Increase of serum uric acid levels associated with APOE ε2 haplotype: a clinico-genetic investigation and in vivo approach

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Abstract
Elevated serum uric acid (SUA)—hyperuricemia—is caused by overproduction of urate or by its decreased renal and/or intestinal excretion. This disease, which is increasing in prevalence worldwide, is associated with both gout and metabolic diseases. Several studies have reported relationships between apolipoprotein E (APOE) haplotypes and SUA levels in humans; however, their results remain inconsistent. This prompted us to investigate the relationship between APOE polymorphisms and SUA levels. Our subjects were 5,272 Japanese men, premenopausal women, and postmenopausal women. Multiple linear regression analyses revealed the ε2 haplotype of APOE to be independently associated with higher SUA in men (N = 1,726) and postmenopausal women (N = 1,753), but not in premenopausal women (N = 1,793). In contrast, the ε4 haplotype was little related to SUA levels in each group. Moreover, to examine the effect of Apoe deficiency on SUA levels, we conducted animal experiments using Apoe knockout mice, which mimics ε2/ε2 carriers. We found that SUA levels in Apoe knockout mice were significantly higher than those in wild-type mice, which is consistent with the SUA-raising effect of the ε2 haplotype observed in our clinico-genetic analyses. Further analyses suggested that renal rather than intestinal underexcretion of urate could be involved in Apoe deficiency-related SUA increase. In conclusion, we successfully demonstrated that the ε2 haplotype, but not the ε4 haplotype, increases SUA levels. These findings will improve our understanding of genetic factors affecting SUA levels.

Keywords Apolipoprotein · Human study · Menopause status · Single-nucleotide polymorphisms (SNPs) · Urate

Introduction
Hyperuricemia is caused by the overproduction of urate or by decreased renal [1, 2] and intestinal [3, 4] urate excretion. This common disease is not only associated with gout but also with other common conditions including hypertension [5, 6] and atherosclerotic cardiovascular diseases [7] as well as kidney diseases [8]. Although recent studies have revealed the pathophysiological importance of urate transporters in urate handling in humans [9, 10], other (non-transporter) genetic factors associated with serum uric acid (SUA) levels have also been reported [11–13]. Moreover, based on the classically known association between hyperuricemia and hyperlipidemia [14, 15], the influence on SUA levels of genetic factors affecting lipid levels in the blood is likely to be involved. One of these is variation in apolipoprotein E (APOE) polymorphisms; however, as described below, their effects on SUA levels have not been conclusive.

The human APOE gene, which is located on chromosome 19q13.2, has two common non-synonymous single-nucleotide polymorphisms (SNPs)—rs429358 (c.334T>C; p.Cys112Arg) and rs7412 (c.472C>T; Arg158Cys) [16]. Given the lack of simultaneous presence of their minor alleles in one haplotype, three haplotypes are defined, named as ε2, ε3, and ε4. Six diplotypes have been observed...
as combinations of these three haplotypes (Table 1). Among the three haplotypes, ε3 is the commonest and recognized as the parent form, corresponding to the wild type (WT).

APOE, a glycoprotein constituted of 299 amino acids, is chiefly distributed in very low-density lipoproteins (VLDLs), chylomicrons, and some high-density lipoproteins (HDLs) [17]. It plays multiple roles in the regulation of lipid and lipoprotein levels in the blood [18]. APOE polymorphisms are also reportedly associated not only with lipoprotein metabolism but with atherosclerotic cardiovascular diseases [19], kidney diseases [20], and neurodegenerative diseases [21–24]. Accordingly, given the observed associations between SUA levels and these disease phenotypes, it is possible that APOE polymorphisms and SUA levels are confounding factors for these disorders. Investigation of the latent relationship between APOE polymorphisms and SUA levels in humans should therefore provide new insights into the pathogenesis of hyperuricemia as well as its associated diseases. Several studies have investigated the relationship between APOE polymorphisms and SUA levels, but their results remain inconsistent. Hitherto, several human studies have reported that the ε2 and ε4 haplotypes may be associated with higher [25–28] and lower [29] SUA levels, respectively, than seen with the ε3 haplotype. In contrast, other studies have reported an association between the ε4 haplotype and higher SUA levels [30, 31]. To enhance our understanding of this unresolved question, we herein aimed to investigate the relationships between APOE polymorphisms and SUA levels in a larger population. We also performed animal experiments using Apoe knockout (KO) mice to examine the effect of total Apoe deficiency on SUA levels in terms of urate excretion from the body.

Methods

Study participants

All the procedures used in human studies were approved by the institutional ethical committees (National Defense Medical College and Nagoya University), and were performed in accordance with the Declaration of Helsinki. All of the Japanese individuals in this study were recruited from participants in the Shizuoka area and Daiko area in the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) [32, 33]. Written informed consent was obtained from all the subjects.

Among the participants, those who were under treatment for or had past histories of gout/hyperuricemia or dyslipidemia, and female participants for whom there was no information about menopause were excluded. Multiple regression analysis was performed on the resulting 5,272 subjects to evaluate the relationships among SUA levels, APOE gene polymorphisms, and other risk factors. Non-HDL cholesterol level was calculated using the following equation: [Non-HDL cholesterol (non-HDL-C) (mg/dL)] = [Total cholesterol (TC) (mg/dL)] – [HDL cholesterol (HDL-C) (mg/dL)].

Genetic analysis

Genomic DNA was extracted from whole peripheral blood cells. Genotyping of the two SNPs (rs429358 and rs7412) in the APOE gene was performed using the TaqMan method (Thermo Fisher Scientific, Waltham, MA, USA) with a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) [34], with minor modifications. To confirm the genotypes, direct sequencing was also performed on more than 200 samples with the following primers: for forward, 5′-CCT ACAATCGGACTGGAG-3′, and for reverse, 5′-CCC GGCCTGGTACACTG-3′. DNA sequencing analysis was performed with a 3130xl Genetic Analyzer (Thermo Fisher Scientific) [34].

Experimental assessment of urate excretion pathways

Animals were handled humanely in accordance with the National Cerebral and Cardiovascular Center’s Guidelines for the Care and Use of Laboratory Animals. The experimental protocol and animal use procedures were approved by the Committee of the National Cerebral and Cardiovascular Center. Male Apoe KO mice [35] (Jackson Laboratories, Bar Harbor, ME, USA) and control WT mice (C57BL6/J; Japan SLC, Shizuoka, Japan) were fed a normal rodent laboratory diet (CE-2; CLEA Japan, Tokyo, Japan).

Concentrations of urate and creatinine in collected serum and urine samples were determined by QuantiChrom Uric Acid Assay Kit (BioAssay Systems, Hayward, CA, USA) and Creatinine Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), respectively. To analyze intestinal urate excretion, mice that had fasted overnight were anaesthetized by intraperitoneal injection of urethane and cannulated with polyethylene tubing (Hibiki Size 8) (Sansyo, Tokyo, Japan) at the upper duodenum and the middle jejunum to make an intestinal loop at the upper half of the intestine, in the

Table 1 Haplotypes of two common variants of human APOE gene

| Haplotypes | rs429358 (Cys112Arg) | rs7412 (Arg158Cys) |
|------------|----------------------|-------------------|
| ε2         | T (Cys)              | T (Cys)           |
| ε3         | T (Cys)              | C (Arg)           |
| ε4         | C (Arg)              | C (Arg)           |
same way as in our previous study [4]. After the intestinal contents had been removed by the slow infusion of saline and air, efflux buffer (saline containing 0.3 mM potassium oxonate) was introduced into the intestinal loop and both ends of the loop were closed with syringes. After the indicated periods, the efflux buffer in the loop was collected using syringes and the urate concentrations were quantified. Intestinal urate excretion was calculated using the following equation: [Intestinal urate excretion] = [Urate concentration in the intestinal loop] × [Volume of efflux buffer in the intestinal loop] × [Length of the whole small intestine]/[Length of the intestinal loop] as previously described [4].

**Statistical analysis**

For statistical analysis calculations in the human studies, R software (version 3.1.1) (http://www.r-project.org/) was used. Student’s t test was employed for comparison of SUA levels in humans. We also carried out a multiple regression analysis to evaluate the independent effect of APOE polymorphisms on SUA levels, adjusting for confounding factors such as serum creatinine levels and non-HDL cholesterol levels. In animal experiments, analyses were performed using JMP software version 12.0 (SAS Institute, Cary, NC, USA). Student’s t test was used for comparison of urate concentrations in mice. All P values were two-tailed, and a P value of <0.05 was considered to be statistically significant.

### Results

#### Effects of APOE haplotypes on SUA levels in humans

To investigate the effects of APOE haplotypes on SUA levels, we examined the associations between APOE haplotypes and SUA levels in 5,272 Japanese individuals. SUA levels in carriers and non-carriers of APOE ε2 and ε4, respectively, are summarized in Tables 2 and 3. The call rates for two SNPs (rs429358 and rs7412) that determine the APOE haplotypes were 100%; these SNPs in the control group were in Hardy–Weinberg equilibrium (P > 0.05). Due to sex differences in SUA levels, which are also affected by menopause, we divided the study participants into three groups: men (N = 1,726), premenopausal women (N = 1,793), and postmenopausal women (N = 1,753). As shown in Table 2, SUA levels were significantly higher in postmenopausal than in premenopausal women (P = 3.1 × 10−52). The ε2 haplotype was associated with higher SUA levels in men (P = 0.033) and in postmenopausal women (P = 0.048); however, interestingly, the ε2 haplotype did not affect SUA levels among premenopausal women (P = 0.61) (Table 2). The ε4 haplotype was not significantly related to SUA levels in men, premenopausal women, or postmenopausal women (Table 3). We therefore conducted further analyses focusing on the ε2 haplotype.

To examine the quantitative effect on SUA levels of harboring the ε2 haplotype, we next performed a multiple linear regression analysis that included variables associated with increased SUA levels. A previous study showed that in healthy adults, the correlation coefficient for an association of non-HDL cholesterol with SUA was higher than those

### Table 2 SUA levels of APOE ε2 carriers and non-ε2 carriers among 5,272 individuals

|                  | Men                                      |         | Premenopausal women |         | Postmenopausal women |         |
|------------------|------------------------------------------|---------|---------------------|---------|----------------------|---------|
|                  | N  | SUA            | P value | N  | SUA            | P value | N  | SUA            | P value |
| ε2 carrier       | 142 | 6.27 ± 1.22 |         | 168 | 4.05 ± 0.91 |         | 171 | 4.68 ± 0.97 |         |
| Non-ε2 carrier   | 1,584 | 6.04 ± 1.24 | 0.033   | 1,625 | 4.08 ± 0.83 | 0.61   | 1,582 | 4.53 ± 0.96 | 0.048   |
| Total            | 1,726 | 6.06 ± 1.24 |         | 1,793 | 4.08 ± 0.84 |         | 1,753 | 4.55 ± 0.96 |         |

Data are expressed as mean ± SD

SUA serum uric acid

### Table 3 SUA levels of APOE ε4 carriers and non-ε4 carriers among 5,272 individuals

|                  | Men                                      |         | Premenopausal women |         | Postmenopausal women |         |
|------------------|------------------------------------------|---------|---------------------|---------|----------------------|---------|
|                  | N  | SUA            | P value | N  | SUA            | P value | N  | SUA            | P value |
| ε4 carrier       | 317 | 6.05 ± 1.15 |         | 370 | 4.09 ± 0.83 |         | 285 | 4.51 ± 0.91 |         |
| Non-ε4 carrier   | 1,409 | 6.06 ± 1.26 | 0.89    | 1,423 | 4.07 ± 0.84 | 0.77   | 1,468 | 4.55 ± 0.97 | 0.45    |
| Total            | 1,726 | 6.06 ± 1.24 |         | 1,793 | 4.08 ± 0.84 |         | 1,753 | 4.55 ± 0.96 |         |

Data are expressed as mean ± SD

SUA serum uric acid

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for associations of triglycerides and other lipid parameters including TC, HDL-C, and LDL-C [36]. Based on this information, we chose non-HDL-C levels as a covariate among available lipid parameters. In the multiple linear regression analysis adjusted for age, body mass index (BMI), serum creatinine levels, and non-HDL-C levels, the e2 haplotype was independently associated with higher SUA levels in men ($P = 0.015$) and postmenopausal women ($P = 0.005$), while this was not in the case in premenopausal women ($P = 0.55$) (Table 4). Although these associative analyses could not uncover the molecular mechanisms lying behind the APOE e2-associated increase of SUA levels, the fact that the APOE E2 protein is defective in its binding ability to the APOE receptors, unlike APOE E4 protein [37] and APOE E3 protein [38], suggests that APOE dysfunction might lead to increased SUA. To address this hypothesis, we further conducted in vivo analyses using male Apoe KO mice as described below.

Effects of Apoe knockout on SUA levels and urate secretion in mice

To examine whether deficiency in Apoe function can affect SUA levels, we performed animal experiments using male Apoe KO mice. Given that like APOE deficiency, e2 homozygosity is also associated with an increased risk of type III hyperlipoproteinemia in humans [36], together with the fact that knock-in mice carrying the human APOE E2 allele in place of the mouse Apoe gene cause type III hyperlipoproteinemia and spontaneous atherosclerosis in mice [39], Apoe KO mice can be a model mimicking e2/e2 carriers.

As expected, SUA levels of Apoe KO mice were significantly higher than those of WT mice ($P = 0.021$) (Fig. 1A). We then investigated the latent mechanisms in terms of urate excretion from the body. As shown in Fig. 1B, there was no significant difference in urate excretion from the intestine between Apoe KO mice and WT mice; however, the urinary urate/creatinine ratios were significantly lower in Apoe KO mice than those in WT mice ($P = 0.022$) (Fig. 1C). There was no difference in body weight between Apoe KO mice and WT mice (28.1 ± 1.0 g vs. 28.0 ± 3.2 g; $P = 0.937$).

Discussion

The present study demonstrates the e2 haplotype, not e4 haplotype, to be associated with higher SUA levels in a Japanese population (Tables 2 and 3). This association was observed

### Table 4 Effect of APOE e2 haplotype and other risk factors on SUA levels in 5,272 individuals

|                      | Men                                      | Premenopausal women | Postmenopausal women |
|----------------------|------------------------------------------|----------------------|-----------------------|
|                      | $\beta$ | SE       | $P$ value | $\beta$ | SE       | $P$ value | $\beta$ | SE       | $P$ value |
| e2 haplotype of APOE | 0.25    | 0.10     | 0.015     | -0.039  | 0.065    | 0.55      | 0.21     | 0.070    | 0.0050    |
| Age                  | -0.0090 | 0.0030   | 0.0020    | -0.0020 | 0.0037   | 0.58      | 0.0020   | 0.0040   | 0.60      |
| BMI                  | 0.070   | 0.010    | $3.4 \times 10^{-12}$ | 0.070   | 0.0060   | $3.5 \times 10^{-28}$ | -0.000012 | 0.000021 | 0.59      |
| Serum creatinine     | 2.59    | 0.23     | $1.7 \times 10^{-29}$ | 2.38    | 0.22     | $2.2 \times 10^{-26}$ | 2.81     | 0.24     | $1.2 \times 10^{-30}$ |
| Non-HDL-C            | 0.0041  | 0.00080  | $1.7 \times 10^{-6}$ | 0.0022  | 0.00070  | 0.001     | 0.0045   | 0.00070  | $4.6 \times 10^{-11}$ |

BMI body mass index, non-HDL-C non-high-density lipoprotein cholesterol, SE standard error
Hence, despite the limited data currently available, the human phenotype in SUA levels associated with e2 haplotype may not be explained by renal dysfunction alone. To address this point, further human studies will be required in addition to biochemical and histological investigations of the kidney using Apoe KO mice and/or such mice with the human APOE e2.

In conclusion, we have demonstrated that the e2 haplotype, not the e4 haplotype, of APOE is associated with higher SUA levels in humans. Results of in vivo experiments suggest that renal underexcretion of urate might be involved in the observed Apeo deficiency-related SUA increase; however, further studies are required to uncover the details of the mechanisms in question. Our findings will enhance our understanding of the genetic factors affecting SUA levels in humans.

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Author contributions MO, MS, and HM conceived and designed this study; MH-S, and NS assisted in the research design; MN, AH, SK, RO, and HM collected and analyzed participants’ clinical data; YT, MS, YK, AN, SS, TH, MN, and HM performed genetic analyses; MO, YT, MS, YK, AN, and HM performed statistical analyses; MO, YT, YY, TT, and HS conducted or supported the animal experiments; MO and HM organized this collaborative study; MS, MA, KT, KI, MH-S, and NS provided intellectual input and assisted with preparation of the manuscript; MO, YT, MS, and HM wrote and revised the manuscript. MO, YT, and MS contributed equally to this work.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.
Ethics approval All procedures used in the human study were approved by the institutional ethical committees at the National Defense Medical College (No. 2914) and Nagoya University (No. 2010-0939-7), and were performed in accordance with the Declaration of Helsinki.

Informed consent Written informed consent was obtained from each subject participating in this study.

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