Genome-wide association study revealed novel loci which aggravate asymptomatic hyperuricaemia into gout

Yusuke Kawamura,1,2 Hirofumi Nakaoka,3 Akiyoshi Nakayama,1,4 Yukinori Okada,5,6,7 Ken Yamamoto,8 Toshihide Higashino,1 Masayuki Sakiyama,1,9 Toru Shimizu,10,11 Hiroshi Ooyama,12 Keiko Ooyama,12 Mitsuo Nagase,13 Yuji Hidaka,14 Yoko Shirahama,8 Kazuyoshi Hosomichi,15 Yuichiro Nishida,16 Ippei Shimoshikiryo,17 Asahi Hishida,18 Sakurako Katsura-Kamano,19 Seiko Shimizu,1 Makoto Kawaguchi,1,20 Hirokazu Uemura,19 Rie Ibusuki,17 Megumi Haru,16 Mariko Naito,18,21 Mikiya Takao,1,22 Mayuko Nakajima,1 Satoko Iwasawa,23 Hiroshi Nakashima,23 Keizo Ohnaka,24 Takahiro Nakamura,25 Blanka Stiburkova,26,27 Tony R Merriman,28 Masahiro Nakatomi,29 Sahoko Ichihara,30 Mitsuhiro Yokota,31 Tappei Takada,32 Tatsuya Saitoh,33,34 Yoichiro Kamatani,6,35 Atsushi Takahashi,6,36 Kokichi Arisawa,19 Toshiro Takezaki,17 Keitaro Tanaka,16 Kenji Wakai,18 Michiaki Kubo,37 Tatsuo Hosoya,38,39 Kimiyoshi Ichida,38,40 Ituro Inoue,3 Nariyoshi Shinomiya,1 Hirotaka Matsuo1

ABSTRACT

Objective The first ever genome-wide association study (GWAS) of clinically defined gout cases and asymptomatic hyperuricaemia (AHUA) controls was performed to identify novel gout loci that aggravate AHUA into gout.

Methods We carried out a GWAS of 945 clinically defined gout cases and 1003 AHUA controls followed by 2 replication studies. In total, 2860 gout cases and 3149 AHUA controls (all Japanese men) were examined. We also compared the ORs for each locus in the present GWAS (gout vs AHUA) with those in the previous GWAS (gout vs normouricaemia).

Results This new approach enabled us to identify two novel gout loci (rs7927466 of CNTN5 and rs9952962 of MIR302F) and one suggestive locus (rs12980365 of ZNF724) at the genome-wide significance level (p<5.0×10−8). The present study also identified the loci of ABCG2, ALDH2 and SLC2A9. One of them, rs671 of ALDH2, was identified as a gout locus by GWAS for the first time. Comparing ORs for each locus in the present versus the previous GWAS revealed three ‘gout vs AHUA GWAS-specific loci (CNTN5, MIR302F and ZNF724) to be clearly associated with mechanisms of gout development which distinctly differ from the known gout risk loci that basically elevate serum uric acid level.

Conclusions This meta-analysis is the first to reveal the loci associated with crystal-induced inflammation, the last step in gout development that aggravates AHUA into gout. Our findings should help to elucidate the molecular mechanisms of gout development and assist the prevention of gout attacks in high-risk AHUA individuals.

Key messages

What is already known about this subject?
► We and others, in past genome-wide association studies (GWASs) of gout cases and normouricaemia controls, have identified multiple gout risk loci that elevate serum uric acid.

What does this study add?
► We performed the first GWAS of clinically defined gout cases and asymptomatic hyperuricaemia (AHUA) controls.
► This new approach identified two novel gout loci and one suggestive locus that aggravate AHUA into gout.

How might this impact on clinical practice or future developments?
► This first discovery of AHUA to gout’ loci using a new GWAS strategy will lead to an understanding of why only a proportion of hyperuricaemia cases develop gout.
► These findings will assist physicians to identify, based on individual genetic differences, AHUA cases who need individually tailored preemptive medicine for gout.

INTRODUCTION

Gout is one of the the most common forms of inflammatory arthritis. It is induced by monosodium urate (MSU) crystals that result from elevated serum uric acid (SUA) level.1 The SUA level is determined by the excretion of uric acid via the urate transporters in the kidney and intestine,2,3 and the
production of uric acid in the liver.2 While both genetic and environmental factors are known to cause hyperuricaemia and gout,46 these diseases are reported to have stronger genetic factors than many other common diseases.10 11 Thus far, a number of genes associated with SUA have been identified by genome-wide association studies (GWASs) of SUA,12-23 such as the urate transporter genes ABCG2 (also known as BCRP) and SLC2A9 (also known as GLUT9). We and others have performed GWASs of gout by comparing the genetic differences between gout cases and normouricaemia controls,24-26 and have identified gout risk loci such as ABCG2 and SLC2A9: these are similar results to those from GWASs of SUA. Not all hyperuricaemia cases develop gout: the reason, we consider, is that there are at least two steps by which normouricaemic individuals develop gout. In the first step, the SUA of normouricaemic individuals elevates, creating asymptomatic hyperuricaemia (AHUA); and in the second step, MSU crystal-induced inflammation is experienced as a gout attack (figure 1). Urate transporters such as ABCG2,27 SLC2A9,28 SLC22A1229 30 and SLC17A131 naturally play important roles in the first step, but there must be other loci for the second step that aggravates AHUA into a gout attack. In this study, for the first time, we performed a gout GWAS using clinically defined gout cases and AHUA controls to identify risk loci that uniquely influence the progression from AHUA to gout, as distinct from causing SUA elevation.

**METHODS**

**Study subjects**

In the present study, we avoided the use of self-reported gout cases and AHUA controls and collected only clinically defined individuals.

All gout cases were clinically diagnosed with primary gout according to the criteria established by the American College of Rheumatology.32 All patients were assigned from Japanese male outpatients at the gout clinics of Midorigaoka Hospital (Osaka, Japan), Kyoto Industrial Health Association (Kyoto, Japan), Ryogoku East Gate Clinic (Tokyo, Japan), Nagase Clinic (Tokyo, Japan), Akasaka Central Clinic (Tokyo, Japan) and Jikei University Hospital (Tokyo, Japan). Patients with inherited metabolic disorders, including Lesch-Nyhan syndrome, were excluded. Finally, 2860 Japanese male gout cases were registered as valid case participants. Of these, 945 cases for GWAS stage were the same patients as reported previously.24 25

As AHUA controls, 3149 individuals were assigned from among Japanese men with a high SUA level (>7.0 mg/dL) without a history of gout, who were obtained from BioBank Japan33 and the Shizuoka, Daiko, Fukuoka, Saga and Kagoshima areas in the Japan Multi-Institutional Collaborative Cohort Study.34 35 The details of participants in this study are shown in the online supplementary table S1.

**Genotyping and quality control**

Genome-wide genotyping was performed using Illumina HumanOmniExpress V.1.0 (Illumina) in 1948 individuals (945 cases and 1003 AHUA controls). The data sets were filtered individually on the basis of single nucleotide polymorphism (SNP) genotype missing call rates (>1%) and the Hardy-Weinberg equilibrium (HWE) in AHUA controls (p<1.0 × 10–6). We confirmed that all the subjects showed high genotype call rates (>98%). Pairwise identity by state was evaluated to identify pairs of individuals with cryptic relatedness.36 We confirmed that there was no pair showing cryptic relatedness greater than expected for second-degree relatives. We performed principal component analysis including our GWAS data set together with HapMap phase II samples37 38 as shown in the online supplementary figure S1, indicating that there are no outliers in our GWAS data. Finally, 569 200 SNPs passed filters for 1948 individuals (945 cases and 1003 controls).

At the first replication (REP1) stage, 1246 gout cases and 1186 AHUA controls were genotyped with a custom genotype platform using iSelect HD Custom Genotyping BeadChips (Illumina) on 897 SNPs, and another 253 gout cases were genotyped with Illumina HumanOmniExpress-24 V.1.0 (Illumina). Selected were 897 SNPs using the following criteria: (1) 1000 SNPs were selected as they showed an association (p<0.001 with Fisher’s exact test) in the GWAS stage with gout cases and AHUA controls. (2) After 103 undesirable SNPs had been eliminated, 897 SNPs were selected as the custom genotype platform. For quality control, the data set was filtered individually on the basis of SNP genotype missing call rates (>1%).
We excluded subjects with low genotype call rates (<98%). Quality controls for 253 gout cases genotyped with Illumina HumanOmniExpress-24 V.1.0 (Illumina) were performed as described previously. 

For REP1 stage, 885 SNPs passed filters for 2685 individuals (1499 cases and 1186 controls) as shown in figure 2. 

As the criteria at the second replication (REP2) stage, 68 SNPs passing the significance threshold at p<1.0×10−4 in the meta-analysis among the GWAS and REP1 stages were used for the subsequent analyses. In addition to SNPs which had already been reported for gout-associated loci (ABCG2, ALDH2 and SLC2A9), we detected top-ranked SNPs among closely located SNPs. We then examined the pairwise linkage disequilibrium (LD) between SNP showing the most significant association and other SNPs. As shown in figure 2, in addition to 3 SNPs of ABCG2, ALDH2 and SLC2A9, we finally selected 12 SNPs that were independent of each other at r²<0.3 (see online supplementary table S2) for the REP2 stage. 

The genotyping for 12 SNPs was performed using an allelic discrimination assay (Custom TaqMan Assay and By-Design, Thermo Fisher Scientific, Waltham, Massachusetts) with a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). We confirmed that all 12 SNPs were of high call rate (>98%) and HWE in AHUA controls (p>0.001). After quality control, a statistical analysis was performed with 1376 individuals (416 gout cases and 960 AHUA controls). The details of participants in this study are shown in online supplementary table S1.

**Comparison with previous GWAS**

The ORs and 95% CIs of gout GWAS with AHUA controls were calculated by meta-analysis with GWAS and REP1 stages in this study, and those of gout GWAS with normouricaemic controls were obtained from our previous study.

**Statistical analysis**

We conducted an association analysis using a 2×2 contingency table based on the allele frequency. For each of the filtered SNPs, the p value of association was assessed using Fisher’s exact test, and the OR and 95% CI were calculated. The quantile-quantile plot and the genomic inflation factor (λ) were used to test for the presence of systematic bias in the test statistics due to potential population stratification. The genomic inflation factor (λ) was 1.013, indicating a subtle inflation of p values (see online supplementary figure S2). The results from GWAS, in the first and second replication stages, were combined by meta-analysis. Inverse-variance fixed-effects model meta-analysis was used for estimating summary OR. Cochran’s Q test and the I² statistic were examined to assess heterogeneity in ORs among the three studies. If heterogeneity was revealed by statistical testing (phet<0.05) or measurement (I² >50%), we implemented a DerSimonian and Laird random-effects model meta-analysis. All statistical analyses were performed using PLINK V.1.07 and the software R V.3.1.1 with GenABEL and meta packages. The genome-wide significance threshold was set at α=5.0×10−8 to reveal any evidence of a significant association.

**RESULTS**

**Association analyses**

The participants for the GWAS stage were genotyped using HumanOmniExpress V.1.0 (Illumina). Nine hundred forty-five clinically defined gout cases and 1003 AHUA controls passed rigorous quality control filtering (figure 2 and online supplementary figures S1 and S2).

The REP1 stage was then carried out by genotyping 885 SNPs, which showed associations at p<1.0×10−3 in the GWAS stage, using a custom genotype platform that employed iSelect HD Custom Genotyping BeadChips (Illumina) in a further 1499...
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Figure 3  Manhattan plots of the present GWAS (Gout vs AHUA). The x-axis shows chromosomal positions and the y-axis shows −log10 p values. The upper purple horizontal line represents the genome-wide significance threshold (p=5.0×10−8). The lower blue line indicates the cut-off level for selecting SNPs for REP1 stage (p=1.0×10−6). The gene names of identified loci are also shown in the figure. AHUA, asymptomatic hyperuricaemia; GWAS, genome-wide association study; REP1, the first replication; SUA, serum uric acid.

We investigated whether or not these identified risk loci are associated with gout susceptibility via SUA elevation (the first step in figure 1). We compared the ORs for each locus in the present GWAS (gout vs AHUA) with those in the previous GWAS25 (gout vs normouricaemia): that is, the 3 loci identified in this study (‘AHUA to gout’ loci; CNTN5, MIR302F and ZNF724) and 10 previously identified risk loci25 (‘normouricaemia to gout’ loci; ABCG2, SLC2A9, CUX2, SLC22A12, GCKR, SLC17A1, HIST1H2BF-HIST1H4E, CNINH-2, NIPAL1 and FAM35A) (figure 5 and online supplementary table S3). Interestingly, when plotted, the ‘AHUA to gout’ loci and ‘normouricaemia to gout’ loci appeared as distinct patterns (figure 5). The ‘normouricaemia to gout’ loci trended under the oblique line, whereas all three ‘AHUA to gout’ loci were located above the oblique line, clearly indicating that these novel loci are associated with distinct mechanisms of gout development that differs from those of 10 known gout loci (see online supplementary table S3).25 It is consistent with the finding that ‘the ratio of the two ORs’ of each locus in the present GWAS (gout vs AHUA) is >1, whereas that of each locus in the previous GWAS (gout vs normouricaemia) is <1 (see online supplementary table S3).25 We also investigated the effect of each locus on SUA using the results from our recent GWAS meta-analysis of SUA with a total of 121 745 Japanese subjects47 and the results from GWAS meta-analysis of SUA with a total of 110 347 individuals of European ancestry within the Global Urate Genetics Consortium (GUGC).25 The association results for each locus are shown in online supplementary table S4. Both results are consistent with those of the present study (shown in figure 5). Furthermore, we also investigated the association results for three gout loci identified in the present GWAS from the result of the gout GWAS (gout vs non-gout) using a total of 69 374 individuals (2115 gout cases and 67 259 controls) of European ancestry within GUGC (see online supplementary table S5).22 The two SNPs that were polymorphic in European populations were evaluated. After Bonferroni correction (p<2.5×10−2=0.05/2), rs12980365 of ZNF724 showed a significant association with gout in persons of European ancestry (p=8.54×10−4, online supplementary table S5).

DISCUSSION

It is well known that gout has stronger genetic contributors than other common diseases, and that only a proportion of AHUA individuals develop gout, but no studies have hitherto reported the differences in genetic background between gout and AHUA. Through the present study, we first performed GWAS with gout cases and AHUA controls and identified two novel loci that have a stronger effect on the second step (AHUA to gout) in the development of gout. The present study detected the loci of ABCG2, SLC2A9 and ALDH2. One of them, rs671 of ALDH2, was identified as a gout-associated SNP in a subsequent fine mapping study.46 The present study therefore identified rs671 of ALDH2 as a gout locus by GWAS for the first time.

To identify additional risk loci, we recruited independent participants comprising 416 gout cases and 960 AHUA controls. Twelve SNPs were selected for the REP2 stage by considering LD among 68 SNPs showing associations at p<1.0×10−6 in the meta-analysis among the GWAS and REP1 stages. Genotyping of these 12 SNPs was performed by TaqMan assay, and the meta-analysis was conducted among the GWAS, REP1 and REP2 stages (figure 2). Online supplementary tables S2A and S2B summarise the GWAS and replication study of three SNPs which have been reported to have gout-associated loci (ABCG2, ALDH2 and SLC2A9) and the 12 SNPs selected for the REP2 stage.

Finally, two novel loci achieved genome-wide significance in the meta-analysis of three stages (figures 3 and 4, table 1): an intronic SNP of CNTN5, rs7927466 (pmeta=5.33×10−8; OR=1.85) and an intergenic SNP located on near MIR302F, rs9952962 (pmeta=1.67×10−8; OR=0.81). In addition, an intergenic SNP nearing ZNF724 (rs12980365) showed a suggestive level of association (pmeta=9.76×10−4; OR=1.77).

Comparison with previous GWASs

We investigated whether or not these identified risk loci are associated with gout susceptibility via SUA elevation (the first step in figure 1). We compared the ORs for each locus in the present GWAS (gout vs AHUA) and with previous GWASs25 (gout vs normouricaemia): that is, the 3 loci identified in this study (‘AHUA to gout’ loci; CNTN5, MIR302F and ZNF724) and 10 previously identified risk loci25 (‘normouricaemia to gout’ loci; ABCG2, SLC2A9, CUX2, SLC22A12, GCKR, SLC17A1, HIST1H2BF-HIST1H4E, CNINH-2, NIPAL1 and FAM35A) (figure 5 and online supplementary table S3). Interestingly, when plotted, the ‘AHUA to gout’ loci and ‘normouricaemia to gout’ loci appeared as distinct patterns (figure 5). The ‘normouricaemia to gout’ loci trended under the oblique line, whereas all three ‘AHUA to gout’ loci were located above the oblique line, clearly indicating that these novel loci are associated with distinct mechanisms of gout development that differs from those of 10 known gout loci (see online supplementary table S3).25 It is consistent with the finding that ‘the ratio of the two ORs’ of each locus in the present GWAS (gout vs AHUA) is >1, whereas that of each locus in the previous GWAS (gout vs normouricaemia) is <1 (see online supplementary table S3).25
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Figure 4  Regional association plots for the six loci identified in the present GWAS (gout vs AHUA). The vertical axis represents $-\log_{10}(p)$ value for assessment of the association of each SNP with gout. The highest association signal in each panel is located on (A) CNTN5, (B) MIR302F as novel loci and (C) ZNF724 as a suggestive locus and (D) ABCG2, (E) ALDH2 and (F) SLC2A9 as known loci. The region within 250 kb from the SNP indicating the lowest p value is shown. Top panel: plots of $-\log_{10} p$ values for the test of SNP association with gout in the GWAS stage. The SNP showing the lowest p value in the meta-analysis is depicted as a pink diamond. Other SNPs are colour coded according to the extent of linkage disequilibrium (measured in $r^2$) with the SNP showing the lowest p value. Middle panel: recombination rates (centimorgans per MB) estimated from HapMap phase II data are plotted. Bottom panel: RefSeq genes. Genomic coordinates are based on NCBI human genome reference sequence build 37. Details of the results for the six loci are also shown in table 1. AHUA, asymptomatic hyperuricaemia; GWAS, genome-wide association study; NCBI, National Center for Biotechnology Information; SNP, single nucleotide polymorphism.

We also identified rs9952962, an SNP near MIR302F as a novel gout locus. MicroRNAs (miRNAs) were discovered in 199358: they are small non-coding RNA molecules which play an important role in regulating gene expression. It is reported that miR-302f is deregulated in gastric cancer60 and that chemotherapy modifies miR-302f expression in esophageal cancer.61 As several miRNAs have been identified as being involved in the pathogenesis of other non-infectious forms of inflammatory arthritis, including rheumatoid arthritis,62 63 some reports have also shown a relationship between miRNA and gouty arthritis.64–66 Our results might suggest that miR-302f affects the inflammation seen in gouty arthritis by modulating gene expression, although further analysis will be needed to elucidate the relationship between them.

We also identified rs12980365, an SNP of ZNF724, as a potential gout locus. However, there are no reports so far on ZNF724. It is of course possible that the identified loci in the present study are just surrogate markers and that other genes including ZNF730 and IPO5P1 near these SNPs are the true risk loci for gout development. The limitation is the lack of imputation because we applied the study design shown in figure 2. Further analysis of larger samples with imputation will be the key to identifying more gout loci.

The unique finding in the present study is that the novel loci for ‘AHUA to gout’ and known loci for ‘normouricaemia to gout’ appear to relate to different molecular mechanisms of gout development (figure 5 and online supplementary table S3). The ‘AHUA to gout’ loci identified in the present study appear to be involved in the second step of development from AHUA to gouty arthritis rather than the ‘normouricaemia to gout’ loci (figure 1). Since only a proportion of hyperuricaemia cases develop gout and most hyperuricaemia cases remain as AHUA cases, the molecules involved in this step are likely to play important roles in innate immunity and/or inflammation in response to MSU deposition.

The frequency of female gout cases is low (1.15% in our data) in Japan, which shows that analysing only male gout patients in the present study should be more appropriate for detecting genetic factors of gout. Because each locus identified in the present GWAS did not show a significant association with SUA in persons of European ancestry or in Japanese individuals (see online supplementary table S4), our findings show that they are not likely to be a locus that is associated with AHUA susceptibility. Interestingly, the present study also demonstrated that rs12980365 of ZNF724 showed a significant association with gout in persons of European ancestry within GUGC, which compared gout and non-gout individuals. This finding suggests that ZNF724 is a novel gout locus that aggravates AHUA into
Table 1

| Allele* | GWAS stage | REP 1 stage§ | REP 2 stage§ | Fixed-effect model | Random-effect model | Cochran’s Q |
|---------|------------|--------------|--------------|--------------------|---------------------|-------------|
| SNP     | A/B        | Chr          | Position†    | Gene               | Case Control OR (95% CI) P value | Case Control OR (95% CI) P value | Case Control OR (95% CI) P value | OR (95% CI) P value |
| rs2728125 | G/A        | 4            | 89 001       | ABCG2              | 0.401 (0.304 to 1.54 (1.35 to 1.76) 1.76×10−10 | 0.400 (0.313 to 1.56 (1.31 to 1.64) 3.3×10−11 | NA NA NA | 1.49 (1.37 to 1.63) 1.49 (1.37 to 1.63) 0.57 0 |
| rs7927466 | A/G        | 11           | 100 141      | CNTN5              | 0.976 (0.953 to 2.19 (2.10 to 2.31) 1.9×10−9 | 0.973 (0.954 to 1.40 (1.30 to 1.47) 1.4×10−8 | NA NA NA | 1.47 (1.33 to 1.64) 4.4×10−10 1.47 (1.34 to 1.66) 0.62 0 |
| rs12980365 | A/G        | 19           | 23 395       | ZNF724             | 0.976 (0.957 to 1.01 (1.01 to 1.03) 1.2×10−3 | 0.974 (0.959 to 1.01 (1.01 to 1.03) 1.2×10−3 | 0.975 (0.948 to 1.01 (1.01 to 1.03) 1.2×10−3 | 1.85 (1.50 to 2.27) 1.85 (1.50 to 2.27) 0.84 0 |

*Allele A is risk-associated allele, and allele B is non-risk-associated allele.

†SNP positions are based on NCBI human genome reference sequence Build 37.4.

‡GWAS stage.

§REP1 stage.

**Meta-analysis of the combined GWAS and replication samples (2860 gout cases and 3149 controls of Japanese men).

AHUA, asymptomatic hyperuricaemia; Chr, Chromosome; GWAS, genome-wide association study; NCBI, National Center for Biotechnology Information; REP1, the first replication; REP2, the second replication; SNP, single nucleotide polymorphism.

Figure 5

Comparison ORs for three gout loci by the present GWAS (gout vs AHUA) with ORs for 10 gout loci by the previous GWAS (gout vs normouricaemia). Dots represent ORs and lines represent 95% CIs.

The first discovery of AHUA to gout loci using a new GWAS strategy will lead to elucidation of the molecular mechanism of gout (see online supplementary table S5). This first discovery of AHUA to gout loci using new GWAS strategy will lead to elucidation of the molecular mechanism of gout (see online supplementary table S5).
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Contributors H Nakaoka, KY, M Kudo, II, NS and H Matsuo conceived and designed this study. Y Kawamura, AN, YO, MS, M Nakatomi, Y Katamati and AT assisted with research design. T Shimizu, HO, K Oyama, M Nagase, YH, T Hosoya, KI and H Matsuo collected and analysed the clinical data of the cases. YO, YN, IS, AH, MH, RI, M Naito, KT Takeaki, KW, K Ohnaka, Y Katamati, AT and M Kudo collected and analysed clinical data of controls. Y Kawamura, AN, KY, T Higashino, MS, YS, SS, M Kawauchi, MT, M Nakajima, NS and H Matsuo performed genetics analysis. H Nakaoka, YO, MS, H Nakashima, TN, M Nakatoki, AT and II performed statistical analysis. Y Kawamura, AN, YO, T Higashino and H Matsuo analysed the data. H Matsuo organised this collaborative study. H Nakaoka, KY, T Higashino, KH, SH, HK, SU, SHASAW, BS, TR, M Nakatoki, S Ichihara, MF, T Taikatu, S Taisho, KA, KI and NS provided intellectual input and assisted with the preparation of the manuscript. Y Kawamura, H Nakaoka, KAN, MS and H Matsuo wrote the manuscript.

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Competing interests None declared.

Patient consent for publication Obtained.

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