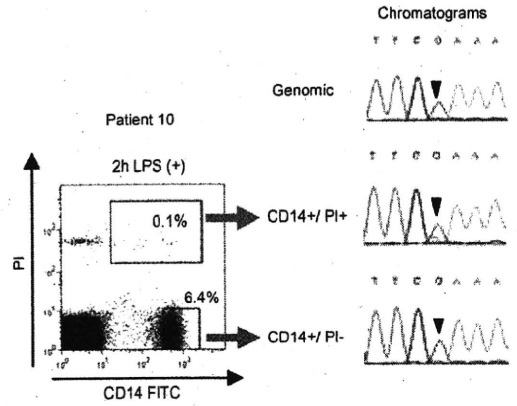

Figure 5. Enrichment of *CIAS1*-mutated monocytes from a *CIAS1* mutation-negative patient. Flow cytometry analysis of PBMCs from patient 10 stimulated with cLPS for 2 hours (left panel), and chromatograms of the *CIAS1* gene at position 1699 from each of the populations of cells (right panel). Numbers in each rectangle are the percentages of total cells.

we sorted CD14-positive/PI-negative cells and CD14-positive/PI-positive cells from cLPS-stimulated PBMCs from patient 7 (Figure 4D) and performed genomic DNA sequencing of each cell population, we found that *CIAS1*-mutated cells were enriched in the PI-positive population, and eliminated from the PI-negative population. Subcloning-based frequency analysis revealed successful enrichment of the mutant G allele, from a frequency of 12.2% in PBMCs to 41.9% in the PI-positive population of dying monocytes, correlating with an enrichment of mutated cells from approximately 25% to more than 80%. The frequency of the mutant allele in PI-negative monocytes that were not undergoing cell death significantly decreased, to 4.9%. These results suggested that cell death was induced exclusively in *CIAS1*-mutated monocytes, and not in normal cells, even though both cell types were exposed to the same extracellular milieu.

Identification of mosaicism in 3 of 4 CIAS1 mutation-negative CINCA patients

Based on the results from patient 7, we set out to identify *CIAS1* mosaicism in the remaining mutation-negative patients by enriching for PI^-, dying monocytes after cLPS stimulation. We stimulated PBMCs from patients 8-11 with cLPS, and while the decrease in monocyte cell number was comparable with normal controls (data not shown), we were able to sort dying (PI^-) from viable (PI^-) monocytes. Subsequent sequencing of the population of monocytes undergoing cell death revealed an overlapping peak on the sequencing chromatogram, indicating mosaicism, in 3 of 4 patients. The nucleotide substitutions were as follows (parentheses indicate the corresponding amino acid change): 790C > T (L264F) in patient 8; 919G > A (G307S) in patient 9; and 1699G > A (E567K) in patient 10 (Figure 5 and Figure S2). Overlapping peaks were not obvious in either chromatogram from unstimulated PBMCs or cLPS-stimulated, PI-negative monocytes. Subcloning analysis of genomic DNA from whole blood revealed that the mutant allele was enriched from 4.3% (2/47) to 19.4% (7/36) in patient 8, 4.3% (2/47) to 11.1% (3/27) in patient 9, and 6.5% (3/46) to 15.2% (7/46) in patient 10 (Table 2). The mutations and corresponding amino acid changes in patients 8, 9, and 10 have not been previously reported as either mutations or SNPs, and were not observed among 100 healthy Japanese donors (data not shown). We confirmed the existence of latent mosaicism in these patients by allele-specific PCR, which can detect mutant alleles at a frequency of 0.6% (Figure 6). We also analyzed 100 healthy controls to exclude the possibility of latent mosaicism among this population, and found no evidence of mutant *CIAS1* alleles (Figure 6 and data not shown). Thus, by selectively inducing cell death with LPS, we successfully diagnosed 3 CAPS patients who had been designated mutation-negative by conventional sequencing. Patient 11 had a phenotype that was marked by severe mental and developmental retardation. When we stimulated PBMCs from patient 11 with cLPS, there was very little cell death of monocytes, and we could not resolve overlapping peaks on the sequencing chromatogram of the population of dying monocytes (data not shown). We generated at least 54 subclones of the entire *CIAS1* coding region from patient 11, and confirmed that this individual did not carry any mutations, even as a mosaicism of *CIAS1*.

Table 2. Frequency of mutant alleles detected in mutation-negative patients

	Patient 8	Patient 9	Patient 10
Site of mutation	790C>T (L264F)	919G>A (G307S)	1699G>A (E567K)
Frequency of mutant allele			
Whole blood	2/47 (4.3%)	2/47 (4.3%)	3/46 (6.5%)
$CD14^+/PI^-$	7/36 (19.4%)	3/27 (11.1%)	7/46 (15.2%)
$CD14^+/PI^-$	2/46 (4.3%)	1/38 (2.6%)	3/48 (6.3%)

Disease-associated mutant CIAS1 induces cell death on THP-1 and spontaneous NF-κB activation

To evaluate whether the newly identified *CIAS1* mutations of this study were relevant to disease manifestation, we examined their ability to rapidly induce necrotic cell death when transiently expressed in human monocytic THP-1 cells (Figure 7A). The mutants identified in the mosaics (patients 7-10, Y570C, L264F,

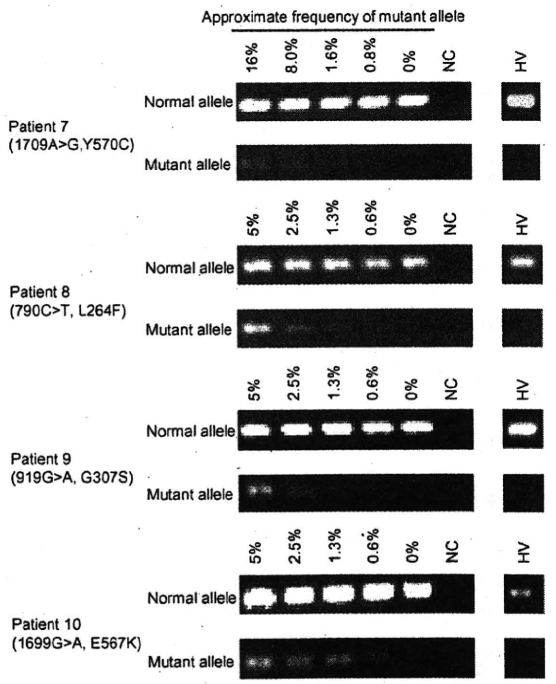

Figure 6. Allele-specific PCR for mutant alleles detected in mosaic patients. PCR was performed with mutant or normal allele-specific primers and the corresponding reverse primer (see Table S1). Dilution series were made by mixing patients' DNA and DNA from an individual who was proved not to have latent mosaicism of CIAS1. Representative results of mosaic patients and 100 healthy volunteers (HV) are shown. NC indicates negative control.

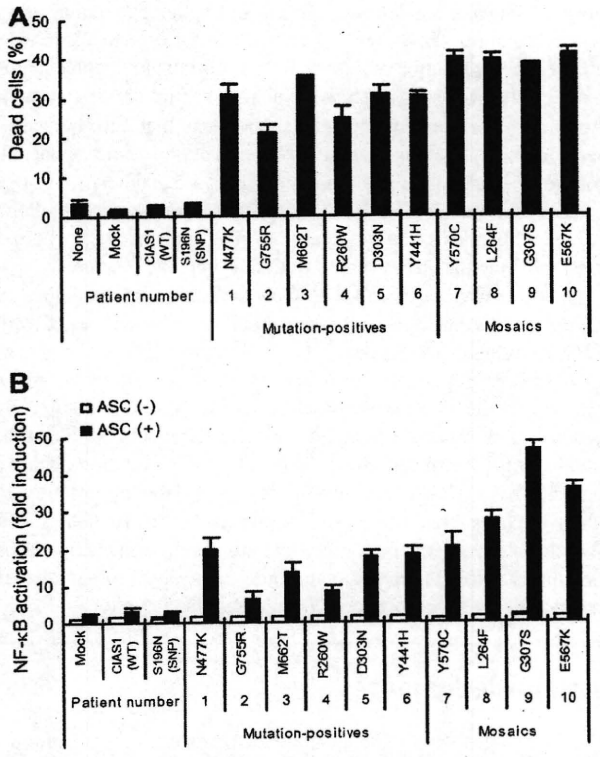

Figure 7. Effect of *CIAS1* mutation on the induction of cell death and ASC-dependent NF-κB activation. (A) 10^6 THP-1 cells were transfected with 0.5 μg of an expression vector for GFP-tagged *CIAS1* wild-type (WT), *CIAS1* SNP S196N, or one of the disease-associated mutants of *CIAS1* (R260W, L264F, D303N, G307S, Y441H, N477K, E567K, Y570C, M662T, and G755R), and incubated with PMA (10 ng/mL) for 4 hours. The percentage of dead cells (7-AAD-positive) among the population of GFP-positive cells is shown. Data represent the means (± SD) of triplicate determinations, and are representative of 3 independent experiments. (B) HEK293FT cells were transfected with 16 ng of an expression vector for *CIAS1*, or one of its mutants, in the presence or absence of 16 ng of an expression vector for ASC. The induction of NF-κB is shown as fold-change compared with cells that were transfected with a control vector without ASC (set equal to one). Values are the means (± SD) of triplicate determinations, and data are representative of 2 independent experiments.

G307S, and E567K) had more potent effects than those of the mutation-positive individuals. Transient coexpression of disease-associated *CIAS1* mutants and ASC in HEK293FT cells also caused enhanced NF-κB reporter activity, compared with the expression of wild-type *CIAS1*, or *CIAS1*-SNP S196N, a disease-unrelated *CIAS1* variant (Figure 7B).[12] Note that NF-κB activation induced by the mutants identified in mosaic patients was higher than that induced by the mutants identified in mutation-positive patients (patient 1-6; Figure 7B). These findings provided additional evidence that mutations of *CIAS1* that are found in both mutation-positive and mosaic patients are functional and related to the development of disease-related symptoms in CAPS patients.

Discussion

In the current study, we demonstrated that monocytes carrying disease-associated *CIAS1* mutations rapidly undergo cell death upon induction of *CIAS1* expression by LPS treatment. Selective induction of monocyte cell death was independent of the disease severity, level of IL-1β production, or therapeutic regimen. Subcloning and analysis of transient expression in cell lines indicated that the ability to induce cell death was specific to disease-associated mutations in *CIAS1*. Consequently, we were able to use this distinct biologic characteristic to enrich for monocytes carrying *CIAS1* mutations. We performed genetic analysis on 4 mutation-negative CAPS patients and found *CIAS1* mosaicism in 3 patients, while in the fourth we were able to exclude the presence of a mutation in the *CIAS1* coding region. These findings suggest that a majority of *CIAS1* mutation-negative patients have disease-associated mutations of *CIAS1* as a latent, low-level mosaicism.

Our results indicated that a small number of monocytes carrying *CIAS1* mutations are sufficient to evoke systemic inflammation in *CIAS1* mosaic patients. The question then arises of how such a small number of mutant cells cause the severe inflammatory responses observed in CAPS patients. As shown in Figure 1, we could not distinguish between *CIAS1*-mutated and nonmutated cells by intracellular IL-1β staining of PBMCs in patient 7 (mosaic patient); both mutated and nonmutated monocytes appeared to have similar levels of IL-1β. In patients who were treated with the IL-1 receptor antagonist anakinra, not only was IL-1β signaling blocked, but IL-1β production in peripheral blood monocytes was also dramatically reduced (Figure 1). This finding indicates that modification of the cytokine milieu due to the production of IL-1β by *CIAS1*-mutated cells, which possess constitutive IL-1β producing activity, may cause up-regulation of IL-1β in nonmutated monocytes, thereby leading to a systemic inflammatory condition. In support of this hypothesis, the addition of a neutralizing anti–IL-1β antibody to cultures of PBMCs from patient 7 reduced the levels of intracellular IL-1β, and addition of exogenous IL-1β to control, nonmutated monocytes induced an up-regulation in intracellular IL-1β (data not shown). In addition, we used transient transfection experiments to demonstrate that all of the *CIAS1* mosaic mutants (Y570C, L264F, G307S, and E567K) have the potential to induce higher NF-κB activity compared with wild-type *CIAS1* (Figure 7); thus, in patients in vivo, these mutations could be highly active, and sufficient to evoke severe systemic inflammation, even when present as a low-level mosaicism.

The clinical symptoms of mosaic patients appear to be milder than those of heterozygous patients. Patient 7 carried a *CIAS1*-Y570C mutation, which is one of the most common *CIAS1* mutations, and is associated with a very severe phenotype including mental retardation and epilepsy.[3,5,43] Patient 7 appeared to have milder symptoms than other reported heterozygous patients carrying the same mutation of *CIAS1*, showing neither mental retardation nor epilepsy, even at 15 years of age. Similarly, patient 8, who had the *CIAS1*-L264F mutation as mosaicism, exhibited a milder phenotype than patients with the heterozygous L264F mutation.[11] Although the *CIAS1*-G307S mutation in patient 9 has not yet been reported, the symptoms of the patient also seemed to be milder than that of a patient reported to have the G307V mutation.[44] The relatively mild phenotypes in mosaic patients, despite the relatively potent effects of their mutations on cellular activity (Figure 7), may be attributable to the lower dose of active mutation. Further study with more CAPS patients with CIAS1 mosaicism and more accurate measurements of mosaicism frequency by real-time PCR could provide clearer view of the correlation between the frequency of mutant allele and disease severity.

The mechanism of LPS-induced monocyte death that we observed remains to be elucidated. When monocytes are treated with LPS, *CIAS1* mRNA is induced immediately, and its encoded protein cryopyrin can be detected within 30 to 60 minutes of treatment.[36] One simple possibility is that the accumulation of LPS-induced mutant cryopyrin in the cytosol mediates necrosis. This is supported by our recent observation that overexpression of a

disease-associated mutant of *CIAS1* in THP-1 cells resulted in rapid necrosis-like cell death in a cathepsin B–dependent manner.[27] The caspase-1 inhibitor YVAD-fmk failed to inhibit LPS-induced monocyte cell death, while it effectively suppressed LPS-induced IL-1β production. Nigericin, a potassium ionophore, induces not only caspase-1–dependent IL-1β/IL-18 release but also rapid necrosis in LPS-primed THP-1 cells.[45] Interestingly, as we observed, the cathepsin B inhibitor CA074-Me inhibited the nigericin-induced necrosis while the caspase-1 inhibitor YVAD-cmk did not.[45] This indicates that a common pathway inducing cathepsin B-dependent necrosis in monocytes exists. Cross-talk between the LPS-TLR4 signaling pathway and the cryopyrin inflammasome to cause monocyte cell death is another possibility.

It was recently reported that the cytoplasmic receptor Ipaf recognizes bacterial flagellin, and induces rapid necrosis of *Salmonella*-infected macrophages.[46,47] It has been proposed that cryopyrin functions as a pattern-recognition receptor[48-50] that mediates inflammation; thus, it is possible that cryopyrin-induced rapid necrotic cell death and subsequent release of various cellular components facilitates local inflammation and prevents intracellular bacterial proliferation. Additional experiments are needed to clarify the mechanism of monocyte cell death observed in *CIAS1*-mutant cells in response to LPS.

While LPS-induced monocyte cell death seemed to be a specific property of *CIAS1* disease-associated mutant cells, the clinical and physiologic relevance of this biologic activity is unknown. The primary etiology of CAPS is considered to be excessive IL-1β production by constitutively activated inflammasomes. Although this hypothesis is supported by the fact that the autoinflammatory symptoms of the syndrome are successfully treated with IL-1β–targeted therapy,[10,29,33-35] it remains unclear whether the unique articular and cartilage manifestations of CAPS can also be attributed to IL-1β overproduction. Histologic analysis of the growth cartilage of CINCA syndrome patients revealed necrosis and disorganized proliferation of chondrocytes, and focal calcification, while infiltration of inflammatory cells was not described.[51] Feldmann et al speculated that the characteristic growth cartilage burst and epiphyseal overgrowth observed among CINCA patients might be due to dysregulated apoptosis of chondrocytes, which express a high amount of *CIAS1*.[4] One possibility is that certain stimuli, probably other than LPS, cause destructive necrosis of chondrocytes, rather than apoptosis, resulting in a loss of regularity of growth cartilage and subsequent bizarre joint destruction. We observed that LPS induces monocyte cell death independently of anti-IL-1β therapy status; thus, careful observation of anakinra-treated patients will provide a more precise understanding of the involvement of mutant *CIAS1*-mediated cell death in CAPS symptoms.

While the strategy we used in the current study was good at detecting single nucleotide substitutions, we cannot exclude the possibility that there were other types of genetic abnormalities present that were not detected, such as mis-splicing and noncoding region mutations. However, it is also possible that mosaicism, with an unequal distribution of mutant cells, is prominent in nonhematopoietic cells or tissues, such as the skin or central nervous system. An analysis of nonhematopoietic tissues may therefore be necessary before concluding that the *CIAS1* mutation is not responsible for the disease symptoms. Importantly, because diagnosis of CAPS is primarily based on clinical symptoms, a reassessment of the patients' histories and a physical re-evaluation is necessary before reestablishing the disease entity of *CIAS1*-unrelated patients.

In summary, we found that monocytes bearing mutations in *CIAS1* rapidly undergo necrosis-like cell death when treated with LPS, enabling us to diagnose *CIAS1* mutation-negative patients as *CIAS1* mosaic patients. Our investigation revealed that for a majority of CAPS patients without detectable *CIAS1* mutations by ordinary genomic sequencing, disease development may be attributable to low-level mosaicism. Not all *CIAS1* mutation-negative patients have *CIAS1* mosaicism, presenting the opportunity to uncover genes other than *CIAS1* as causative genes for CAPS. Our findings also raise the possibility that low-level mosaicism in other hereditary autoinflammatory syndromes may play a role in disease development, in the absence of detectable gene mutations.

Acknowledgments

We thank our CAPS patients and their parents for their participation. We thank Dr W. Strober (National Institutes of Health, Bethesda, MD) for critical reading of the manuscript and suggestions, and Dr S. Teramukai (Kyoto University, Kyoto, Japan) for advice on statistical analysis.

This study was supported in part by the Morinaga Hoshi-Kai; the Sapporo Bioscience Foundation; Ministry of Education, Science, Sports, and Culture; and the Ministry of Health, Labor, and Welfare, Japan.

Authorship

Contribution: M.S. performed research and wrote the paper. R.N. and N.K. designed the research, wrote the paper, and analyzed data. A.F. and H. Tanizaki performed research. K.T., T. Imagawa, T. Iehara, H. Takada, T.M., H. Tanaka, H.K., K.K., and S.K. treated the patients and analyzed data. I.O. and T.Y. performed research and discussed results. S.A. wrote the paper and discussed results. T.H., Y.M., and T.N designed the research.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Ryuta Nishikomori, MD, PhD, Department of Pediatrics, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan; e-mail: rnishiko@kuhp.kyoto-u.ac.jp.

References

1. Neven B, Prieur AM, Petty RE. Cryopyrin-associated periodic syndromes. In: Cassidy JT, Petty RE, Laxer RM, Lindsley CB, eds. Textbook of pediatric rheumatology 5th edition. Philadelphia, PA: Elsevier Saunders; 2005:671-675.
2. Prieur AM, Griscelli C, Lampert F et al. A chronic, infantile, neurologic, cutaneous and articular (CINCA) syndrome. A specific entity analysed in 30 patients. Scand J Rheumatol Suppl. 1987;66: 57-68.
3. Aksentijevich I, Nowak M, Mallah M et al. De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. Arthritis Rheum. 2002;46:3340-3348.
4. Feldmann J, Prieur AM, Quartier P et al. Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. Am J Hum Genet. 2002;71:198-203.
5. Neven B, Callebaut I, Prieur AM et al. Molecular basis of the spectral expression of CIAS1 mutations associated with phagocytic cell-mediated autoinflammatory disorders CINCA/NOMID, MWS, and FCU. Blood. 2004;103:2809-2815.
6. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial

cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet. 2001;29:301-305.

7. Aganna E, Martinon F, Hawkins PN et al. Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. Arthritis Rheum. 2002;46: 2445-2452.
8. Dodé C, Le Du N, Cuisset L et al. New mutations of CIAS1 that are responsible for Muckle-Wells syndrome and familial cold urticaria: a novel mutation underlies both syndromes. Am J Hum Genet. 2002;70:1498-1506.
9. Aróstegui JI, Aldea A, Modesto C et al. Clinical and genetic heterogeneity among Spanish patients with recurrent autoinflammatory syndromes associated with the CIAS1/PYPAF1/NALP3 gene. Arthritis Rheum. 2004;50:4045-4050.
10. Goldbach-Mansky R, Dailey NJ, Canna SW et al. Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. N Engl J Med. 2006;355:581-592.
11. Aksentijevich I, D Putnam C, Remmers EF et al. The clinical continuum of cryopyrinopathies: novel CIAS1 mutations in North American patients and a new cryopyrin model. Arthritis Rheum. 2007;56:1273-1285.
12. Saito M, Fujisawa A, Nishikomori R et al. Somatic mosaicism of CIAS1 in a patient with chronic infantile neurologic, cutaneous, articular syndrome. Arthritis Rheum. 2005;52:3579-3585.
13. Aksentijevich I, Remmers EF, Goldbach-Mansky R, Reiff A, Kastner DL. Mutational analysis in neonatal-onset multisystem inflammatory disease: Comment on the articles by Frenkel et al and Saito et al. Arthritis Rheum. 2006;54:2703-2704.
14. Saito M, Fujisawa A, Nishikomori R et al. Reply. Arthritis Rheum. 2006;54:2704-2705.
15. Kwiatkowska J, Wigowska-Sowinska J, Napierala D, Slomski R, Kwiatkowski DJ. Mosaicism in tuberous sclerosis as a potential cause of the failure of molecular diagnosis. N Engl J Med. 1999; 340:703-707.
16. Kluwe L, Mautner V, Heinrich B et al. Molecular study of frequency of mosaicism in neurofibromatosis 2 patients with bilateral vestibular schwannomas. J Med Genet. 2003;40:109-114.
17. Youssoufian H, Pyeritz RE. Mechanisms and consequences of somatic mosaicism in humans. Nat Rev Genet. 2002;3:748-758.
18. Danielson PB, Kristinsson R, Shelton RJ, Laberge GS. Separating human DNA mixtures using denaturing high-performance liquid chromatography. Expert Rev Mol Diagn. 2005;5:53-63.
19. Emmerson P, Maynard J, Jones S, Butler R, Sampson JR, Cheadle JP. Characterizing mutations in samples with low-level mosaicism by collection and analysis of DHPLC fractionated heteroduplexes. Hum Mutat. 2003;21:112-115.
20. Ting JP, Davis BK. CATERPILLER: a novel gene family important in immunity, cell death, and diseases. Annu Rev Immunol. 2005;23:387-414.
21. Inohara N, Chamaillard M, McDonald C, Nunez G. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem. 2005;74:355-383.
22. Dowds TA, Masumoto J, Zhu L, Inohara N, Nunez G. Cryopyrin-induced interleukin 1beta secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. J Biol Chem. 2004;279:21924-21928.
23. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity. 2004;20:319-325.
24. Mariathasan S, Newton K, Monack DM et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature. 2004; 430:213-218.
25. Janssen R, Verhard E, Lankester A, Ten Cate R, van Dissel JT. Enhanced interleukin-1beta and interleukin-18 release in a patient with chronic infantile neurologic, cutaneous, articular syndrome. Arthritis Rheum. 2004;50:3329-3333.
26. Manji GA, Wang L, Geddes BJ et al. PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappa B. J Biol Chem. 2002;277:11570-11575.
27. Fujisawa A, Kambe N, Saito M et al. Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. Blood. 2007;109:2903-2911.
28. Takada H, Kusuhara K, Nomura A et al. A novel CIAS1mutation in a Japanese patient with chronic infantile neurological cutaneous and articular syndrome. Eur J Pediatr. 2005;164:785-786.
29. Matsubayashi T, Sugiura H, Arai T, Oh-ishi T, Inamo Y. Anakinra therapy for CINCA syndrome with a novel mutation in exon 4 of the CIAS1 gene. Acta Paediatr. 2006;95:246-249.
30. Tanaka H, Waga S, Kakizaki Y, Sugimoto K, Nomura K, Yokoyama M. Chronic urticaria associated with aseptic meningitis: an atypical urticarial vasculitis? Acta Paediatr Jpn. 1997;39:64-68.
31. Kagami S, Saeki H, Kuwano Y, Imakado S, Tamaki K. A probable case of Muckle-Wells syndrome. J Dermatol. 2006;33:118-121.
32. Kawashima H, Sato A, Nisimata S et al. A case report of neonatal onset multisystemic inflammatory disease treated with continuous hemodiafiltration and steroid pulse therapy. Ther Apher Dial. 2007;11:232-234.
33. Hoffman HM, Rosengren S, Boyle DL et al. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. Lancet. 2004;364: 1779-1785.
34. Hawkins PN, Lachmann HJ, Aganna E, McDermott MF. Spectrum of clinical features in Muckle-Wells syndrome and response to anakinra. Arthritis Rheum. 2004;50:607-612.
35. Lovell DJ, Bowyer SL, Solinger AM. Interleukin-1 blockade by anakinra improves clinical symptoms in patients with neonatal-onset multisystem inflammatory disease. Arthritis Rheum. 2005;52: 1283-1286.
36. O'Connor W Jr., Harton JA, Zhu X, Linhoff MW, Ting JP. Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory mediator with NF-kappa B suppressive properties. J Immunol. 2003;171:6329-6333.
37. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000;406:782-787.
38. Poltorak A, He X, Smirnova I et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282:2085-2088.
39. Qureshi ST, Lariviere L, Leveque G et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4) J Exp Med. 1999;189:615-625.
40. Kadowaki N, Ho S, Antonenko S et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med. 2001;194:863-869.
41. Martinon F, Agostini L, Meylan E, Tschopp J. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. Curr Biol. 2004;14:1929-1934.
42. Kuida K, Lippke JA, Ku G et al. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science. 1995; 267:2000-2003.
43. Rösen-Wolff A, Quietzsch J, Schroder H, Lehmann R, Gahr M, Roesler J. Two German CINCA (NOMID) patients with different clinical severity and response to anti-inflammatory treatment. Eur J Haematol. 2003;71:215-219.
44. Matsubara T, Hasegawa M, Shiraishi M et al. A severe case of chronic infantile neurologic, cutaneous, articular syndrome treated with biologic agents. Arthritis Rheum. 2006;54:2314-2320.
45. Hentze H, Lin XY, Choi MS, Porter AG. Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. Cell Death Differ. 2003;10:956-968.
46. Franchi L, Amer A, Body-Malapel M et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat Immunol. 2006;7:576-582.
47. Miao EA, Alpuche-Aranda CM, Dors M et al. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nat Immunol. 2006;7:569-575.
48. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 2006; 440:237-241.
49. Mariathasan S, Weiss DS, Newton K et al. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 2006;440:228-232.
50. Kanneganti TD, Ozoren N, Body-Malapel M et al. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature. 2006;440:233-236.
51. De Cunto CL, Liberatore DI, San Roman JL, Goldberg JC, Morandi AA, Feldman G. Infantile-onset multisystem inflammatory disease: a differential diagnosis of systemic juvenile rheumatoid arthritis. J Pediatr. 1997;130:551-556.

ARTHRITIS & RHEUMATISM
Vol. 60, No. 1, January 2009, pp 242–250
DOI 10.1002/art.24134

Role of the *NOD2* Genotype in the Clinical Phenotype of Blau Syndrome and Early-Onset Sarcoidosis

Ikuo Okafuji,[1] Ryuta Nishikomori,[1] Nobuo Kanazawa,[2] Naotomo Kambe,[3] Akihiro Fujisawa,[1] Shin Yamazaki,[1] Megumu Saito,[1] Takakazu Yoshioka,[1] Tomoki Kawai,[1] Hidemasa Sakai,[1] Hideaki Tanizaki,[1] Toshio Heike,[1] Yoshiki Miyachi,[1] and Tatsutoshi Nakahata[1]

Objective. **Blau syndrome and its sporadic counterpart, early-onset sarcoidosis (EOS), share a phenotype featuring the symptom triad of skin rash, arthritis, and uveitis. This systemic inflammatory granulomatosis is associated with mutations in the *NOD2* gene. The aim of this study was to describe the clinical manifestations of Blau syndrome/EOS in Japanese patients and to determine whether the *NOD2* genotype and its associated basal NF-κB activity predict the Blau syndrome/EOS clinical phenotype.**

Methods. **Twenty Japanese patients with Blau syndrome/EOS and *NOD2* mutations were recruited. Mutated *NOD2* was categorized based on its basal NF-κB activity, which was defined as the ratio of NF-κB activity without a *NOD2* ligand, muramyldipeptide, to NF-κB activity with muramyldipeptide.**

Results. **All 9 mutations, including E383G, a novel mutation that was identified in 20 patients with Blau syndrome/EOS, were detected in the centrally located NOD region and were associated with ligand-independent NF-κB activation. The median age of the patients at disease onset was 14 months, although in 2 patients in Blau syndrome families (with mutations R334W and E383G, respectively) the age at onset was 5 years or older. Most patients with Blau syndrome/EOS had the triad of skin, joint, and ocular symptoms, the onset of which was in this order. Clinical manifestations varied even among familial cases and patients with the same mutations. There was no clear relationship between the clinical phenotype and basal NF-κB activity due to mutated *NOD2*. However, when attention was focused on the 2 most frequent mutations, R334W and R334Q, R334W tended to cause more obvious visual impairment.**

Conclusion. ***NOD2*** **genotyping may help predict disease progression in patients with Blau syndrome/EOS.**

Sarcoidosis is a systemic inflammatory disease with unknown etiology, but it can be clinically characterized by swelling of the bilateral hilar lymph nodes and histologically defined by the presence of noncaseating epithelioid cell granulomas. A special subtype called early-onset sarcoidosis (EOS; MIM no. 609464) occurs in children younger than 4 years of age and is characterized by a distinct triad of skin, joint, and eye disorders without apparent pulmonary involvement (1). An autosomal-dominant disease with clinical manifestations similar to those of EOS has been recognized as Blau syndrome (MIM no. 186580) (2,3). The gene responsible for Blau syndrome has been mapped close to the inflammatory bowel disease 1 (*IBD1*) locus by linkage analysis (4), and later the nucleotide-binding oligomerization domain 2 gene (*NOD2*) was identified by Miceli-Richard et al to be responsible for Blau syndrome (5). In the study by Miceli-Richard et al, 2 European patients with EOS had no mutation in *NOD2*; therefore, it remained

Supported by the Ministry of Education, Science, Sports, and Culture, Japan.

[1]Ikuo Okafuji, MD, Ryuta Nishikomori, MD, PhD, Akihiro Fujisawa, MD, PhD, Shin Yamazaki, PhD, Megumu Saito, MD, PhD, Takakazu Yoshioka, MD, Tomoki Kawai, MD, Hidemasa Sakai, MD, Hideaki Tanizaki, MD, Toshio Heike, MD, PhD, Yoshiki Miyachi, MD, PhD, Tatsutoshi Nakahata, MD, PhD: Kyoto University Graduate School of Medicine, Kyoto, Japan; [2]Nobuo Kanazawa, MD, PhD: Kyoto University Graduate School of Medicine, Kyoto, and Wakayama Medical University, Wakayama, Japan; [3]Naotomo Kambe, MD, PhD: Kyoto University Graduate School of Medicine, Kyoto, and Chiba University Graduate School of Medicine, Chiba, Japan.

Address correspondence and reprint requests to Ryuta Nishikomori, MD, PhD, Department of Pediatrics, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: rnishiko@kuhp.kyoto-u.ac.jp.

Submitted for publication March 19, 2008; accepted in revised form September 5, 2008.

controversial whether Blau syndrome and EOS have the same etiology.

In 2004, we encountered a 27-year-old Japanese man with multiple lichenoid papules. He was almost blind, exhibited camptodactyly, and had a continuous low-grade fever. This case of sporadic systemic granulomatosis with clinical features of EOS showed the same *NOD2* mutation, the arginine-to-tryptophan substitution at amino acid 334 (R334W), as that detected in Blau syndrome (6). Therefore, we expanded this report (6) and retrospectively examined cases of EOS in Japan and observed that 9 of 10 patients with EOS had *NOD2* mutations (7). Until recently, other investigators have also confirmed that Blau syndrome and EOS are clinically and genetically identical across various ethnic groups (8–10).

NOD2 activates NF-κB after recognizing a signal from a bacterial cell wall component, muramyldipeptide, in the cytoplasm of monocytes, and thus can work as an intracellular sensor of bacteria (11,12). *NOD2* has a tripartite domain structure consisting of 2 amino-terminal domains (termed caspase activation and recruitment domains) that are composed of protein–protein interaction cassettes, 1 centrally located NOD, and carboxy-terminal leucine-rich repeats (LRRs) (13). Using assays of NF-κB activity, an impaired ligand-dependent response was demonstrated for 3 Crohn's disease–associated mutations located in *NOD2* LRRs (14,15), whereas enhanced ligand-independent NF-κB activity was demonstrated for *NOD2* alleles associated with Blau syndrome and EOS (5,7,16). However, it remains unknown how increased basal NF-κB activity derived from gain-of-function mutations in *NOD2* affects the pathogenesis of Blau syndrome/EOS and whether a genotype–phenotype correlation exists between the clinical manifestations or onset of Blau syndrome/EOS and *NOD2* mutations.

Because Blau syndrome/EOS is so rare, very few reports are in the literature. Therefore, it was worthwhile to conduct a nationwide survey limited to patients with a specific ethnic background, such as Japanese patients. In this study, we precisely documented the clinical manifestations in a cohort of Japanese patients with Blau syndrome/EOS and *NOD2* mutations, including 9 previously reported cases (7), and explored the genotype–phenotype correlation to the basal NF-κB activity associated with each mutation, especially focusing on the correlation of visual impairment with the most frequent mutations, R334W and R334Q.

PATIENTS AND METHODS

Patients and clinical information. Among patients with clinically diagnosed Blau syndrome/EOS, the 20 patients with *NOD2* mutations were included in this study (7,17–20). None of these mutations were identical to the reported single-nucleotide polymorphisms (SNPs) of *NOD2*, nor were they detected in 100 Japanese healthy volunteers. Clinical information and patient histories were collected from medical records and by direct interviews of the patients and their attending physicians. The presence of each symptom was established as follows: a) persistent or repeated transient skin lesions without definite cause were determined, b) persistent or repeated transient arthritis without definite cause was determined, c) uveitis was diagnosed by an ophthalmologist, and d) remittent or intermittent fever without definite cause was determined under close examination at the time of hospital admission. The age at disease onset was defined as the age of the patient when any of the above-mentioned symptoms appeared.

Clinical evaluation was performed primarily when individual symptoms first appeared that were hardly affected by treatment or disease duration. The severity of visual impairment was assessed in accordance with the World Health Organization definition (21). Briefly, moderate visual impairment was defined as visual acuity between 6/18 and 3/60, and severe visual impairment was defined as acuity of 3/60 or less in the better eye with best correction, as previously described (9). Written informed consent was obtained from the patients and their families, and the study protocol was in accordance with the guidelines of the Institutional Review Board of Kyoto University Hospital.

Genetics analysis. Genomic DNA was extracted from the peripheral blood of the patients, and sequencing of all exons and exon–intron junctions of *NOD2* was performed as previously described (7).

Generation of *NOD2* mutants and NF-κB luciferase assay. Expression plasmids of *NOD2* and its mutants were subcloned into the p3xFLAG-CMV vector, as previously described (7). Blau syndrome/EOS–associated mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as described previously (7). The ability of each construct to induce NF-κB activity was assessed by dual luciferase reporter assay in HEK 293 human embryonic kidney cells, as previously described (7).

Other analyses. We determined the age at the time of this survey, the age at onset of each symptom, and the *NOD2* genotype for all patients as well as the distribution of age at disease onset. Next, we analyzed the relationship between age at disease/symptom onset and basal NF-κB activity due to mutated *NOD2*. Basal NF-κB activity was defined as the ratio of NF-κB reporter activity without muramyldipeptide to NF-κB reporter activity with muramyldipeptide, as determined using the in vitro NF-κB luciferase assay described above. The activity was arbitrarily categorized as low (<0.3), moderate (0.3–0.5), and high (>0.5). Finally, we analyzed the relationship between visual impairment (normal, moderate, severe) and basal NF-κB activity (low, moderate, high) due to individual mutated *NOD2* genes, particularly the 2 most frequent mutations, R334W and R334Q. We did not perform statistical analysis because of the limited number of patients.

Table 1. Demographic and clinical characteristics of the patients with Blau syndrome/early-onset sarcoïdosis*

Patient/ age/sex	Genotype	Fever		Skin rash		Arthritis		Uveitis		Visual acuity		Ref.
		Age at onset	Type	Age at onset	Type	Age at onset	Type	Age at onset	Type	OD	OS	
1/15/F†	E383G	2 yr 3 mo	Int	8 mo	LP/SE/EN	3 yr	Poly	11 yr	A/P	20/50	20/67	
2/48/F†	E383G	5 yr	Per	5 yr	LP/SE/EN	11 yr	Poly	11 yr	A/P	HM	Null	
3/36/F	H496L	–	–	1 yr	LP/SE	3 yr	Poly	5 yr	A/P	20/20	20/20	7
4/16/M	R334Q	1 yr 8 mo	Int	6 mo	LP/SE	1 yr 8 mo	Poly	1 yr 10 mo	A/P	20/22	20/22	
5/19/M	R334Q	2 yr 7 mo	Per	1 yr 4 mo	LP/SE/EN	10 mo	Poly	5 yr	A/P	20/50	20/20	17
6/8/F	R334Q	–	–	–	–	3 yr	Poly	–	–	20/20	20/20	
7/8/M	T605P	–	–	7 mo	LP/SE	1 yr 6 mo	Poly	3 yr 3 mo	A/P	20/25	20/50	7
8/18/F	D382E	–	–	3 yr 4 mo	LP/SE	4 yr	Poly	5 yr 4 mo	A/P	20/20	20/25	7, 18
9/13/M	R334W	8 mo	Per	1 yr 3 mo	LP/SE/EN	8 mo	Poly	1 yr 8 mo	A/P	20/29	20/33	
10/32/M	R334W	2 yr	Int	2 yr	LP/SE	1 yr 3 mo	Poly	6 yr	A/P	Blind, 20 yr	Blind, 20 yr	6, 7
11/21/F	R334W	2 yr 1 mo	Per	2 yr 1 mo	LP/SE	6 yr	Poly	4 yr	A/P	20/670	20/330	7, 19
12/33/M	R334W	–	–	2 yr	LP/SE	–	–	13 yr	A/P	20/29	20/20	7
13/31/F	R334W	–	–	2 yr 6 mo	LP/SE	8 yr	Poly	3 yr 6 mo	A/P	20/100	20/200	7
14/10/F†	R334W	1 yr	Per	1 yr	LP/SE	1 yr	Poly	2 yr	A/P	20/40	Null	
15/46/F†	R334W	–	–	44 yr	LP/SE	8 yr	Poly	3 yr	A/P	Blind, 28 yr	Blind, 28 yr	
16/16/M†	R334W	–	–	6 yr	SE	1 yr	Oligo	6 yr	A/P	20/13	20/13	20
17/18/F†	R334W	–	–	12 yr	SE	8 yr	Oligo	12 yr	A/P	20/40	20/25	20
18/8/M	M513T	2 yr 10 mo	Int	2 yr 8 mo	SE	2 yr 9 mo	Poly	2 yr 11 mo	A	20/17	20/17	7
19/15/F	N670K	1 yr 8 mo	Int	5 mo	LP/SE/EN	1 yr 8 mo	Poly	3 yr	A/P	20/200	20/200	7
20/7/M	C495Y	1 yr	Int	1 yr	LP/SE	1 yr	Poly	–		20/20	20/20	

* Patient 5 also had left ventricular dysfunction and pulmonary hemorrhage due to bronchial granuloma. Patient 10 also had interstitial pneumonia. Patient 11 also had hepatosplenomegaly and parotid swelling. Patient 18 also had renal calcification. OD = right eye; OS = left eye; yr = years; mo = months; Int = intermittent; LP = multiple lichenoid papules; SE = scaly erythematous plaques; EN = erythema nodosum–like lesion; Poly = polyarticular; A = anterior; P = posterior; Per = persistent; HM = hand motion; Oligo = oligoarticular.
† Familial case.

RESULTS

Genotype and basal NF-κB activity. The study population comprised 9 male patients and 11 female patients, with a median age of 17 years (range 7–48 years) and a median disease duration of 15 years (range 5–43 years). Fourteen of these 20 cases were sporadic (EOS), and 6 were familial (Blau syndrome). The familial cases were in 3 unrelated families; 2 families (patients 14 and 15 and patients 16 and 17, respectively) had Blau syndrome/EOS symptoms in 2 generations, and 1 family (patients 1 and 2) had Blau syndrome/EOS symptoms in 3 generations. The most frequent heterozygous mutation of *NOD2* was R334W (1000C>T), which was recognized in 2 familial and 5 sporadic cases (total of 9 cases), followed by R334Q (1001G>A) in 3 sporadic cases, and E383G (1148A>G, a novel amino acid substitution) in 2 familial cases (in 1 family). H496L (1487A>T), T605P (1813A>C), D382E (1146C>G), M513T (1538T>C), N670K (2010C>A), and C495Y (1484G>A) were detected in 1 sporadic case each (Table 1).

Nine mutations were identified in the centrally located NOD region (Figure 1a) and were associated with increased basal NF-κB activity in the absence of muramyldipeptide (Figure 1b), which is consistent with the finding of a previous study on Blau syndrome/EOS–associated *NOD2* mutations (16). We also confirmed that 100 healthy control subjects and their genotyped asymptomatic relatives did not have these amino acid substitutions. Therefore, we concluded that these *NOD2* mutations (amino acid substitutions) detected in patients with Blau syndrome/EOS were not SNPs but rather were disease-causing mutations.

Disease onset. The defining characteristic of EOS is its onset in children younger than age 4 years (1). In the present study, despite the median age at disease onset of 14 months, the first clinical symptoms developed at age 5 years or older in 2 patients (patients 2 and 17, who were members of different Blau syndrome families) with the E383G mutation and the R334W mutation, respectively (Table 2). In patient 2, skin rash developed at age 5 years; in patient 17, arthritis developed at age 8 years (Table 1).

The earliest presenting symptom was skin rash in 13 patients (65%), arthritis in 8 patients (40%), and ocular symptoms in 1 patient (patient 15, who had familial Blau syndrome with the R334W mutation) (Table 1). Approximately 95%, 95%, and 90% of pa-

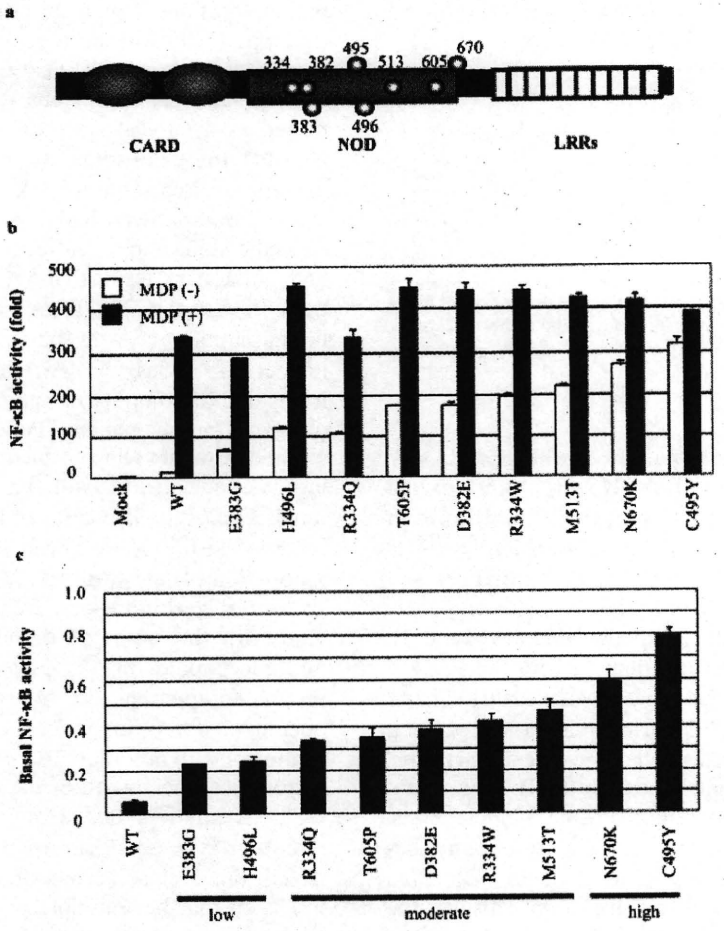

Figure 1. Biologic effects of *NOD2* mutants discovered in patients with Blau syndrome/early-onset sarcoidosis (EOS). **a,** Schematic presentation of NOD-2 protein. Numbers indicate the positions of mutated amino acid residues identified in our cohort. **b,** Increased basal NF-κB activity due to different mutated *NOD2* genes in patients with Blau syndrome/EOS. HEK 293T cells were cotransfected with a *NOD2* mutant together with the NF-κB reporter plasmid and internal control plasmid, and NF-κB reporter activity was measured after 12 hours of incubation with or without muramyldipeptide (MDP; 5 μg/ml). Mock vector and wild-type (WT) *NOD2* were used as controls. Bars show the mean and SD of normalized data (mock without muramyldipeptide = 1) from triplicate cultures. Results are representative of 3 independent experiments. **c,** Basal NF-κB activity due to mutated *NOD2* in patients with Blau syndrome/EOS. Bars show the mean and SD results from 3 independent experiments. CARD = caspase activation and recruitment domain; LRRs = leucine-rich repeats.

tients, respectively, had skin, joint, and ocular symptoms. Consistent with the previous report (1), a triad of skin, joint, and ocular symptoms developed (in this order) in many patients with Blau syndrome/EOS. The median age at onset of rash, arthritis, and uveitis was 24 months, 33 months, and 4.5 years, respectively (Table 2).

The triad of symptoms. All except 1 patient (patient 6 [with the R334Q mutation]) had skin manifestations. Consistent with a previous report (22), the most frequent skin symptom was scaly erythematous plaques with multiple lichenoid papules. Several patients (patients 1 and 2 with the E383G mutation, patient 5

Table 2. Age of the patients at the onset of disease and symptoms*

Age, years	Disease onset (n = 20)	Symptom onset			
		Fever (n = 11)	Rash (n = 19)	Arthritis (n = 19)	Uveitis (n = 18)
0	6 (30)	1 (9)	4 (21)	2 (11)	0 (0)
1	5 (25)	4 (36)	5 (26)	7 (37)	2 (11)
2	4 (20)	5 (45)	5 (26)	1 (5)	2 (11)
3	3 (15)	0 (0)	1 (5)	3 (16)	4 (22)
4	0 (0)	0 (0)	0 (0)	1 (5)	1 (6)
≥5	2 (10)	1 (9)	4 (21)	5 (26)	8 (44)

* Values are the number (%). The median age at disease onset was 1 year 2 months; the median age at onset of fever and rash was 2 years; the median age at onset of arthritis was 2 years 9 months; the median age at onset of uveitis was 4 years 6 months.

with the R334Q mutation, patient 9 with the R334W mutation, and patient 19 with the N670K mutation) had erythema nodosum–like lesions on their lower limbs in addition to solid lichenoid eruptions. Notably, 3 patients (patients 16 and 17 with the R334W mutation and patient 18 with the M513T mutation) showed only scaly erythematous plaques without lichenoid papules (Table 1).

All except 1 patient (patient 12 with the R334W mutation) had joint lesions (polyarticular arthritis in 17 patients and oligoarticular arthritis in 2 [patients 16 and 17]) (Table 1). Both patients with oligoarticular arthritis, who had familial Blau syndrome with the R334W mutation, had camptodactyly without obvious synovial cysts. Camptodactyly with synovial cysts is frequently described as a typical joint sign in patients with Blau syndrome/EOS (10). A consequence of arthritis was the use of a wheelchair for daily mobility in 2 patients (patient 5 with the R334Q mutation and patient 10 with the R334W mutation).

All except 2 patients (patient 6 with the R334Q mutation who also lacked skin eruptions and patient 20 with the C495Y mutation) had ocular lesions. The lesions were bilateral, although visual acuity was asymmetric, as in previous studies (22,23). Moreover, 17 (89%) of all 18 patients with ocular lesions had panuveitis, while only 1 patient (patient 18 with mutation M513T) had anterior uveitis, which demonstrated the predominance of panuveitis over anterior uveitis. Ocular symptoms were the last of the triad to develop in 15 of the 18 patients and the first to develop in only 1 patient (patient 15 with mutation R334W).

Clinical features other than the triad of symptoms. It is noteworthy that 11 patients (55%) experienced fever at a median age of 24 months, almost simultaneously with skin and/or joint symptoms (Table 1). Five patients had persistent fever reaching 38–40°C, and 6 patients had intermittent fever. In particular, in 1 patient (patient 9 with mutation R334W) the disease developed with intermittent fever (which then became persistent fever over the next 6 months) and finger joint swelling. In only 1 previous report (10), fever is mentioned as a clinical symptom of Blau syndrome/EOS, although there are some case reports in which fever was present at disease onset (24).

Four patients had involvement of organs other than the skin, joints, and eyes (Table 1). Two patients had pulmonary lesions (interstitial pneumonitis in patient 10 with the R334W mutation and bronchial granuloma in patient 5 with the R334Q mutation). Bilateral hilar lymph nodes, which are identified by chest radiography and/or computed tomographic scanning, were not observed in any patient. Patient 11 with the R334W mutation exhibited hepatosplenomegaly and parotid swelling (19), and patient 18 with the M513T mutation exhibited renal calcification. No cases of large-vessel vasculitis were observed in this cohort, even though vasculitis has been reported in patients with EOS (25–27).

Triggering factors. BCG vaccination was associated with the onset of disease (i.e., development of multiple papules on the extremities) in 2 patients, although no apparent infection or vaccination was clearly documented in other patients of our cohort. In 1 patient (patient 7 with mutation T605P) who had papules on the extremities, the spread of papules was from the site of BCG vaccination. In the other patient (patient 1 with mutation E383G), Gianotti disease was initially diagnosed, but a close review of her medical history later indicated that her multiple papules were a symptom of Blau syndrome/EOS.

Relationship between the onset of disease/symptoms and basal NF-κB activity due to mutated *NOD2*. Because disease duration and treatment varied among patients, we focused on the onset of disease and of each clinical symptom (i.e., fever, rash, arthritis, and uveitis). We evaluated the relationship between age at the onset of disease/symptoms and basal NF-κB activity due to mutated *NOD2* (defined as the ratio of NF-κB activity without a *NOD2* ligand, muramyldipeptide, to NF-κB activity with muramyldipeptide for each mutated *NOD2*). The calculated basal NF-κB activity ranged from 0.23 to 0.79 (mean 0.42) for mutated *NOD2* and was 0.05 for wild-type *NOD2* (Figure 1c).

Because the number of patients with each *NOD2* mutation was limited, we arbitrarily categorized basal NF-κB activity as low (<0.3), moderate (0.3–0.5), and high (>0.5). According to these criteria, mutations E383G and H496L were associated with low activity; mutations R334Q, T605P, D382E, R334W, and M513T were associated with moderate activity; and mutations

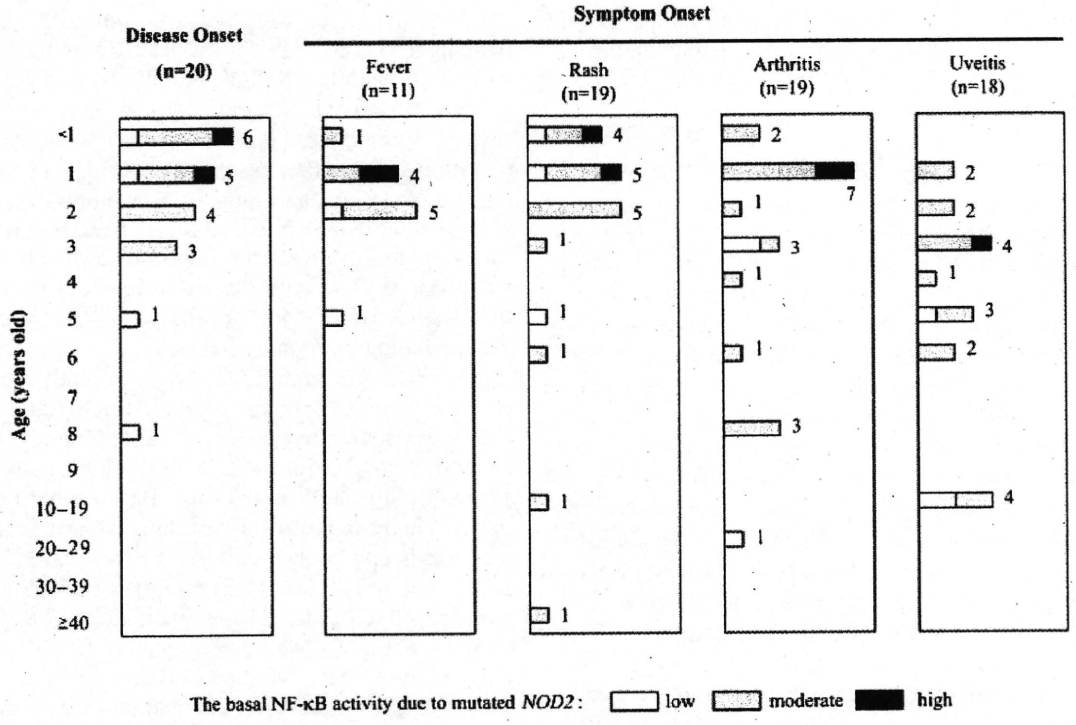

Figure 2. Relationship between age at disease or symptom onset and basal NF-κB activity due to mutated *NOD2*. Among the 9 patients without fever, 8 had moderate and 1 had low basal NF-κB activity. One patient without rash had moderate basal NF-κB activity, and 1 patient without arthritis had moderate basal NF-κB activity. Of 2 patients without uveitis, 1 had high and the other had moderate basal NF-κB activity.

N670K and C495Y were associated with high basal NF-κB activity. Our limited number of patients was insufficient to detect a correlation between the defined basal NF-κB activity and the onset of disease, fever, rash, arthritis, and uveitis (Figure 2). Notably, the age at onset of symptoms varied markedly between patients with the same R334W mutation, even in familial cases (Table 1).

Relationship between visual impairment and basal NF-κB activity due to mutated *NOD2*. The most relevant morbidity associated with Blau syndrome/EOS is ocular involvement, which is usually refractory to conventional treatment. Thus, we next explored the relationship between visual impairment and basal NF-κB activity. There was no clear correlation when the analysis included all recruited patients (Table 3). When we focused on the most frequent genotypes R334Q and R334W, between-genotype differences in visual impairment were observed (Table 4). Basal NF-κB activity was higher in patients with the R334W mutation than in those with the R334Q mutation (Figure 1c). None of the 3 patients with the R334Q mutation had visual impairments, while 4 of 9 patients with the R334W mutation

Table 3. Correlation between visual impairment and basal NF-κB activity*

	Visual impairment			Disease duration, median (range) years
	Normal	Moderate	Severe	
Basal NF-κB activity				
Low	2	0	1	35 (15–43)
Moderate	11	2	2	15 (5–43)
High	1	1	0	10.5 (6–15)

* Except where indicated otherwise, values are the number of patients.

Table 4. Correlation between visual impairment and the 2 most frequent genotypes*

	Visual impairment			Disease duration, median (range) years
	Normal	Moderate	Severe	
Present study				
R334Q	3	0	0	15 (5–19)
R334W	5	2	2	19 (9–43)
Previous study (9)				
R334Q	8	0	0	12 (3–26)
R334W	8	2	1	16 (5–44)

* Except where indicated otherwise, values are the number of patients.

had visual impairments. This result suggests that patients with the R334W mutation were more likely to have visual impairments than were those with the R334Q mutation (Table 4).

DISCUSSION

Blau syndrome/EOS is a rare systemic granulomatosis that has been associated with *NOD2*. In this study, patients with Blau syndrome/EOS and *NOD2* mutations were retrospectively recruited nationwide in Japan, to determine whether the *NOD2* genotype and its functional abnormality predict the Blau syndrome/EOS clinical phenotype. This study is the first to investigate the correlation between the *NOD2* genotype and its functional abnormality and the Blau syndrome/EOS clinical phenotype. Our findings suggest that *NOD2* genotyping may help predict disease progression in patients with Blau syndrome/EOS, although the clinical severity of Blau syndrome/EOS was not clearly associated with basal NF-κB activity due to mutated *NOD2* among the limited number of patients we studied.

The classic Blau syndrome/EOS symptom triad is skin rash, arthritis, and uveitis. Corresponding clinical manifestations include widespread erythematous papules, polyarthritis with boggy synovial swellings, and panuveitis (1,9,10,23), which were also identified in the present study. Rose et al described 2 patients who also had 1 episode of erythema nodosum–like lesions during the course of the disease (9). In our cohort, 5 patients had erythema nodosum–like lesions, suggesting that this should be recognized as one of the skin manifestations associated with Blau syndrome/EOS.

In the current study, 55% of the patients had fever, which always accompanied at least 1 symptom of the classic triad. Arostegui et al also reported that 50% of their cohort had recurrent or persistent fever (10). These findings suggest that fever is one of the important symptoms of Blau syndrome/EOS and is the reason why Blau syndrome/EOS is misdiagnosed as systemic-onset juvenile idiopathic arthritis (JIA). In fact, patient 11 in our study (who had the R334W mutation) experienced persistent fever reaching 40°C and received aggressive immunosuppressive therapy, because systemic-onset JIA was initially diagnosed. This case alerts us to the possibility that patients with Blau syndrome/EOS can sometimes have fever, and that Blau syndrome/EOS can resemble systemic-onset JIA.

Bilateral hilar lymph nodes, which are often seen in adult sarcoidosis, are not observed in Blau syndrome/EOS, but this does not mean that pulmonary lesions do not occur in patients with Blau syndrome/EOS. In fact, 2 patients (patient 5 [with the R334Q mutation] and patient 10 [with the R334W mutation]) had pulmonary lesions; in particular, patient 10 had the first reported case of sporadic EOS in association with the *NOD2* mutation (6). Another case of Blau syndrome/EOS with pulmonary lesions and interstitial pneumonitis, but not bilateral hilar lymph nodes, has also been reported (28). These findings suggest the importance of following up patients with Blau syndrome/EOS to check for not only the classic triad of symptoms but also other abnormalities, including pulmonary lesions.

Blau syndrome/EOS, which usually occurs in children younger than age 4 years, developed at 5 years and 8 years, respectively, in 2 patients in the present study (patient 2 [with the E383G mutation] and patient 17 [with the R334W mutation]). Because both of these patients had a family history of skin rash/arthritis/uveitis, they had been closely monitored by their parents as well as by their physicians. Therefore, it is unlikely that any symptoms that occurred when the patients were younger than 4 years of age were overlooked in these 2 cases. In the literature, there is 1 case of Blau syndrome in which skin rash, persistent fever, and camptodactyly started to develop at age 18 years (10). These findings indicate that the onset of Blau syndrome/EOS can be at age 5 years or older, and that disease onset in a patient younger than 4 years should not be considered requisite for a diagnosis of Blau syndrome/EOS.

In our cohort, the age at disease/symptom onset, organ involvement, and severity of Blau syndrome/EOS varied substantially even within affected families and between individuals with the same *NOD2* mutation (e.g., R334W). In other genetic disorders, identical mutations have been associated with phenotypic variation in unrelated individuals, within a family, and even in monozygotic twins (29). Phenotypic variation in Blau syndrome/EOS has been reported in monozygotic twins; therefore, nongenetic factors such as environmental conditions and/or infectious agents might be involved in phenotypic variation (24). Interestingly, in 2 of our cases, BCG vaccination was an obvious triggering factor. In addition, a previous report noted that cutaneous lesions first arose after BCG vaccination in a patient with Blau syndrome/EOS (30). The BCG vaccine contains muramyldipeptide, a ligand for NOD-2 protein (11,12), which is interesting from a pathophysiologic point of view. However, BCG vaccination did not always cause the onset of disease in patients with Blau syndrome/EOS, because most patients in our cohort were vaccinated with BCG according to the immunization protocol used in areas of

Japan where the risk for tuberculosis was high. An unknown endogenous ligand for NOD-2 could influence disease onset and/or progression, similar to uric acid as an endogenous cryopyrin/NLRP3 ligand (31). The potential roles of endogenous ligands, pathogen-associated molecular patterns, and/or danger-associated molecular patterns in disease pathogenesis remain to be elucidated.

Although increased basal NF-κB activity due to mutated *NOD2* has been proposed as an etiology of Blau syndrome/EOS, how such activity causes the characteristic symptoms remains unclear. We hypothesized that if increased basal NF-κB activity is the key to the pathophysiology of this disease, it should be related to disease severity or disease progression. Unfortunately, there was no clear correlation between basal NF-κB activity and the onset of disease/symptoms. However, patients with mutated *NOD2* and low basal NF-κB activity tended to experience complications, e.g., arthritis and uveitis, at a later age. This finding raises the possibility that basal NF-κB activity may affect disease progression rather than disease onset. Given that NOD-2 protein signals through MAPK/ERK as well as the NF-κB pathway (32), the possibility cannot be excluded that the MAPK/ERK activation potential of each *NOD2* genotype might also be correlated with disease severity or progression.

From the perspective of quality of life, the ocular manifestations of Blau syndrome/EOS require the closest attention (33). In a previous study, one-third of patients with Blau syndrome/EOS and *NOD2* mutations had a poor or extremely poor visual outcome, and the progression of visual field loss was independent of the particular *NOD2* mutant and was not associated with disease duration (9). In our cohort, however, patients with the R334W mutation experienced more visual impairment than did patients with the R334Q mutation, although 4 patients with the R334W mutation were from 2 families (patients 14 and 15 and patients 16 and 17, respectively). Therefore, familial genetic and environmental factors could easily influence the phenotype. Thus, in order not to favor our hypothesis, we excluded patients 15 and 17 from the analysis, and the trend was still evident. This observation was consistent with the findings of Rose et al (9), although those investigators did not address this issue. These findings suggest that *NOD2* genotyping could help predict the course of eye disease in patients with Blau syndrome/EOS, especially those with the R334Q mutation or the R334W mutation.

The relationship between visual impairment and basal NF-κB activity also remains a matter for discussion. Our data showed that visual impairments were more severe in patients with the R334W mutation than in those with the R334Q mutation, which seems to be consistent with the hypothesis that higher basal NF-κB activity causes more severe disease or more disease progression. However, no ocular symptoms have developed during the 6 years since disease onset in patient 20 (with the C495Y mutation and the highest basal NF-κB activity in our cohort), although ocular symptoms developed in another patient with the same genotype (10). Also, in patient 2, who had the E383G mutation and the lowest basal NF-κB activity, severe visual impairment occurred when she was in her late twenties. These findings contradict our hypothesis that *NOD2* genotypes with higher basal NF-κB activity are associated with severe disease. However, Blau syndrome/EOS was promptly diagnosed in patient 20 with the C495Y mutation, who luckily was under the care of the same pediatric rheumatologist who treated patient 19 (who had the N670K mutation) and was treated with systemic steroid therapy. Patient 2 (who had the E383G mutation) subsequently received inappropriate immunosuppressive therapy, because the patient refused steroid treatment. Furthermore, patient 10 (with the R334W mutation), who had no obvious systemic inflammatory findings and did not receive systemic steroid therapy, became blind at 20 years of age. These findings raise the possibility that the extent of visual impairment could be modified by therapy.

Finally, we were not able to prove a link between the clinical severity of Blau syndrome/EOS and basal NF-κB activity in the whole cohort, possibly because of the restricted number of patients and because of the differences in treatment among patients. Therefore, a prospective study involving a sufficient number of patients to allow analysis of each genotype–phenotype correlation would be required to test our hypothesis. Given that there is no standard treatment protocol for Blau syndrome/EOS, some predictors of disease progression, especially progression of visual impairment, would have great benefit for clinicians. We observed a difference in the development of visual impairment only between patients with the R334W mutation and those with the R334Q mutation, which provides a clue that predicts the development of visual impairment in patients with the R334W and R334Q mutations. We also believe that understanding the mechanisms of how *NOD2* acts in disease pathogenesis should help in discovering therapeutic targets for the treatment of Blau syndrome/EOS.

ACKNOWLEDGMENTS

We appreciate the invaluable assistance of the following physicians, who kindly provided materials and allowed us to study their patients: Drs. Sonoko Nagai, Takenosuke Yuasa, Akira Manki, Yoshihiko Sakurai, Mitsuru Nakajima, Hiroko Kobayashi, Ikuma Fujiwara, Hiroyuki Tsutsumi, Shuji Takei, Kumiko Nakao, Yoshikazu Otsubo, Kouichi Ohta, Kazunaga Agematsu, Hiroaki Azukisawa, Hiroyuki Murota, and Kenji Katamura.

AUTHOR CONTRIBUTIONS

Dr. Nishikomori had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Okafuji, Nishikomori, Heike, Miyachi, Nakahata.
Acquisition of data. Okafuji, Fujisawa, Saito, Yoshioka, Kawai, Sakai, Tanizaki.
Analysis and interpretation of data. Okafuji, Nishikomori.
Manuscript preparation. Okafuji, Nishikomori, Kanazawa, Kambe.
Statistical analysis. Yamazaki.

REFERENCES

1. Hetherington S. Sarcoidosis in young children. Am J Dis Child 1982;136:13–5.
2. Blau EB. Familial granulomatous arthritis, iritis, and rash. J Pediatr 1985;107:689–93.
3. Jabs DA, Houk JL, Bias WB, Arnett FC. Familial granulomatous synovitis, uveitis, and cranial neuropathies. Am J Med 1985;78:801–4.
4. Ohmen JD, Yang HY, Yamamoto KK, Zhao HY, Ma Y, Bentley LG, et al. Susceptibility locus for inflammatory bowel disease on chromosome 16 has a role in Crohn's disease, but not in ulcerative colitis. Hum Mol Genet 1996;5:1679–83.
5. Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Hafner R, et al. CARD15 mutations in Blau syndrome. Nat Genet 2001;29:19–20.
6. Kanazawa N, Matsushima S, Kambe N, Tachibana T, Nagai S, Miyachi Y. Presence of a sporadic case of systemic granulomatosis syndrome with a CARD15 mutation. J Invest Dermatol 2004;122: 851–2.
7. Kanazawa N, Okafuji I, Kambe N, Nishikomori R, Nakata-Hizume M, Nagai S, et al. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-κB activation: common genetic etiology with Blau syndrome. Blood 2005;105:1195–7.
8. Rose CD, Doyle TM, McIlvain-Simpson G, Coffman JE, Rosenbaum JT, Davey MP, et al. Blau syndrome mutation of CARD15/NOD2 in sporadic early onset granulomatous arthritis. J Rheumatol 2005;32:373–5.
9. Rose CD, Wouters CH, Meiorin S, Doyle TM, Davey MP, Rosenbaum JT, et al. Pediatric granulomatous arthritis: an international registry. Arthritis Rheum 2006;54:3337–44.
10. Arostegui JI, Arnal C, Merino R, Modesto C, Carballo MA, Moreno P, et al. NOD2 gene–associated pediatric granulomatous arthritis: clinical diversity, novel and recurrent mutations, and evidence of clinical improvement with interleukin-1 blockade in a Spanish cohort. Arthritis Rheum 2007;56:3805–13.
11. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003; 278:8869–72.
12. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2: implications for Crohn's disease. J Biol Chem 2003;278:5509–12.
13. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB. J Biol Chem 2001;276:4812–8.
14. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599–603.
15. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603–6.
16. Chamaillard M, Philpott D, Girardin SE, Zouali H, Lesage S, Chareyre F, et al. Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases. Proc Natl Acad Sci U S A 2003;100:3455–60.
17. Yotsumoto S, Takahashi Y, Takei S, Shimada S, Miyata K, Kanzaki T. Early onset sarcoidosis masquerading as juvenile rheumatoid arthritis. J Am Acad Dermatol 2000;43(5 Pt 2):969–71.
18. Ukae S, Tsutsumi H, Adachi N, Takahashi H, Kato F, Chiba S. Preschool sarcoidosis manifesting as juvenile rheumatoid arthritis: a case report and a review of the literature of Japanese cases. Acta Paediatr Jpn 1994;36:515–8.
19. Sakurai Y, Nakajima M, Kamisue S, Nishimura Y, Ueda T, Miyagawa S, et al. Preschool sarcoidosis mimicking juvenile rheumatoid arthritis: the significance of gallium scintigraphy and skin biopsy in the differential diagnosis. Acta Paediatr Jpn 1997;39:74–8.
20. Kurokawa T, Kikuchi T, Ohta K, Imai H, Yoshimura N. Ocular manifestations in Blau syndrome associated with a CARD15/Nod2 mutation. Ophthalmology 2003;110:2040–4.
21. Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, et al. Global data on visual impairment in the year 2002. Bull World Health Organ 2004;82:844–51.
22. North AF Jr, Fink CW, Gibson WM, Levinson JE, Schuchter SL, Howard WK, et al. Sarcoid arthritis in children. Am J Med 1970;48:449–55.
23. Lindsley CB, Petty RE. Overview and report on international registry of sarcoid arthritis in childhood. Curr Rheumatol Rep 2000;2:343–8.
24. Milman N, Andersen CB, Hansen A, van Overeem Hansen T, Nielsen FC, Fledelius H, et al. Favourable effect of TNF-α inhibitor (infliximab) on Blau syndrome in monozygotic twins with a de novo CARD15 mutation. APMIS 2006;114:912–9.
25. Rotenstein D, Gibbas DL, Majmudar B, Chastain EA. Familial granulomatous arteritis with polyarthritis of juvenile onset. N Engl J Med 1982;306:86–90.
26. Gross KR, Malleson PN, Culham G, Lirenman DS, McCormick AQ, Petty RE. Vasculopathy with renal artery stenosis in a child with sarcoidosis. J Pediatr 1986;108:724–6.
27. Rose CD, Eichenfield AH, Goldsmith DP, Athreya BH. Early onset sarcoidosis with aortitis: "juvenile systemic granulomatosis?" [published erratum appears in J Rheumatol 1990;17:575]. J Rheumatol 1990;17:102–6.
28. Becker ML, Martin TM, Doyle TM, Rose CD. Interstitial pneumonitis in Blau syndrome with documented mutation in CARD15. Arthritis Rheum 2007;56:1292–4.
29. Wolf U. Identical mutations and phenotypic variation [review]. Hum Genet 1997;100:305–21.
30. Osborne GE, Mallon E, Mayou SC. Juvenile sarcoidosis after BCG vaccination. J Am Acad Dermatol 2003;48(5 Suppl):S99–102.
31. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 2006;440:237–41.
32. Pauleau AL, Murray PJ. Role of Nod2 in the response of macrophages to Toll-like receptor agonists. Mol Cell Biol 2003; 23:7531–9.
33. Fink CW, Cimaz R. Early onset sarcoidosis: not a benign disease. J Rheumatol 1997;24:174–7.