厚生労働省科学研究費補助金（難治性疾患克服研究事業）

総合研究報告書

Muckle-Wells 症候群における NLRP3 体細胞モザイクの検出に関する研究

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研究要旨

CAPS は軽症の家族性寒冷蕁麻疹（familial cold autoinflammatory syndrome, FCAS）、中等症の Muckle-Wells 症候群、重症の CINCA 症候群（chronic infantile neurologic cutaneous articular syndrome）の 3 つの疾患の総称である。サンガー法による遺伝子解析では NLRP3 ヘテロ変異を認めない CINCA 症候群において NLRP3 体細胞モザイクの存在が報告されている。Muckle-Wells 症候群においても CINCA 症候群同様に、サンガー法による遺伝子解析では NLRP3 ヘテロ変異を検出できない患者が存在する。研究代表者らにより確立された次世代シーケンサーを用いた NLRP3 体細胞モザイクの迅速診断を用い、Muckle-Wells 症候群 2 人において NLRP3 体細胞モザイクを検出した。NLRP3 ヘテロ変異を認めない Muckle-Wells 症候群の一部は NLRP3 体細胞モザイクにより発症していると考えられた。

A．研究目的

Muckle-Wells 症候群は、自然免疫に関わる NLRP3 遺伝子の機能獲得型変異によって発症する自己炎症疾患である。常染色体優性遺伝形式で発症し家族歴を有することが多いが、de novo の変異も認める。NLRP3 ヘテロ変異を認めない Muckle-Wells 症候群においても CINCA 症候群同様に、NLRP3 体細胞モザイクで発症している可能性が考えられる。そのため、研究代表者らが確立した次世代シーケンサーによる NLRP3 体細胞モザイク検出法を用いて、Muckle-Wells 症候群における NLRP3 体細胞モザイク検出を試みた。

B．研究方法

全血または PBMC より抽出された DNA 検体を用いて NLRP3 の全エクソンおよびそのエクソン-イントロン接合部位を、１４アンプリコンにわけ、2 段階の PCR 法で増幅、患者タグをつけた後、次世代シーケンサー（Roche 454 Genome Sequencer FLX）を用いてそれぞれ５００リード以上を解析した。健常人５０人での遺伝子解析結果をもとに、シーケンスエラーと真のモザイク変異とを統計的に判別した。そして臨床的に Muckle-Wells 症候群疑い患者の全血または PBMC より DNA を抽出し、次世代シーケンサーを用いて NLRP3 体細胞モザイクの有無を検討した。また認められた変異を機能解析することによって病的意義を検討した。

（倫理面への配慮）

遺伝子解析を行うにあたり、京都大学大学院医学研究科・医学部及び医学部附属病院 医の倫理委員会に、“ヒト原発性免疫不全症の臨床的遺伝子診断”の申請を行い、承認を得ている（G-432）。その内容を忠実に順守して研究を行っている。

C．研究結果

症例 1 は 15 歳女児、新生児期より蕁麻疹様発疹を認め、5 歳頃から発熱、両膝関節炎などから全身型若年性特発性関節炎と診断された。軟骨過形成や難聴、髄膜炎は認めない。15 歳時、Muckle-Wells 症候群を疑われたが、ダイレクトシーケンスにて NLRP3 ヘテロ変異を認めなかった。次世代シーケンサーで NLRP3 モザイクの有無を検討したところ、NLRP3 体細胞モザイク変異 c.1699G>A、p.Glu567Lys を検出し、変異アリル頻度は 5.6%であった。症例 2 は 13 歳男児、8 歳頃から軽度難聴を指摘され、しばしば頭痛、関節痛、年に 1 回ほど蕁麻疹様発疹を認めていた。軟骨過形成は認めなかった。血液検査にて炎症反応は持続しており、自己炎症疾患が疑われ、遺伝子検査を施行した。ダイレクトシーケンスにて NLRP3 遺伝子に変異を認めたが、変異波形が極端に小さく、モザイクが疑われ、次世代シーケンサーにて変異アリル頻度 35.1%の NLRP3 体細胞モザイク変異 c.1000A>G、p.Ile334Val を検出した。最終診断は Muckle-Wells 症候群と考えられた。両症例において認められた変異は機能解析により病的意義が確認された。

D．考察

CINCA 症候群同様に Muckle-Well 症候群においても NLRP3 体細胞モザイクにより発症していることが示された。NLRP3 ヘテロ変異を認めない Muckle-Wells 症候群においては次世代シーケンサー等による NLRP3 体細胞モザイクの検索が重要と考えられた。

E．結論

Muckle-Well 症候群 2 人において NLRP3 体細胞モザイクを検出した。

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G．知的財産権の出願・登録状況（予定も含む）

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他

該当なし

III. 研究成果の刊行に関する一覧表

書籍

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原寿郎	原発性免疫不全症候群	永井良三 大田健	今日の治療と看護(改訂第3版)	南江堂	日本		印刷中

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Ⅳ. 研究成果の刊行物・別刷

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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).

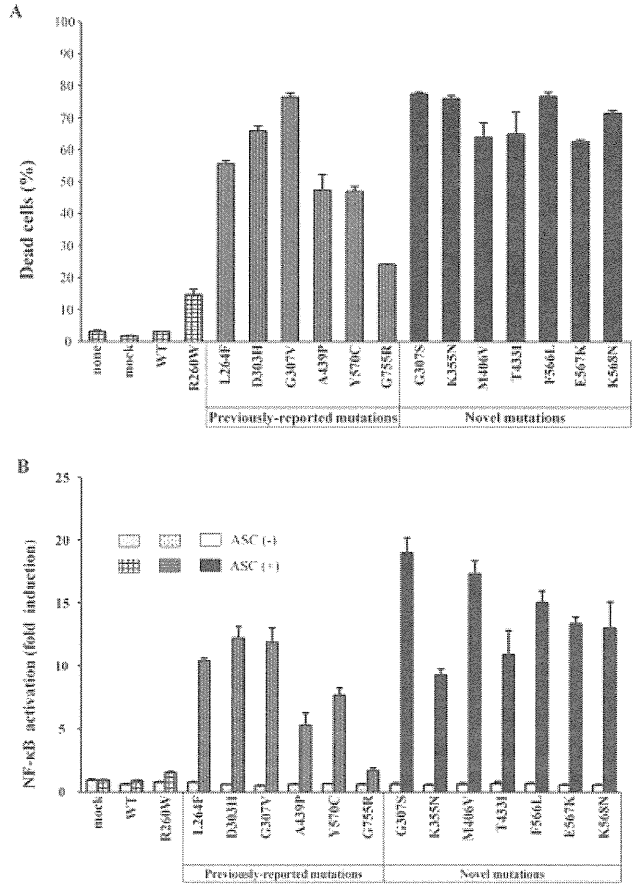

Figure 1. In vitro functional assessment of the identified *NLRP3* mosaicism mutations. **A,** Necrotic cell death of THP-1 cells induced by the identified somatic *NLRP3* mosaicism mutations. Green fluorescent protein (GFP)–fused mutant NLRP3 was transfected into THP-1 cells. The percentage of dead 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

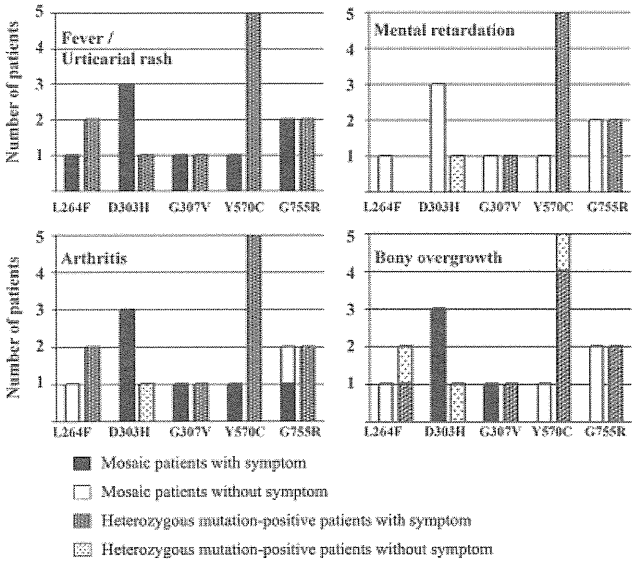

Figure 2. Comparison of the clinical profiles of patients carrying somatic *NLRP3* mutations and patients carrying the same mutation, but with germline status. Clinical profiles of patients with mosaicism and those of patients with heterozygous germline mutations are compared for each protein variant (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.