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A Novel Missense Mutation in the Nuclear Factor- κ B Essential Modulator (NEMO) Gene Resulting in Impaired Activation of the NF- κ B Pathway and a Unique Clinical Phenotype Presenting as MRSA Subdural Empyema

Gene A. Devora¹, Lijun Sun², Zhijian Chen², Nicolai S. C. van Oers³, Eric P. Hanson^{4,5}, Jordan S. Orange⁴, and M. Teresa de la Morena¹

¹Division of Allergy and Immunology, Department of Pediatrics and Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-8859, USA

²Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA

³Department of Immunology and Microbiology, University of Texas Southwestern Medical Center, Dallas, TX, USA

⁴Children's Hospital of Philadelphia Research Institute, Philadelphia, PA, USA

Abstract

Introduction—We describe a previously unreported 437 T→G missense mutation producing a V146G substitution in the first coiled-coil (CC1) domain of nuclear factor- κ B essential modulator (NEMO) in a 9-month-old boy with ectodermal dysplasia with immunodeficiency who presented with methicillin-resistant *Staphylococcus aureus* subdural empyema. We performed in vitro experiments to determine if this novel mutation resulted in impaired NF- κ B signaling.

Methods—I κ B α phosphorylation experiments were performed using a Jurkat T cell line lacking endogenous NEMO expression that was transfected with vectors containing either the wild type or the patient's V146G mutation. The cells were stimulated with TNF- α to activate the NF- κ B pathway. Phosphorylated I κ B α was detected by immunoblotting with anti-phospho-I κ B α antibodies. Peripheral blood mononuclear cells from the patient were stimulated with TNF- α or anti-CD3 and anti-CD28. Impaired I κ B α degradation was detected using antibodies against the I κ B α protein.

Results—While TNF- α stimulation resulted in I κ B α phosphorylation in NEMO-deficient Jurkat cells reconstituted with wild-type NEMO, cell transfected with the V146G mutant exhibited a 75% reduction in phospho-I κ B α . Peripheral blood mononuclear cells from the patient showed impaired degradation of I κ B α after stimulation when compared with normal controls.

Conclusions—The patient's V146G mutation results in impaired NF- κ B activation in vitro. The mutation extends the known N-terminal boundary within the CC1 domain that produces an ectodermal dysplasia phenotype, and defines an infectious susceptibility previously unappreciated in ectodermal dysplasia with immunodeficiency (methicillin-resistant *S. aureus* subdural empyema), broadening the clinical spectrum associated with the disease.

Keywords

NEMO; immunodeficiency; ectodermal; dysplasia; mutation; MRSA

Introduction

Nuclear factor- κ B essential modulator (NEMO) is an essential component of the NF- κ B signaling pathway [1]. It is a part of the I κ B kinase (IKK) complex, which also contains the IKK α and IKK β catalytic subunits [1–4]. Signaling through innate immune receptors, including TLR5, TNF- α , and T- and B cell antigen receptors, promotes the activation of the IKK complex, resulting in I κ B α phosphorylation and degradation, enabling NF- κ B translocation into the nucleus and activation of gene transcription [5–8].

Amorphic mutations that result in a nonfunctional NEMO protein are uniformly lethal in males [9]. Hypomorphic mutations, however, most often present with ectodermal dysplasia with immunodeficiency (ED-ID) [9]. These patients are classically susceptible to mycobacterial infections, recurrent infections with pyogenic bacteria, and abnormal immunoglobulin production in the setting of variable T and B cell defects [9–12]. Ectodermal dysplasia results from impaired activation of NF- κ B after ligation of the ectodysplasin A receptor, a member of the TNF receptor family [11].

Impaired activation of NF- κ B through a variety of other immunoreceptors resulting from hypomorphic mutations of NEMO is now known to produce a broad spectrum of clinical phenotypes that has recently been reviewed [9]. We describe a novel mutation in NEMO in a child with ED-ID who presented with an MRSA subdural empyema. The clinical relevance of the mutation was unknown. To our knowledge, hypomorphic NEMO mutations have never been associated with methicillin-resistant *Staphylococcus aureus* (MRSA) subdural empyemas. The purpose of this study was to determine if this mutation resulted in impaired NF- κ B signaling. Impaired signaling would suggest a novel, hypomorphic NEMO mutation that extends the repertoire of known mutations that produce an ectodermal dysplasia phenotype, and defines an infectious susceptibility previously unappreciated in ED-ID (MRSA subdural empyema), broadening the known clinical spectrum associated with the disease.

Materials and Methods

Case Report

The patient is a full-term male who presented at 9 months of age with methicillin resistant *S. aureus* (MRSA) subdural empyema. Laboratory studies demonstrated leukocytosis (WBC 19,200 cells/ μ L) with eosinophilia (2,496 cells/ μ L). Lymphocyte subpopulations are indicated in Table 1. Immunoglobulin evaluation revealed undetectable levels of IgG (<200 mg/dL; normal 269–913 mg/dL) and IgM (<25 mg/dL; normal 27–80 mg/dL) and elevated levels of IgA (124 mg/dL; normal 8–54 mg/dL) (Table 2). The patterns of both immunoglobulin levels and lymphocyte subpopulations have been described in patients with hypomorphic NEMO mutations.

Initial analysis revealed decreased absolute numbers and percentages of CD3⁺ and CD4⁺ T cells, normal CD8⁺ T cell and CD16/56⁺ NK cell absolute numbers and percentages, and normal numbers of B cells with elevated percentages. Repeat analysis showed elevated CD3⁺ and CD8⁺ absolute numbers with normal percentages, normal CD4⁺ T cells and CD16/56⁺ NK cells absolute numbers and percentages, and high CD19⁺ B cell absolute

numbers with normal percentages. This pattern has been described in patients with mutations in NEMO

Immunoglobulin levels: undetectable levels of IgG and IgM with elevated levels of IgA. This pattern has also been described in patients with NEMO mutations

At 11 months of age, he was admitted for failure to thrive, diarrhea, rash, and eosinophilia. Physical exam was notable for both height and weight at the 25th percentile, generalized erythematous pruritic maculopapular rash, sparse hair, generalized lymphadenopathy, and a single conical-shaped incisor. RAG-1 and RAG-2 sequences were normal. His clinical features, presentation, and laboratory studies were characteristic of ED-ID. Subsequent sequencing of the NEMO gene identified a previously unreported 437 T→G missense mutation producing a valine to glycine substitution at amino acid position 146 (V146G) in the CC1 of NEMO. This mutation was not found in the general population as a single nucleotide polymorphism when searched utilizing the National Center for Biotechnologies (NCBI) databases that are publically available. Also, the nucleotide change within the coding region of NEMO is predicted to be a non-conservative change by BLOSUM50.

I κ B α Phosphorylation Experiments

A mammalian expression vector containing NEMO was mutagenized to introduce the patient's 437 T→G alteration into the NEMO cDNA. Wild type or previously described NEMO-deficient Jurkat cells transfected with a vector control or plasmids encoding either wild type or V146G mutant NEMO were stimulated with TNF- α for 0, 5, and 10 min [13]. Lysates were prepared, and NEMO expression and I κ B α phosphorylation was detected by immunoblotting with antibodies against NEMO and phospho-I κ B α , respectively. Amounts of wild type and V146G mutant NEMO were determined by densitometry scanning. NF- κ B activation was assessed by detecting the levels of phosphorylated I κ B α before and after ligand stimulation and normalized to the levels of NEMO.

I κ B α Degradation Experiments

Peripheral blood mononuclear cells (PBMCs) from the patient and a healthy control were isolated and stimulated with either TNF- α or through the T cell receptor with antibodies against CD3 and CD28, respectively. PBMCs were isolated from whole blood via centrifugation using Ficoll-Hypaque lymphocyte separation media. Cells were stimulated and lysed after 0, 10, and 60 min for TNF α activation and 0, 30, and 60 min for T cell receptor (TCR) activation. Lysates were immunoblotted with rabbit polyclonal anti-I κ B α as described previously [9, 14]. Actin was used as a loading control and detected using anti-actin antibodies. Densitometry of immunoblot bands was performed using Image J software (NIH—shareware). To normalize the I κ B α signal to the loaded protein per lane, the densitometric value for the actin band was used as the denominator for the I κ B α band value from that particular lane. In this way, the I κ B α present relative to the amount of protein loaded per lane could be estimated and is represented graphically to supplement the actual immunoblot.

Results

To determine that impaired NF- κ B activation was caused by the patient's specific NEMO mutation, a previously described cellular reconstitution technique was used as outlined above. Stimulation of parental Jurkat T cells (expressing endogenous NEMO) revealed I κ B α phosphorylation at 5 and 10 min after TNF- α stimulation (Fig. 1, lanes 2–3). NEMO-deficient Jurkat cells transfected with vector control alone showed no phosphorylation of I κ B α (Fig. 1, lanes 4–6). Reconstituting these NEMO-deficient Jurkat cells with wild-type

NEMO restored I κ B α phosphorylation after TNF- α treatment (Fig. 1, lanes 7–9). Transfecting the cells with the mutant V146G NEMO construct was unable to restore I κ B α phosphorylation to those noted with wild-type NEMO, reaching only 25–35% of wild-type responses using normalized values for NEMO expression (Fig. 1, lanes 10–12).

To confirm that this novel NEMO mutation impaired NF- κ B activation in the patient's own cells, PBMCs from the patient were stimulated with TNF- α . Prior to stimulation with TNF- α , levels of I κ B α in both control cells and the patient's cells were detectable (Fig. 2, lanes 1 and 4). After 10 and 60 min of stimulation with TNF- α , however, the I κ B α in control cells degraded, but that in the patient PBMC did not (Fig. 2a, lanes 2–3 and 4–6). Protein loading controls for each lane was determined by stripping the immunoblot and reprobing for actin. Although there was less protein loaded in the control, there were equivalent amounts across the individual timepoints in both patient and control. To normalize for protein loading, densitometric analyses were performed and the I κ B α was normalized to actin. This confirmed that I κ B α was degraded after stimulation in control, but not patient cells (Fig. 2a—right). Since many NEMO mutants appear to also affect TCR signaling, patient PBMCs were isolated from whole blood and subjected to activation using anti-CD3 and anti-CD28 antibodies. Similar to the TNF α stimulation of PBMC, patient's cells failed to degrade I κ B α when compared with control (Fig. 2b). In this experiment protein loading per lane as demonstrated by stripping the immunoblot and reprobing for actin was equivalent, and a densitometric evaluation illustrates that the patient's PBMCs failed to degrade I κ B α after activation (Fig. 2b—right).

Discussion

Although a complete functional mutant of NEMO is incompatible with life, numerous hypomorphic mutations with some preserved functionality have been described in different patients [1, 5, 7]. We describe a patient with ED-ID with a previously unreported hypomorphic mutation within the CC1 domain who presented with MRSA subdural empyema.

Our studies describe the functional significance of the patient's V146G mutation. TNF- α stimulation of NEMO-deficient Jurkat cells demonstrated no phosphorylation of I κ B α and reconstitution with the patient's mutant V146G NEMO gene was unable to restore I κ B α phosphorylation. In contrast, wild-type Jurkat cells and NEMO-deficient Jurkat cells transfected with a wild-type NEMO construct exhibited I κ B α phosphorylation. These experiments indicate that the patient's NEMO mutation results in impaired NF- κ B signaling. These findings were corroborated using the patient's own PBMCs, which demonstrated impaired I κ B α degradation compared with healthy controls after TNF- α induced stimulation of the TNF receptor or stimulation of the TCR.

A recent publication characterizing 72 patients with NEMO mutations reported that all patients presented with immunodeficiency, but only 77% had ED [9]. Mutations resulting in ED were clustered around three regions that did not include the leucine zipper domain or N-terminal ends of the CC1 and CC2 domains [9,15].

A previously reported L153R mutation within the CC1 domain is associated with ED-ID, and functional studies demonstrated that this mutation impairs toll-like receptor signaling functions, impaired I κ B α degradation, and impaired apoptosis [9]. To date, the L153R mutation was the farthest described N-terminal mutation resulting in ED [9]. This is relevant as other N-terminal mutations, including D113N and L80P, were not associated with the ED phenotype.

This novel mutation extends the known N-terminal boundary within the CC1 domain that produces an ED phenotype. It also defines an infectious susceptibility previously unappreciated in ED-ID (MRSA intracranial infection).

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Fig. 1.

IκBα phosphorylation in NEMO-deficient Jurkat cells transfected with wild type or V146G mutant NEMO expressing vectors. Wild type or NEMO-deficient Jurkat cells transfected with vector control or plasmids encoding wild type or V146G mutant NEMO were stimulated with TNF-α for 0, 5, and 10 min. Lysates were prepared, and NEMO expression and IκBα phosphorylation were detected by immunoblotting with antibodies against NEMO and phospho-IκBα, respectively. Amounts of wild type and V146G mutant NEMO in the samples were determined by densitometry scanning. The levels of phospho-IκBα were normalized to the levels of NEMO. There was impaired IκBα phosphorylation in NEMO-deficient Jurkat cells transfected with V146G mutant NEMO compared with wild-type NEMO.

Fig. 2.

Impaired I κ B α degradation in immune receptor stimulated V146G mutant peripheral blood mononuclear cells from the patient. PBMCs from the patient and a healthy control were stimulated with TNF- α or a combination of anti-CD3 and anti-CD28. **a** The cells stimulated with TNF- α were lysed at 0, 10, and 60 min, and cell lysates were immunoblotted with antibodies specific for I κ B α . There was impaired TNF- α -induced I κ B α degradation in the patient's cells compared with healthy controls. **b** The population of patient T cells from unpurified PBMCs was stimulated with anti-CD3 and anti-CD28. These showed impaired TNF- α -induced I κ B α degradation in the patient's cells compared with healthy controls. Normalized densitometric analyses are shown on the right of immunoblot (see "Materials and Methods" for details)

Table 1

Lymphocyte Subpopulation Analysis with Normal Age References

Cell type	Initial, cells/mL (%)	Repeat, cells/mL (%)	Normal, cells/mL (%)
WBC	19,200	26,000	6,000–14,000
CD3 ⁺	1627 (42.6%)	8083 (68%)	1,900–5,900 (49–76%)
CD4 ⁺	699 (18%)	3898 (33%)	1,400–4,300 (31–56%)
CD8 ⁺	917 (24%)	4082 (35%)	500–1,700 (12–24%)
CD16/56 ⁺	195 (5%)	660 (5.6%)	160–950 (3–15%)
CD19 ⁺	1876 (49%)	3026 (25%)	610–2,600 (14–37%)

Table 2

Immunoglobulin Levels with Normal Age References

Immunoglobulin	Level (mg/dL)	Normal (mg/dL)
IgG	<200	269–913
IgA	124	8–54
IgM	<25	27–80