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H：知的財産権の出願・登録状況
(予定を含む)

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

研究成果の刊行に関する一覧表

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

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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

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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 sarcoidosis*

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-

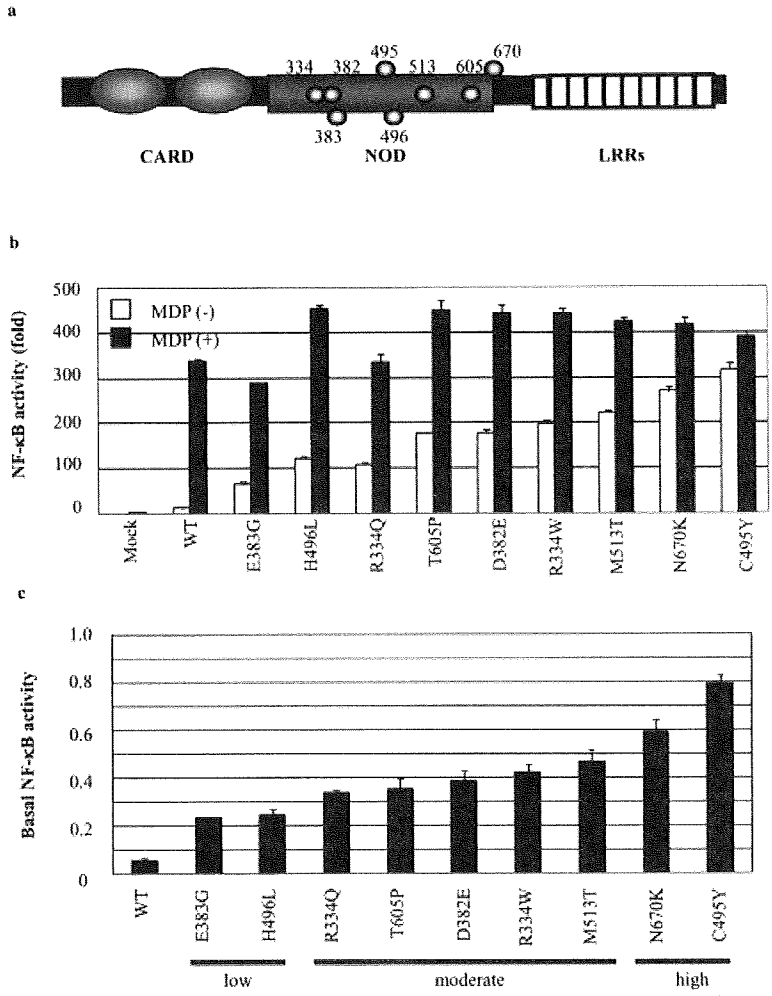

Figure 1. Biologic effects of *NOD2* mutants discovered in patients with Blau syndrome/early-onset sarcoidosis (EOS). **a,** Schematic presentation of NOD-2 protein. Numbers indicate the positions of mutated amino acid residues identified in our cohort. **b,** Increased basal NF-κB activity due to different 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

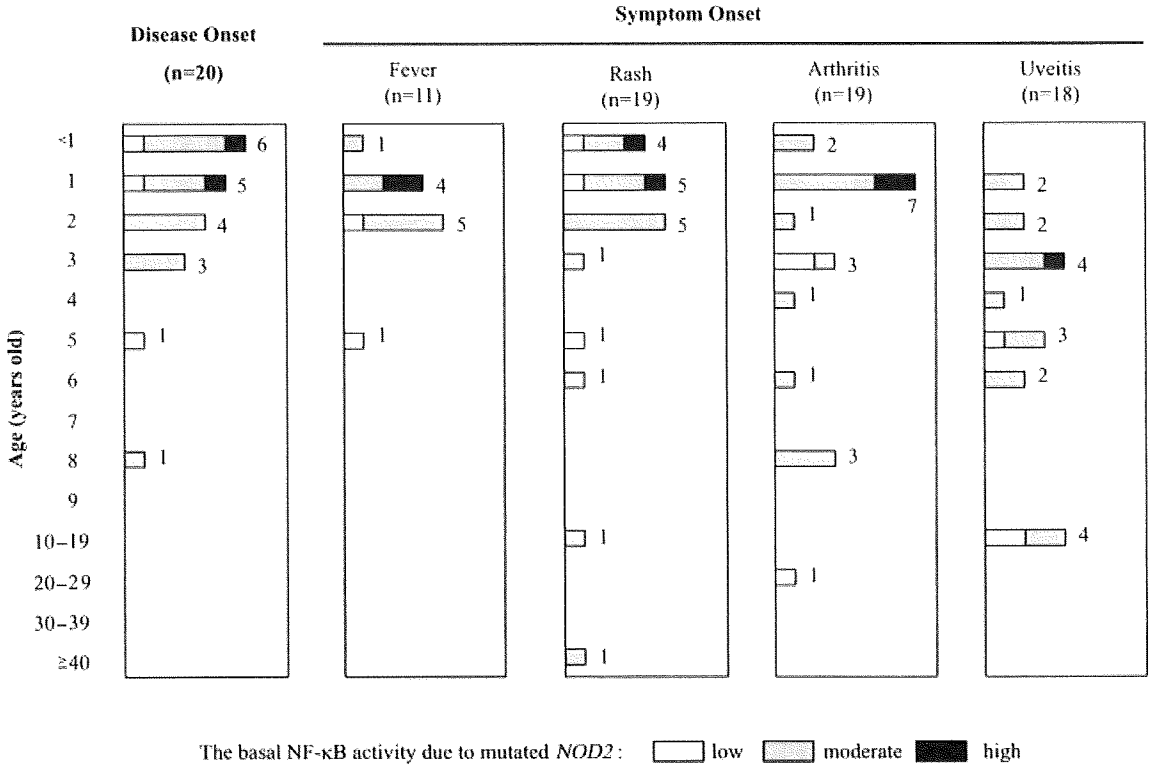

Figure 2. Relationship between age at disease or symptom onset and basal NF-κB activity due to mutated *NOD2*. Among the 9 patients without fever, 8 had moderate and 1 had low basal NF-κB activity. One patient without rash had moderate basal NF-κB activity, and 1 patient without arthritis had moderate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

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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.

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TABLE 2 Details of response to sequential treatments where applicable ($n = 10$)

No.	Severity of disease	First treatment		Second treatment		Third treatment	
1	Severe	Amlodopine	×	Nifedipine	√	–	–
2	Moderate	Amlodopine	×	GTN	×	–	–
3	Moderate	Amlodopine	×	GTN	×	–	–
4	Severe	Nifedipine	×	Amlodopine	×	–	–
5	Severe	Nifedipine	×	Amlodopine	×	GTN	√
6	Moderate	Nifedipine	×	GTN	×	–	
7	Severe	GTN	×	Amlodopine	×	Nifedipine	√
8	Moderate	Nifedipine	×	GTN	√	–	–
9	Severe	Amlodopine	×	Nifedipine	×	GTN	×
10	Moderate	Amlodopine	√	GTN	√	–	–

×: no response/inadequate response; √: response.

Overall, GTN patches were effective in 55% of the treated patients. Efficacy was better than that of nifedipine and amlodipine (33 *vs* 25% response rate, respectively), but small numbers and retrospective analysis does not allow statistical comparison. Response was similar in primary and secondary RP. Children with severe RP had a better response to nifedipine and amlodopine than children with moderate disease. The sub-group with severe disease was more likely to be using a disease-modifying drug, which may have had an impact. However, numbers are too small for any conclusion to be drawn from this.

Application of GTN patches allows removal if adverse events occur. Together with absence of tablets, this may make treatment with GTN attractive in paediatric practice. All patients received Deponit GTN patches. Alternative brands may not have adequate skin adhesion when cut into quarters for this off-license use.

GTN patches, nifedipine and amlodipine offer symptomatic relief for patients with moderate primary/secondary RP. Further studies, including head-to-head trials, are needed to determine if one agent is superior. Meanwhile, GTN patches offer an alternative to oral calcium channel blockers for symptomatic relief of paediatric RP.

Rheumatology key message

- GTN patches are an efficacious treatment option in paediatric RP.

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A case of early-onset sarcoidosis with a six-base deletion in the *NOD2* gene

SIR, We present the first case of early-onset sarcoidosis (EOS, MIM no. 609464) with a six-base deletion in the *NOD2* gene, resulting in the replacement of one amino acid and the deletion of two additional amino acids. All previous mutations reported for EOS and Blau syndrome (BS, MIM no. 186580) were single-base substitutions that resulted in the replacement of a single amino acid [1–3].

The patient was a Japanese male born after an uncomplicated pregnancy and delivery. His family had no symptoms of skin lesions, arthritis or uveitis. At 5 years of age, he was diagnosed with bilateral severe uveitis. He became blind in both eyes during adolescence. He had swollen ankles without pain during childhood,

and developed arthritis in his both knees and ankles at 15 years of age. At 30 years, a skin rash had developed on his extremities after his first BCG vaccination. The skin lesions were scaly erythematous plaques with multiple lichenoid papules and some pigmentation. At the same age, camptodactyly without obvious synovial cysts of the hands was observed, and the deformity in all fingers developed by 35 years. At 41 years, he had low-grade fever for 1 year. He had no pulmonary lesions. His laboratory investigations showed normal white blood cell count, mildly elevated CRP (1.0 mg/dl) and ESR (20 mm/h). A skin biopsy from his left forearm revealed non-caseating granulomas without lymphocyte infiltration. There were no indications of infection by *Mycobacterium*.

The clinical symptoms and pathological findings on the biopsied skin indicated that the patient suffered from EOS. It has been reported that EOS and BS have a common genetic aetiology due to mutations in the *NOD2* gene that cause constitutive Nuclear Factor (NF)-κB activation [4, 5]. Thus we analysed the *NOD2* gene from the patient to look for mutations that might correlate with the pathology of EOS. A written informed consent was obtained from the patient and his families, according to the protocol of the institutional review board of Kyoto University Hospital and in accordance with the Declaration of Helsinki. Genomic sequencing analysis of the patient's *NOD2* gene showed the presence of a heterozygous deletion of six bases in exon 4, which resulted in c.1493_1498delAACTGT, p.E498V, 499–500del (Fig. 1A). The mutation was novel and was not identified in 100 normal controls. A genome alignment of *NOD2* among several species showed that E498, L499 and L500 are conserved from zebrafish to human (Fig. 1B). These data strongly suggested that the identified deletion of six bases in the *NOD2* gene is not a single nucleotide polymorphism (SNP), but is probably responsible for EOS in the patient.

Previous studies report that *NOD2* mutations causing EOS/BS show constitutive activation of NF-κB [6–8]. Therefore, we investigated the level of NF-κB activity associated with the new mutation identified here. First, we confirmed the level of mRNA expression of the mutated allele by subcloning analysis of *NOD2*-cDNA, which showed that the mutated allele was expressed as well as the wild type allele (data not shown). We then evaluated the ability of the *NOD2* mutant to constitutively activate NF-κB by using an *in vitro* reporter system in HEK293T cells transfected with both *NOD2* mutants and NF-κB reporter plasmids (Fig. 1C). The deletion mutant demonstrated almost five times more NF-κB activity than wild type without muramyl dipeptide (MDP) stimulation. Western blot analysis confirmed that NOD2 mutant protein expression was similar to that of wild type (Fig. 1C). Thus, like other mutations of *NOD2* identified previously, the deletion mutant identified here also showed constitutive activation of NF-κB.

The mechanism underlying EOS/BS has not been totally understood, although two pathways downstream from *NOD2* have been identified: NF-κB activation through receptor-interacting protein (RIP) like interacting caspase-like apoptosis regulatory protein kinase (RICK) and MAP kinase activation through the caspase recruitment domain 9 (CARD9) [9]. We previously tested 10 *NOD2* missense mutations that have been identified in our cohort of EOS/BS patients in Japan, and all of them demonstrated constitutive activation of NF-κB [3]. By analysing this newly identified deletion mutant, we have further confirmed the importance of constitutive activation of NF-κB by mutated *NOD2* for the pathogenesis of EOS/BS. We would like to emphasize the

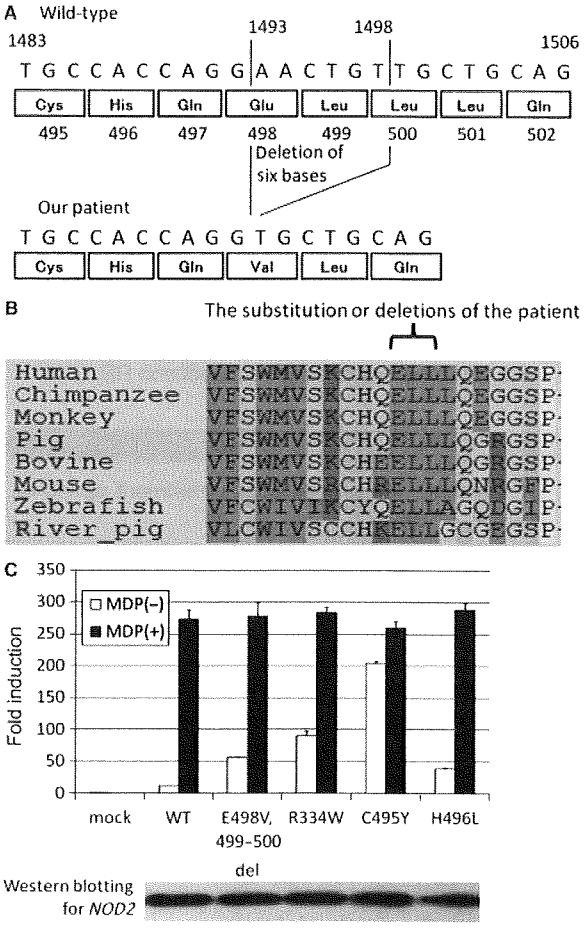

FIG. 1 (**A**) Summary of the mutations identified in our patient. (**B**) NOD2 protein alignment among different species on the mutated amino acids. (**C**) NF-κB reporter assay using the *NOD2* deletion mutant. *In vitro* NF-κB reporter assays were performed as previously described [1, 3, 6, 7]. Mock vector, wild type *NOD2* (WT) and three *NOD2* variants (R334W, C495Y, H496L) derived from EOS/BS patients, were used as controls. Values represent the mean of normalized data (mock without MDP = 1) of triplicate cultures, and error bars indicate S.D. Shown is one representative result of three independent experiments. Protein expression levels of *NOD2* mutants analysed by western blotting are shown in the bottom panel.

usefulness of the NF-κB reporter assay with mutant *NOD2* for observing its role in EOS/BS, although the MAP kinase activation pathway and other possible pathways need to be evaluated to more completely understand the pathogenesis of the *NOD2* mutation in EOS/BS.

We have identified the first deletion mutation in the *NOD2* gene responsible for EOS/BS, and the mutant showed constitutive activation of NF-κB, which is one of the key features that lead to the pathogenesis of EOS/BS.

Rheumatology key message

- A six-base deletion in *NOD2* gene causes EOS.

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Comment on: Hepatotoxicity rates do not differ in patients with rheumatoid arthritis and psoriasis treated with methotrexate

SIR, We read with interest the recent article by Amital *et al.* [1] that compared hepatotoxicity rates in PsA and RA patients treated with MTX based on the evaluation of standard liver function tests. The authors conclude that the incidence of hepatotoxicity does not differ between the two disease groups after adjusting for the cumulative dose of MTX.

Several studies in MTX-treated psoriasis patients have reported that isolated abnormalities of liver enzymes (i.e. alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase) were poor predictors of the severity of liver histopathology. The authors state that the combined sensitivity of aspartate aminotransferase, alanine aminotransferase and bilirubin for detecting an abnormal liver biopsy has been rated at 0.86 based on a previous study [2]. This figure implies that 14% of those with normal liver function tests will have undetected hepatic disease. Larger studies have suggested that 30–50% of the psoriasis patients on MTX have normal standard liver function test results despite histology showing fibrosis and cirrhosis [3]. The lack of correlation between liver enzymes and hepatic fibrosis and cirrhosis has been the major factor leading to the recommendation that liver biopsies be done to monitor potential hepatotoxicity. In this study, the liver function tests were performed with varying frequency which could allow abnormal liver function tests to be missed. The authors acknowledge that the rates of other hepatotoxic agents such as alcohol use and the occurrence of other hepatic comorbidities were not known. We believe that these are significant confounding variables, which make the interpretation of the results of this study difficult. The British Association of Dermatologists recommends serial monitoring

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Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells

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ABSTRACT **Satellite cells are myogenic stem cells responsible for the postnatal regeneration of skeletal muscle. Here we report the successful *in vitro* induction of Pax7-positive satellite-like cells from mouse embryonic stem (mES) cells. Embryoid bodies were generated from mES cells and cultured on Matrigel-coated dishes with Dulbecco's modified Eagle medium containing fetal bovine serum and horse serum. Pax7-positive satellite-like cells were enriched by fluorescence-activated cell sorting using a novel anti-satellite cell antibody, SM/C-2.6. SM/C-2.6-positive cells efficiently differentiate into skeletal muscle fibers both *in vitro* and *in vivo*. Furthermore, the cells demonstrate satellite cell characteristics such as extensive self-renewal capacity in subsequent muscle injury model, long-term engraftment up to 24 wk, and the ability to be secondarily transplanted with remarkably high engraftment efficiency compared to myoblast transplantation. This is the first report of transplantable, functional satellite-like cells derived from mES cells and will provide a foundation for new therapies for degenerative muscle disorders.—Chang, H., Yoshimoto, M., Umeda, K., Iwasa, T., Mizuno, Y., Fukada, S., Yamamoto, H., Motohashi, N., Yuko-Miyagoe-Suzuki, Takeda, S., Heike, T., Nakahata, T. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. *FASEB J.* 23, 1907–1919 (2009)**

Key Words: long-term engraftment · secondary transplantation · high engraftment efficiency · self-renewal

DUCHENNE MUSCULAR DYSTROPHY (DMD; ref. 1) is a progressive, lethal muscular disorder (2) with no effective cure despite extensive research efforts. DMD results from mutations in the X-linked *dystrophin* gene (3). Dystrophin and its associated proteins function to link the intracellular actin cytoskeleton of muscle fibers to laminin in the extracellular matrix (4), thereby protecting myofibers from contraction-induced damage (5). Skeletal muscle fibers are continuously regenerated following exercise and injuries when satellite cells (6) are induced to differentiate into myoblasts that form myotubes and replace the damaged myofibers (7, 8). This muscular regeneration is observed at a much higher frequency in DMD patients (9). Continuous damage to myofibers and constant activation of resident satellite cells due to loss of dystrophin leads to the exhaustion of the satellite cells (10, 11), and the eventual depletion of satellite cells is primarily responsible for the onset of DMD symptoms.

Successful transplantation of normal satellite cells into the skeletal muscle of DMD patients may enable *in situ* production of normal muscle tissue and create a treatment option for this otherwise fatal disease. A recent report has shown that the transplantation of satellite cells collected from mouse muscle tissues can produce muscle fibers with normal dystrophin expression in mdx mice (12-14), a model mouse for DMD (15). This study suggests that stem cell transplantation may be a viable therapeutic approach for the treatment of DMD (16).

Satellite cells are monopotent stem cells that have the ability to self-renew and to differentiate into myoblasts and myotubes to maintain the integrity of skeletal muscle (17). Satellite cells lie dormant beneath the basal lamina and express transcription factors such as Pax3 (13, 18) and Pax7 (19). Pax7, a paired box transcription factor, is particularly important for satellite cell function. A recent study of *Pax7-null* mice revealed that Pax7 is essential for satellite cell formation (19) and that the *Pax7-null* mice exhibit a severe deficiency in muscle fibers at birth and premature mortality with complete depletion of the satellite cells. Surface markers such as M-cadherin and c-met (20) are also expressed by satellite cells. However, these markers are not specific to satellite cells because they are also expressed in the cerebellum (21) and by hepatocytes (22). To specifically identify quiescent satellite cells, a

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novel monoclonal antibody, SM/C-2.6, has recently been established (23). Satellite cells purified with this antibody regenerate muscle fibers on implantation into mdx mice (15).

The use of satellite cells for clinical therapies would require the establishment of a reliable source of these cells. Embryonic stem (ES) cells are totipotent stem cells that are able to differentiate into various types of somatic cells *in vitro*. While mouse embryonic stem (mES) cells can be readily induced to differentiate into muscle fibers (24, 25) and the myogenicity of human ES cells was recently validated (26), the induction of mES cells into functional satellite cells has not been reported. Here we have successfully induced mES cells to generate cells expressing Pax7 *in vitro* by forming embryoid bodies (EBs). These ES cell-derived (ES-derived) Pax7-positive cells can be enriched using the SM/C-2.6 antibody (23) and possess a great potential for generating mature skeletal muscle fibers both *in vitro* and *in vivo*. The Pax7-positive cells display a self-renewal ability that can repopulate Pax7-positive cells *in vivo* in the recipient muscles following an injury. Furthermore, these ES-derived Pax7-positive cells could engraft in the recipient muscle for long periods, up to 24 wk, and could also be serially transplanted. These results indicate that ES-derived Pax7-positive cells possess satellite cell characteristics. This is the first report of effective induction of functional satellite cells from mES cells, and these novel findings may provide a new therapeutic approach for treatment of DMD.

MATERIALS AND METHODS

Cell culture

D3 cells, mES cells (27) that ubiquitously express the *EGFP* gene under the *CAG* promoter (28) (a gift from Dr. Masaru Okabe, Osaka University, Osaka, Japan), were used in this study. ES cells were maintained on tissue culture dishes (Falcon) coated with 0.1% gelatin (Sigma, Oakville, CA, USA), in DMEM (Sigma) supplemented with 15% fetal bovine serum (FBS; Thermo Trace, Melbourne, Australia), 0.1 mM 2-mercaptoethanol (Nakalai Tesque, Japan), 0.1 mM nonessential amino acids (Invitrogen, Burlington, CA, USA), 1 mM sodium pyruvate (Sigma), penicillin/streptomycin (50 μg/mL), and 5000 U/ml leukemia inhibitory factor (Dainippon Pharmaceutical Co., Japan).

In vitro differentiation of ES cells into a muscle lineage

To induce EB formation, undifferentiated ES cells were cultured in hanging drops for 3 d at a density of 800 cells/20 μl of differentiation medium, which consisted of DMEM supplemented with penicillin/streptomycin, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptonethanol, 5% horse serum (HS), and 10% FBS. EBs were transferred to suspension cultures for an additional 3 d (d 3+3). Finally, the EBs were plated in differentiation medium in 48-well plates (Falcon) coated with Matrigel (BD Bioscience, Bedford, MA, USA). The medium was changed every 5 d.

Immunofluorescence and immunocytochemical analysis

Immunostaining of cultured cells and recipient mouse tissues were carried out as described previously (29). Briefly, the left tibialis anterior (LTA) muscle of the recipient mouse was fixed with 4% paraformaldehyde and cut into 6 μm cross sections using a cryostat, and samples were fixed for 5 min in 4% paraformaldehyde (PFA) in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After incubation in 5% skim milk for 10 min at room temperature to block nonspecific antibody binding, cells were incubated for 12 h at 4°C with anti-mouse monoclonal antibodies. Antibodies used in this study were mouse anti-Pax7, which was biotinylated using a DSB-X Biotin Protein Labeling Kit (D20655; Molecular Probes, Eugene, OR, USA), mouse anti-Pax3 (MAB1675, MAB2457; R&D Systems, Minneapolis, MN, USA), rabbit anti-mouse Myf5 (sc-302; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-mouse M-cadherin (205610; Calbiochem, San Diego, CA, USA), mouse anti-myosin heavy chain (MHC; 18-0105; Zymed Laboratories, San Francisco, CA, USA; reacts with human, rabbit, rat, mouse, bovine, and pig skeletal MHC), mouse anti-mouse myogenin and mouse anti-mouse Myo-D1 (M3559, M3512; Dako, Carpinteria, CA, USA), monoclonal rabbit anti-mouse laminin (LB-1013; LSL, Tokyo, Japan), and mouse anti-mouse dystrophin (NCL-DYS2; Novocastra Laboratories, Newcastle-upon-Tyne, UK). Cy3-labeled antibodies to mouse or rabbit IgG, fluorescein isothiocyanate-labeled antibodies to mouse or rabbit IgG (715-005-150, 711-165-152; Jackson ImmunoResearch Laboratory, Bar Harbor, ME, USA), or Alexa 633-labeled goat anti-rabbit IgG (A21070; Invitrogen, Molecular Probes) were applied as secondary antibodies. Hoechst 33324 (H3570; Molecular Probes) was used for nuclear staining. The samples were examined with a fluorescence microscope (Olympus, Tokyo, Japan) or an AS-MDW system (Leica Microsystems, Wetzlar, Germany). Micrographs were obtained using an AxioCam (Carl Zeiss Vision, Hallbergmoos, Germany) or the AS-MDW system (Leica Microsystems). In sections of muscles transplanted with ES-derived satellite cells, the number of GFP-positive muscle fascicles and GFP/Pax7-double-positive cells were counted, per field, at ×100. More than 10 fields in each tissue sample were observed. To prevent nonspecific secondary antibody binding to Fc receptors, all immunostaining of frozen sections used the Vector® M.O.M™ Immunodetection Kit (BMK-2202; Vector Laboratories, Burlingame, CA, USA).

PCR analysis

Total RNA was isolated from cultured cells in 48-well plates, using TRIzol reagent (Invitrogen). The following specific primers were used for PCR:

Pax3, sense, 5′-AACACTGGCCCTCAGTGAGTTCTAT-3′, and antisense, 5′-ACTCAGGATGCCATCGATGCTGTG-3′; Pax7, sense, 5′-CATCCAGTGCTGGTACCCCACAG-3′, and antisense, 5′-CTGTGGATGTCACCTGCTTGAA-3′; Myf5, sense, 5′-GAGCTGCTGAGGGAACAGGTGG-3′, and antisense, 5′-GTTCTTTCGGGACCAGACAGGG-3′; MyoD, sense, 5′-AGGCTCTGCTGCGCGACCAG-3′, and antisense, 5′-TGCAGTCGATCTCTCAAAGC-3′; myogenin, sense, 5′-TGAGGGAGAAGCGCAGGCTCAAG-3′, and antisense, 5′-ATGCTGTCCACGATGGACGTAAGG-3′; M-cadherin, sense, 5′-CCACAAACGCCTCCCCTACCC-3′, and antisense, 5′-GTCGATGCTGAAGAACTCAGGGC-3′; C-met, sense, 5′-GAATGTCGTCCTACACGGCCAT-3′, and antisense, 5′-CACTACACAGTCAGGACACTGC-3′; GAPDH, sense, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, and antisense, 5′-TGTTGGGGGCCGAGTTGGGATA-3′. AmpliTaqGold (Applied