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RIP3 as a diagnostic and severity marker for Stevens-Johnson syndrome and toxic epidermal necrolysis

Akito Hasegawa, MD, Satoru Shinkuma, MD, PhD, Ryota Hayashi, MD, PhD, Natsumi Hama, MD, Hideaki Watanabe, MD, PhD, Manao Kinoshita, MD, PhD, Youichi Ogawa, MD, PhD, Riichiro Abe, MD, PhD

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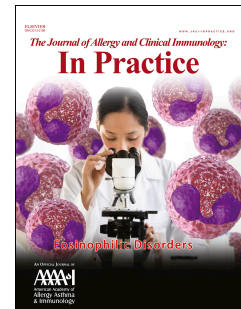
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1 **Supplementary Information**

2

3 **RIP3 as a diagnostic and severity marker for Stevens-Johnson syndrome and toxic**

4 **epidermal necrolysis**

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7 **Methods**

8 **Cell culture and induction of necroptosis**

9 HaCaT cells were purchased from COSMO BIO (Tokyo, Japan) and cultured in
10 Dulbecco's modified Eagle's medium (Gibco, Waltham, MA), supplemented with 10% fetal
11 bovine serum (Gibco), 100 unit/mL penicillin, and 100 µg/mL streptomycin (Sigma, St. Louis,
12 MO), in an incubator at 37 °C under 5% CO₂. Cells were treated with 40 ng/mL TNF-α (T
13 (Sigma), 100 µM Smac-mimetics (S) (BioVision, Milpitas, CA), and 40 µM z-VAD-fmk (Z)
14 (R&D Systems, Minneapolis, MN) to induce necroptosis.

16 **RNA isolation and quantitative real-time PCR**

17 Total RNA was isolated from HaCaT cells cultured with necroptosis inducers for 24 h,
18 using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was transcribed into cDNA
19 using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the
20 manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was
21 performed using the THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). The
22 sequences of primers used in the study were: *RIP3* forward primer,

23 5'-CCTTCCAGGAATGCCTACCA-3'; *RIP3*-reverse primer,
24 5'-CGTGGAGACAGCAGCATTCA-3'; *β-actin* forward primer,
25 5'-AGAAAATCTGGCACCACACC-3'; *β-actin* reverse primer,
26 5'-GGGGTGTGTAAGGTCTCAA-3'. Total RNA concentration in each sample was
27 normalized to *β-actin* mRNA levels. The expression of *RIP3* is presented as the ratio of the
28 *RIP3* mRNA to *β-actin* mRNA. Data were analyzed by the delta-delta Ct method.

29

30 **Western blot analysis**

31 HaCaT cells were lysed in a buffer containing 0.5% Triton X-100 with protease and
32 phosphatase inhibitor cocktails. Insoluble material was removed by centrifugation. Lysates
33 were boiled in sample buffer for 10 min and electrophoresed on a 4–12% SDS-polyacrylamide
34 gel (Invitrogen). The resolved proteins were transferred to PVDF or nitrocellulose membranes.
35 The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing Tween-20
36 (TBS-T) for 30 min and then incubated overnight at 4 °C with the appropriate primary antibody
37 prepared in the blocking solution. Antibodies against pMLKL (1:1000, Abcam, Cambridge,
38 UK), MLKL (1:1000, Abcam), pRIP3 (1:1000, Cell Signaling Technology, Danvers, MA), and

RIP3 (1:1000, Abcam) were used. After a minimum of 10 h incubation, the membranes were washed thrice with TBS-T for 5 min and incubated in secondary antibodies for 1 h at room temperature. The membranes were then washed with TBS-T for 5 min and imaged using LuminoGraph I (ATTO, Tokyo, Japan).

Cell viability assay

Cell viability was evaluated using the RealTime-GloTM MT cell Viability Assay kit (Promega, Madison, WI). HaCaT cells were treated in 96-well plates with TNF- α , Smac-mimetics, and z-VAD-fmk (T/S/Z), then incubated at 37 °C in a 5% CO₂ incubator. Luminescence in each well was measured at 3-h intervals for 24 h.

Patient selection

Patients diagnosed with Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) (n = 22), erythema multiforme (EM) minor (n = 5), EM major (n = 19), or maculopapular exanthema (MPE) (n = 6) were enrolled for the study. In patients presenting with a skin rash that was indistinguishable from MPE, EM, or SJS/TEN at the time of blood

55 collection (SJS/TEN: n = 2, EM minor: n = 1, EM major: n = 5, MPE: n = 2), final diagnoses
56 were made based on skin manifestations and histopathological findings. All TEN patients had
57 been clearly diagnosed as TEN when blood for the RIP3 assay was drawn. The patients for
58 whom any medication had been initiated during the period 1 month prior to the enrollment and
59 who did not have any episode of infection were classified into the “medication-caused” group,
60 whereas those who did not receive any medication during this period but had had a clear
61 episode of infection were classified into the “infection-caused” group. Patients for whom the
62 cause of disease was not clear were classified into the “unknown” group. The patients in the
63 infection-caused group were diagnosed with either herpes simplex virus (EM minor: n = 1, EM
64 major: n = 1) or *Mycoplasma pneumoniae* (SJS/TEN: n = 4). Patients with drug-induced
65 hypersensitivity syndrome (DIHS)/drug rash with eosinophilia and systemic symptoms
66 (DRESS) (n = 4), bullous pemphigoid (BP) (n = 8), and pemphigus vulgaris (PV) (n = 6) were
67 enrolled as disease controls. DIHS/DRESS is a severe cutaneous adverse drug reaction
68 characterized by herpesviruses reactivation. In the pathomechanisms of DIHS/DRESS, the
69 association of necroptosis has not been evident.^{E1} Controls (five healthy adults) had no history
70 of drug hypersensitivity. The details of the study were fully explained to each patient or his/her

guardian, after which written informed consent was obtained. The study protocol was approved by the Ethics Committees of Niigata University, Showa University, and Yamanashi University. The study was conducted in accordance with the Declaration of Helsinki.

Laboratory tests and evaluation of clinical scores

White blood cell (WBC) counts, C-reactive protein (CRP) levels, body temperatures (BT), numbers of mucosal involvement sites, numbers of injured organs, and histopathological parameters of the patients were evaluated. The worst level for each clinical parameter was used for determining the correlation with the serum RIP3 level. We performed skin biopsies on all patients except one EM minor patient. In histopathological analysis, we assigned the patients to three groups depending on the number of dead keratinocytes observed in a field of view of hematoxylin and eosin stained samples at $\times 200$ magnification; the three groups had 0, 0-10, and >10 dead cells, respectively.

Measurement of serum RIP3 levels by enzyme-linked immunosorbent assay (ELISA)

Serum RIP3 levels were determined at the first visit before the initiation of treatment

and after clinical recovery using an ELISA kit, according to the manufacturer's instructions (Cusabio, Wuhan, China).⁷

Immunofluorescence

Skin samples were obtained from non-bullous erythematous lesions of SJS/TEN, EM, and MPE patients. Cryostat sections or HaCaT cells were fixed with 4% paraformaldehyde for 10 min and blocked with 10% goat serum for 30 min at room temperature. Immunolabeling was performed with polyclonal anti-RIP3 (1:100, Novus Biologicals, Littleton), anti pRIP3 (1:100, Cell Signaling Technology), or anti pMLKL (1:100, Signalway Antibody, College Park, MD) antibody overnight at 4 °C, followed by incubation with secondary antibody for 30 min at room temperature in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:100, Vector Laboratories, Burlingame, CA). Images were acquired using a Keyence BZ-X710 all-in-one fluorescence microscope (Keyence, Osaka, Japan). For immunofluorescence staining of frozen sections, we changed the green color of RIP3 to red using the BZ-X image converter (Keyence).

Statistical analyses

Statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for the R environment (The R Foundation for Statistical Computing, Vienna, Austria).^{E2} Independent group comparisons between serum RIP3 levels and clinical parameters were determined using the Mann–Whitney U test. Correlations with clinical data were assessed by Spearman's rank correlation test. The Wilcoxon signed-rank test was used to compare pairs of related samples. Statistical significance was assumed at $P < 0.05$.

References

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- E2. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013;48:452-8.

Supplementary Figures

FIG E1. RIP3 release from necroptotic keratinocytes.

A, Cell viability of HaCaT cells treated with TNF- α (40 ng/mL), Smac-mimetics (100 μ M), and Z-VAD (40 μ M) for 24 h. Cell viability was measured for 3 h for 24 h. The viability of T/S/Z treated HaCaT cells was significantly decreased compared to that of non-treated control cells. $**P < .01$. **B**, Western blot detection of RIP3, phosphorylated RIP3, MLKL, and phosphorylated MLKL in HaCaT cells treated with TNF- α (40 ng/mL), Smac-mimetics (100 μ M), and Z-VAD (40 μ M) for 24 h. **C-D**, Immunofluorescent staining of HaCaT cells treated with TNF- α (40 ng/mL), Smac-mimetics (100 μ M), and Z-VAD (40 μ M) for 24 h. Cells were stained with phosphorylated MLKL (red) and phosphorylated RIP3 (green). Scale bar = 50 μ m. **(B-D)** In T/S/Z treated HaCaT cells, phosphorylation of RIP3 and MLKL was detected. **E**, Expression of RIP3 mRNA. Values are normalized to nontreated controls. The mRNA expression of *RIP3* was increased in T/S/Z treated HaCaT cells over that in non-treated control cells. $*P < .05$. **F**, Concentrations of RIP3 in the supernatants of HaCaT cells treated with TNF- α (40 ng/mL), Smac-mimetics (100 μ M), and Z-VAD (40 μ M) for 48 h. $*P < 0.05$.

135

136 **FIG E2.** Comparison of serum RIP3 levels between SJS/TEN group and autoimmune blistering
137 disorders groups.

138 **A,** Serum RIP3 levels compared between SJS/TEN and autoimmune blistering disorders. The
139 serum RIP3 levels were significantly higher in the SJS/TEN group than in the BP (n = 8) and
140 PV (n = 6) groups (BP: $P = .006$; PV: $P < .001$). $**P < .01$. Notably, serum RIP3 levels of 2
141 patients in SJS/TEN group whose diagnosis was unclear because there was no observable skin
142 detachment at the time of blood collection were higher (420.3 pg/mL, 247.6 pg/mL) than those
143 of BP or PV patients who had blisters or erosion (BP: 222.3 ± 220.7 , PV: 53.3 ± 119.8). In
144 addition, no significant differences were observed between the levels in BP or PV group and
145 those of healthy controls (BP: $P = 0.05$; PV: $P < 0.73$)

146

147 **FIG E3.** Associations between serum RIP3 levels and clinical and laboratory parameters.

148 **A,** Serum RIP3 levels in cases of medication-caused and infection-caused disease. There was
149 no significant difference between the groups. Boxes represent medians with interquartile ranges.

150 **B,** Correlation between serum RIP3 levels and WBC counts. Statistical analysis by Spearman's

151 rank correlation test.

152

153 **FIG E4.** Clinical course of a patient with TEN.

154 **A,** Changes in serum RIP3 levels and detachment areas with representative photos.

155 The patient was a 67-year old male. Vildagliptin and metformin hydrochloride combined was
156 initiated for diabetes mellitus, and 2 weeks later, erythema and erosions appeared on the entire
157 body. The skin detachment area at the first analysis was 15%. Mucosal symptoms were
158 observed in the genital area and oral mucosa and the patient had mild kidney dysfunction. The
159 SCORTEN was 3 and the predicted mortality rate was 33%. The serum RIP3 level was 505.6
160 pg/mL at day 0. Although steroid pulse therapy was initiated, skin detachment spread further
161 and serum RIP3 level increased to 896.3 pg/mL. Because the reactivity to steroid pulse therapy
162 was poor, IVIg therapy (400 mg/kg/day for 5 days) was performed immediately after the steroid
163 pulse therapy and treatment with oral corticosteroid (1 mg/kg/day) was started. However, the
164 skin detachment area spread to 60% of the body and the serum RIP3 level increased to 2205
165 pg/mL at the time the IVIg therapy was finished. Because the patient developed catheter
166 infection, a second course of IVIg therapy was initiated, and disease activity gradually

167 decreased. After recovery, the serum RIP3 level decreased to 341.1 pg/mL. Thirty-nine days
168 after the initiation of treatment, the patient was completely healed and oral corticosteroid was
169 discontinued. The serum RIP3 level decreased to 300.0 pg/mL.

170 **Table E1.** Patient attributes and outcomes

Variable	SJS/TEN	EM major	EM minor	MPE	DRESS/DIHS
n	22	19	5	6	4
Sex, male/female	12/10	8/11	2/3	2/4	2/2
Age (years), median (range)	61 (7–89)	65 (16–82)	65 (16–82)	57 (35–84)	66 (35–73)
Cause					
Drug	14 (64%)	11 (58%)	5 (100%)	4 (67%)	4 (100%)
Infection	6 (27%)	4 (21%)	0 (0%)	2 (33%)	0 (0%)
Unknown	2 (9%)	4 (21%)	0 (0%)	0 (0%)	0 (0%)
BT >38 ° C	15 (68%)	9 (38%)	0 (0%)	2 (33%)	2 (50%)
Mucosal involvement					
Eyes	15 (68%)	7 (37%)	1 (20%)	2 (33%)	1 (25%)
Lips/Mouth	22 (100%)	13 (68%)	4 (80%)	1 (17%)	0 (0%)
Genital area/Urinary tract	12 (55%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)
Organ dysfunction					
Kidney	9 (41%)	3 (16%)	0 (0%)	0 (0%)	0 (0%)
Liver	12 (55%)	8 (42%)	0 (0%)	0 (0%)	4 (100%)
Lung	2 (9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Gastrointestinal tract	3 (14%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)
Laboratory examination					
WBC count (/μL), median (range)	7850 (1790–24200)	8610 (2730–16630)	7230 (5530–10130)	5600 (3540–12460)	7850 (2710–10290)
CRP (mg/dL), median (range)	9.54 (0.22–25.4)	2.6 (0.09–11.1)	0.7 (0.1–0.88)	1.31 (0.1–4.46)	1.78 (1.43–2.91)

Abbreviations: BT, body temperature; CRP, C-reactive protein; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash with eosinophilia and systemic symptoms; EM, erythema multiforme; MPE, maculopapular exanthema; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; WBC, white blood cells

