ABSTRACT

The molecular pathogenesis of natural killer/T-cell lymphoma (NKTCL) is not well understood. We conducted whole-exome sequencing and identified Janus kinase 3 (JAK3) somatic–activating mutations (A572V and A573V) in 2 of 4 patients with NKTCLs. Further validation of the prevalence of JAK3 mutations was determined by Sanger sequencing and high-resolution melt (HRM) analysis in an additional 61 cases. In total, 23 of 65 (35.4%) cases harbored JAK3 mutations. Functional characterization of the JAK3 mutations support its involvement in cytokine-independent JAK/STAT constitutive activation leading to increased cell growth. Moreover, treatment of both JAK3-mutant and wild-type NKTCL cell lines with a novel pan-JAK inhibitor, CP-690550, resulted in dose-dependent reduction of phosphorylated STAT5, reduced cell viability, and increased apoptosis. Hence, targeting the deregulated JAK/STAT pathway could be a promising therapy for patients with NKTCLs.

SIGNIFICANCE:

Gene mutations causing NKTCL have not been fully identified. Through exome sequencing, we identified activating mutations of JAK3 that may play a significant role in the pathogenesis of NKTCLs. Our findings have important implications for the management of patients with NKTCLs.

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patients as there is currently no accepted standard first-line treatment for NKTCLs. Despite multiagent chemotherapy and involved-field radiotherapy, the 5-year overall survival rate is approximately 9% for non-nasal NKTCLs and 42% for nasal NKTCLs (2, 3). There is thus an urgent need to identify novel genetic aberrations and potential treatment targets in NKTCLs.

In this study, we conducted whole-exome sequencing to identify somatic mutations in protein-coding genes of NKTCL tumors to shed light on their pathogenesis and to uncover potential new therapeutic targets, which are urgently needed.

**RESULTS**

**Identification and Validation of JAK3 Mutations**

Whole-exome sequencing was successfully conducted on fresh-frozen NKTCLs and paired blood samples from 4 different patients. The average coverage of each base in the targeted regions was 111-fold; 84% of the bases were represented at least 20 times (Supplementary Table S1) and a total of 208 somatic mutations were identified in 201 genes (Supplementary Table S2). Known somatic mutations in NKTCLs, such as TP53, KRAS, and NRAS (4), identified by exome sequencing were further validated by Sanger sequencing in the same tumors (Supplementary Table S2).

Interestingly, several somatic heterozygous Janus kinase (JAK) mutations were found in 2 separate samples. One tumor harbored both JAK1Y652D and JAK3A572V mutations, whereas the other tumor harbored a JAK3A573V mutation. The JAK3A572V and JAK3A573V mutations were located at exon 12, in the Janus homology domain 2 (JH2) that negatively regulates the Janus homology domain 1 (JH1) kinase activity (Fig. 1). The JAK1Y652D mutation was located in the JH2 domain as well. All 3 missense mutations were predicted by PolyPhen to be damaging (5).

To determine the prevalence of JAK1 and JAK3 mutations in NKTCLs, we Sanger sequenced an additional 61 NKTCL formalin-fixed, paraffin-embedded (FFPE) cases. In total, we found mutations in JAK3 in 23 of 63 (35.4%) cases (Supplementary Table S3) and for JAK1, besides the case with concomitant JAK1Y652D and JAK3A572V mutations described above, no additional mutations were identified. Among the patients with JAK3 mutations, there were 17 heterozygous JAK3A572V, 2 homozygous JAK3A573V, 2 heterozygous JAK3A572V, 1 homozygous JAK3A573V, and 1 heterozygous with both JAK3A572V and JAK3A573V mutations. These results were further confirmed by high-resolution melt (HRM) analysis (Supplementary Fig. S1). The presence of nonmalignant stroma (our samples contained at least 50% tumor content) or tumor subclones makes it difficult to assess whether these “heterozygous” tumors might actually represent a mixture of JAK3 homozygous–mutated cancer cells admixed with normal tissue. As such, it is possible that the number of homozygous tumors reported is actually an underestimate and that this value should be regarded as a lower limit.

We also conducted Epstein-Barr virus–encoded RNA (EBER) testing on all cases. Apart from 4 older cases that cannot be interpreted, all but one case were positive for EBER, regardless of JAK3 mutation status. The single EBER-negative case was a cutaneous deposit taken from a patient with EBER-negative nasal NKTCLs (Supplementary Table S3). In parallel, 50% of extra-nasal cases possessed JAK3 mutations and 31.7% of nasal cases had JAK3 mutations. This latter difference was not statistically significant (Supplementary Table S3).

**JAK3A572V-Activating Mutations Confer Cytokine-Independent Growth**

Interleukin (IL)-2 is an essential cytokine required for the proliferation and activation of NK cells (6). JAK1 and JAK3 mediate IL-2 receptor signaling through phosphorylation of STAT transcription factors (7). In line with the functional importance of the activating JAK3 mutations identified, we tested whether JAK3 mutations could confer IL-2-independent growth to the NKTCL cell line (NK-S1) that harbors a homozygous JAK3A572V mutation. JAK-mutant (NK-S1) cells showed IL-2-independent growth (Fig. 2A) and constitutive phosphorylation of both JAK3 and STAT5 (Fig. 2B). In contrast, JAK3 wild-type KHYG-1 cells were clearly IL-2-dependent (Fig. 2C and D). Importantly, NK-S1 cells treated with JAK3 siRNAs exhibited a significant reduction in cell proliferation and also decreased autophosphorylation of JAK3 and STAT5 (Fig. 3A). Reciprocally, KHYG-1 cells transiently overexpressing a mutated JAK3 (JAK3A573V) cDNA showed IL-2–independent proliferation and autophosphorylation of JAK3 and STAT5 (Fig. 3B). These results indicate that the
JAK3-activating mutations are gain-of-function alleles and contribute to the constitutive activity of the JAK/STAT pathway in an IL-2–independent manner.

**Effects of CP-690550 on NKTCL Cell Lines**

To further confirm the involvement of JAK/STAT signaling in the survival of NKTCLs, we next evaluated the effect of a pan-JAK inhibitor, CP-690550, in NK-S1, KHYG-1, and K562 cells. As expected, both the NK-S1 and KHYG-1 cells showed a reduction of phosphorylated STAT5 (Fig. 4A) and cell viability in a dose-dependent fashion (Fig. 4B). Furthermore, Annexin V staining revealed that the reduction of NK-S1 viability was due to an increase in cellular apoptosis (Fig. 4C). However, this phenomenon was not observed in the K562 cells in which STAT5 phosphorylation is dependent on BCR/ABL1 (8) and not JAK3 (Fig. 4A and B).

**DISCUSSION**

The JAK/STAT pathway is a key component in normal hematopoiesis. The JAK family of tyrosine kinases comprises 4 members: JAK1, JAK2, JAK3, and TYK2. Among these 4 members, JAK3 signaling is specifically related to T-cell development and proliferation (8) with loss-of-function mutations resulting in severe combined immunodeficiency characterized by the lack of T and NK cells (9). Recent data suggest that mutations resulting in persistent activation of JAK/STAT signaling are involved in the pathogenesis of T-cell acute lymphoblastic lymphoma/leukemia, cutaneous TCL, mantle cell lymphoma, acute megakaryoblastic leukemia, and myeloproliferative diseases (8, 10–16).

Besides hematologic neoplasia, nonhematologic cancers, including breast, stomach, and lung cancer, have also been found to harbor JAK3 mutations (17, 18). To date, transforming ability of the activating mutations of JAK3 (such as P132T, L156P, R172Q, E183G, Q501H, M511I, A572V, A573V, R657Q, and V722I) has been previously validated in Ba/F3 cells (8, 12, 19, 20). In line with these observations, we identified the presence of activating JAK3 mutations in 35% of NKTCL tumors. The JAK3A572V and JAK3A573V mutations found in our samples were located at the JH2 pseudokinase domain that is known to have an autoinhibitory effect on the JH1 kinase domain. Cellular studies revealed that the NK-S1 cells harboring the homozygous JAK3A572V mutation are able to proliferate in cell culture without IL-2 stimulation, with constitutive expression of both phosphorylated JAK3 and STAT5. Cornejo and colleagues (21) showed that when JAK3A572V retroviral-transduced bone marrow cells were transplanted into C57BL/6 and BALB/c mice, there was a constitutive activation of JAK/STAT signaling which led to the development of fatal polyclonal T-cell lymphoproliferative disorder. In accordance, transiently overexpressing a JAK3A572V in a JAK3 wild-type NKTCL cell line (KHYG-1) resulted in IL-2–independent cell proliferation and the activation of JAK/STAT signaling pathways. Thus, it is conceivable that the JAK3 mutation may play an important role in the pathogenesis of NKTCLs.

CP-690550, a novel pan-JAK inhibitor, has recently been shown to inhibit adult TCL/leukemia (ATLL) cells (22) and ATLL xenograft tumors and is currently in phase III trials for the treatment of rheumatoid arthritis (23).
Consistent with the high frequency of JAK3 mutations (35%) in NKTCLs, use of CP-690550 in the JAK3-mutant NKTCL cell line showed inhibition in the phosphorylation of STAT5 along with reduced cell viability. These data are compelling and suggest a potential target for this otherwise fatal disease.

In summary, our studies identified, for the first time, frequent JAK3 mutations in NKTCLs. They also indicated that targeting the JAK/STAT pathway in this disease is a potentially effective therapeutic approach that warrants further investigation.

**METHODS**

**Tissue Samples**

Matched fresh-frozen tissue and peripheral blood samples were obtained from 4 consented patients with NKTCLs for whole-exome sequencing. The JAK3, JAK1, JAK2, and TYK2 mutation status in these
JAK3 Mutations Identified in NKTCLs

**RESEARCH BRIEF**

**Figure 4.** Effects of CP-690550 on NKTCL cell lines. A, NK-S1, KHYG-1, and K562 cells were treated with CP-690550 for 48 hours, and the effect on STAT5 phosphorylation was evaluated by Western blotting. B, cell viability was analyzed by MTS assay after the cells were treated with their respective treatment for 72 hours. C, drug-induced apoptosis was evaluated by Annexin V-FITC (fluorescein isothiocyanate) staining, followed by flow cytometric analysis. Both NK-S1 and KHYG-1 cells showed a dose-dependent reduction in STAT5 phosphorylation. Treatment with CP-690550 resulted in reduced cell viability of NK-S1 and KHYG-1 cells but not in K562 cells. Experiments were repeated at least 3 times. Data were analyzed by paired t test, and values significantly different from control are shown as *, **, ***.

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**Preparation of Genomic DNA**

DNA of frozen tissue and paired blood samples was isolated using a DNeasy blood and tissue mini kit and a QIAmp DNA blood midi kit (Qiagen), respectively, according to the manufacturer’s instruction. For FFPE samples, genomic DNA was extracted from one or two 10-μm slices from each sample by removal of paraffin followed by proteinase K digestion according to standard procedures. DNA was then extracted using a DNeasy blood and tissue mini kit (Qiagen).

**Whole-Exome Sequencing and Identification of Candidate Mutations**

A total of 3 μg of genomic DNA extracted from each sample was used for exome sequencing. Captured DNA libraries were sequenced with the Illumina GAIIx Genome Analyzer, yielding 150 (2 × 75) base pairs from the final library fragments. We used Burrows Wheeler Aligner to align the sequence reads to the human reference genome NCBI built 37.1 (hg19) and then we ran SamTools to remove PCR duplicates. To detect single-nucleotide variants (SNV), we used a discovery pipeline based on the Genome Analyzer Toolkit (GATK). Our pipeline first recalibrates the base qualities and realigns the sequence reads around micro-indels. The next step uses a GATK Unified Genotyper that does the consensus calling to identify SNVs. Only well-mapped reads (mapping quality ≥30, number of mismatches within a 40-bp window ≤3) were used as input to the genotyper. We retained SNVs that passed additional quality filters (a quality by depth ≥5, a variant depth ≥5, a normal depth ≥5) and discarded any SNV close to a micro-indel or to several other SNVs. We compared our variants against the common polymorphisms present in dbSNP 131 and in the 1000 genomes databases to discard any common SNPs. All variants retained following this step were considered to be novel. Several gene transcript annotation databases (CCDS, RefSeq, Ensembl, and UCSC) were used for transcript identification and for determining the amino acid change. Only SNVs in exons or in canonical splice sites were further analyzed. Amino acid changes corresponding to SNVs were annotated according to the largest transcript of the gene.

**Mutation Validation by HRM and Sanger Sequencing**

Sanger sequencing and HRM (25, 26) were used to confirm the JAK3 and JAK1 mutations identified and validate their prevalence in our NKTCL patient population. The primer sequences used for validation are listed in Supplementary Table S4. For Sanger sequencing, PCR was carried out with Platinum Taq Polymerase (Life Technologies, catalog number 10966-083) and cycled at 95°C for 10 seconds; 30 cycles of 95°C for 30 seconds; 60°C for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. Sequencing PCR was carried out with ABI BigDye Terminator v3.1 (Life Technologies, catalog number 4337457) and cycled at 96°C for 1 minute; 29 cycles of 96°C for 10 seconds; 50°C for 5 seconds, and 60°C for 4 minutes. The resulting products were run on an ABI 3730 DNA analyzer. For HRM analysis, SsoFast EvaGreen Supermix (Bio-Rad, catalog number 172-5200) was used in the amplification of the sample amplicons using the JAK3 HRM primers at a final temperature of 65°C for 10 minutes; 40 cycles of 94°C for 15 seconds; 58°C for 15 seconds, and 72°C for 1 minute.
concentration of 600 nmol/L on a Bio-Rad CFX96 Real-Time PCR detection system in replicates. The cycling and melting conditions were as follows: 1 cycle of 98°C for 2 minutes; 39 cycles of 98°C for 5 minutes; 58°C for 10 minutes; 1 cycle of 95°C for 30 minutes; and a melt from 72°C to 95°C increasing at 0.2°C/s. The HRM curves were analyzed with the Bio-Rad Precision Melt Analysis Software. HRM difference curves deviating from the wild-type curve were considered to be harboring a mutation.

Cell Lines

NK-S1, established from a previously described NKTCL xenograft (27), was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated FBS and 10% equine serum. KHYG-1 was obtained from the Japanese Collection of Research BioResources (28) and cultured in RPMI medium (Life Technologies) supplemented with heat-inactivated FBS (10%), equine serum (10%), and 200 IU/mL of recombinant human IL-2 (Novartis). K562 (CCL-234) was purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% heat-inactivated FBS and 10% equine serum. The coding exons of JAK3 were fully sequenced in these 3 cell lines, and we confirmed that only NK-S1 harbored a homozygous JAK3A572V mutation.

Cell Line Transfections

JAK3 siRNA or control siRNA (Dharmacon) were transfected into NK-S1 cell line using RNAiMAX (Invitrogen) according to the manufacturer’s protocols. MIGR1 expression vectors containing full-length wild-type JAK3 or JAK3A572V mutant were generously provided by Dr. Brian Druker (Howard Hughes Medical Institute, Chevy Chase, MD; ref. 8). Transient overexpression of these 2 constructs in KHYG-1 cells was then generated using Effectene Transfection Reagent (Qiagen Inc.).

Cell Viability and Apoptosis Assays

Cells were seeded at 2 × 10^4 cells/100 μL/well in 96-well plates and treated with or without CP-690550 (Selleck Chemical, S5001) at various concentrations before being subjected to MTS assay (Promega). The extent of drug-induced apoptosis was evaluated by Annexin V-FITC (BD Biosciences) staining. Acquisition of the data were conducted on FACSCalibur flow cytometer (BD Biosciences).

Western Blot

Cells were harvested at indicated time intervals after IL-2 or CP-690550 treatment. Total proteins were extracted with lysis buffer, resolved by SDS-PAGE gels, and blotted onto a nitrocellulose membrane. After blocking, membranes were probed with primary antibodies against phospho-STAT5 (Cell Signaling, catalog number 9363), phospho-JAK3 (Cell Signaling, catalog number 5031), JAK3 (Cell Signaling, catalog number 3775), and β-actin followed by either peroxidase-conjugated antibody or anti-rabbit secondary antibody. Signals were visualized with enhanced chemiluminescence (ECL; Amersham).

Disclosure of Potential Conflicts of Interest

S. Rozen is employed by Duke University as Associate Professor Track V. No potential conflicts of interests were disclosed by the other authors.

Authors’ Contributions


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REFERENCES

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