Inherited Genetic Susceptibility to Nonimmunosuppressed Epstein-Barr Virus-associated T/NK-cell Lymphoproliferative Diseases in Chinese Patients*

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Summary: Epstein-Barr virus (EBV) T/NK-cell lymphoproliferative diseases are characterized by clonal expansion of EBV-infected T or NK cells, including chronic active EBV infection of T/NK-cell type (CAEBV+T/NK), EBV-associated hemophagocytic lymphohistiocytosis (EBV+HLH), extranodal NK/T-cell lymphoma of nasal type (ENKTL), and aggressive NK-cell leukemia (ANKL). However, the role of inherited genetic variants to EBV+T/NK-LPDs susceptibility is still unknown. A total of 171 nonimmunosuppressed patients with EBV+T/NK-LPDs and 104 healthy donors were retrospectively collected and a targeted sequencing study covering 15 genes associated with lymphocyte cytotoxicity was performed. The 94 gene variants, mostly located in UNC13D, LYST, ITK, and PRF1 genes were detected, and mutations covered 28/50 (56.00%) of CAEBV-T/NK, 31/51 (60.78%) of EBV+HLH, 13/28 (46.42%) of ENKTL, and 13/48 (27.09%) of ANKL. Most mutations represented monoallelic and missense. Three-year overall survival rate of patients with CAEBV-T/NK and EBV+HLH was significantly lower in patients with germline mutations than in those without germline mutations (P=0.0284, P=0.0137). Our study provided novel insights into understanding a spectrum of nonimmunosuppressed EBV+T/NK-LPDs with respect to genetic defects associated with lymphocyte cytotoxicity and reminded us that the gene sequencing may be an auxiliary test for diagnosis and risk stratification of EBV+T/NK-LPDs.

Key words: germline mutation; EBV-associated T/NK-cell lymphoproliferative disease; hemophagocytic lymphohistiocytosis; primary immunodeficiencies; lymphocyte cytotoxicity; gene sequencing

Epstein-Barr virus (EBV), which belongs to the Gammaherpesvirinae subfamily, exists in more than 90% of the human adult population worldwide. It is most commonly recognized as the etiologic agent of a variety of human diseases[1]. Primary infections are generally asymptomatic in childhood or result in self-limiting infectious mononucleosis in healthy adolescents and adults. During primary infection, EBV first targets oral epithelial cells, and then promiscuously infects B cells[2]. Afterwards, EBV selectively persists in memory B cells and causes lifelong latent infection. Although lytic replication can be prevented by sophisticated immune responses, host immune system cannot eliminate EBV completely. EBV also infects T or nature killer (NK) cells which do not express CD21, but the underlying mechanism remains elusive. Recurrent and persistent EBV infections in T/NK-cells are rare and usually life-threatening[3]. Of note, EBV-associated T/NK-cell lymphoproliferative diseases (EBV+T/NK-LPDs) are a variety of diseases that characterized by clonal expansion of EBV-infected T or NK cells. In a broad sense, EBV+T/NK-LPDs have a vast spectrum from reactive to neoplastic processes, and from indolent to aggressive or fulminant forms, at least including EBV-associated hemophagocytic lymphohistiocytosis (EBV+HLH)[4], chronic active EBV infection of T/NK-cell type (CAEBV+T/NK)[3],

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extranodal NK/T-cell lymphoma of nasal type (ENKTL)\textsuperscript{[8]}, and aggressive NK-cell leukemia (ANKL)\textsuperscript{[5, 6]}. The spectrum of diseases has overlapping manifestations and is associated with poor outcomes, thereby leading to diagnostic confusions and therapeutic challenges. CAEBV-T/NK has a strong tendency to eventually progress into EBV+HLH or overt malignancies including ENKTL, ANKL, and peripheral T-cell lymphoma\textsuperscript{[7]}. Furthermore, previous studies had demonstrated that EBV+T/NK-LPDs were more prevalent in East Asian populations\textsuperscript{[8]}, and many patients presented with refractory and recurrent elevated EBV loads, suggesting that specific host genetic defects that impair anti-EBV immunity may play a role in the pathogenesis of EBV+T/NK-LPDs.

Germline mutations related to lymphocyte cytotoxicity have been substantially revealed in various Mendelian recessive primary immunodeficiencies (PIDs), including familial HLH (FHL), FHL syndromes with hypopigmentation, and EBV-susceptible lymphoproliferative syndromes (LPS). To date, at least 12 disease-causing genes have been identified, including PRF1, UNC13D, STX11, STXBP2, RAB27A, LYST, AP3B1, SH2D1A, XIAP, MAGT1, ITK, and CD27 (table 1). These genes mainly involve in perforin/granzyme-mediated cytotoxic pathway and other essential signal pathways that cytotoxic lymphocytes required. Functionally, perforin (encoded by PRF1) is synthesized in cytotoxic lymphocytes and enveloped in secretory vesicles, which are responsible for cytotoxic lymphocyte-triggered apoptosis\textsuperscript{[9]}. UNC13D, STX11, STXBP2, RAB27A, LYST, and AP3B1 encode proteins that involve in vesicle trafficking of cytotoxic granules towards target cells\textsuperscript{[10–13]}, SH2D1A, XIAP, MAGT1, ITK, and CD27 encode proteins that regulate activation and proliferation of cytotoxic lymphocytes\textsuperscript{[14, 15]}. Moreover, GZMB, GNLY, and SRGN are also genes which involve in perforin/granzyme-mediated cytotoxic pathway (table 1). Granzyme B (encoded by GZMB) and granulysin (encoded by GNLY) co-localize with perforin. Of note, granzyme B is critical for cytotoxic lymphocyte-triggered apoptosis\textsuperscript{[16]}. Serglycin (encoded by SRGN) is a glycoprotein that binds to granzyme B to form a complex entering target cells. Thus, defects in GZMB, GNLY, and SRGN may also be reasons for antiviral immunodeficiency and immunosurveillance dysfunction. Defects in such genes have been associated with FHL type 2–5, albinism-associated PIDs, and EBV-susceptible PIDs\textsuperscript{[4, 17, 18]}. However, mutations in those genes in EBV+T/NK-LPDs have been seldom reported and are still largely unknown, especially in adult cases. Considering that most patients presented decreased anti-EBV immunity and immunosurveillance, we hypothesized genetic variants associated with lymphocyte cytotoxicity may play a role in the pathogenesis of EBV+T/NK-LPDs. Here, using the next-generation sequencing (NGS) technology, we systematically performed targeted sequencing in 15 genes of peripheral blood specimens from 177 patients with EBV+T/NK-LPDs along with 104 healthy donors. Our study improves the understanding of the disease mechanism and provides novel insight into the clinical practice of EBV+T/NK-LPDs.

1 MATERIALS AND METHODS

1.1 Patients and Samples

This study was approved by the institutional review board of Tongji Hospital. We conducted a retrospective genetic analysis on 177 nonimmunosuppressed individuals who were diagnosed with EBV+T/NK-LPD from February 2012 to March 2019. We also

| Causative gene | Associated disease/immunodeficiencies | Function | Referenced coding sequence |
|----------------|--------------------------------------|----------|-----------------------------|
| PRF1           | FHL-2                                | Pore formation | NM_005041.4 |
| UNC13D         | FHL-3                                | Vesicle priming | NM_199242.2 |
| STX11          | FHL-4                                | Vesicle fusion | NM_003764.2 |
| STXBP2         | FHL-5                                | Vesicle fusion | NM_006949.3 |
| RAB27A         | GS-2                                 | Vesicle docking | NM_183236.2 |
| LYST           | CHS                                  | Vesicle trafficking | NM_000813.1 |
| AP3B1          | HPS-2                                | Vesicle trafficking | NM_003664.4 |
| GZMB           | N/A                                  | Target cell apoptosis | NM_004131.5 |
| GNLY           | N/A                                  | Target cell apoptosis | NM_006433.3 |
| SRGN           | N/A                                  | Forming a complex with granzyme B | NM_002727.3 |
| SH2D1A(SAP)    | XLP-1                                | Regulating intracellular signaling | NM_002351.4 |
| XIAP(BIRC4)    | XLP-2                                | Regulating intracellular signaling | NM_001204041.1 |
| MAGT1          | XMEM                                 | TCR signaling | NM_032121.5 |
| ITK            | ITK deficiency                       | TCR-mediated activation | NM_005546.3 |
| CD27           | CD27 deficiency                      | Generation and long-term maintenance of T cells | NM_001242.4 |

N/A, not applicable; FHL, familial hemophagocytic lymphohistiocytosis; GS, Griscelli syndrome; CHS, Chediak-Higashi syndrome; HPS, Hermansky-Pudlak syndrome; XLP, X-linked lymphoproliferative disease; XMEM, X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia; TCR, T-cell receptor
collected samples from 104 healthy donors. Specimens were obtained and analyzed to determine EBV-infected lymphocyte cell types for diagnostic purposes. The dominant infected cell types were identified as those with higher EBV DNA levels than the unfractionated peripheral blood mononuclear cells (PBMCs) based on real-time PCR and/or ≥0.2% EBER-positive cells by fluorescence in situ hybridization (FISH). Patients with EBV-LPDs were divided into 4 clinical subgroups: EBV+HLH (n=50), systematic CAEBV-T/NK (n=51), EBV-positive ENKTL (n=28), and EBV-positive ANKL (n=48). EBV+HLH was defined according to HLH guidelines\(^\text{[4]}\). CAEBV-T/NK was defined on referring to the criteria proposed by Kimura et al and Okano et al\(^\text{[19, 20]}\), including cases that eventually progressed to overt HLH manifestation or malignancies during the follow-up period. Localized forms of CAEBV-T/NK such as hydroa vacciniform-like lymphoproliferative disorder and severe mosquito bite allergy were excluded. Diagnoses of ENKTL and ANKL were made according to the 2016 WHO classification\(^\text{[35]}\). Genomic DNA of peripheral blood samples and matched exfoliated cells of oral mucosa was extracted with QIAamp® DNA Blood Mini kit (Qiagen, Germany) according to the manufacturer’s instructions. Prognostic data were available in 50 EBV+HLH patients and 33 CAEBV-T/NK. All these patients did not receive allogeneic hematopoietic stem cell transplantation (allo-HSCT) before progression into accelerated phase.

### 1.2 Targeted High-throughput Sequencing

Using hg19/GRCh37 as reference, a sequencing panel targeting the coding sequences (CDS) with 5 intronic base pairs around exons in PRFI, UNC13D, STXII, STXB2, RAB27A, LYST, AP3B1, SH2D1A, XIAP, MAGT1, ITK, and CD27 was designed. The details of the gene panel are shown in table 1 (Ion AmpliSeq Designer, Thermo Fisher Scientific, USA). Overall, 298 amplicons, covering 99.27% of the 30.41-kb targeted gene region, were analyzed per sample, multiplexed in two library preparations, using 20 ng of input Genomic DNA. Enriched templates were sequenced by Ion Torrent Personal Genome Machine (PGM) (Thermo Fisher Scientific, USA) and aligned with default settings using the Ion Torrent Suite Software v5.0. Germline mutations were filtered by “Germline mutation-low stringency” mode in variant calling process. Called variants were analysed with online Ion Reporter Software v5.6 for further annotations. We performed sequencing on patients according to standard procedures under the following conditions: (1) CDS and canonical ±1/2 splice sites in GZMB, GNYL, and SRGN for each sample; (2) c.118-307G>A and c.118-308C>T in UNC13D for each sample which reduced the expression of encoding Munc13-4; (3) all non/poorly amplified (<20×) CDS and canonical ±1/2 splice sites; (4) validation and identification of germline/somatic and monoallelic/biallelic mutation status.

### 1.3 Bioinformatic Analyses

Variant frequencies were investigated by the genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/), and 1000 Genomes (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes). The variants detected from the patients were included if: (1) variants with allele frequency less than 0.01 according to the gnomAD; (2) confirmed as germline mutations by matched exfoliated cells of oral mucosa using Sanger sequencing then.

### 1.4 Statistical Analyses

Statistical analyses in this study were evaluated by SPSS 18.0 (International Business Machine Corp., USA). Differences were analysed using Student’s t test or Wilcoxon rank sum test for continuous variables and Pearson Chi-square test or Fisher’s exact test for categorical variables. Progression-free survival (PFS) time of CAEBV-T/NK was defined as the time from the onset to the start of accelerated phase (progression into EBV+HLH, overt malignancies, or severe complications). The Kaplan-Meier method and log-rank test were used for survival analysis. A two-sided P-value <0.05 was considered statistically significant for all analyses.

### 2 RESULTS

We collected a total of 177 EBV+T/NK-LPD patients including CAEBV-T/NK (n=50), EBV+HLH (n=51), ENKTL (n=28), and ANKL (n=48). There were 115 males and 62 females. Age at onset of EBV+T/NK-LPDs ranged from 1 to 83 years old. The median age of onset was 28 years in EBV+HLH group, 31 in CAEBV-T/NK group, 34 in ENKTL group, and 31 in ANKL group, respectively. A total of 110/177 (62.14%) EBV+T/NK-LPD patients were children or young adults less than 30 years of age. The characteristics of patients recruited in this study are summarized in supplementary table 1 (table S1). The median depth of NGS was 431×. Coverage of all targeted bases ranged from 98.4% to 100% at 1×, from 93.6% to 99.7% at 20×, and from 91.3% to 98.3% at 100×, respectively. A total of 94 potential pathogenic germline variants were identified and validated by Sanger sequencing (table S2). Mutations were detected in 28/50 (56.00%) in CAEBV-T/NK, 31/51 (60.78%) in EBV+HLH, 13/28 (46.42%) in ENKTL, 13/48 (27.09%) in ANKL, and 10/104 (9.6%) in healthy donor (fig. 1 and fig. 2A). Almost all individuals with mutations only harboured monoallelic mutations. An overwhelming proportion of mutations represented missense in all categories (fig. 2B). Gross deletion and frameshift deletions were more common in EBV+HLH and ANKL. Deep intronic mutation c.118-308C>T
in \textit{UNC13D} was detected in one case of CAEBV-T/NK. Other types of mutation represented nonsense, insertion, in-frame deletion, stop loss, or canonical ±1/2 splice site variant was not detected. In addition, digenic mutations were more common in CAEBV-T/NK (4/51) than in others (fig. 2A). Mutations were most frequently located in genes that involved in vesicle trafficking, especially in \textit{UNC13D} and \textit{LYST}. No mutation was found in \textit{SH2D1A} or \textit{MAGT1}.

Several different recurrent variants were identified in this study, including c.1232G>A (p.Arg411Gln), and c.2588G>A (p.Gly863Asp) in \textit{UNC13D}, c.8368A>C (p.Lys2790Gln) in \textit{LYST}, c.674G>A (p.Arg225Gln) in \textit{PRF1}, and c.1741C>T (p.Arg581Trp) in \textit{ITK}. Schematic locations of mutations in \textit{UNC13D}, \textit{LYST}, \textit{PRF1}, and \textit{ITK} are illustrated in fig. 3. Mutations were dispersedly distributed in \textit{UNC13D}, but most of them located in the region that interacted with Rab27a (residues 240–543), Munc13 homology domain 1 (MHD2, residues 788–895), and C2 domain B (C2B, residues 912–1019). Notably, variant c.2588G>A (p.Gly863Asp) in \textit{UNC13D} was considered as a founder mutation in Chinese population in previous reports\cite{21}. Mutations in \textit{LYST} were all not located in identified domains, and the preponderant recurrent variant c.8368A>C (p.Lys2790Gln) accounted for...
33.3% (14/41) LYST mutations. Mutations in PRF1 also distributed separately, but most of them affected membrane attack complex/perforin domain (MACPF, residues 27–375). Among them, the infrequent variant c.272C>T (p.Ala91Val) in PRF1 was thought to be not clinically neutral and be susceptible to FHL-2 by substantial reports[22]. This variant had been seldom identified in East Asian patients previously but was detected in a patient with ANKL here.

There were no significant differences regarding either EBV titers or age of onset between patients with and without mutations in CAEBV-T/NK (P=0.8807, P=0.9652) and EBV+HLH (P=0.4567, P=0.7611) (fig. 4A and 4B). While individuals with mutations had a poor prognosis as compared with individuals without mutations, as reflected by the survival curve (fig. 4C and 4D). Three-year overall survival in both CAEBV-T/NK and EBV+HLH with mutations was lower than that without these germline mutations (P=0.0284, P=0.0137).

3 DISCUSSION

In this retrospective study, we performed sequencing targeted in 15 genes related to lymphocyte cytotoxicity in 177 patients with a spectrum of EBV+T/NK-LPDs, concentrating on revealing potential disease-relative mutations and specific mutation patterns of EBV+T/NK-LPDs.

The mutation patterns of EBV+T/NK-LPDs demonstrated in our study provided us a novel insight into such spectrum of diseases. Mutations associated with functional defects of lymphocyte cytotoxicity in EBV+T/NK-LPDs were more prevalent than suspected previously, though the frequencies varied among different categories. Mutations were detected in nearly or more than half of the patients with EBV+HLH, CAEBV-T/NK and ENTKL, and in
20%–30% of the patients with ANKL. Remarkably, most mutations were “milder” type of mutations representing monogenic, monoallelic, and missense, rather than “severer” type of mutations representing di/trigenic, biallelic, and truncated, respectively. In addition, hotspot mutations determined in Caucasian, Korean, Saudi Arabian and Turkish populations, including c.766C>T (p.Arg256*) and c.754-1G>C in \textit{UNC13D}, c.1430C>T (p.Pro477Leu) in \textit{STXBP2}, c.369_376delAGTGGCGCinsTGG (p.Val124Glyfs) and c.802C>T (p.Gln268*) in \textit{STX11} were all not detected\cite{10, 23–25}. Most variants here showed much ethnic and geographical differences.

HLH is a disease that comprises a primary form and a secondary form. The primary form was commonly deemed to be following an autosomal recessive mode of inheritance, indicating that patients with primary HLH harbour biallelic mutations in disease-causing genes, and manifest initial symptoms in early infancy. By contrast, the secondary form may develop as a result due to viral infections, immunosuppressed conditions, hematological malignancies, or autoimmune conditions. However, we identified a proportion of the EBV+HLH (12/51, 23.52%) patients harbouring monoallelic missense mutations in this study. Such atypical forms of congenital defects are not definitely explained by current genetic insights. It may be difficult to distinguish EBV-triggered later-onset primary HLH from EBV-associated secondary HLH for adult patients carrying monoallelic mutations. Moreover, severer di/trigenic monoallelic mutations were much more common in EBV+HLH than in other subcategories of EBV+T/NK-LPDs. Those phenomena indicate that the boundary between primary HLH and secondary HLH may be ambiguous. In some individuals with monoallelic mutations, although the lymphocyte cytotoxicity is impaired, the residual cytotoxic activity may be sufficient to prevent hosts escaping HLH manifestations in infancy and even keeping disease-free states for many years. Therefore, it may be an interpretation for detection of mutation on few of the healthy donors without occurrence of HLH status. HLH susceptibility and the severity of the resultant HLH manifestations may be ranked in terms of the severity of genetic defects. In accordance with our findings, Zhang et al illustrated that hypomorphic
mutations in HLH-causing genes correlated with later-onset HLH with more indolent course in adult patients, and almost all the mutations were either missense mutations or splice-site changes\[30\]. They also found that polygenic defects in the cytotoxic pathway led to synergistic function effects on granule-mediated lymphocyte cytotoxicity, which contributed to the development of HLH\[27\]. Furthermore, Sepulveda also elegantly showed that accumulation of genetic lesions might increase the likelihood of developing of HLH manifestations and cancers\[28, 29\]. The significance of genetic mutations could not be simply considered as diagnosis evidence due to the complex factors involved in disease. First, what degree the gene defects contribute to disease happening were not so clear in recent study, even the most concerned \(TP53\) gene. The clinical diagnosis should be thoroughly considered not only based on the gene mutations, but also the clinical manifestation and some other laboratory findings. What’s more, similar as the “double-hit” theory in hereditary disease, these germline genetic defects may only provide a predisposition to disease process, in which condition the disease may not appear, when lacking the second-hit event from more genetic defects or from environment.

Mutations were also detected in 46.42% ENKTL cases and 27.09% ANKL cases, most of which represented monoallelic and missense. Interestingly, several previous studies had revealed a potential relationship between genetic lesions in lymphocyte cytotoxicity and cancer predisposition\[28, 30–32\]. Löfstedt et al found that heterozygous mutations in HLH-causing genes might be a risk factor for cancer. They considered that the cancer predisposition might imply haploinsufficiency of cytotoxic lymphocyte-mediated immunosurveillance of cancer in carriers of these mutations\[30\]. Chia found that inherited temperature-sensitive missense mutations in \(PRF1\) were linked to hematological malignancy in childhood or adolescence\[31\]. Thus, germline mutations in genes associated with lymphocyte cytotoxicity that impair the immunosurveillance, may play a more important role than expected in the tumorigenesis of ENKTL and ANKL. Further investigations based on an extended sequencing panel and study cohort are required to elucidate the impact of genetic defects on ENKTL and ANKL.

Genetic defects in CAEBV-T/NK had been seldom reported previously. Only a few studies showed that germline mutations in \(PRF1\), \(STXB2\), and \(UNC13D\) were detected in few CAEBV cases\[33–35\]. In this study, 56.00% CAEBV-T/NK patients harboured mutations in HLH-associated genes, most of which represented monogenic, monoallelic, and missense. Genetic findings demonstrated that mutations in genes associated with lymphocyte cytotoxicity may play an important role in the development of CAEBV-T/NK. CAEBV-T/NK may be a dynamic disease to some degree characterized by a duality of anti-EBV immunodeficiency and immunosurveillance dysfunction, and has a tendency towards either EBV\(^{+}\)HLH or overt malignancies.

Thus, we speculate that EBV\(^{+}\)T/NK-LPDs may be partly a biologic continuum rather than completely discrete entities. Genetic defects impairing lymphocyte cytotoxicity may play a contributing role in the development of such diseases when patients were challenged by the EBV infection and other environmental stresses. The severity of genetic lesions is generally associated with the clinical phenotype of onset. Severer genetic defects are more likely to lead to a fulminant inherited form than an indolent neoplastic form. Nonetheless, since the nosogenesis of EBV\(^{+}\)T/NK-LPDs is quite complicated, it is difficult to precisely predict the ultimate outcome of a healthy-looking individual with a specific mutation status.

Prognostic data showed that patients in mutated group had an inferior clinical outcome compared with patients in non-mutated group. OS time of EBV\(^{+}\)HLH and CAEBV-T/NK significantly decreased in mutated group as compared with that in non-mutated group, due to a higher risk of death in early stage of disease. The results suggested that the prevention and timely control of early severe complications of EBV\(^{+}\)HLH are vital in the treatment of individuals with mutations and allo-HSCT, if possible, should be conducted for individuals with mutations in prodromal phase of CAEBV-T/NK.

Nonetheless, in clinical practice, the definite impact of a specific germline mutation must be comprehensively evaluated according to the guidelines proposed by the American College of Medical Genetics and Genomics (ACMG)\[36\]. Precise prediction of the ultimate outcome of a healthy-looking individual with a specific mutation status will be difficult due to the complexity. Patients in mutated group had a more inferior clinical outcome. However, this result may be biased since all the patients were recruited from a single center. In addition, some other factors such as age of onset, sex, or therapeutic strategy should be taken into consideration. Further studies should be conducted with an expanded patient cohort to cope with this problem.

In conclusion, our study provided a novel insight into understanding a spectrum of EBV\(^{+}\)T/NK-LPDs with respect to genetic defects associated with lymphocyte cytotoxicity and demonstrated that gene sequencing was recommended to be used as an auxiliary test for assistant diagnosis and risk stratification of EBV\(^{+}\)T/NK-LPDs.

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Conflict of Interest Statement
All authors have read the journal’s policy on disclosure of potential conflicts of interest and declare that they have no competing interests.

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