SHORT COMMUNICATION

Novel *JAK3*-Activating Mutations in Extranodal NK/T-Cell Lymphoma, Nasal Type

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Inhibition of the Janus kinase (JAK)—STAT pathway has been implicated as a treatment option for extranodal natural killer/T-cell lymphoma, nasal type (NTCL). However, JAK-STAT pathway alterations in NTCL are variable, and the efficacy of JAK-STAT pathway inhibition has been poorly evaluated. *JAK3* mutation and *STAT3* genetic alterations were investigated by direct sequencing and immunohistochemistry in 84 patients with newly diagnosed NTCL. Five of 71 patients with NTCL (7.0%) had *JAK3* mutations in the pseudokinase domain: two *JAK3*^{A573V}, two *JAK3*^{H583Y}, and one *JAK3*^{G589D} mutation. Proliferation of Ba/F3 cells transduced with novel *JAK3* mutations (*JAK3*^{H583Y} and *JAK3*^{G589D}) was independent of IL-3 and was inhibited by the *JAK3* inhibitor tofacitinib (means $\pm$ SD drug concentration causing a 50% inhibition of the desired activity, 85 ± 10 nmol/L and 54 ± 9 nmol/L). Ribbon diagrams revealed that these *JAK3* pseudokinase domain mutations were located at the pseudokinase-kinase domain interface. Although phosphorylated *STAT3* was overexpressed in 35 of 68 patients with NTCL (51.4%), a *STAT3* mutation (p.Tyr640Phe; *STAT3*^{Y640F}) at the SRC homology 2 domain was detected in 1 of the 63 patients (1.5%). A *STAT3* inhibitor was active against *STAT3*-mutant SNK-6 and YT cells. Novel *JAK3* mutations are oncogenic and druggable in NTCL. The *JAK3* or *STAT3* signal was altered in NTCL, and pathway inhibition might be a therapeutic option for patients with *JAK3*- or *STAT3*-mutant NTCL. (*Am J Pathol* 2017, 187: 980–986; <http://dx.doi.org/10.1016/j.ajpath.2017.01.004>)

Natural killer (NK)/T-cell lymphoma, nasal type (NTCL) is a subtype of Epstein-Barr virus (EBV)—positive and highly aggressive mature NK/T-cell neoplasm with extranodal presentation.^{1,2} The survival outcome of advanced disease is poor; therefore, discovery of new druggable targets is critical.³ Although Janus kinase (JAK)—STAT pathway activation has been suggested as the oncogenic mechanism in NTCL,^{4–6} several novel oncogenes outside the JAK-STAT pathway have been discovered.^{7,8} Although the frequency of *JAK3* mutations in NTCL has been variable (0% to 35%),^{5,9,10} next-generation sequencing revealed a low *JAK3* mutation frequency (0% to 5%).^{6–8} A *JAK3* inhibitor was effective against NK-S1 cells with the *JAK3*^{A572V} mutation⁵ and against MEC04 cells with the *JAK3*^{A573V} mutation⁹ *in vitro* and *in vivo*; however, its ability to inhibit other *JAK3* mutant subtypes has not been elucidated. We investigated *JAK3*

mutations and *STAT3* genetic alterations in NTCL and discovered novel *JAK3*-activating mutations that were oncogenic and druggable by the *JAK3* inhibitor tofacitinib.

Materials and Methods

Patients

Eighty-four patients were newly diagnosed with NTCL based on the World Health Organization classifications^{1,2}

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between January 2001 and December 2011 at Seoul National University Hospital. Formalin-fixed, paraffin-embedded (FFPE) tissue samples were prepared and tested for JAK-STAT pathway alterations. This study was approved by the Institutional Review Board of Seoul National University Hospital (H1404-071-572).

JAK3 and STAT3 Genetic Alterations

Direct sequencing of PCR products was performed bidirectionally to detect *JAK3* mutations on exons 13 and 16 and *STAT3* mutation on exon 21. Briefly, genomic DNA was isolated from FFPE tumor tissue using a Maxwell 16 FFPE Plus Tissue LEV DNA Purification kit (Promega, Madison, WI). *JAK3* and *STAT3* exons were amplified from genomic DNA using EconoTaq PLUS GREEN 2× premix (Lucigen, Middleton, WI) and then sequenced bidirectionally using the ABI3730 DNA sequencer (Applied Biosystems, Carlsbad, CA). The primer pairs used in the present study were as follows: *JAK3* A572V and A573V mutations on exon 13 (forward: 5'-GCAGGTCTGTGAGC-ACAAAAT-3', reverse: 5'-ACTGTCTCCAGCCATGCAC-ACG-3'), *JAK3* V722I mutation on exon 16 (forward: 5'-AGGCGCAGACACTTAGCTTG-3', reverse: 5'-CAAAG-TGGGGGTTTCGGAGAC-3'), and *STAT3* Y640F and D661Y mutations on exon 21 (forward: 5'-TTTCCGAA-TGCCTCCTCCTTGG-3', reverse: 5'-GAGATGACCTAG-CTGTAGGTTCC-3').

Retrovirus Packaging and Transduction

Retroviral vectors harboring novel *JAK3* mutations were generated through site-directed mutagenesis and were introduced into Ba/F3 and NIH-3T3 cells to test transformation capacity. The coding region of *JAK3* (NM_000215.3) was amplified and cloned into vector TOPO TA (Invitrogen, Grand Island, NY). Mutations of *JAK3* with 1847 T > C and 1866 A > G in the coding region were generated by site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) with mutation-specific primers according to the manufacturer's instructions. The Platinum-E retroviral packaging cell line was seeded in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum. The following day, the cells were transfected with the *JAK3* mutant construct in the pMXs-Puro vector (Cellbio Labs, San Diego, CA) and the packaging plasmids pCL-eco using Eugene6 transfection reagents (Roche, Indianapolis, IN). Viral supernatants were collected 48 to 72 hours later. Ba/F3 and NIH-3T3 cells in 6-well plates or 10-cm dishes were inoculated with 1 mL of viral supernatant in 1 mL of medium with polybrene (4 µg/mL). The transduced cells were selected using puromycin.

Soft Agar Assay

Cells were suspended in medium that contained 0.3% select agar and plated on a bottom layer of medium that contained

1% select agar [2% agar was melted in a microwave oven, mixed 1:1 with medium (2 × Dulbecco's modified Eagle's medium with 20% FBS, 2% P/S mixture, and 2% glutamine)] in a 6-well plate. To enable colony formation, plates were incubated at 37°C for 2 weeks. The number of colonies that grew in the 6-well plates after 2 weeks was counted in triplicate and normalized to NIH-3T3 cells.

Immunohistochemistry and Immunoblotting

Two-millimeter-diameter core samples were taken from representative FFPE tissue blocks of patients' tumors. Tumor cell content was evaluated by board-certified pathologists (Y.K.J., S.J.N., and C.W.K.) (Table 1). Immunohistochemical staining for phosphorylation of STAT3 (pSTAT3) was performed using anti-pSTAT3 antibody (Cell Signaling Technology, Danvers, MA). Part of the unstained tumor area was used as a negative staining control. Immunoreactivity was recorded as a total percentage of positive cells for pSTAT3. Only tumor cells were counted; if the number of cells with strong nuclear staining for pSTAT3 was >2% of all tumor cells, then the tumor had a high level of expression. Antibodies to total STAT3 and pSTAT3 (Cell Signaling Technology) were used for Western blotting, and the blots were subsequently washed, transferred to membranes, and developed using Enhanced Lumi Light Western Blotting Substrate (Roche, Indianapolis, IN).

Cell Culture and Cell Viability Assay

SNK-6 and NKL cells were kindly provided by Drs. Norio Shimizu (Tokyo Medical and Dental University, Tokyo, Japan) and Masao Seto (Aichi Cancer Center, Aichi, Japan), respectively. YT cells were purchased from the German Resource Center for Biological Material (Braunschweig, Germany). The *JAK3* inhibitor tofacitinib or the *STAT3* inhibitor Stattic was added to each well at specified concentrations (tofacitinib and Stattic; Selleck Chemical, Houston, TX). The plates were incubated at 37°C in a 5% CO₂ atmosphere incubator for 48 or 72 hours. For Ba/F3 with tofacitinib, the cells were incubated without IL-3 for 72 hours; for NTCL with Stattic, the cells were incubated without IL-2 for 48 hours. Growth inhibition assays were assessed using the CCK-8 colorimetric assay (Dojindo Laboratories, Kumamoto, Japan). For IL-3-independent growth assays, Ba/F3 cells transfected with the *JAK3* mutant plasmid were cultured in the absence of IL-3 for 6 days. The number of viable cells was determined by trypan blue exclusion assay.

JAK3 Homology Modeling

A *JAK3* homology model was generated using the SWISS-MODEL server.¹¹ TYK2 structure (Protein Data Bank identification 4OLI)¹² was used as the modeling template. The stereochemical quality of the constructed models was validated using a Ramachandran plot generated by

Table 1 Patient Characteristics

Characteristic	Finding
Age, years (<i>n</i> = 84)	
Median	49
Range	8–79
Presentation (<i>n</i> = 84)	
UAT	58 (69)
NUAT	26 (31)
B symptoms (<i>n</i> = 83)	
No	49 (59)
Yes	34 (41)
ECOG performance status (<i>n</i> = 82)	
0–1	60 (73)
≥2	22 (27)
EBV (<i>n</i> = 84)	
Positive	84 (100)
Negative	0 (0)
Local tumor invasiveness (<i>n</i> = 84)	
No	48 (57)
Yes	36 (43)
Ann Arbor stage (<i>n</i> = 82)	
I, II	50 (61)
III, IV	32 (39)
IPI scores (<i>n</i> = 82)	
0–1	44 (54)
2	8 (10)
3	15 (18)
4–5	15 (18)
JAK3 mutation in exon 13 (<i>n</i> = 71)	
Negative	66 (93)
Positive	5 (7)
JAK3 mutation in exon 16 (<i>n</i> = 68)	
Negative	68 (100)
Positive	0 (0)
STAT3 mutation (<i>n</i> = 63)	
Negative	62 (98)
Positive	1 (2)
STAT3 phosphorylation (<i>n</i> = 68)	
Low	33 (49)
High	35 (51)
Tumor cell content, % (<i>n</i> = 83)	
Median	35
Range	1–64

Data are presented as *n* (%) of patients unless otherwise indicated.
EBV, Epstein-Barr virus; ECOG, Eastern Cooperative Oncology Group; IPI, International Prognostic Index; NUAT, non–upper aerodigestive tract; UAT, upper aerodigestive tract.

PROCHECK. Ribbon diagrams were generated using the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA).

Results

Patient Characteristics

Tumor tissues from 84 patients with NTCL were examined for the presence of *JAK3* mutations within the pseudokinase domain (JH2) and *STAT3* alterations (mutation within the

SRC homology 2 domain and phosphorylation). Approximately two-thirds of patients with NTCL presented with involvement of the upper aerodigestive tract (*n* = 58), and two-fifths of patients presented with advanced disease (*n* = 32) (Table 1).

JAK3 Mutations

We identified three *JAK3* mutations on exon 13 in 5 of 71 patients (7.0%) who had amplified PCR products from FFPE tumor tissues. Tumor cell content ranged from 1% to 64%. The *JAK3*^{A573V} mutation was identified in two patients with non–upper aerodigestive tract NTCL (Figure 1A and Table 2). The following two novel heterozygous missense mutations were identified in *JAK3* exon 13: histidine-to-tyrosine substitution at codon 583 (*JAK3*^{H583Y}, c.1847C>T, NM_000215.3) (*n* = 2) and glycine-to-aspartic acid substitution at codon 589 (*JAK3*^{G589D}, c.1866G>A) (*n* = 1) (Figure 1A). The *JAK3*^{G589D} mutation was somatic (Supplemental Figure S1). The *JAK3* mutations in exon 16, such as *JAK3*^{V722I}, were not observed in 68 patients with NTCL. Overall, the *JAK3* mutation frequency in NTCL was 7.0% in our study (Supplemental Table S1).^{5–10}

Oncogenic Properties of Novel JAK3 Mutations

We generated retroviral vectors expressing wild-type *JAK3* or *JAK3*^{H583Y}, *JAK3*^{G589D}, or *JAK3*^{A572V} mutations and inoculated NIH-3T3 or Ba/F3 cells. Ba/F3-*JAK3*^{H583Y} and Ba/F3-*JAK3*^{G589D} cells exhibited IL-3–independent growth (Figure 1B), whereas NIH-3T3-*JAK3*^{H583Y} and NIH-3T3-*JAK3*^{G589D} cells exhibited anchorage-independent growth (Figure 1C) similar to the transforming activity of *JAK3*^{A572V} mutant cells.^{5,8} Then, we tested whether the *JAK3* inhibitor tofacitinib could inhibit the growth of *JAK3*^{H583Y}- and *JAK3*^{G589D}-transduced Ba/F3 cells. Tofacitinib significantly inhibited Ba/F3-*JAK3*^{H583Y} and Ba/F3-*JAK3*^{G589D} cell growth; it also inhibited Ba/F3-*JAK3*^{A572V} growth but to a lesser extent (Figure 1D).

Structural Modeling of Novel JAK3 Mutations

Ribbon diagrams of the *JAK3* JH2 and kinase (JH1) domains revealed that H583 in the αC-helix and G589 in the β4-sheet were located in the N-lobe of the JH2 domain (Figure 1E), similar to other tumor-associated *JAK3* JH2 mutations, such as A572V and A573V. The *JAK3*^{H583Y} and *JAK3*^{G589D} mutation sites were closer to the JH1 domain than the *JAK3*^{A572V} and *JAK3*^{A573V} mutation sites (Figure 1F).

STAT3 Mutation and Phosphorylation

Strong expression of pSTAT3 in the nucleus of ≥2% of all tumor cells was observed in 35 of 68 patients (51.4%) (Figure 2A). Although clinical variables were comparable regardless of pSTAT3 expression, tumors with high

Figure 1 Novel *JAK3* mutations in natural killer/T-cell lymphoma, nasal type. **A:** Electropherogram of *JAK3* mutations in patients 1, 3, and 5. **B:** IL-3-independent proliferation of Ba/F3 cells transduced with retrovirus containing a *JAK3* mutation. **C:** NIH-3T3-JAK3, NIH-3T3-A572V, NIH-3T3-H583Y, and NIH-3T3-G589D cell growth was measured by colony formation in soft agar. **D:** Cell viability assays with various tofacitinib concentrations in Ba/F3 cells. Ba/F3-JAK3^{H583Y} and Ba/F3-JAK3^{G589D} cells are sensitive to the *JAK3* inhibitor. Drug concentration causing a 50% inhibition of the desired activity (IC₅₀) values are provided for each case. **E** and **F:** Homology models of the *JAK3* pseudokinase and kinase domains. H583Y and G589D are located at the interface between the two domains (marked with red boxes in **E**, enlarged in **F.**). **P* < 0.05 versus NIH-3T3-JAK3.

pSTAT3 expression had a trend toward advanced disease stage (Supplemental Table S2). The pSTAT3 status did not affect overall survival (*P* = 0.137). *STAT3* mutation (p.Tyr640Phe; *STAT3*^{Y640F}) at the SRC homology 2 (SH2) domain was found in 1 of the 63 tumor samples (1.5%) (Figure 2B). Growth of YT and SNK-6 cells harboring *STAT3*^{Y640F} and *STAT3*^{D661Y} mutations, respectively, were inhibited by the *STAT3* inhibitor Stattic (Figure 2C) but not

Table 2 *JAK3* or *STAT3*-Mutant Natural Killer/T-Cell Lymphoma, Nasal Type

Patient No.	Sex/age, years	Primary site	Stage	First-line treatment	Response to treatment	PFS, months	OS, months	<i>JAK3</i> mutation	<i>STAT3</i> mutation	Phosphorylated <i>STAT3</i>	Tumor cell content, %
1	F/49	NUAT	I	CHOP	SD	4.07	11.67	A573V	WT	High	35
2	M/37	NUAT	IV	IMEP	PD	2.43	2.83	A573V	NA	Low	15
3	M/68	NUAT	III	CHOP	NA	NA	NA	H583Y	WT	Low	49
4	M/47	UAT	IV	BVP	PD	0.6	0.7	H583Y	NA	Low	28
5	F/42	UAT	I	IMEP	CR	29.27	29.27	G589D	WT	Low	49
6	M/57	UAT	IV	IMEP	PD	2.03	3.36	WT	Y640F	Low	25

BVP, bleomycin, vincristine, prednisolone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisolone; CR, complete remission; F, female; IMEP, ifosfamide, methotrexate, etoposide, and prednisolone; M, male; NA, not available; NUAT, non-upper aerodigestive tract; OS, overall survival; PD, progressive disease; PFS, progression free survival; SD, stable disease; UAT, upper aerodigestive tract; WT, wild type.

Figure 2 *STAT3* alterations in natural killer/T-cell lymphoma, nasal type (NTCL). **A:** Immunohistochemical staining for phosphorylated *STAT3* (p*STAT3*) reveals low p*STAT3* and high p*STAT3* expression. **B:** Electropherogram of *STAT3* mutations in tumor from patient 6, YT cells, and SNK6 cells. **C:** Cell viability assay after treatment of NTCL cells with various concentrations of Stattic. YT and SNK6 cells harboring *STAT3* mutations were more sensitive to the *STAT3* inhibitor than HANK1 or SU-DHL6. **D:** *STAT3* phosphorylation status in various cell lines. OCI-Ly3 and SU-DHL6 derived from diffuse large B-cell lymphoma were used as control cells. Data are expressed as means ± SD of three independent experiments (**C**). Original magnification, ×400. DLBCL, diffuse large B-cell lymphoma; IC₅₀, drug concentration causing a 50% inhibition of the desired activity.

by tofacitinib (Supplemental Figure S2). Stattic was not toxic to HANK1 cells with wild-type *STAT3*, which have relatively lower p*STAT3* (Tyr705) levels than SNK-6 and YT cells (Figure 2D). Diffuse large B-cell lymphoma cells without *STAT3* mutation were used as controls; OCI-Ly3 cells with high p*STAT3* (Tyr705) expression were more sensitive to Stattic than SU-DHL6 cells with low p*STAT3* (Tyr705) expression (Figure 2, C and D).

Discussion

Our study found that *JAK3*-activating mutations were rare (7.0%) in patients with NTCL. We identified novel *JAK3*^{H583Y} and *JAK3*^{G589D} mutations that were oncogenic and targetable alterations. In addition, the *STAT3* mutation in the SH2 domain also might be a promising target for inhibition as an NTCL treatment.

Since the first report of an oncogenic *JAK3* mutation,⁵ reports of its mutation frequency in NTCL has been variable. In contrast to the studies reporting high mutation frequencies (21% to 35%),^{5,9} subsequent studies suggested a much lower mutation frequency ranging from 0% to 5.1%,^{6,8,10} which was comparable to our result (Supplemental Table S1). Although the *JAK3*^{A573V} mutation (which is the second-most common) was identified in our

patients, the *JAK3*^{A572V} and *JAK3*^{V722I} mutations were not observed, in contrast to previous reports.^{5,9} This difference in *JAK3* mutation frequency might be attributable to the ethnic variation in *JAK3* mutations in NTCL. In addition, technical issues in detecting mutations and variation in tumor content might have contributed to reported differences in mutation frequency.

In this study, novel *JAK3*^{H583Y} and *JAK3*^{G589D} mutations with oncogenic potential that were somatic and not seen in COMSIC and public databases were identified in three patients. Anchorage- or IL-3-independent growth of *JAK3*^{H583Y} and *JAK3*^{G589D}-mutant cells was observed in the NIH-3T3 or Ba/F3 cell lines, which suggested these novel *JAK3* mutations had oncogenic properties. *JAK3* mutant cells exhibited 6.2- to 46-fold greater sensitivity to tofacitinib compared with *JAK3* wild-type cells. Considering the efficacy of tofacitinib for inhibiting *JAK3* in Ba/F3-*JAK3*^{H583Y} and *JAK3*^{G589D} cells and inhibiting *JAK3* through prevention of EZH2 phosphorylation in NTCL,¹³ *JAK3* inhibition might be clinically relevant for treating *JAK3*-mutant NTCL. In addition, structural modeling found that the novel *JAK3* mutations were located in the *JAK3* pseudokinase domain. The novel *JAK3* mutations were closer to the JH1 domain compared with the *JAK3*^{A572V} and *JAK3*^{A573V} mutations, which might render the novel *JAK3* mutations more oncogenic and sensitive to tofacitinib. These

results suggest that novel *JAK3* mutations in the N-lobe of the JH2 domain are druggable target oncogenes located at the interface of the JH2 and JH1 domains, similar to other cancer-associated *JAK3* mutations.¹² Considering JH2-mediated negative regulation of *JAK3*,¹⁴ our pathogenic *JAK3* mutations in the JH2 domain might disrupt a negative signal between JH2-JH1 domains, resulting in *JAK3* kinase activation and sensitivity to inhibition.⁵ Tofacitinib is a potent inhibitor of *JAK1* and *JAK3* but not *JAK2* and has been used as monotherapy for treatment of rheumatoid arthritis.¹⁵ In addition, tofacitinib has clinical activities against *JAK3*-mutant primary mediastinal B-cell lymphoma as combination therapy¹⁶ and *STAT3*-mutant T-cell large granular lymphocytic leukemia as monotherapy.¹⁷ Clinical and *in vitro* activities of *JAK3* inhibition warrant further investigation in *JAK3*-mutant lymphoid neoplasms, including NTCL.

STAT3 genetic alterations were evaluated because of the oncogenic role of constitutive *STAT3* signal activation in NTCL.^{4,18} Although more than half of the patients had strong p*STAT3* expression, the previously identified *STAT3*^{Y640F} mutation^{6–8,19} was found in only one patient, which was a relatively lower frequency than in other published studies (*STAT3* mutation frequencies in the SH2 domain were reported as 5.9%,⁶ 7.6%,⁷ and 26%⁸). *STAT3* activation was adversely associated with survival in patients with diffuse large B-cell lymphoma,²⁰ whereas p*STAT3* did not correlate with survival in patients with NTCL. Stattic, a *STAT3* inhibitor, was effective against *STAT3*-mutant NTCL cells but less effective in *STAT3* wild-type NTCL cells with high p*STAT3* expression in our study. Similarly, NTCL patient-derived MEC04 cells with high p*STAT3* expression were inhibited by high concentrations of a *STAT3* inhibitor.⁴ Interestingly, *JAK1/2* inhibitors that disrupted the *STAT3*-activating signal inhibited NTCL cells with high p*STAT3* expression⁴ as well as *STAT3*-mutant NTCL cells,⁶ which suggested a therapeutic option for patients with NTCL.

In conclusion, the frequency of *JAK3* mutations in the JH2 domain was relatively low in NTCL (mean, 11.8%; range, 0% to 35.4%) in contrast to a previous report.⁵ We identified novel *JAK3*^{H583Y}- and *JAK3*^{G589D}-activating mutations that were oncogenic and sensitive to a *JAK3* inhibitor. Although the frequency of *STAT3* mutations was low in patients with NTCL, *STAT3*-mutant NTCL cells were sensitive to a *STAT3* inhibitor but not to a *JAK* inhibitor. These results indicated that *JAK3* and *STAT3* mutations are candidate therapeutic targets in patients with NTCL. Future efforts should be directed to evaluate the efficacy of *JAK3* or *STAT3* inhibitors in patients with NTCL with *JAK3*- or *STAT3*-activating mutations.

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S.H.S., S.K., and T.M.K. designed the research; Y.K.J., S.J.N., and C.W.K. reviewed slides and collected tumor tissues; S.H.S. and S.K. performed experiments and/or analyzed data; T.M.K., B.K., D.-W.K., and D.S.H. provided samples; H.H.P. performed structural modeling; S.H.S., S.K., and T.M.K. wrote the manuscript; all authors contributed to data interpretation and approved the final manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.01.004>.

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