MYC/BCL2/BCL6 triple hit and TP53 deletion in a case of high-grade B cell lymphoma receiving CAR T cell immunotherapy

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ABSTRACT
High-grade B cell lymphoma with MYC, BCL2, and BCL6 rearrangements (HGBL-TH) is rare and often portends poor outcome after standard chemoimmunotherapy. Adoptive chimeric antigen receptor (CAR) T cell therapy is a new paradigm for the treatment of refractory/relapsed (r/r) lymphomas, but its therapeutic effects in treating HGBL-TH remain inconclusive. Here, we report a patient with HGBL-TH who failed to achieve complete remission (CR) and progressed rapidly after first-line and second-line therapies. Furthermore, he was resistant to the CAR19 and CAR22 T cell cocktail following autologous hematopoietic stem cell transplantation (ASCT), and disease progressed again after the anti-B cell maturation antigen (BCMA) CAR T cell immunotherapy. Comprehensive analyses were performed to explore the inherent mechanism for resistance, which indicated that tumor-derived antigen escape, clonal evolution, and T cell defects were involved in it. This is the first report of an HGBL-TH patient with TP53 deletion and additional germline (PIM1) and somatic mutations (TP53, KMT2D, and IGLL5) who was treated with ASCT, CD19/22 CAR T cell cocktail therapy and BCMA CAR T cell immunotherapy. The clinical evolution and genetic features of this case may help to explain the underlying mechanism of treatment resistance and provide novel insights into lymphoma treatment. Trial registration: ChiCTR-OPN-16009847

BACKGROUND
Immunotherapy has become one of the most effective treatments for refractory/relapsed (r/r) B cell lymphoma. In particular, chimeric antigen receptor (CAR) T cell immunotherapy has recently been found to be a highly effective treatment for r/r diffuse large B cell lymphoma (DLBCL). Several studies have reported the safety and efficacy of CD19/22 CAR T cell cocktail immunotherapy alone and human CAR T cell therapy and expect to provide insights into the therapeutic strategy for such patients.

CASE REPORT
A 38-year-old man presented to a local hospital in September 2018 with intermittent abdominal pain for 1 month. He was diagnosed with IVA stage (Ann Arbor staging
system) primary abdominal DLBCL. Immunohistochemistry (IHC) indicated that BCL2, BCL6, CD19, CD20, CD10, C-MYC, and TP53 were positive in his initial diagnosed lymph node, whereas CD3, CD5, and CD30 were negative, consistent with germinal center B origin. He received a standard dose of R-CHOP (rituximab, cyclophosphamide, vincristine, adriamycin, and prednisone) for six cycles but experienced progressive disease. The patient then received intensified induction chemotherapy of one cycle of R-ESHAP (rituximab, etoposide, cytarabine, cisplatin, and methylprednisolone), but the disease progressed again.

The patient was referred to our hospital in March 2019 to receive ASCT and CAR T cell therapy. Before treatment, target antigen expression for CAR T therapy (CD19, CD20, CD22, and BCMA) was confirmed in initially diagnosed lymph nodes by immunohistochemical staining (online supplemental figure 1A). He was enrolled in a clinical trial of sequential cocktail infusion of murine anti-CD19 and anti-CD22 CAR T cells following ASCT. The patient was given a standard dose of the BEAM regimen (300 mg/m² bis-carmustine, −6 days; 200 mg/m² etoposide, −5 to −2 days; 400 mg/m² cytarabine, −5 to −2 days; and 140 mg/m² melphalan, −1 day) as myeloablative chemotherapy. CAR19 and CAR22 T cells were infused 6–7 days (May 8, 2019–May 9, 2019) after autologous stem cell infusion (day 0, May 2, 2019). The structure (figure 1A), manufacture, and preparation of CAR T cells and hematopoietic stem cells were described as previously. The patient had stable disease on day +58 (June 27, 2019), as evaluated by enhanced abdominal CT compared with that on the day before stem cell collection. PET/CT scan on day +71 (July 12, 2019) indicated that mediastinum and abdominal lymphomas had progressed, and new lesions had appeared in the groin. Subsequently, the patient received sintilimab injection (an anti-PD-1 antibody) plus other immunotherapeutic drugs (rituximab, chidamide, bortezomib, and dexamethasone) from day +78 (July 19, 2019) to day +99 (August 9, 2019). At the same time, the disease continued to progress rapidly. After ASCT and CAR19/22 T cell cocktail therapy, the CD19 antigen was lost (figure 1B,C). Nevertheless, IHC indicated positive expression of BCMA in tumor cells. The patient was then transferred to another hospital to receive an infusion of human-derived anti-BCMA CAR T cells for compassionate use. Unfortunately, the disease remained refractory. The timeline of the main events in the clinical treatment of this patient is presented in online supplemental table 1.

Before CAR T cell treatment, various analyses were performed to study the molecular features of the patient. Rearrangements of the monoclonal receptor genes IGKA and IGKB were detected in the bone marrow (online supplemental figure 1B). Next-generation sequencing (NGS) was performed on his initial diagnosed formalin-fixed paraffin-embedded inguinal lymph node biopsy. One germline mutation (PIM1, c.403G>A, p.Glu135Lys, 49.40%) and three somatic mutations (TP53 missense mutation, c.706T>G, p.Tyr236Asp, 80.6%; KMT2D frameshift mutation, c.15547del, p.Val5184Cysfs*59, 47.0%; IGLL5 missense mutation, c.64G>A, p.Glu22Lys, 47.58%) were detected. Germine DNA was extracted from leukocytes. Sanger sequencing confirmed that the patient carried the PIM1 mutation in a heterozygous state (online supplemental figure 1C). After CAR T cells infusion, large amounts of tumor-derived ascites appeared, and FISH analysis found MYC, BCL2, and BCL6 (triple-hit) rearrangements with TP53 gene deletion (figure 1D) on day +81 (July 22, 2019). In addition, karyotypic analysis suggested a complex karyotype as follows: 92–95.XXY.t(3;14)(q27;q22)×2,der(8)(t;2;8) (p12;q24)×2,11,add(11)(q23),del(17)(p13) ×2,der(18)(t;14;18)(q32;q21)×2,6;+mar[cp10] (online supplemental figure 1E).

After sequential infusion of anti-CD19 and anti-CD22 CAR T cells, grade 1 cytokine release syndrome (CRS) was observed, and interleukin-6 and ferritin increased slightly and transiently (figure 1E). Neither the anti-CD19 nor the anti-CD22 CAR transgene expanded or persisted in this case (figure 1F), compared with the previously reported average level in our center, and dropped quickly to baseline on day +17 (May 19, 2019). With CAR T cells disappearing quickly, white cell counts and lymphocytes returned to their previous levels on day +17 (figure 1G). Flow cytometry analysis of ascites showed that CD19 and CD22 markers were positively expressed in lymphoma cells on day +78 (July 19, 2019), but CD19 antigen was lost by 1 month later, on day +110 (August 20, 2019) (figure 1B,C).

To explore the mechanisms underlying this persistent refractoriness, we performed additional genetic and transcriptional studies retrospectively. In addition to NGS performed on tumor biopsy at the initial diagnosis, peripheral blood circulating tumor DNA (ctDNA) NGS was performed twice and revealed potential clonal evolution (figure 2A,B). When the patient’s abdominal pain had been relieved after CAR T cell therapy, on day +40 (June 11, 2019), ctDNA NGS indicated a secondary primary clone of BCL2 mutation (c.87G>C, p.Glu29Asp). One month later, when lymphoma had progressed and abdominal pain had reappeared, on day +75 (July 16, 2019), a primary tumor-derived clone with TP53 missense mutation, KMT2D truncating mutation, and IGLL5 missense mutation reamplified rapidly, and a new subclone with BCL2 and DDX3X mutations emerged.

In contrast, patient T cells sorted by CD3+ magnetic beads (Miltenyi, CD3+ MicroBeads) from peripheral blood mononuclear during hematopoietic stem cell collection before the ASCT were used for transcriptional analysis. The GO and KEGG analysis of differentially expressed genes between the patient and two healthy donors indicated enrichment in T cell co-stimulation and activation pathways and in JAK/STAT, NF-KB and MAPK signaling pathways (figure 2C,D). To explore the immune responses in which key genes were involved, an immune gene set for CAR T cell characterization (nCounter CAR-T Characterization
Panel, NanoString) was identified by gene set enrichment analysis (GSEA), and Pearson’s correlation coefficient was used as a metric. GSEA (Pearson) indicated that genes whose expression was positively correlated with PIM1 were enriched in T cell activation and costimulatory molecules (figure 2E), and those positively correlated with BCL2 were enriched in T cell exhaustion and migration (figure 2F). Moreover, the expression of the PIM1 gene was significantly correlated with that of the T cell exhaustion gene PD1 (figure 2G,H). In addition, the expression of immune-related proteins in the patient’s T cells was relatively lower than that in healthy controls (figure 2I).

**DISCUSSION**

HGBL-DH/TH is a phenotypically and genetically heterogeneous disease with a dismal prognosis. Several studies
Figure 2  DNA and RNA high-throughput sequencing results and analysis. (A) Schematic models of evolutionary progression before and after CAR T cell infusion. Germline clones, primary dominant clones, secondary dominant clones, and subclones are represented in creamy white, blue, red, and gray shapes, respectively. (B) References and VAFs of DNA mutations investigated by NGS. An NGS panel with 157 target genes was employed for analysis of the FFPE sample. The mean amplicon coverage of the patient’s FFPE sample was 11,204.9×, and its uniformity of coverage (Pct >0.2*mean) was 95.29%. A panel with 171 target genes was employed for analysis of peripheral blood ctDNA samples. ctDNA NGS was performed by duplex unique molecular identifier sequencing technology. The mean coverage of the target region are 151,775.17× and 116,533.09×. The mean coverage of DS211 on the target region was 1911.26× and 1734.61×. (C) The histograms show the top 13 GO-BP enrichment results of differentially expressed genes between the patient and two healthy donors. The x-axis represents the enriched gene count, and the intensities of different colors represent the p value. (D) The bubble diagram shows the top 11 KEGG enrichment items of differentially expressed genes between the patient and two healthy donors. The x-axis represents the gene ratio, and the intensities of different colors represent the p value. (E) GSEA of the PIM1 gene. GSEA for T cell activation sets and T cell costimulatory molecule sets based on Pearson correlation with PIM1 expression level. Bottom: the plot of all genes. Y-axis, the value of the ranking metric; x-axis, the RANK for all genes. (F) GSEA of the BCL2 gene. GSEA for T cell exhaustion sets and T cell migration sets based on Pearson correlation with BCL2 expression level. Bottom: the plot of all genes. Y-axis, the value of the ranking metric; x-axis, the rank for all genes. (G) The correlation plot showed a strong correlation between the expression of PIM1 and PD1 (r=0.9958, p<0.05, Pearson test). (H) Positive correlation between the expression of PIM1 and PD1 (r=0.9958, p<0.05, Pearson test). (I) Heat map of RNA expression on T cell activation-related and exhaustion-related genes. Columns represent the patient and two healthy donors. FFPE, formalin-fixed paraffin-embedded; GSEA, gene set enrichment analysis; NGS, next-generation sequencing; VAFs, variant allele frequencies.
have recently revealed MYC/BCL2/BCL6 rearrangements and TP53 mutations as independent indicators of prognosis in patients with DLBCL treated with standard chemotherapy. And TP53 mutation predispose to a poor outcome in children with B-ALL treated with hCD19 CAR T cells.\(^1\) The NCCN guidelines have recommended clinical trials such as CAR T cell immunotherapy. However, the mechanism underlying its resistance to various therapies remains inconclusive.

First, tumor antigen escape is often seen in failure for long-term disease control in CAR T cell therapy. In this case, although CD19 antigen expression was lost after treatment, infusions of CD19-targeted and CD22-targeted CAR T cell products cocktails might partially overcome this phenomenon. Interestingly, clonal evolution with the appearance of new BCL2 mutation and subclones with DDX3X and BCL2 mutations was detected in the plasma ctDNA after CAR T cell therapy. The BCL2 mutation in the secondary clones might be related to T cell dysfunction (figure 2E,F). Furthermore, It is highly suspected that this case was a genetic composite, with features of EZB-MYC\(^+\) and A53 according to the 2020 LymphGen algorithm.\(^1\) This composite genetic subtype exhibits relatively lower immune signatures and is associated with poorer outcomes, as confirmed by RNA-seq (figure 2H).

In conclusion, the pathogenic molecular background, antigen escape, and clonal evolution may contribute to resistance to CAR T cell therapy in this case. Moreover, it is worth noting that plasma ctDNA NGS is useful as a noninvasive method to monitor the clonal evolution process.

Second, intrinsic T cell function is vital for patient response to CAR T therapy. Specific characteristics of CAR T cell subtypes in the infusion product are correlated with better therapeutic results.\(^1\) Enhanced transcription of genes associated with T cell activation and memory T cells may predict a better response to T cell expansion and persistence. Furthermore, Lyn, Jun, and Daf\(^2\) found that a functional deficiency in c-Jun mediates dysfunction in exhausted human T cells which indicates that deficiency of crucial genes may extensively influence T cell function. Patients with no response (NR) have a poor response in terms of the persistence and expansion of cellular kinetics. PIM1 plays an essential role in regulating signal transduction cascades, which promote cell survival, proliferation, and drug resistance. In addition, PIM1 somatic mutation was identified as one of the potential early drivers of lymphomagenesis.\(^1\) PIM1 (c.403G>A, p.Glu135Lys, legacy Identifier in COSMIC: COSM1161628) is frequently reported in DLBCL in somatic states, and this mutation was predicted to be damage by FATHMM and SIFT. However, in this case, the patient carried the PIM1 germline mutation in the heterozygous state, whether the PIM1 germline mutation was associated with the lack of amplification or persistence of CAR T cells, and the abnormal transcriptional features of the patient’s CD3\(^+\) T cells in terms of T cell antigen presentation, activation, and costimulation pathways has yet to be determined. Unfortunately, this patient passed away and could only be studied retrospectively from RNA sequencing. For patients with immunodeficiency-related mutations, allogeneic HSCT can be considered a better option than autologous HSCT, which can compensate for the defects of intrinsic lymphocyte immunodeficiency. According to NCCN Guidelines V.1.2021 for acute myeloid leukemia (age ≥18 years), patients with a family history of leukemia, hematologic cancer or abnormalities together with the presence of genetic mutations should be considered for germline testing or genetic counseling. It is strongly recommended that patients with a germline variant allele frequency of 40%–60% be referred for germline testing. However, germline variants in CAR T cell immunotherapy have been poorly studied; further research is needed to investigate the impact and may be applied for guiding clinical therapy and donor selection.

In conclusion, several studies have recently revealed MYC/BCL2/BCL6 rearrangements and TP53 mutations as independent indicators of prognosis in patients with DLBCL treated with standard chemotherapy; EZB-MYC\(^+\) and A53 subtypes are associated with poorer prognosis; and the germline mutation of PIM1 (c.403G>A, p.Glu135Lys) is reported for the first time and is highly suspected that this germline mutation was related to T cell defect. The factors previously make this an intractable case and may contribute to CD19 antigen loss and T cell defect. Tumor-derived antigen escape and T cell dysfunction, two interactive factors, are major challenges to CAR T cell therapy. Tumor burden and antigen density can positively stimulate T cell expansion, which in turn increases the risk and severity of CRS. Such a dilemma should be carefully considered for each patient. We propose that precise monitoring of tumor genetic abnormalities and T cell function should be conducted during CAR T cell therapy. Antigen loss, clonal evolution, and T cell defects might appear simultaneously and contribute to resistance in combination.

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Contributors JW analyzed the data and wrote the manuscript. ZS and JX took care of the patient. JW, YG, LY, and WZ revised the manuscript and were in charge of the manuscript’s final approval. KS, MZ, ZZ, JW, LC, QL, and CH performed the experiments. NW and LH provided clinical information. YX, MX, and JZ directed the research.

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