Materials and methods

Immunohistochemical analysis. The samples for immunohistochemistry were paraffin-embedded and the thickness of the sections was 3 μm. The samples were fixed in 10% formalin solution at room temperature for 6-8 h. The immunohistochemical analysis was performed using the immunohistochemistry autostainer manufactured by Roche Diagnostics (model BENCHMARK-XT). The primary antibodies were supplied by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. The secondary antibodies were supplied by Roche Diagnostics. The primary antibodies were incubated at 37˚C for 32 min, and the secondary antibodies were incubated at 37˚C for 8 min. All antibodies were ready to use without dilution. A Leica light microscope (Leica Microsystems, Inc.) was used to assess the samples. Representative images are shown in Fig. 2. The immunohistochemical analysis was performed by the Department of Pathology in The Affiliated Hospital of Yangzhou University (Yanzhou, China). As primary CD20-negative diffuse large B-cell tumors are rare, in order to further verify the results, the immunohistochemical results were reviewed by the Department of Pathology of Jiangsu Province Hospital (First Affiliated Hospital of Nanjing Medical University, Nanjing, China).

Intensity of PD-1/PD-L1$^+$ cell staining. Assessment of cell staining was performed by a biomedical laboratory company named SINO-US Diagnostics Lab, Tianjin. According to the study by Kwon *et al* (1), PD-L1 expression was evaluated based on the intensity and proportion of cells showing membranous staining and/or cytoplasmic staining, and was scored as follows: 0, negative (no or any staining in <10% of cells); 1, weak; 2, moderate; and 3, strong staining in >10% of cells. The numbers of PD-1$^+$ cells were assessed semi-quantitatively and scored as follows: 0, no positive cells/high-power field (HPF); 1, <10 positive cells/HPF; 2, 10-30 positive cells/HPF; 3, >30 positive cells/HPF on average.

Next-generation sequencing. The next-generation sequencing was performed by a biomedical laboratory company named Burning Rock Dx and the sample number was RS20052643FFP.

Targeted DNA sequencing. DNA of FFPE samples was extracted (QIAamp DNA FFPE tissue kit; Qiagen GmbH) and the DNA concentration was measured by QubitdsDNA assay (Thermo Fisher Scientific, Inc.). The gDNA quality was assessed to make sure that A260/A280 was within the range of 1.8 to 2.0. At the time of biopsy, 10 ml peripheral blood was obtained, stored in tubes containing ethylenediaminetetraacetic acid and incubated at room temperature for 2 h. The supernatant was transferred to 15-ml centrifuge tubes and centrifuged for 10 min at 16,000 x g and 4˚C. The supernatant was then transferred to a new tube and stored at 80˚C for further analysis. Circulating cfDNA was recovered from 4-5 ml plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen GmbH). Quantification of cfDNA was assessed with the Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Inc.). A minimum of 50 ng cfDNA was required for construction of a next-generation sequencing library.

For patients with available DNA, targeted DNA sequencing was performed. DNA was profiled using a capture-based targeted sequencing panel (Burning Rock Biotech, Ltd.), targeting 112 genes and spanning 314 K of Human genomic regions. The concentration of the DNA samples was measured with the Qubit dsDNA assay to make sure that genomic DNA was 14-18 pM. Fragments of 200-400 bp in size were selected with beads (Agencourt AMPure XP kit; Beckman-Coulter, Inc.), followed by hybridization with the capture probes baits, hybrid selection with magnetic beads and PCR amplification. A bioanalyzer high-sensitivity DNA assay was then used to assess the quality and size range. Available indexed samples were then sequenced on a Miseq (Illumina, Inc.) with paired end reads.

Sequencing data analysis. Sequencing data were mapped to the human genome (hg19) using BWA aligner 0.7.10 (2) [PCR duplicate reads were removed before base substitution detection. Local alignment optimization and variant calling was performed using GATK v3.2-2 (3). DNA translocation analysis was performed using both Tophat2 (4) and Factera 1.4.3 (5). Insert size distribution and library complexity of each sample were computed to assess the level of DNA degradation. Different mutation calling thresholds were applied on samples with different DNA quality to avoid false-positive mutation calls due to DNA damage. SNV and indels identified were annotated using the dbNSFP (v30a), COSMIC (v69) and dbSNP (snp138) databases. Variants with a global minor allele frequency >1.0% in 1000Genome Project (Phase3; http://www.1000genomes.org/data) were considered as common SNPs and removed.

Integrative Genomics Viewer (Broad Institute) was used to visualize variants aligned against the reference genome to confirm the accuracy of the variant calls by checking for possible strand biases and sequencing errors. Copy number variation (CNV) analysis was performed by normalizing and read counts from each target region, and the gene-level CNV was assessed by a z-test.

Fluorescence in situ hybridization. In The Affiliated Hospital of Yangzhou University (Yangzhou, China), the postoperative tumor was diagnosed as CD20-negative diffuse large B-cell lymphoma (DLBCL). The specimen was sent to Jiangsu Province Hospital (The First Affiliated Hospital of Nanjing Medical University) for re-examination and FISH was performed simultaneously to determine whether it was double-hit DLBCL.

Flow cytometry. Flow cytometry was also performed by SINO-US Diagnostics Lab, Tianjin. In total, 5 ml of the patient's bone marrow fluid was taken, anticoagulated with heparin, treated with red blood cell lysate, labeled with relevant antibodies and washed with PBS. Lymphoma-associated antigen expression was detected by BC Navios multicolor flow cytometry (Beckman Coulter GmbH). The CD45/SSC gating method was used to analyze the data. Representative flow cytometry histograms/dot plots are shown in Fig. S1.

References

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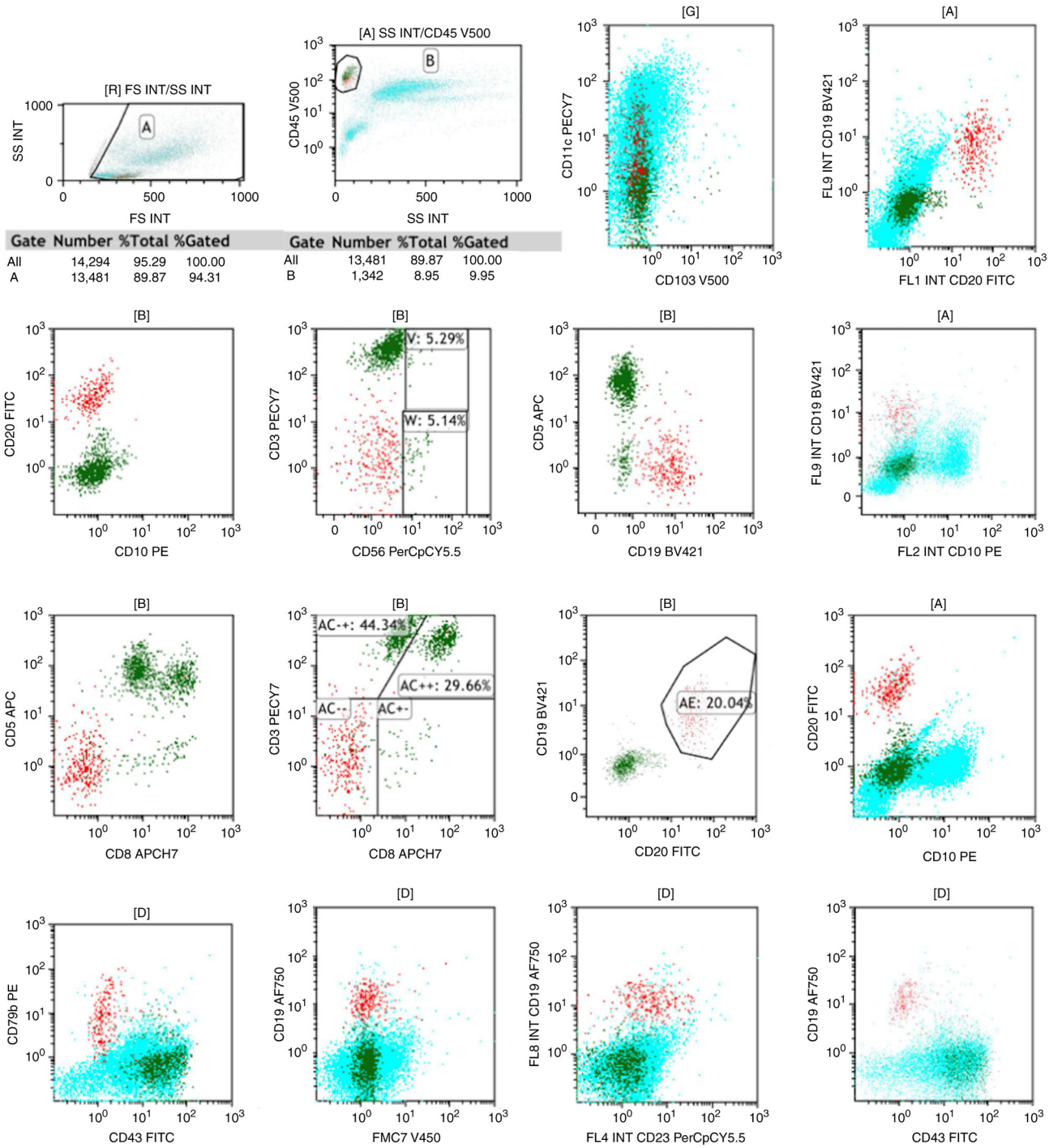
Figure S1.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SS INT
FS INT
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[A] SS INT/CD45 V500
CD45 V500
SS INT
B
Gate Number %Total %Gated
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B 1,342 8.95 9.95
[G]
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CD103 V500
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FL1 INT CD20 FITC
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CD20 FITC
CD10 PE
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CD3 PECY7
CD56 PerCpCY5.5
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W: 5.14%
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CD19 BV421
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FL2 INT CD10 PE
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CD5 APC
CD8 APCH7
[B]
CD3 PECY7
CD8 APCH7
AC-+: 44.34%
AC++: 29.66%
AC--
AC+-
[B]
CD19 BV421
CD20 FITC
AE: 20.04%
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CD19 AF750
CD43 FITC

Figure S1. Continued. Flow cytometry analysis of the bone marrow. Mature lymphocytes accounted for 9.95% of the nucleated cells, and 74% of the lymphocytes were CD3-positive T cells. The ratio of CD4/ CD8 was 1.49. NK cells that showed CD16 or CD56 positive and CD3 negative accounted for 5.14% of the lymphocytes. CD3-positive with CD56-positive lymphocytes account for 5.29%. CD19-positive B cells accounted for 20.04% of lymphocytes. The ratio of lymphocyte populations was as above, and no clonal abnormalities were seen in B lymphocytes.