

Frequent somatic mosaicism of *NEMO* in T cells of patients with X-linked anhidrotic ectodermal dysplasia with immunodeficiency

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Somatic mosaicism has been described in several primary immunodeficiency diseases and causes modified phenotypes in affected patients. X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is caused by hypomorphic mutations in the *NF-κB essential modulator (NEMO)* gene and manifests clinically in various ways. We have previ-

ously reported a case of XL-EDA-ID with somatic mosaicism caused by a duplication mutation of the *NEMO* gene, but the frequency of somatic mosaicism of *NEMO* and its clinical impact on XL-EDA-ID is not fully understood. In this study, somatic mosaicism of *NEMO* was evaluated in XL-EDA-ID patients in Japan. Cells expressing wild-type *NEMO*, most of

which were derived from the T-cell lineage, were detected in 9 of 10 XL-EDA-ID patients. These data indicate that the frequency of somatic mosaicism of *NEMO* is high in XL-EDA-ID patients and that the presence of somatic mosaicism of *NEMO* could have an impact on the diagnosis and treatment of XL-EDA-ID patients. (*Blood*. 2012;119(23):5458-5466)

Introduction

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is a disease with clinical features including hypohidrosis, delayed eruption of teeth, coarse hair, and immunodeficiency associated with frequent bacterial infections.¹⁻⁵ The gene responsible for XL-EDA-ID has been identified as *NF-κB essential modulator (NEMO)*.⁶⁻⁸ *NEMO* is necessary for the function of IκB kinase, which phosphorylates and degrades IκB to activate NF-κB.⁹⁻¹⁰ Defects in *NEMO* cause various abnormalities in signal transduction pathways involving NF-κB, and affect factors such as the IL-1 family protein receptors, the TLRs, VEGFR-3, receptor activator of nuclear factor κB (RANK), the ectodysplasin-A receptor, CD40, and the TNF receptor I.⁷ Whereas a complete loss of *NEMO* function in humans is believed to cause embryonic lethality,¹¹ *NEMO* mutations in XL-EDA-ID patients are hypomorphic,⁸ causing a partial loss of *NEMO* functions.

In XL-EDA-ID, *NEMO* defects lead to diverse immunologic features including susceptibility to pathogens, impaired Ab response to polysaccharides,^{2,4,12} hypogammaglobulinemia,¹³ hyper IgM syndrome,¹⁴ and impaired NK-cell activity,¹⁵ with a large degree of variability in phenotypes among the patients. For example, approximately one-tenth of XL-EDA-ID patients exhibit reduced mitogen-induced proliferation of T lymphocytes.¹² Moreover, one-fourth suffer from inflammatory disor-

ders such as inflammatory bowel disease and rheumatoid arthritis,¹² although the inflammatory process usually relies on NF-κB activation.¹⁶ One explanation for this clinical variability is that the XL-EDA-ID phenotype is *NEMO* genotype-specific. Although the XL-EDA-ID database reported by Hanson et al succeeds to some extent in linking the specific clinical features to *NEMO* genotype,¹² the penetrance of some clinical features is not high and the mechanism accounting for this variability is unknown.

Recently, we have reported a case of spontaneous reversion mosaicism of the *NEMO* gene in XL-EDA-ID, which showed an atypical phenotype involving decreased mitogen-induced T-cell proliferation along with decreased CD4 T cells (patient 1).¹⁷ There have been no subsequent reports on somatic mosaicism in XL-EDA-ID, and its prevalence and impact on the clinical features of the disease is unknown. In this study, we describe the younger brother of patient 1, who suffered from XL-EDA-ID with the same mutation and somatic reversion mosaicism of *NEMO*. Patient 2 showed intriguing laboratory findings in that mitogen-induced T-cell proliferation varied in accordance with the rate of detected reversion in the peripheral blood. These 2 cases led us to perform a nationwide study of XL-EDA-ID patients in Japan that revealed a high incidence of somatic mosaicism of *NEMO*.

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Table 1. Clinical and genetic features of XL-EDA-ID patients

Patient	Mutation	Ectodermal dysplasia	Mitogen-induced proliferation	Infections	Complications	Therapy	Sex chromosome chimerism
1	Duplication	+	Reduced	Sepsis (S.P. and P.A.)	Chronic diarrhea	IVIG	100% XY
				Disseminated M.A.C.	Failure to thrive	RFP, CAM, AMK, EB	
				Skin abscess (S.A.)	Small intestinal stenosis	Rifabutin	
				Invasive <i>Aspergillus</i>	Lymphedema		
2	Duplication	+	Reduced	Sepsis (<i>E coli</i>)	Failure to thrive	IVIG, ST, EB, CAM	99.8% XY 0.2% X
				Disseminated M.S.		Rifabutin, SCT	
3	D311E	–	Normal	Disseminated B.C.G.		IVIG, INH	100% XY
				Sepsis (S.P.)		RFP, SCT	
4	A169P	+	Normal	Meningitis (S.P.)	IBD	IVIG, ST, PSL	99% XY
					Interstitial pneumonia	CyA, MTX, Infliximab	
					Rheumatoid arthritis		
5	L227P	+	Normal	Recurrent pneumonia	IBD	ST, mesalazine	Not done
				Pyogenic coxitis		Infliximab	
				Recurrent otitis media			
6	R182P	+	Not done	Recurrent otitis media	IBD	ST, mesalazine	99.8% XY 0.2% X
				UTI, Recurrent stomatitis			
				Subepidermal abscess			
7	R175P	+	Normal	Recurrent sepsis (S.P.)		IVIG	100% XY
8	Q348X	+	Normal	Disseminated B.C.G.	IBD	IVIG, ST	100% XY
9	R175P	+	Normal	Recurrent pneumonia	IBD	IVIG	100% XY
				Recurrent otitis media		5-aminosalicylic acid	
				Kaposi varicelliform eruption			
10	1167 ins C	+	Normal	Sepsis and Enteritis (E.A)	Failure to thrive	IVIG, SCT	Not done
				Sepsis (C.G.)	Pyloric stenosis, colon polyps		
				UTI (K.P.)			

S.P. indicates *Streptococcus pneumoniae*; P.A., *Pseudomonas aeruginosa*; IVIG, intravenous immunoglobulin infusion; M.A.C., *Mycobacterium avium* complex; S.A., *Staphylococcus aureus*; *E coli*, *Escherichia coli*; ST, trimethoprim-sulfamethoxazole; M.S., *Mycobacterium szulgai*; AMK, amikacin; EB, ethambutol; CAM, clarithromycin; SCT, stem cell transplantation; B.C.G., Bacille de Calmette et Guerin; INH, isoniazid; RFP, rifampicin; IBD, inflammatory bowel disease; PSL, prednisolone; CyA, cyclosporine A; MTX, methotrexate; UTI, urinary tract infection; E.A., *Enterobacter aerogenes*; C.G., *Candida glabrata*; and K.P., *Klebsiella pneumoniae*.

Methods

Informed consent

Informed consent was obtained from the patients and their families following the Declaration of Helsinki according to the protocol of the Internal Review Board of Kyoto University, which approved this study.

Patients

Patient 1 was an XL-EDA-ID patient with a duplication mutation of the *NEMO* gene spanning intron 3 to exon 6. This patient has been reported previously¹⁷ and died from an *Aspergillus* infection at the age of 4. Patient 2, born at term, was the younger brother of patient 1. This patient was also diagnosed as XL-EDA-ID with the same duplication mutation as patient 1 by genetic study. He received trimethoprim-sulfamethoxazole prophylaxis and a monthly infusion of immunoglobulin from the age of 1 month. The patient maintained good health and had a body weight of 7899g at 6 months when he started to fail to thrive. Except for poor weight gain, patient 2 appeared active with a good appetite, negative C-reactive protein, normal white blood cell counts, and no apparent symptoms. At 19 months of age, *Mycobacterium szulgai* was detected by venous blood culture, and the patient was treated with multidrug regimens including ethambutol, rifabutin, and clarithromycin based on the treatment of systemic *Mycobacterium avium* complex infection. The patient responded well to the treatment and his weight increased from 7830g to 9165g within a month after the treatment was initiated. Patient 2 received an unrelated cord blood cell transplantation at 26 months of age, containing 8.5×10^7 nucleated cells/kg (4.4×10^5 CD34⁺ cells/kg), which was matched at 5 of 8 loci: mismatches occurred at 1 HLA-B and 1 HLA-C allele (according to serology), and at 1 HLA-A, 1 HLA-B, and 1 HLA-C allele (according to DNA typing). The preconditioning regimen consisted of fludarabine (30 mg/m²/d) on days –7 to –3, melphalan (70 mg/m²/d) on days –6 to –5, and rabbit anti-thymocyte globulin (2.5 mg/kg/d) on days –6 to –2. At

first, Tacrolimus (0.024 mg/kg/d) was used to prevent GVHD, but this was switched to cyclosporin A (3 mg/kg/d) on day 9 because of drug-induced encephalopathy. Neutrophil ($> 0.5 \times 10^9/L$) and platelet ($> 50 \times 10^9/L$) engraftment were examined on days 13 and 40, respectively. Although CD19⁺ cells (2042/μL, 94% donor chimerism), CD56⁺ cells (242/μL, 97% donor chimerism), and monocytes (557/μL, 69% donor chimerism) were successfully generated, CD3⁺ cells were not detected in the peripheral blood by day 54. The patient suffered from septic shock and died on day 60. Patients 3 to 10 were XL-EDA-ID patients recruited nationwide in Japan. Clinical details of patients 3, 4, and 10 have been reported previously.^{18–20} These patients had clinical phenotypes characteristic of XL-EDA-ID such as ectodermal dysplasia, innate and/or acquired immunity defects, and susceptibility to pyogenic bacteria and *Mycobacterium* infection. Every patient had a mutation in the *NEMO* gene that caused reduced NF-κB activation in a *NEMO* reconstitution assay, as described in “Proliferation of *NEMO*^{normal} and *NEMO*^{low} T cells.” Patient profiles are listed in Table 1.

Flow cytometric analysis

NEMO intracellular staining was performed as previously described.¹⁷ The cells were stained for the following lineage markers before staining for *NEMO*: CD4, CD8, CD14, CD15, CD19, CD56, CD45RA (BD Biosciences/BD Pharmingen), and CCR7 (R&D Systems Inc). Intracellular staining of human IFN-γ, TNF-α, and *NEMO* was performed as previously described.¹⁸ The stained cells were collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the FlowJo software (TreeStar).

Reporter assay

Wild-type and mutant *NEMO* cDNAs were generated from a healthy volunteer and the recruited XL-EDA-ID patients by RT-PCR; the cDNAs were subcloned into the p3xFLAG-CMV14 vector (Sigma-Aldrich). *NEMO* null rat fibroblast cells (kindly provided by Dr S. Yamaoka, Department of Molecular Virology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan) were plated at a density of

3×10^4 cells/well in a 24-well culture dish and were transfected with 40 ng of NF- κ B reporter plasmid (pNF- κ B-Luc; BD Biosciences/BD Clontech), 2 ng of *NEMO* mutant expression construct, 10 ng of internal control for the normalization of transfection efficiency (pRL-TK; Toyo Ink), and 148 ng of mock vector using FuGENE HD Transfection Reagent (TOYO-B-Net) according to the manufacturer's protocol. Twelve hours after transfection, the cells were stimulated with 15 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich) for 4 hours and the NF- κ B activity was measured using the PicaGene Dual SeaPansy assay kit (TOYO-B-Net). Experiments were performed in triplicate and firefly luciferase activity was normalized to *Renilla* luciferase activity.

Subcloning analysis of cDNA

Cell sorting of the various cell lineages was performed by FACS Vantage (BD Biosciences). The purity of each lineage was $> 95\%$. The cDNA from sorted cells was purified and reverse transcribed by Super Script III (Invitrogen) with random hexamers and amplified by the proofreading PCR enzyme KOD, as previously described.^{17,21} The PCR primers used were NEMO2 (5'-AGAGACGAAGGAGCACAAAGCTGCCTTGAG-3') and NEMO3 (5'-ACTGCAGGGACAATGGTGGGTGCATCTGTC-3'). The PCR products were subcloned using a TA cloning kit (Invitrogen) and sequenced by ABI 3130xl Genetic analyzer (Applied Biosystems). To determine whether additional mutations occurred in revertant subclones that had wild-type sequence in the original mutation site, the entire coding region of the *NEMO* gene was sequenced and an additional mutation was considered present when the same mutation was detected in multiple subclones.

Allele-specific PCR

The mRNA purified from sorted T cells and monocytes was reverse-transcribed by SuperScript III (Invitrogen) with the gene-specific primer NEMO2 and amplified by the proofreading PCR enzyme KOD (Toyobo) using the primers NEMO3 and NEMO 4 (5'-TGTGGACACGCAGT-GAAACGTGGTCTGGAG-3'). The PCR products were used as templates for allele-specific PCRs with Ex Taq polymerase (Takara Bio). Mutant and wild-type *NEMO* DNA was generated from each *NEMO* expression plasmid, mixed at graded ratios, and used as controls. PCR conditions and primer sequences are listed in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Proliferation of NEMO^{normal} and NEMO^{low} T cells

To obtain PHA-induced T-cell blasts, PBMCs were stimulated with PHA (1:100; Invitrogen) and cultured in RPMI 1640 supplemented with 5% FCS and recombinant human IL-2 (50 IU/mL; kindly provided by Takeda Pharmaceutical Company) at 37°C for 7 days. Subcloning analysis of the cDNA obtained from the T-cell blasts was performed as described in "Subcloning analysis of cDNA."

Results

Reversion mosaicism of NEMO occurred in siblings with similar immunologic phenotypes

We previously reported patient 1 with a duplication mutation of the *NEMO* gene spanning intron 3 to exon 6, who was diagnosed as XL-EDA-ID at 1 year of age after suffering from recurrent infections.¹⁷ At first, genetic diagnosis of the patient was difficult because the expression of aberrant *NEMO* mRNA was masked by the expression of normal *NEMO* mRNA by the revertant cells. Flow cytometric analysis of intracellular *NEMO* expression revealed cells with normal (NEMO^{normal}) and reduced (NEMO^{low}) levels of *NEMO* expression, indicating the presence of reversion mosaicism of the *NEMO* gene, and further analysis revealed that

the *NEMO* mutation was disease-causing. PCR across the mutated region and sequencing of the PCR products revealed a duplication extending from intron 3 to exon 6, which was confirmed by Southern blot analysis. Additional copy number analysis of the *NEMO* gene of patient 1 and his mother excluded the possibility of a complex chromosomal aberration such as multiple duplication of the *NEMO* gene (supplemental Figure 1). Furthermore, polymorphism analysis using variable number tandem repeats on NEMO^{normal} and NEMO^{low} cells from patient 1 revealed that these cells were derived from the same origin (supplemental Table 2), indicating that the *NEMO* gene mosaicism was less likely because of amalgamation. The genomic analysis of the NEMO^{normal} cells revealed a complete reversion of the *NEMO* gene with no additional mutations. The clinical phenotype of patient 1 was combined immunodeficiency with a reduced number of T cells and mitogen-induced proliferation (Tables 2-3). We previously determined that reduced *NEMO* expression in the mutant T cells caused impairment of T-cell development and mitogen-induced proliferation.

Patient 2, the younger brother of patient 1, was diagnosed as XL-EDA-ID with the same duplication mutation as his brother. Flow cytometric analysis of intracellular *NEMO* expression performed at diagnosis showed that most of his PBMCs had reduced *NEMO* expression (Figure 1A). At 2 months of age, when most of the T cells were NEMO^{low}, absolute counts of the patient's T cells and the mitogen-induced proliferation of the patient's PBMCs were comparable with those of the healthy controls (Figure 1A-B; Table 2). These findings indicated that the *NEMO* mutation had no effect on T-cell development and mitogen-induced proliferation during early infancy in patient 2.

NEMO^{normal} T cells gradually increased as patient 2 grew older, while the absolute count of NEMO^{low} T cells decreased (Figure 1A-B). Accordingly, normal full-length *NEMO* cDNA, which had been undetectable in cord blood, was detectable in the patient's peripheral blood at 12 months of age. However, while NEMO^{normal} T cells were increasing, mitogen-induced T-cell proliferation started to decrease (Table 3), and the patient started to show poor weight gain from 6 months of age. When patient 2 was 17 months old, a blood culture revealed an *M szulgai* bacteremia. At this time, the absolute count of NEMO^{normal} T cells peaked, and NEMO^{low} T cells were at a minimum. He began to gain weight after anti-*Mycobacterium* medication was initiated, although NEMO^{normal} T cells started to decrease and NEMO^{low} T cells began to increase (Figure 1B). When the patient was 23 months old, mitogen-induced T-cell proliferation was still low and a roughly equal number of NEMO^{low} and NEMO^{normal} T cells were detected (Table 3). Overall, as patient 2 grew older, NEMO^{normal} T cells increased as the total number of T cells and the mitogen-induced T-cell proliferation decreased, similar to what had occurred in patient 1 at a similar age.

Various analyses were performed to compare the immunologic phenotype of NEMO^{low} and NEMO^{normal} T cells in detail. Both NEMO^{normal} and NEMO^{low} CD4⁺ T cells carried a diverse V β repertoire, but CD8⁺ T cells had a skewed V β repertoire regardless of *NEMO* expression level (Figure 1C). Surface marker analysis revealed that most of the NEMO^{normal} T cells were CD45RA⁻/CCR7⁻ and most of the NEMO^{low} T cells were CD45RA⁺/CCR7⁺ (Figure 1D). The NEMO^{normal} T cells produced similar amounts of IFN- γ and TNF- α as healthy control cells, while the production of these cytokines were reduced in NEMO^{low} T cells (Figure 1E-F). Taken together, these data implied that the immunologic phenotype of T cells from patient 2 converged with that of patient 1 as patient 2 grew older.

Table 2. Surface marker analysis of peripheral mononuclear cells of patients 1 and 2

	Patient 1	Patient 2	Healthy controls
Age at analysis	2 y	2 mo	19 mo
CD3	1503	2366	1014
CD4	292	1583	374
CD8	1160	783	547
TCR $\alpha\beta$	1386	2295	439
TCR $\gamma\delta$	109	74	574
CD4 ⁺ CD45RA	58	1336	105
CD4 ⁺ CD45RO	263	307	266
CD8 ⁺ CD45RA	1178	783	297
CD8 ⁺ CD45RO	361	21	250
CD4 ⁺ CD25	80	427	93
CD19	1200	941	1543
CD20	1189	931	1536
CD19 ⁺ Sm-IgG	7	18	17
CD19 ⁺ Sm-IgA	15	4	14
CD19 ⁺ Sm-IgM	1171	910	1505
CD19 ⁺ Sm-IgD	1171	906	1495
CD16	912	176	24
CD56	908	176	24

Surface markers expressed by XL-EDA-ID patients' PBMCs are shown as absolute counts per microliter of peripheral blood. Healthy control values are based on children aged 1 to 6 years and are shown as the mean \pm SD. Sm indicates the surface membrane.

High incidence of somatic mosaicism of the *NEMO* gene in XL-EDA-ID patients

It is worth noting that somatic reversion mosaicism of the *NEMO* gene occurred in both of the 2 XL-EDA-ID siblings carrying a duplication mutation. To determine whether a high frequency of reversion is a specific event for this type of *NEMO* duplication mutation²²⁻²⁵ or if the reversion of the *NEMO* gene occurs commonly in XL-EDA-ID patients, we recruited an additional 8 XL-EDA-ID patients from throughout Japan (Table 1) and analyzed the presence of *NEMO* reversion. These patients had various combinations of clinical phenotypes characteristic of XL-EDA-ID such as ectodermal dysplasia, innate and acquired immunity defects, and susceptibility to pyogenic bacteria and *Mycobacterium* infections. Every patient had a mutation of the *NEMO* gene with reduced NF- κ B activation potential, as evaluated in a *NEMO* reconstitution assay (Figure 2).

Among the 8 patients, only patient 3 had a large proportion of *NEMO*^{low} cells by flow cytometric analysis. The majority of patient 3's PBMCs were *NEMO*^{low}, whereas 10% of the patient's CD8⁺ cells were *NEMO*^{normal} (Figure 3A). This patient was identified as carrying the D311E mutation. Because missense mutations of the *NEMO* gene often do not result in the reduced expression of *NEMO* protein, subcloning and sequencing analysis was performed on the *NEMO* cDNA isolated from the remaining patients,

and 6 of the 7 patients had normal *NEMO* subclones (Table 3). Expansion of maternal cells after fetomaternal transfusion was ruled out in these patients by FISH analysis with X and Y probes (Table 1).

Additional genetic analysis of the entire coding region of the *NEMO* gene was performed on *NEMO*^{normal} cells from patient 3 and on reverted subclones from the other patients, except for patient 10 who had already received stem cell transplantation. The *NEMO* gene in these samples had reverted to wild-type with no additional mutations (Figure 3B and data not shown). To specifically determine in which cell lineages the reversion occurred, subcloning and sequencing analysis of cDNA in various cell lineages was performed. This analysis revealed that all the revertant cells were of the T-cell lineage and that no reversion occurred in monocytes and very little occurred in B cells (Table 4). Allele-specific PCR confirmed that reversion occurred in T cells but not in monocytes (Figure 4).

Selective advantage of *NEMO*^{normal} cells in XL-EDA-ID carriers

The high frequency of somatic mosaicism in T cells of XL-EDA-ID patients indicated a strong selective advantage of wild-type *NEMO* T cells over T cells carrying mutant *NEMO*. To confirm this hypothesis, *NEMO* cDNA analysis was performed on various cell lineages from the mothers of the patients who are heterozygous for *NEMO* mutation and thus have mosaicism

Table 3. Immunologic analysis of patients 1 and 2

	Patient 1	Patient 2 (treated with IVIG)	
Age at analysis, mo	9	9	20
Serum immunoglobulin levels, g/L (control)			
IgG	10.63 (4.51-10.46)	8.44 (4.51-10.46)	10.37 (7.15-9.07)
IgA	1.36 (0.14-0.64)	1.88 (0.14-0.64)	3.93 (0.22-1.44)
IgM	0.4 (0.33-1.00)	0.17 (0.33-1.00)	0.20 (0.34-1.28)
Age at analysis	2 y	2 mo	23 mo
T-cell proliferation, SI (control)	9.3 (206.9 \pm 142.5)	55.3 (64.8 \pm 8.1)	7.2 (89.4 \pm 31.2)

Control values of serum immunoglobulin levels are based on children aged either 7 to 9 months or 1 to 2 years and are shown as the mean \pm SD. The T-cell proliferation assay was performed as described previously¹⁷ with at least three healthy adults as controls. SI indicates stimulation index; and IVIG, 2.5 g of monthly IV immune globulin infusion.

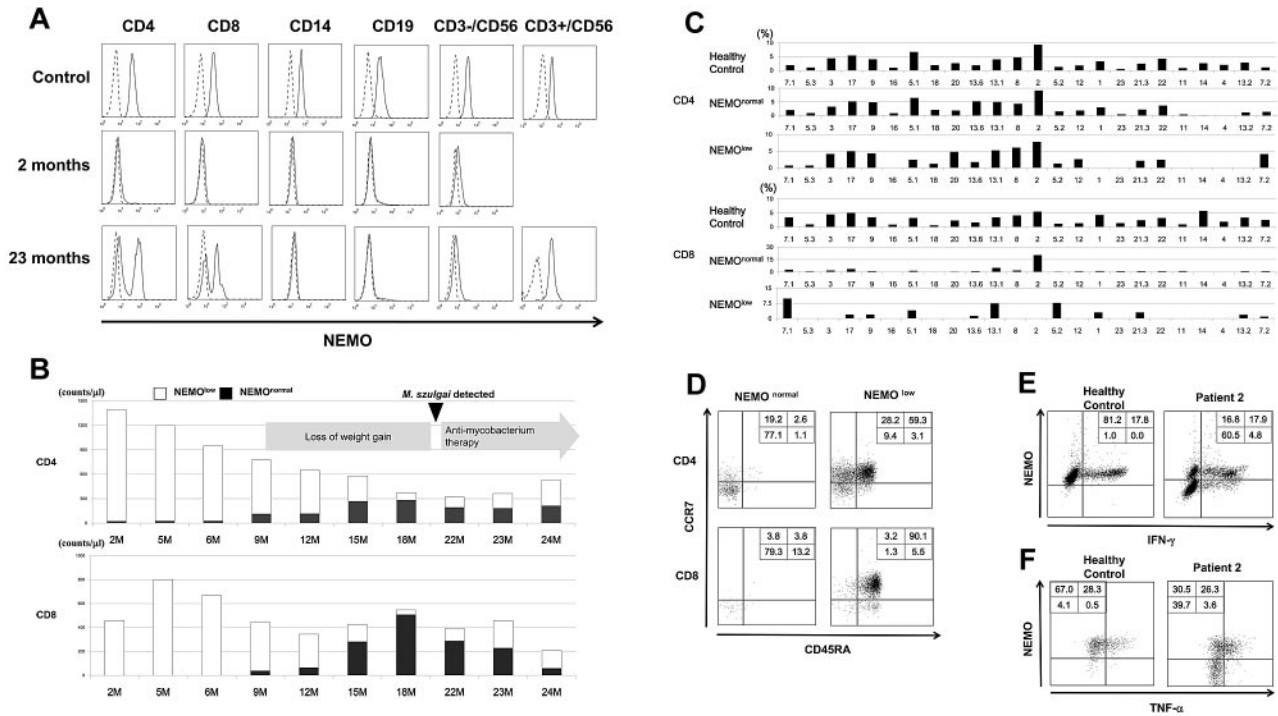


Figure 1. Identification and characterization of *NEMO* revertant T cells in patient 2. (A) Intracellular expression of *NEMO* in various PBMC lineages from a healthy adult control and patient 2 were evaluated by flow cytometry. For the patient, results of the analyses performed at 2 months and 23 months are shown. Solid lines indicate staining with the anti-*NEMO* mAb, and dotted lines indicate the isotype control. (B) Time-course variations in the absolute count of *NEMO*^{normal} and *NEMO*^{low} T cells in patient 2. M indicates age in months. (C) TCR-V β repertoire analysis of the patient's CD4⁺ and CD8⁺ T cells. PBMCs from the patient and a healthy adult control were stained for the TCR-V β panel, CD4, CD8, and *NEMO*, and analyzed by flow cytometry. (D) Phenotype analysis of T cells in patient 2. PBMCs from the patient and a control were stained for the expression of *NEMO*, CCR7, CD45RA, and CD4 or CD8. Data shown were gated on *NEMO*^{normal} or *NEMO*^{low} CD4⁺ or CD8⁺ cells. (E-F) Cytokine production from *NEMO*^{normal} and *NEMO*^{low} T cells. PBMCs from the patient and a control were stimulated with PMA and ionomycin for 6 hours and stained for intracellular (E) IFN- γ or (F) TNF- α along with *NEMO*. Cells shown are gated on the CD3⁺ population.

because of X-chromosome inactivation. This analysis assumes that the percentage of cDNA for wild-type *NEMO* reflects the percentage of cells expressing wild-type *NEMO*. A high proportion of

wild-type *NEMO* cDNA was observed in T cells from the mothers of patients 1/2, 3, 8, and 10, although wild-type *NEMO* cDNA was not predominant in T cells from the mother of patient 4 (Table 5).

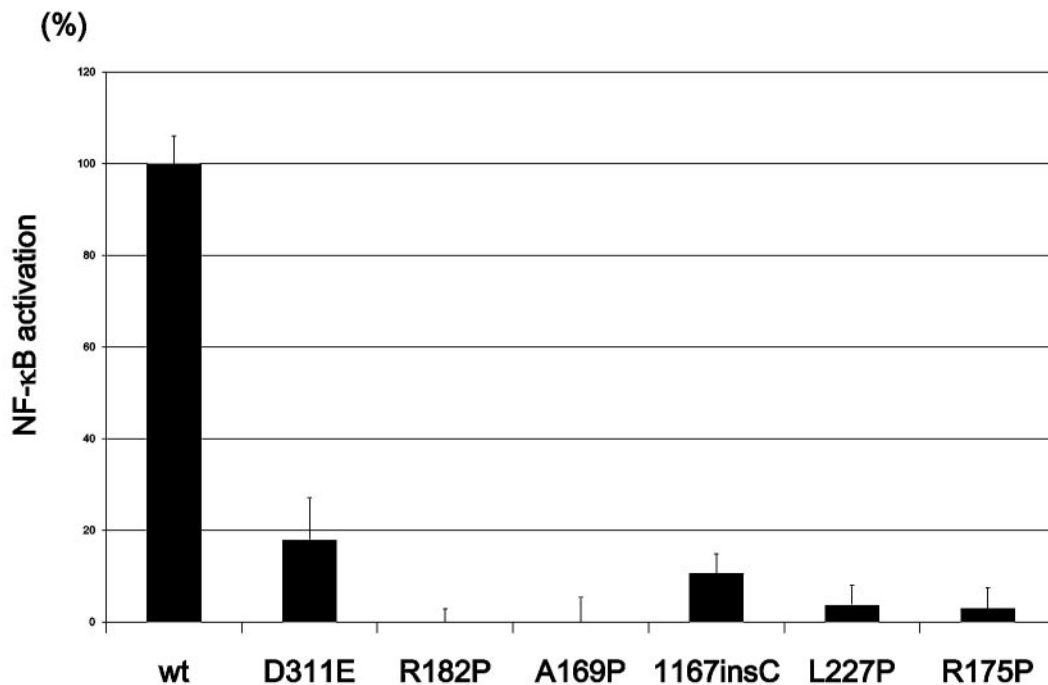


Figure 2. NF- κ B transactivation by *NEMO* mutants from the XL-EDA-ID patients. NF- κ B transactivation induced by *NEMO* mutants in the XL-EDA-ID patients. Mock vectors and wild-type (wt) *NEMO* were used as controls. The NF- κ B activation index of *NEMO* variants were calculated as (NF- κ B activation by each *NEMO* variant – NF- κ B activation of the mock vector)/(NF- κ B activation by wild-type *NEMO* – NF- κ B activation of the mock vector). The data shown are the mean \pm SD of triplicate wells and are representative of 3 independent experiments with similar results.

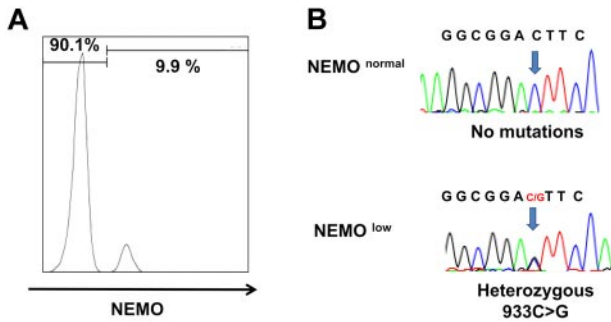


Figure 3. *NEMO* revertant T cells in patient 3. (A) Intracellular expression of *NEMO* in CD8⁺ cells from patient 3. (B) Sequencing chromatograms of DNA from *NEMO*^{normal} or *NEMO*^{low} CD8⁺ cells of patient 3. Arrows indicate the mutated base position at c. 931.

Similarly, there was an apparent high proportion of wild-type *NEMO* cDNA in monocytes and B cells from the mothers of patients 1/2, 8, and 10 (Table 5). These findings suggested a general selective advantage of *NEMO*^{normal} cells over *NEMO*^{low} cells in vivo, especially in T cells.

Proliferation capacity of *NEMO*^{normal} and *NEMO*^{low} T cells

T-cell proliferation stimulated by mitogens such as PHA is usually not reduced in XL-EDA-ID patients. However, the emergence of *NEMO*^{normal} cells coincided with a reduction in mitogen-induced proliferation in patient 2. To further determine the effect of *NEMO*^{normal} cells on mitogen-induced proliferation of peripheral T cells, the proportions of T cells carrying the wild-type and mutant were examined before and after PHA stimulation in XL-EDA-ID patients and their mothers (Table 6). In patients 2, 4, and 8, the percentage of the *NEMO*^{normal} cells decreased after PHA stimulation, while *NEMO*^{normal} cells prevailed in patient 9. In the mothers of patient 4 and 10, the percentage of *NEMO*^{normal} cells increased after PHA stimulation, while the percentage of the *NEMO*^{normal} cells decreased in the mother of patient 3. These results indicated that the *NEMO* mutation does not directly affect the mitogen-induced proliferation capacity of T cells and factors other than the *NEMO* genotype determine the proliferation capacity of *NEMO*^{normal} and *NEMO*^{low} T cells.

Discussion

Somatic reversion mosaicism has been described in several disorders affecting the hematopoietic system, the liver, and the skin.^{23,26} Reports of somatic reversion cases have been particularly abundant in patients with immunodeficiency diseases, including Wiskott-

Aldrich syndrome (WAS)²⁷ and SCID, which occur because of mutations in the interleukin receptor common γ chain,²⁸ CD3 ζ ,²⁹ *RAG-1*³⁰, and *ADA* genes.³¹ Patients with somatic reversion mosaicism may present with significantly milder clinical phenotypes compared with nonrevertant patients with the same germline mutation, although this is not always the case.²⁶ One common feature in most cases where the somatic reversion mosaicism has been observed is a strong in vivo selective advantage of the revertant cells that express the wild-type gene product. One of the most intensively investigated diseases associated with somatic reversion mosaicism is WAS.³²⁻³⁴ A report showed that up to 11% of WAS patients have presented with somatic reversion mosaicism.³³

In our investigation, 9 of 10 XL-EDA-ID patients presented with somatic mosaicism. Two of the 9 were cases of reversion from a duplication mutation, while the others exhibited true back-reversion from a substitution or insertion mutation. This finding calls for caution when diagnosing XL-EDA-ID patients. Because the existence of a *NEMO* pseudogene makes it difficult to perform genetic analysis using genomic DNA, diagnosis of the disease is often confirmed by sequencing analysis of *NEMO* cDNA, and the presence of somatic mosaicism can cause misdiagnosis of XL-EDA-ID patients either when *NEMO*^{normal} cells make up the majority of the patients' PBMCs or when the cDNA of the mutated *NEMO* gene cannot be amplified by PCR.¹⁷ In fact, mutated *NEMO* cDNA could not be amplified from the PBMCs of patient 2 even when *NEMO*^{normal} cells were absent (during early infancy), and only wild-type *NEMO* cDNA was amplified after the appearance of *NEMO*^{normal} cells (data not shown), probably because of the instability of the mutated *NEMO* mRNA. Flow cytometric analysis of intracellular *NEMO* protein is of help in identifying the *NEMO*^{low} cells in some patients, but the technique is not applicable when the *NEMO* mutation does not cause reduced expression of *NEMO* protein. Thus, some cases of XL-EDA-ID patients with reversion may be difficult to diagnose.

The high frequency of somatic mosaicism observed in XL-EDA-ID patients indicates a strong in vivo selective advantage for *NEMO*^{normal} cells, which express the wild-type gene product. Patient 2 presented with a high mutant T-cell count at birth that gradually decreased over time (Figure 1B). This finding indicates that wild-type *NEMO* expression is critical for the survival of certain cell lineages, including T cells, after birth. On the other hand, no *NEMO*^{normal} monocytes and very few *NEMO*^{normal} B cells were detected in the recruited XL-EDA-ID patients (Table 4). This specific feature is similar to other somatic reversion mosaicism seen in primary immunodeficiency patients²⁶ and indicates that the expression of *NEMO* is less critical for the survival of monocytes or B cells compared with that of T cells. There is also an apparent

Table 4. Analysis of *NEMO* gene mosaicism in various cell lineages for each patient

Patient	Mutation	Age at analysis	CD4, % (proportion)	CD8, % (proportion)	CD14, % (proportion)	CD19, % (proportion)
1	Duplication	2 y	90	100	0	4.0
2	Duplication	15 mo	45	66	0	4.0
3	D311E	3 y	2.4	9.9	0	1.2
4	A169P	12 y	0 (0/19)	24 (9/37)	0 (0/19)	0 (0/47)
5	L227P	3 y	0 (0/25)	0 (0/35)	0 (0/30)	0 (0/25)
6	R182P	4 y	18 (5/28)	17 (9/52)	0 (0/27)	0 (0/33)
7	R175P	6 y	0.4 (1/25)	39 (11/28)	0 (0/28)	0 (0/25)
8	Q348X	8 y	38 (6/16)	47 (9/19)	0 (0/33)	0 (0/25)
9	R175P	15 y	30 (9/30)	36 (12/33)	0 (0/23)	0 (0/14)
10	1167 ins C	9 mo				PBMC 9.3 (4/43)

For patients 1 to 3, data represent the percentages of *NEMO*^{normal} cells in each lineage, as assessed by flow cytometry. For patients 4 to 10, the ratio indicates the number of wild-type *NEMO* clones in various cell lineages as compared with the total number of clones analyzed, based on subcloning and sequencing analysis.

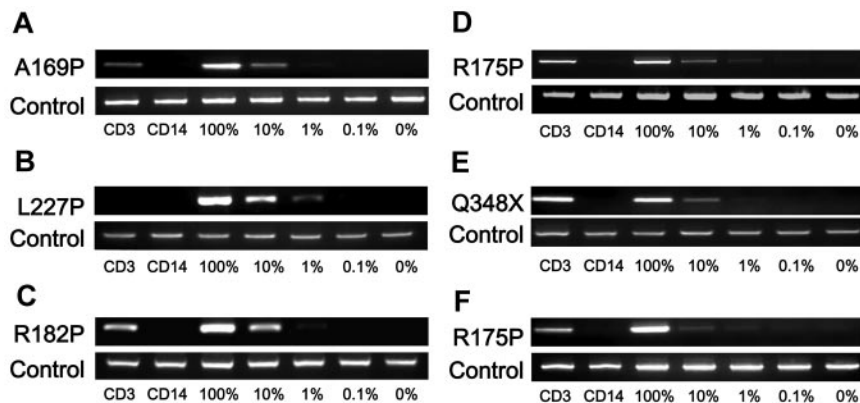


Figure 4. NEMO reversion selectively occurs in T cells of XL-EDA-ID patients. Allele-specific PCR for *NEMO* on CD3⁺ or CD14⁺ cells from (A) patient 4, (B) patient 5, (C) patient 6, (D) patient 7, (E) patient 8, and (F) patient 9. Numbers below each figure indicate the percentages of wild-type *NEMO* cDNA mixed with each mutant. Primers used in each PCR are shown on the left.

concordance between the degree of the disruption of *NEMO* gene and the proportion of reverted *NEMO*^{normal} cells compared with *NEMO*^{low} cells. The high proportion of reverted T cells seen in patients 1 and 2 as well as in patient 8 was associated with a highly disruptive mutation of the *NEMO* gene (ie, a duplication mutation in patients 1 and 2, and a truncation mutation in patient 8). In addition, the highly selective X-chromosome inactivation observed in the mothers of XL-EDA-ID patients indicated a strong selective advantage for *NEMO*^{normal} cells over *NEMO*^{low} cells. It is also noteworthy that reverted T cells were not detected in patient 5, who carried an L227P mutation that was not localized to either of the functional domains in the *NEMO* protein. Other reported cases with the same mutation presented with polysaccharide-specific humoral immunodeficiency and autoinflammatory diseases, but were spared complications such as cellular immunodeficiency and susceptibility to *Mycobacterium* (similar to patient 5).^{4,8} This may reflect the fact that the L227P mutation in *NEMO* has less influence on T-cell growth than *NEMO* mutations that occur in functional domains, and suggests that reversion of the mutation has little impact on T-cell survival. Although the number of cases in our study is limited, it appears that the more disruptive *NEMO* mutations favor the survival of *NEMO*^{normal} cells after reversion and X-chromosome inactivation.

Regarding the gradual decline in the number of *NEMO*-deficient T cells, one candidate trigger could be infection. Because the dominance of the memory phenotype and the skewed TCR

repertoire among CD8⁺ T cells in *NEMO*^{normal} cells were observed in both patients 1 and 2 (Figure 1C and Mizukami et al¹⁸), continuous infection of pyogenic bacteria in patient 1 and *M szulgai* in patient 2 could be a reason for the emergence of *NEMO*^{normal} cells and the elimination of *NEMO*^{low} cells. The decrease in *NEMO*^{normal} cells and restoration of *NEMO*^{low} cells after anti-mycobacterial therapy in patient 2 support this hypothesis. In the case of patient 1, the predominance of *NEMO*^{normal} T cells with an effector/memory phenotype at diagnosis (Table 4 and Mizukami et al¹⁸) is likely to be the result of chronic infection, and it is possible that *NEMO*^{low} cells were predominant during his early infancy. Because some reports have indicated that TNF- α -induced programmed cell death of several cell types, including a human T-cell line, was enhanced by hypomorphic *NEMO* mutations,^{12,35} and considering our finding that the levels of TNF- α expressed in revertant T cells were similar to levels in healthy control T cells in vitro (Figure 1F), TNF- α produced from these cells in response to infection could be involved in mutant T-cell elimination.

Unexpectedly, T-cell proliferation in patient 2 was equivalent to that of normal controls at the age of 2 months and was reduced after *NEMO*^{normal} T cells increased (Figure 1B; Table 3). This finding indicates that the *NEMO*^{low} T cells did not have intrinsically impaired mitogen-induced proliferation. One reasonable explanation for the reduced proliferation observed after the increase in *NEMO*^{normal} T cells is a reduction in the absolute number of T cells (naive T cells in particular), probably because of the infection.

Table 5. Expression of mutant *NEMO* in various cell lineages for the mother of each XL-EDA-ID patient

Sample	Mutation	Analysis	Subtype	Mutant type, % (proportion)
Mother of patients 1 and 2	Duplication	FACS	CD3	0
			CD14	0
			CD19	0
Mother of patient 3	D311E	FACS	CD3	13
			CD3 ⁻	54
			Subcloning	CD3
			CD3 ⁻	55 (12/22)
Mother of patient 4	A169P	Subcloning	CD3	52 (11/21)
			CD14	58 (11/19)
			CD19	42 (5/12)
Mother of patient 8	Q348X	Subcloning	CD3	0 (0/26)
			CD14	17 (3/18)
			CD19	0 (0/18)
Mother of patient 10	1167insC	Subcloning	CD3	18 (7/39)
			CD14	12 (5/43)
			CD19	27 (12/44)

Data are shown as either the percentages of *NEMO*^{normal} cells, as assessed by flow cytometry, or as the ratio of clones containing wild-type *NEMO* to the total number of clones, as analyzed by subcloning and sequencing analysis.

Table 6. Expression of mutant NEMO in CD3-positive cells and PHA blasts

Sample	Mutations	Analysis	Subtype	Mutant type, % (proportion)
Mother of patient 3	D311E	FACS	CD3	13
			PHA blast	47
		Subcloning	CD3	22 (6/27)
Mother of patient 4	A169P	Subcloning	PHA blast	48 (11/23)
			CD3	52 (11/21)
Mother of patient 8	Q348X	Subcloning	PHA blast	18 (9/49)
			CD3	0 (0/26)
Mother of patient 10	1167insC	Subcloning	PHA blast	0 (0/21)
			CD3	18 (7/39)
Patient 2	Duplication	FACS	PHA blast	9 (4/43)
			CD3	73
Patient 4	A169P	Subcloning	PHA blast	93
			CD3	79 (19/24)
Patient 8	Q348X	Subcloning	PHA blast	100 (37/37)
			CD3	56 (18/32)
Patient 9	R175P	Subcloning	PHA blast	100 (16/16)
			CD3	87 (34/39)
			PHA blast	0 (0/28)

PHA blasts were obtained by incubating PBMCs with PHA and soluble IL-2 for 7 days. Data are shown as either the percentages of NEMO^{normal} cells, as assessed by flow cytometry, or as the ratio of clones containing wild-type NEMO to the total number of clones, as analyzed by subcloning and sequencing analysis.

Therefore, to identify other mechanisms underlying reduced T-cell proliferation, the impact of *NEMO* mutation on PHA-induced T-cell proliferation was indirectly examined in vitro by comparing the response of NEMO^{normal} and NEMO^{low} cells derived from XL-EDA-ID patients and their mothers. After PHA stimulation and proliferation, the proportion of NEMO^{low} T cells increased in patients 2, 4, and 8, while the opposite result was observed in patient 9 and in the mother of patient 4 (Table 6). Although the precise mechanism is unclear, a reduction in the proportion of NEMO^{normal} cells after PHA stimulation would reflect the lower proliferative capacity of NEMO^{normal} cells compared with that of NEMO^{low} cells, which may be another explanation for the reduced T-cell proliferation observed in patient 2 at 23 months of age when NEMO^{normal} T cells were dominant. In the reports on reversion mosaicism of *IL2RG* gene mutations,^{28,36} the restoration of T-cell function and clinical symptoms varied among patients. Therefore, other factors besides the genotype of the mutations, such as the developmental stage where reversion occurred and the frequency of reversion, affect the clinical impact of somatic mosaicism of *NEMO* gene mutations.

In this study, the effect of somatic mosaicism of the *NEMO* gene on clinical phenotype could not be fully evaluated. However, cytokines produced by revertant T cells could influence the development of clinical symptoms of XL-EDA-ID, such as inflammatory bowel disease. In a mouse model, intestinal epithelial cell-specific inhibition of NF- κ B through the conditional ablation of NEMO resulted in the development of chronic bowel inflammation sensitized intestinal epithelial cells to TNF- α -induced apoptosis.³⁷ In this model, the first phase of intestinal inflammation was initiated by epithelial cell death and was followed by a second phase of TNF- α -induced intestinal inflammation, the latter being dependent on T cells. Another report showed that HSCT in XL-EDA-ID patients exacerbated the patients' inflammatory bowel disease.³⁸ Indeed, in patient 4, the percentage of reverted T cells was reduced after repeated administrations of anti-TNF α blocking Ab, and the symptoms of inflammatory bowel disease improved.¹⁸ Considering this evidence, somatic mosaicism in T cells might be an important factor leading to inflammatory disease in XL-EDA-ID patients with defective NF- κ B activation. However, our study did not show a tight association between inflammatory bowel disease and somatic mosaicism, and further investigation is needed to

determine whether the NEMO^{normal} T cells play a role in inflammatory processes in XL-EDA-ID.

In conclusion, this study has identified a high frequency of somatic mosaicism in XL-EDA-ID patients, particularly in T cells, and has revealed important insights into human T-cell immunobiology in XL-EDA-ID. Although we could not demonstrate the clinical impact of somatic mosaicism in XL-EDA-ID patients, our findings suggest that care is required when making molecular diagnoses of XL-EDA-ID, and might shed light on the mechanisms underlying the variability in the clinical manifestation of XL-EDA-ID and facilitate the search for appropriate treatments.

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Authorship

Contribution: Tomoki Kawai wrote the manuscript and performed research; R.N., T.Y., T.N., and T.H. edited the manuscript and supervised this work; K.I., Y.M., N.T., H.S., M.S., and Y.T. cultured cells; and T. Mizukami, H.N., Y.K., A.Y., T. Murata, S.S., E.I., H.A., Toshinao Kawai, C.I., S.O., and M.K. treated patients and analyzed data.

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References

- Pinheiro M, Freire-Maia N. Ectodermal dysplasias: a clinical classification and a causal review. *Am J Med Genet*. 1994;53(2):153-162.
- Abinun M, Spickett G, Appleton AL, Flood T, Cant AJ. Anhidrotic ectodermal dysplasia associated with specific antibody deficiency. *Eur J Pediatr*. 1996;155(2):146-147.
- Sitton JE, Reimund EL. Extramedullary hematopoiesis of the cranial dura and anhidrotic ectodermal dysplasia. *Neuropediatrics*. 1992;23(2):108-110.
- Schweizer P, Kalhoff H, Horneff G, Wahn V, Diekmann L. [Polysaccharide specific humoral immunodeficiency in ectodermal dysplasia. Case report of a boy with two affected brothers]. *Klin Padiatr*. 1999;211(6):459-461.
- Abinun M. Ectodermal dysplasia and immunodeficiency. *Arch Dis Child*. 1995;73(2):185.
- Zonana J, Elder ME, Schneider LC, et al. A novel X-linked disorder of immune deficiency and anhidrotic ectodermal dysplasia is allelic to incontinentia pigmenti and due to mutations in IKK-gamma (NEMO). *Am J Hum Genet*. 2000;67(6):1555-1562.
- Courtois G, Smahi A, Israel A. NEMO/IKK gamma: linking NF-kappa B to human disease. *Trends Mol Med*. 2001;7(10):427-430.
- Doffinger R, Smahi A, Bessia C, et al. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. *Nat Genet*. 2001;27(3):277-285.
- Rothwarf DM, Zandi E, Natoli G, Karin M. IKK-gamma is an essential regulatory subunit of the I-kappaB kinase complex. *Nature*. 1998;395(6699):297-300.
- Yamaoka S, Courtois G, Bessia C, et al. Complement cloning of NEMO, a component of the I-kappaB kinase complex essential for NF-kappaB activation. *Cell*. 1998;93(7):1231-1240.
- Smahi A, Courtois G, Vabres P, et al. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature*. 2000;405(6785):466-472.
- Hanson EP, Monaco-Shawver L, Solt LA, et al. Hypomorphic nuclear factor-kappaB essential modulator mutation database and reconstitution system identifies phenotypic and immunologic diversity. *J Allergy Clin Immunol*. 2008;122(6):1169-1177.
- Orange JS, Jain A, Ballas ZK, Schneider LC, Geha RS, Bonilla FA. The presentation and natural history of immunodeficiency caused by nuclear factor kappaB essential modulator mutation. *J Allergy Clin Immunol*. 2004;113(4):725-733.
- Jain A, Ma CA, Liu S, Brown M, Cohen J, Strober W. Specific missense mutations in NEMO result in hyper-IgM syndrome with hypohidrotic ectodermal dysplasia. *Nat Immunol*. 2001;2(3):223-228.
- Orange JS, Brodeur SR, Jain A, et al. Deficient natural killer cell cytotoxicity in patients with IKK-gamma/NEMO mutations. *J Clin Invest*. 2002;109(11):1501-1509.
- Sebban H, Courtois G. NF-kappaB and inflammation in genetic disease. *Biochem Pharmacol*. 2006;72(9):1153-1160.
- Nishikomori R, Akutagawa H, Maruyama K, et al. X-linked ectodermal dysplasia and immunodeficiency caused by reversion mosaicism of NEMO reveals a critical role for NEMO in human T-cell development and/or survival. *Blood*. 2004;103(12):4565-4572.
- Mizukami T, Obara M, Nishikomori R, et al. Successful treatment with infliximab for inflammatory colitis in a patient with X-linked anhidrotic ectodermal dysplasia with immunodeficiency. *J Clin Immunol*. 2012;32(1):39-49.
- Imamura M, Kawai T, Okada S, et al. Disseminated BCG infection mimicking metastatic nasopharyngeal carcinoma in an immunodeficient child with a novel hypomorphic NEMO mutation. *J Clin Immunol*. 2011;31(5):802-810.
- Tono C, Takahashi Y, Terui K, et al. Correction of immunodeficiency associated with NEMO mutation by umbilical cord blood transplantation using a reduced-intensity conditioning regimen. *Bone Marrow Transplant*. 2007;39(12):801-804.
- Saito M, Nishikomori R, Kambe N, et al. Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. *Blood*. 2008;111(4):2132-2141.
- Yang TP, Stout JT, Konecki DS, Patel PI, Alford RL, Caskey CT. Spontaneous reversion of novel Lesch-Nyhan mutation by HPRT gene rearrangement. *Somat Cell Mol Genet*. 1988;14(3):293-303.
- Zhang LH, Jenssen D. Reversion of the hprt mutant clone SP5 by intrachromosomal recombination. *Carcinogenesis*. 1992;13(4):609-615.
- Monnat RJ Jr, Chiaverotti TA, Hackmann AF, Maresh GA. Molecular structure and genetic stability of human hypoxanthine phosphoribosyltransferase (HPRT) gene duplications. *Genomics*. 1992;13(3):788-796.
- Rautenstrauss B, Liehr T, Fuchs C, et al. Mosaicism for Charcot-Marie-Tooth disease type 1A: onset in childhood suggests somatic reversion in early developmental stages. *Int J Mol Med*. 1998;1(2):333-337.
- Wada T, Candotti F. Somatic mosaicism in primary immune deficiencies. *Curr Opin Allergy Clin Immunol*. 2008;8(6):510-514.
- Ariga T, Kondoh T, Yamaguchi K, et al. Spontaneous in vivo reversion of an inherited mutation in the Wiskott-Aldrich syndrome. *J Immunol*. 2001;166(8):5245-5249.
- Stephan V, Wahn V, Le Deist F, et al. Atypical X-linked severe combined immunodeficiency due to possible spontaneous reversion of the genetic defect in T cells. *N Engl J Med*. 1996;335(21):1563-1567.
- Rieux-Laucat F, Hivroz C, Lim A, et al. Inherited and somatic CD3zeta mutations in a patient with T-cell deficiency. *N Engl J Med*. 2006;354(18):1913-1921.
- Wada T, Toma T, Okamoto H, et al. Oligoclonal expansion of T lymphocytes with multiple second-site mutations leads to Omenn syndrome in a patient with RAG1-deficient severe combined immunodeficiency. *Blood*. 2005;106(6):2099-2101.
- Hirschhorn R. In vivo reversion to normal of inherited mutations in humans. *J Med Genet*. 2003;40(10):721-728.
- Wada T, Schurman SH, Jagadeesh GJ, Garabedian EK, Nelson DL, Candotti F. Multiple patients with revertant mosaicism in a single Wiskott-Aldrich syndrome family. *Blood*. 2004;104(5):1270-1272.
- Stewart DM, Candotti F, Nelson DL. The phenomenon of spontaneous genetic reversions in the Wiskott-Aldrich syndrome: a report of the workshop of the ESID Genetics Working Party at the XIII Meeting of the European Society for Immunodeficiencies (ESID). Budapest, Hungary October 4-7, 2006. *J Clin Immunol*. 2007;27(6):634-639.
- Davis BR, Yan Q, Bui JH, et al. Somatic mosaicism in the Wiskott-Aldrich syndrome: molecular and functional characterization of genotypic revertants. *Clin Immunol*. 2010;135(1):72-83.
- Yamaoka S, Inoue H, Sakurai M, et al. Constitutive activation of NF-kappa B is essential for transformation of rat fibroblasts by the human T-cell leukemia virus type I Tax protein. *EMBO J*. 1996;15(4):873-887.
- Speckmann C, Pannicke U, Wiech E, et al. Clinical and immunologic consequences of a somatic reversion in a patient with X-linked severe combined immunodeficiency. *Blood*. 2008;112(10):4090-4097.
- Nenci A, Becker C, Wullaert A, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*. 2007;446(7135):557-561.
- Fish JD, Duerst RE, Gelfand EW, Orange JS, Bunin N. Challenges in the use of allogeneic hematopoietic SCT for ectodermal dysplasia with immune deficiency. *Bone Marrow Transplant*. 2009;43(3):217-221.