資料は、厚生労働記者会、大学記者会、科学記者会、文部科学記者会の会員報道機関等に配布しております。

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Email: info@regenerativemedicinehw.info
ウェブ:http://www. regenerativemedicinehw.info

＜別紙１＞

厚生労働科学研究費補助金
難病・がん等の疾患分野の医療の実用化研究事業(再生医療関係研究分野)
研究評価委員会　委員名簿

	氏　名	所　属・役　職
	(◎:委員長、○:委員長代理)	
	あらい　けんいち 新井　賢一	東京大学　名誉教授
	うちだ　かずなり 内田　和成	早稲田大学大学院　商学研究科　教授
	かなざわ　いちろう 金澤　一郎	国際医療福祉大学大学院　教授
	こうさか　しんいち 髙坂　新一	(独)国立精神・神経医療研究センター　神経研究所　所長
	さるた　たかお 猿田　享男	慶應義塾大学　名誉教授
◎	せのお　けんいちろう 妹尾　堅一郎	特定非営利活動法人　産学連携推進機構　理事長
	たけなか　とういち 竹中　登一	日本製薬団体連合会　前会長
	ながい　りょうぞう 永井　良三	東京大学大学院　医学系研究科　教授
	まちの　さく 町野　朔	上智大学生命倫理研究所　所長代行
○	みくりや　たかし 御厨　貴	東京大学先端科学技術研究センター　教授
	みやた　みつる 宮田　満	日経BP社　医療局主任編集委員
	やまぐち　すぐる 山口　英	奈良先端科学技術大学院大学　教授
	やまもと　たかふみ 山本　貴史	株式会社　東京大学TLO　代表取締役社長

(敬称略・五十音順)

＜別紙２＞

報道機関説明会

1. 会見日時: 平成24年2月7日(火)11:00～11:50

2. 会見場所: 東京大学医科学研究所　総合研究棟8F　セミナールーム
（地図は別紙3を参照）

3. 出席者(予定):

	東京大学医科学研究所	清野 宏	所長
	東京大学医科学研究所	中井 謙太	教授
	東京大学医科学研究所	諸田 清	事務部長
研究分担者	京都大学 再生医科学研究所	中辻 憲夫	教授
研究分担者	京都大学 iPS 細胞研究所	中畑 龍俊	副所長
研究分担者	東京女子医科大学	大和 雅之	教授
研究評価委員	東京大学	新井 賢一	名誉教授
研究評価委員	上智大学生命倫理研究所	町野 朔	所長代行

4. 発表概要: 「再生医療臨床実現化ハイウェイ研究事業」に関する概要説明

5. 注意事項: 説明会へのご参加を希望される方は、下記の様式にご記入の上、2月6日(月)正午までに事務局宛にFAX又はメールにてお申し込みください。また、説明会終了後に個別取材をご希望の方は、事前に事務局宛にご連絡ください。ご連絡いただいた順に当日14時までの間で時間調整をさせていただきます。
なお、当日は御名刺をご持参いただき、会場受付にお渡しください。

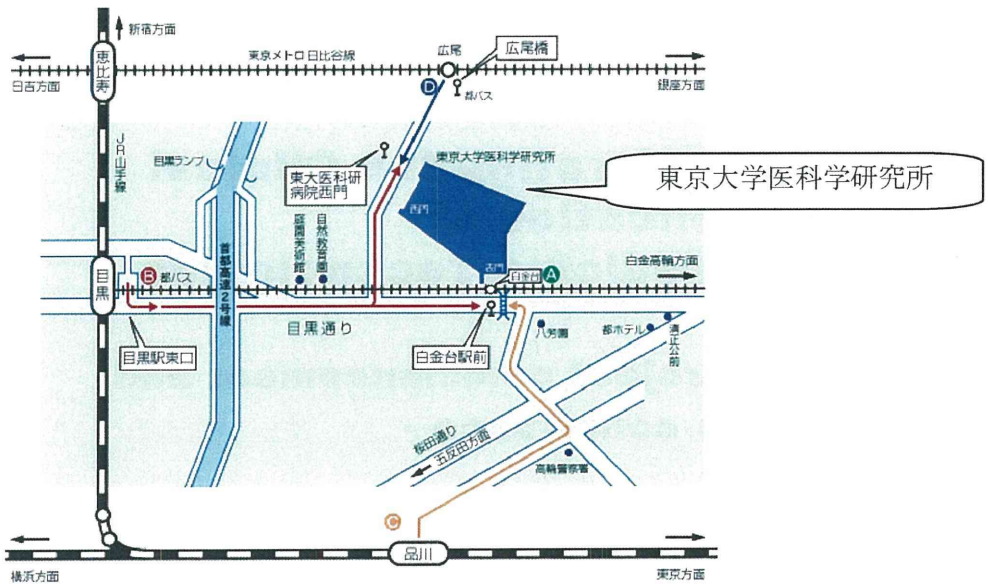
－－

「再生医療臨床実現化ハイウェイ研究事業」報道機関説明会　出席希望

御社名	
出席者名	
同伴者名	（計　　名）
個別取材	希望する　・　希望しない
撮影有無	撮影あり　・　撮影なし

＜別紙３＞

【東京大学医科学研究所　地図】



【校内建物配置図】

※会見場所（総合研究棟）は⑧の建物になります。また、１階ドアはセキュリティのため施錠されておりますので、備え付けの電話にて【内線：75511】までご連絡をお願い致します。

厚労科研費「再生医療臨床実現化ハイウェイ研究事業」～事業開始のお知らせと報道機関説明会の開催案内～
＜お問い合わせ先＞東京大学医科学研究所　再生医療ハイウェイ事務局
TEL:　03-5449-5511　FAX: 03-5449-5133　Email: info@regenerativemedicinehw.info
ウェブ:　http://www. regenerativemedicinehw.info

THE UNIVERSITY OF TOKYO

THE INSTITUTE OF MEDICAL SCIENCE
THE UNIVERSITY OF TOKYO

January 27th, 2012
The Institute of Medical Science,
The University of Tokyo, Japan

Project for Accelerating the Clinical Application of Regenerative Medicine Technologies

- Information for project commencement and press conference -

The Institute of Medical Science, the University of Tokyo (IMSUT; Dean: Hiroshi KIYONO) today announced the launch of the "Project for Accelerating the Clinical Application of Regenerative Medicine Technologies" with grants from the Ministry of Health, Labour and Welfare of Japan (Grants for Health and Labour Sciences Research). The outstanding team members are listed in appendix A.IMSUT will have its first press conference on February 7th, when the project leader, Professor Kenta NAKAI, will explain the goal and the plans of the project.

The Project aims to achieve the earliest possible clinical application of regenerative medicine technologies using safe, effective and high-quality human stem cells by building a collaborative platform among researchers through a network centric research infrastructure. Also in the project, the basis of an "open innovation" environment allowing research institutes to continuously create innovative technologies will be developed. To support this activity, an advisory committee has been formed with members from various sectors as listed in appendix B.

Contact: Highway Project Office at The Institute of Medical Science, the University of Tokyo
TEL: +81-3-5449-5511 FAX: +81-3-5449-5133 Email: info@regenerativemedicinehw.info
Web: http://www. regenerativemedicinehw.info

APPENDIX-A

【Project Team】

- Leader

Kenta NAKAI	Professor, Laboratory of Functional Analysis In Silico, Human Genome Center, The Institute of Medical Science, The University of Tokyo

- Members

Tatsutoshi NAKAHATA	Professor and Deputy Director of Center for iPS Cell Research and Application (CiRA), Head of Clinical Application Department, Kyoto University
Norio NAKATSUJI	Director, Institute for Integrated Cell-Material Sciences, Professor, Institute for Frontier Medical Sciences, Kyoto University
Kohji NISHIDA	Professor and Chairman, Department of Ophthalmology, Graduate School of Medicine, Osaka University
Hideyuki OKANO	Professor, Department of Physiology, School of Medicine, Keio University
Masayo TAKAHASHI	Team Leader, Laboratory for Retinal Regeneration, Center for Developmental Biology(CDB), Riken Kobe Institute
Akihiro UMEZAWA	Director, Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development
Masayuki YAMATO	Professor, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University
Shinya YAMANAKA	Professor and Director of the Center for iPS Cell Research and Application (CiRA), Head of Clinical Application Department, Kyoto University

- Advisor

Akifumi MATSUYAMA	Director, Department of Somatic Stem Cell Therapy and Health Policy, Foundation for Biomedical Research and Innovation

APPENDIX-B

[The Advisory Committee]

(* Chair, **Vice Chair)

Kenichi ARAI	Professor Emeritus, The University of Tokyo, Founding President, Asia-Pacific International Molecular Biology Network (A-IMBN)
Ichiro KANAZAWA	Medical Supervisor for Royal Families, Imperial Household Agency, Dean, Graduate School, International University of Health & Welfare
Shinichi KOHSAKA	Director-General, National Institute of Neuroscience, National Center of Neurology and Psychiatry
Saku MACHINO	Professor, Institute of Bioethics, Sophia University
Takashi MIKURIYA**	Professor, Research Center for Advanced Science and Technology, The University of Tokyo
Mitsuru MIYATA	Executive Lead Writer, Webmaster of Biotechnology Japan, Nikkei Business Publications, Inc.
Ryozo NAGAI	Professor and Chairman, Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo
Takao SARUTA	Emeritus Professor, Keio University
Ken SENOH*	President and Chairperson, The Industry-Academia Collaboration Initiative (Non Profit Organization)
Toichi TAKENAKA	Former Chairman of the Federation of Pharmaceutical Manufacturers' Associations of Japan
Kazunari UCHIDA	Professor, the Graduate School of Commerce, Waseda University
Suguru YAMAGUCHI	Professor, the Graduate School of Information at the Nara Institute of Science and Technology
Takafumi YAMAMOTO	CEO & President, TODAI TLO, Ltd.

２０１１年1月２７日ホームページ開設