Large granular lymphocyte leukemia (LGL L) is a group of proliferative disorders of cytotoxic T cells or natural killer (NK) cells frequently complicated with cytopenia and autoimmune phenomena.\(^\text{1,2}\) In the current World Health Organization (WHO) classification, T-cell large granular lymphocytic leukemia (T-LGL L)\(^\text{3}\) and chronic lymphoproliferative disorders of NK cells (CLPD-NK) are included in this category.\(^\text{4}\) There has been controversy concerning the nature of these diseases, whether they are reactive processes or neoplasms. Recently, recurrent somatic mutations in the Src homology 2 (SH2) domain of the signal transducer and activator of transcription 3 (STAT3) gene have been found in T-LGL L and CLPD-NK,\(^\text{5,6}\) leading to constitutive activation of STAT3 and dysregulation of genes downstream of STAT3. Because it is suggested that clinical characteristics of LGL L differ among different ethnic groups,\(^\text{7–9}\) these findings prompted us to investigate mutations in STAT3 in an Asian cohort of LGL L, including familial cases.

### Materials and Methods

**Patients.** Patients categorized with LGL L, that is, T-LGL L and CLPD-NK, were selected. The criteria for diagnosis of each disease were based on the WHO classifications of 2008.\(^\text{3,4}\) The T-LGL L was diagnosed as follows: large granular lymphocyte (LGL) morphology with typical surface phenotypes with CD2, CD3, T-cell receptor (TCR) \(\alpha\beta\) or \(\gamma\delta\), CD8 and CD16/56, TCR \(\gamma\)-chain monoclonal rearrangement and a LGL count over 2000/\(\mu\)L. In some cases with a LGL count less than 2000/\(\mu\)L, characteristic features other than cell count and persistent clinical features for more than 6 months were recognized.\(^\text{10}\) The CLPD-NK was characterized by a LGL count over 700/\(\mu\)L with a phenotype of CD2\(^+\) CD3\(^-\) CD56\(^-\)/CD16\(^+\) TCR\(^-\) for more than 6 months duration. Epstein–Barr virus (EBV) was uniformly negative in cells with T-LGL L and CLPD-NK. In the control groups, aggressive NK-cell leukemia (ANKL) had the cellular characteristics of EBV-positive LGL with CD2\(^+\) CD3\(^-\) CD56\(^-\)/CD16\(^+\) TCR\(^-\), with the main involved sites being bone marrow, peripheral blood, liver and/or spleen with a diffuse pattern, as well as frequent associations with liver dysfunction, hemophagocytosis and a rapidly deteriorating clinical course.\(^\text{11}\) The EBV-associated T-cell lymphoproliferative disorders (T-LPD) were diagnosed using the features of EBV-positive atypical LGL with a phenotype of CD2\(^+\) CD3\(^-\) TCR\(^+\), monoclonal TCR \(\gamma\)-chain rearrangements, hepatosplenic involvements with acute onset of generalized symptoms, liver dysfunction and coagulopathy.

The study protocol was approved by the Institutional Review Board of Shinshu University School of Medicine and performed in accordance with the Declaration of Helsinki.

**DNA isolation, polymerase chain reaction (PCR) and direct sequencing analysis.** Stored mononuclear cells, isolated from peripheral blood drawn after informed consent had been provided and stored at \(-80^\circ\)C, were analysed. In a patient, anticogulated peripheral blood was used.

Extraction of genomic DNA was performed using a QIAamp DNA blood mini-kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. The sequences of exons 19 to 24, which encode the SH2 domain of STAT3,
were amplified by PCR using the primers\(^5\) and the PCR products were subjected to direct sequencing in both directions on an automatic DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA).

Genomic DNA was also extracted from non-hematopoietic cells of hairs or nails to examine whether the mutation was derived from the germline mutation in familial LGL L cases. **Allele-specific PCR (AS-PCR).** Because Y604F and D661Y mutations of STAT3 were recognized by direct sequencing analysis of this cohort, allele-specific PCR assays for these mutations were performed with primers designed as described previously.\(^6\)

**Allele-specific quantitative PCR (AS-qPCR).** Mutation-specific primers and universal primers were designed according to a previous report\(^6\) and TaqMan probes were designed as follows: 5′-ttccctcctgtgccg-3′ for Y640F; 5′-taagaccacagctgcctg-3′ for D661Y; and 5′-aaagcagcagctgaa-3′ for total copy numbers including mutant and wild-type alleles. Here, 50 μL of the AS-qPCR reaction mixture contained 100 ng of genomic DNA, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μmol/L each primer and 0.25 μmol/L TaqMan probe. The AS-qPCR was performed using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The reaction conditions were as follows: 50°C for 2 min; 95°C for 10 min; and 50 cycles of 95°C for 15 s and 60°C for 1 min. To construct plasmids carrying the wild-type STAT3, the PCR products obtained by amplification of genomic DNA from a normal control using 5′-TTAAATGCCAGGAACATGGAAAAT-3′ and 5′-AAAAAATGCCCCGATGGAGGTGGATGCC-3′ and 5′-AAAAATTAATGCCCCGATGGAGGTGGATGGAAT-3′ primers\(^9\) were cloned into a pTA2 vector using the TA cloning kit (Target Clone, TOYOBO, Osaka, Japan). Plasmids carrying both STAT3 mutations were heterozygous. In total, nine of 22 patients with STAT3 mutations in our T-LGL L and CLPD-NK cohorts were positive for mutations. Therefore, the frequencies of STAT3 mutations in our T-LGL L and CLPD-NK cohorts were 47.6% and 27.2%, respectively (\(P = 0.31\)). All of these mutations were heterozygous. In total, nine of 22 patients with TCR γ-chain-type T-LGL L and three of six T-LGL L patients with γ6TCR type were positive for mutations. Mutations in the SH2 domain of the STAT3 gene were not found in ANKL or EBV T-LPD patients using either direct sequencing or AS-qPCR. Samples from 50 Japanese healthy controls were examined using AS-qPCR and all were negative for Y604F and D661Y mutations. Three T/NK cell lines, Jurkat, NKL and NK92, were negative for mutations in the SH2 domain.

**Statistical analysis.** Comparison between groups was carried out using the Mann–Whitney U-test, Kruskal–Wallis H-test, Wilcoxon t-test, Fisher’s exact test or Chi-squared test as appropriate. These analyses were performed with JMP software (version 9; SAS Institute, Cary, NC, USA).

**Results**

**Identification of STAT3 SH2 domain mutations.** A total of 53 patients were analyzed in the present study (Table 1). They consisted of 42 patients with T-LGL L (22 with γβ TCR type, six with γ8 TCR type and 14 undetermined) and 11 patients with CLPD-NK. All patients with T-LGL L showed a monoclonal pattern of TCR gene rearrangement detected using PCR methods and/or Southern blot analyses. In the control groups, five patients with ANKL and two patients with EBV-T lymphoproliferative disorders (LPD) (one with γβ TCR type and one with γδ TCR type) were also analyzed. Cells of two patients with γβTCR-type T-LGL L were positive for CD4. Some of the patients were reported previously.\(^8\)\(^-\)\(^16\)

| Table 1. Clinical features of patients with LGL leukemia |
|-----------------|-----|-----|
|                | Japanese cohort | Hong Kong cohort |
|----------------|-----------------|-----------------|
| T-LGL L (n = 17) |                 |                 |
| Age, median     | 67 (22–80)      |                 |
| (range) (years) |                 | 54 (17–75)      |
| Sex (male/female) | 7/10           | 6/5             |
| Spilomelagacy | 2 (18%)         | 1 (9%)          |
| WBC, median     | 9 (2.1–16.7)    | 9.4 (2.5–17.2)  |
| (range) (×10⁹/L) |                 | 4.7 (1.8–15.7)  |
| LGL count, median (range) | 40 (17–91) | 48 (16–61) |
| (%)             | 33 (8–87)       |                 |
| Neutropenia     | 9 (53%)         |                 |
| (18%)           |                 | 8/18 (44%)      |
| Rheumatoid      | 1 (6%)          |                 |
| (0%)            |                 | 0               |
| Arthritis       |                 | 0               |
| (0%)            |                 | 0               |
| Hemolytic anemia|                 | 0               |
| (0%)            |                 | 0               |
| Pure red cell   |                 | 12 (48%)        |
| (0%)            |                 | 0               |
| Neutropenia     | 0               | 2 (18%)         |
| (1%)            |                 | 1 (4%)          |
| Symptomatitc    |                 | 6 (55%)         |
| (17%)           |                 |                 |

\(^\dagger\)Require treatments including blood transfusion, CLPD-NK, chronic lymphoproliferative disorder of natural killer cells; LGL, large granular lymphocyte; T-LGL L, T-cell large granular lymphocytic leukemia; WBC, white blood cell.

First, all patients were subjected to direct sequencing of the SH2 domain of the STAT3 gene and two mutations, Y604F and D661Y, were identified (Table 2). Y604F was recognized in two patients with T-LGL L and D661Y in three patients with CLPD-NK. Next, using AS-PCR, 18 additional patients among the 48 patients negative for the mutations by direct sequencing were found to be positive for mutations of Y604F and/or D661Y (Table 2). All five patients positive for the mutations by direct sequencing were confirmed to be positive using AS-qPCR. In total, 20 T-LGL L and three CLPD-NK patients were positive for mutations. Therefore, the frequencies of STAT3 mutations in our T-LGL L and CLPD-NK cohorts were 47.6% and 27.2%, respectively (\(P = 0.31\)). All of these mutations were heterozygous. In total, nine of 22 patients with TCR γ-chain-type T-LGL L and three of six T-LGL L patients with γ6TCR type were positive for mutations. Mutations in the SH2 domain of the STAT3 gene were not found in ANKL or EBV T-LPD patients using either direct sequencing or AS-qPCR. Samples from 50 Japanese healthy controls were examined using AS-qPCR and all were negative for Y604F and D661Y mutations. Three T/NK cell lines, Jurkat, NKL and NK92, were negative for mutations in the SH2 domain.

**STAT3 mutation and clinical parameters.** The relationships of STAT3 mutations and clinical parameters are shown in Table 3. Among the patients with T-LGL L and CLPD-NK, anemia was more prevalent in patients with the mutations than in those without and pure red cell aplasia (PRCA) was significantly associated with STAT3 mutation. Patients with STAT3 mutations also tended to need more treatments, such as corticosteroids, cyclosporine, cyclophosphamide or red blood cell transfusions (Table 3).

Pure red cell aplasia with STAT3 mutation was recognized in 13 T-LGL L and two CLPD-NK. Comparing these 15 patients with the other seven LGL L-associated PRCA patients who were negative for STAT3 mutation, the clinical features...
Table 2. Detection and comparison of STAT3 gene mutations using direct sequencing, AS-PCR and AS-qPCR methods

| UPN | Direct sequencing | AS-PCR | AS-PCR | AS-qPCR | AS-qPCR |
|-----|------------------|--------|--------|---------|---------|
| J1  | -                | +      | -      | 3.42    | ND      |
| J2  | -                | +      | -      | 2.16    | ND      |
| J3  | -                | -      | +      | 6.81    | 1.11    |
| J8  | -                | -      | +      | ND      | 0.96    |
| J9  | Y640F            | +      | -      | 21.3    | ND      |
| J12 | -                | -      | +      | NA      | NA      |
| J13 | -                | -      | +      | ND      | 0.57    |
| J19 | D661Y            | +      | +      | 6.35    | 25.90   |
| J22 | D661Y            | -      | +      | ND      | 34.60   |
| J32 | -                | -      | +      | ND      | 0.03    |
| J34 | -                | -      | -      | ND      | 1.97    |
| J35 | -                | -      | -      | ND      | 0.10    |
| J38 | D661Y            | -      | +      | ND      | 34.50   |
| H1  | -                | +      | -      | 1.24    | ND      |
| H3  | -                | -      | +      | ND      | 12.10   |
| H4  | -                | -      | +      | ND      | 0.62    |
| H5  | -                | -      | +      | ND      | 7.27    |
| H10 | -                | -      | +      | ND      | 1.79    |
| H16 | -                | -      | +      | ND      | 0.15    |
| H17 | -                | +      | -      | 0.61    | ND      |
| H22 | -                | -      | +      | ND      | 0.13    |
| H26 | D661Y            | -      | +      | ND      | 17.80   |
| H30 | -                | -      | +      | ND      | 0.18    |
| NC  | -                | -      | -      | ND      | ND      |

AS-PCR, allele-specific PCR; AS-qPCR, allele-specific quantitative PCR; NA, not available; NC, normal control; ND, not detected; PCR, polymerase chain reaction; STAT3, signal transducer and activator of transcription 3. −, negative for the mutation(s); +, positive for the mutations.

did not differ (data not shown), although those with mutations showed a trend towards a younger age than those without mutations (median age, 52.5 years and 77.5 years, respectively; P = 0.071). Regarding other disorders, two patients were associated with aplastic anemia (AA) and two other patients had myelodysplastic syndrome (MDS). Two of these patients, one with AA and one with MDS, were positive for STAT3 mutations.

Allele-specific quantitative PCR analysis of STAT3 mutations. Samples positive for STAT3 gene mutations using either the direct sequencing method or AS-PCR were subjected to AS-qPCR and the ratio of the mutated allele in relation to the whole DNA was calculated (Table 2). The levels of mutated alleles varied from 17.8% to 34.6% in the patients positive for the mutations by direct sequencing and from 0.03% to 12.1% in the patients positive only by AS-PCR. In five T-LGL L patients, subsequent samples were also examined using AS-qPCR. The mutation was persistently detected at levels of 0.96% at diagnosis and 0.70% after 7 months in one patient (unique patient number [UPN] J8), at 21.3% at diagnosis and 28.5% after 6 months in another patient (UPN J9) and 34.6% at diagnosis and 21.5% after 58 months in one other patient (UPN J22). Two other familial patients are described below.

Large granular lymphocyte leukemia with two STAT3 mutations. Two patients, one with T-LGL L and the other with CLPD-NK, possessed both Y640F and D661Y mutations. To examine the relationships of the mutational status in each patient, amplified PCR products were subcloned and sequenced. There were no clones positive for both Y640F and D661Y mutations among the 46 clones analyzed in each case (data not shown). Therefore, the two samples consisted of two distinct clones with different mutations.

Familial cases of T-LGL L and STAT3 mutation. Among the patients with T-LGL L, two were related, the index case (UPN J1) and her daughter (UPN J3), whose clinical information had been previously described in part. (12) They developed T-LGL L separately 5 years apart, complicated by PRCA and neutropenia. The index case was treated with cyclosporine and then cyclophosphamide, resulting in a complete response. This patient was positive for Y640F mutation by AS-PCR. After 8 years of treatment, the mutation was no longer detected. The second case (UPN J3) possessed two mutations, Y640F and D661Y. Y640F and D661Y mutations were detected at levels of 6.81% and 1.11% at diagnosis, respectively, and at 6.55% and 1.37% after 120 months of treatment with cyclosporine. Because the Y640F mutation was shared between the two, we examined the possibility of germline mutation in these patients. Both patients were negative for the mutation in non-hematopoietic cells (data not shown).

Discussion

In the present cohort, two mutations, Y604F and D661Y, were found in the SH2 domains of the STAT3 gene. The frequencies of mutations in Asian cases of T-LGL L and CLPD-NK were rather high compared with those in reports on Western cohorts, (5,6) although mutations in addition to these two were also detected in previously reported studies. (5,6) Several clinical differences have been suggested between Asian and Western LGL L populations. (7,9) In Asian populations, PRCA is the most common hematological complication of T-LGL L and CLPD-NK, in contrast to the situation in Western countries where rheumatoid arthritis and infections due to neutropenia are more frequent in cases of LGL L. (1,17) In a subset of bone marrow failure syndrome such as MDS and AA, STAT3-mutated T-cell subclones were found, suggesting contributions.
of STAT3-mutated T cells to immune dysregulation in these disorders. Significant associations of STAT3 SH2 domain mutations and PRCA in the present study further imply their roles in PRCA, another type of immune-mediated cytopenia. LGL L is a major cause of secondary PRCA and because of the diagnostic difficulties of LGL L the importance of LGL L in PRCA as an etiology might have been underestimated. These differences might be partly due to ethnicity and it is intriguing to explore the contributing factors, such as the genetic background. It is also conceivable that STAT3-related deregulation derived from STAT3 mutations in T or NK cells is a major contributor to PRCA, especially among Asians.

One of the major advantages of the discovery of STAT3 mutations in LGL L is that it enables definition of the neoplastic characteristics of at least some portion of the patients with T- or NK-cell-type LGL L. In the present series, more than 70% of patients were identified as having mutations using AS-PCR. Jerez et al. showed that as little as 10% of the mutant population could be detected using AS-PCR, however, it is unclear how much of the increased LGL population possesses the mutation in each patient. In addition, clonal drift is a common phenomenon in LGL L and the relationship between STAT3 mutation and clonal drift needs to be further elucidated.

In NK/T-cell leukemia associated with EBV, such as ANKL or EBV T-LPD, showing LGL morphology, no STAT3 mutation was found. EBV-driven and other mechanisms besides activating STAT3 mutations could cause proliferation of these cells. In T-LGL L and CLPD-NK with STAT3 mutation, STAT3 activation led to multiple gene deregulation and treatment with STAT3 inhibitors induced the apoptosis of leukemic cells. These observations were not limited to the STAT3-mutated cases; similar findings were also recognized in STAT3-non-mutated cases with T-LGL L/CLPD-NK, but not in normal controls. Although the details of STAT3-related pathways were not examined in the present STAT3-non-mutated patients, the mechanisms of proliferative and/or anti-apoptotic characteristics of leukemic cells in non-mutated cases might be similar to those in STAT3-mutated patients through alternative changes other than STAT3 SH2 domain mutations.

Two related patients with T-LGL L were analyzed and found to possess the same STAT3 mutation of Y640F. Their phenotypes of LGL leukemic cells were both CD3+ CD8– CD16– CD56+ TCRβ+, but their Vβ usage differed. The STAT3 mutations in these patients were somatic because they were not detected in non-hematopoietic cells. A familial predisposition to LGL L might be rare but has been reported and further studies of such cases should provide clues to understand the pathogenesis of LGL L.

Among 23 patients with STAT3 mutations, two (8.7%) possessed both Y604F and D661Y mutations. This combination of mutations was also previously recognized in a patient. Using subcloning and sequencing we showed that the mutations are not located on the same allele. There were no apparent clinical differences between these two patients and other cases of LGL L data not shown; although the number of patients was too small to draw any definitive conclusions.

In summary, mutations in the SH2 domain of the STAT3 gene were frequently recognized in T-LGL L and CLPD-NK and closely associated with PRCA in Asian LGL L.

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