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Case report

A sporadic case of CTLA4 haploinsufficiency manifesting as Epstein–Barr virus-positive diffuse large B-cell lymphoma

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Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) is a coinhibitory receptor that plays an essential role in maintaining immune system homeostasis by suppressing T-cell activation. We report a sporadic case of CTLA4 haploinsufficiency in a patient with Epstein–Barr virus-positive diffuse large B-cell lymphoma and subsequent benign lymphadenopathy. A missense mutation in exon 2 of the CTLA4 gene (c.251T>C, p.V84A) was found in the patient's peripheral blood and buccal cell DNA, but not in her parents' DNA. CTLA4 expression decreased in the peripheral regulatory T cells upon stimulation, whereas CTLA4 and PD-1-positive T cell subsets increased, possibly to compensate for the defective CTLA4 function. This case suggests that some adult lymphoma patients with no remarkable medical history have primary immune disorder. As immune-targeted therapies are now widely used for the treatment of malignancies, it is increasingly important to recognize the underlying primary immune disorders to properly manage the disease and avoid unexpected complications of immunotherapies.

Keywords: Epstein–Barr virus, diffuse large B-cell lymphoma, CTLA4, haploinsufficiency, germline mutation

INTRODUCTION

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) is a homodimeric coinhibitory receptor that plays an essential role in maintaining immune system homeostasis by suppressing T-cell activation. CTLA4 is expressed on the surface of activated T cells and inhibits the secondary signal activation from antigen-presenting cells by competitively binding to CD80 and CD86 with CD28. CTLA4 is also constitutively expressed on regulatory T cells and is required for their inhibitory function. Antibodies targeting CTLA4 promote anti-tumor immunity and have been applied in cancer immunotherapy.[1,2]

In 2014, an autosomal dominant immune dysregulation syndrome caused by CTLA4 haploinsufficiency was reported in two studies.[3,4] The cause was heterozygous germline mutations in *CTLA4*, and in addition to various autoimmune symptoms, the patients demonstrated hypogammaglobulinemia and recurrent infections as a consequence of the decreased T-cell repertoire, autoimmune cytopenia, and

B-cell exhaustion. Although a worldwide cohort study of *CTLA4* mutation carriers has been reported,[5] the detailed disease mechanisms and clinical features have not been fully elucidated because of its rarity.

We report a sporadic case of CTLA4 haploinsufficiency in a patient with Epstein–Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL) and subsequent benign lymphadenopathy. Although the patient had no history of autoimmune disease or specific infections, her uncommon clinical course led us to perform genetic screening for congenital immune dysfunction, and a missense germline mutation in *CTLA4* was identified.

MATERIALS AND METHODS

Ethical statement

Written informed consent was received from the patient and her parents for the genomic analysis in accordance with the ethical standards of the Declaration of Helsinki and the

institutional review board of Kyoto University Hospital.

Immunohistochemistry

Immunohistochemistry was performed with antibodies against the following proteins: CD20 (346595, BD Biosciences, San Jose, CA), CD3 (349201, BD Biosciences), CD30 (clone Ber-H2, Roche, Mannheim, Germany), CD15 (Carb-3, Dako, Santa Barbara, CA), Ki-67 (clone MIB-1, Dako), EBV LMP-1 (CS-1-4, Dako), EBV EBNA2 (PE2, Dako), CTLA4 (sc-376016, Santa Cruz Biotechnology, Santa Cruz, CA), and FOXP3 (clone PCH101, eBioscience, San Diego, CA). Immunohistochemistry was performed using an automatic immunostainer (BenchMark, Ventana Medical Systems, Tucson, AZ and BOND-III system, Leica Microsystems, Bannockburn, IL) in accordance with the manufacturer's instructions. Epstein–Barr virus detection was performed by *in situ* hybridization using an EBV-encoded small non-polyadenylated RNA probe on an automated system (Ventana Medical systems for Figure 1B, g, and Leica Microsystems for Figure 2B, e).

Flow cytometric analysis

For intracellular CTLA4 staining, peripheral blood mononuclear cells were stimulated at 37°C for 16 hours with anti-CD3/CD28-activating Dynabeads (Life Technologies, Oslo, Norway). After removing the beads by magnetic separation, cells were stained with PC7-conjugated anti-CD4 (clone, SFCI12T4D11 (T4), Beckman Coulter), and fixed and permeabilized with Fixation/Permeabilization kit (eBioscience). Cells were then stained with Alexa Fluor 647-conjugated anti-FOXP3 (clone 236A/E7, eBioscience) and PE-Cy5-conjugated CTLA4 (clone BNI3, BD Biosciences) antibod-
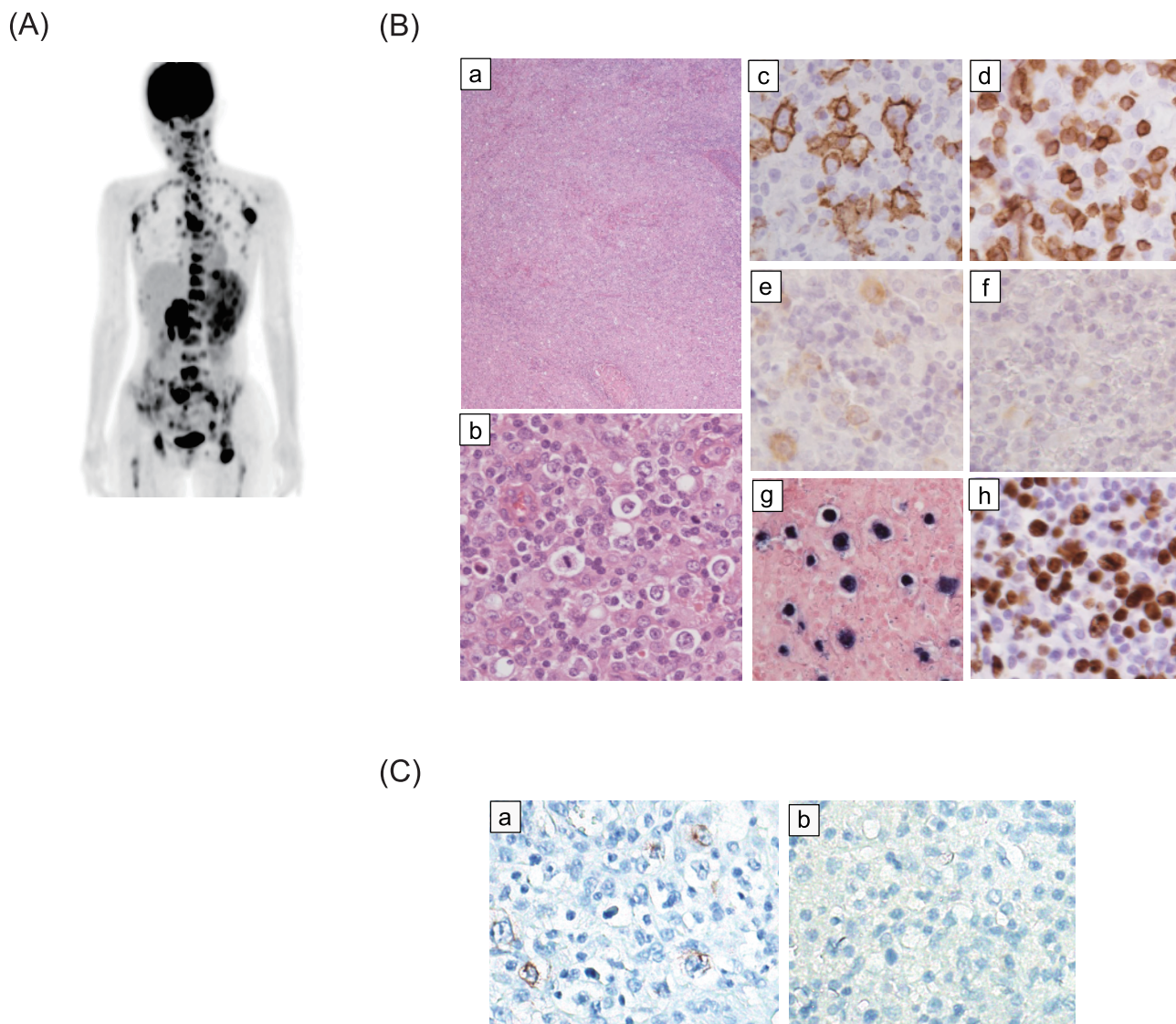
Fig. 1.
(**A**) PET-CT of the patient at the time of initial presentation. (**B**) Histological images of the right axillar lymph node leading to a diagnosis of DLBCL. (**a**, **b**) Hematoxylin and eosin, (**c**) CD20, (**d**) CD3, (**e**) CD30, (**f**) CD15, (**g**) EBER-ISH, (**h**) Ki-67. Original magnification, (**a**) × 40; (**b-h**) × 400. (**C**) Evaluation of the EBV protein expression in the lymphoma tissue. (**a**) LMP-1, (**b**) EBNA2. Original magnification, × 400.

(A)

(B)
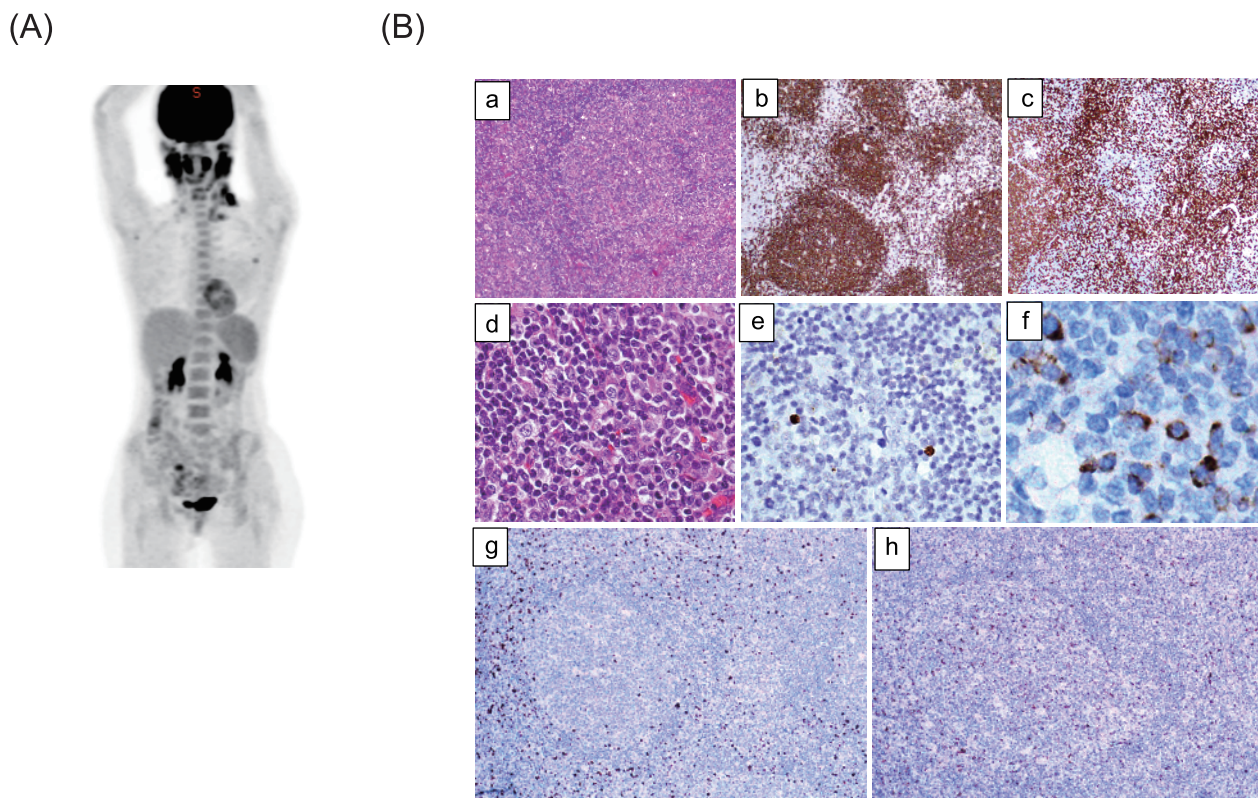
Fig. 2.
(*A*) PET-CT of the patient at the time of recurrence of lymph node swelling. (*B*) Histological images of the cervical lymph node, which led to a diagnosis of reactive follicular hyperplasia. (*a*, *d*) Hematoxylin and eosin, (*b*) CD20, (*c*) CD3, (*e*) EBER-ISH, (*f*, *h*) CTLA4, (*g*) FOXP3. Original magnification, (*a-c*, *g*, *h*) × 40; (*d-f*) × 400.

ies, and were analyzed using a flow cytometer.

Phenotypic analysis was performed using a flow cytometer (FACSLyric, BD Biosciences, San Jose, CA) with FITC-conjugated CD56 (clone HCD56; BioLegend, San Diego, CA), PE-conjugated CD14 (clone M5E2, BioLegend), PerCP/Cy5.5-conjugated CD19 (clone HIB19, BioLegend), PE/Cy7-conjugated CD8 (clone SK1, BioLegend), APC or PerCP/Cy5.5-conjugated CD4 (clone RPA-T4, BioLegend), APC/Cy7-conjugated CD3 (clone SK7, BioLegend), FITC-conjugated CD45RA (clone HI100, BioLegend), PE/Cy7-conjugated CD25 (clone BC96, BioLegend), APC-conjugated CD38 (clone HB-7, BioLegend), APC-conjugated CTLA-4 (clone BNI3, BioLegend), PE-conjugated PD-1 (clone MIH4, BD Pharmingen, Palo Alto, CA), PE-conjugated CCR7 (clone 150503, BD Pharmingen), and PE-conjugated FOXP3 (clone 236A/E7, eBioscience). For CTLA4 and FOXP3, intracellular staining was performed using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) in accordance with the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Peripheral blood mononuclear cells were separated from the peripheral blood of the patient and two healthy donors using a Ficoll-Paque density gradient (Cedarlane), and total T cells were collected by negative selection using MACS Cell Separation Technology (Miltenyi Biotec, Bergisch Gladbach,

Germany). Total RNA was extracted using the RNeasy Mini kit, and complementary DNA (cDNA) was synthesized using a SuperScript III First-Star and Synthesis system (Life Technologies, Carlsbad, CA, USA). qRT-PCR was performed using TB Green Premix Ex Taq II (Takara Bio, Otsu, Japan). The primers used for the amplification are listed in Table 1. The expression levels of *FOXP3*, *CTLA4*, and *PDCD1* were normalized to that of *ACTB*.

CASE REPORT

A 28-year-old female with a history of only mild atopic dermatitis presented with fatigue, high fever, and multiple swollen lymph nodes. [18F]-Fluorodeoxy-d-glucose positron

Table 1. Primers used for qRT-PCR.

Gene	Primers
FOXP3 (forward)	5'-CACTGCTGGCAAATGGTGTC-3'
FOXP3 (reverse)	5'-GCTGCTCCAGAGACTGTACC-3'
CTLA4 (forward)	5'-CATGATGGGGAATGAGTTGACC-3'
CTLA4 (reverse)	5'-TCAGTCCTTGGATAGTGAGGTTC-3'
PDCD1 (forward)	5'-TGGCATTTGCTGAACGCATTT-3'
PDCD1 (reverse)	5'-TGCAGCCAGGTCTAATTGTTTT-3'
ACTB (forward)	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'
ACTB (reverse)	5'-TGGCACCCAGCACAATGAA-3'

emission tomography (FDG-PET)-computed tomography (CT) revealed systemic lymphadenopathy, splenomegaly, and multiple bone lesions (Figure 1A). Laboratory data included increases in lactate dehydrogenase (LDH) to 1,127 IU/L (normal range, 117–236 IU/L), soluble interleukin-2 receptor (sIL-2R) to 10,500 U/mL (145–519 U/mL), and β2-microglobulin to 4.6 µg/mL (1.0–1.9 µg/mL). Serum IgG and IgA moderately decreased to 651 mg/dL (870–1,700 mg/dL) and 28 mg/dL (110–410 mg/dL), respectively, whereas IgM was normal (78 mg/dL; normal range 35–220 mg/dL). The patient's serum was negative for human immunodeficiency virus-1 antibody.

Histological analysis of the biopsied right axillar lymph node first led to a diagnosis of classic Hodgkin lymphoma (cHL), but the diagnosis was later revised to EBV-positive DLBCL with a T-cell-rich large B-cell lymphoma-like pattern (Figure 1B). The large tumor cells were positive for CD20, CD30 (weak, 30%), and EBV-encoded small RNA (EBER)-*in situ* hybridization (ISH), and negative for CD3 and CD15. Ki-67 was positive in 80% of the tumor cells. The tumor cells expressed EBV latent membrane protein 1 (LMP-1), but lacked EBV nuclear antigen 2 (EBNA2), and exhibited a type 2 latency pattern (Figure 1C). The patient was treated with one cycle of ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) and six cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone), and achieved complete remission.

Two years later, the patient developed cervical lymph node swelling (Figure 2A). Although the relapse of DLBCL was suspected, histological analysis of the biopsied cervical lymph node revealed only reactive follicular hyperplasia with a few, small EBER-positive cells (Figure 2B, a-e). The patient had normal blood cell counts with a relatively low lymphocyte count of 590/µL (normal range, 400–3,700/µL) and normal lymphocyte subsets (CD3+ T cells, 72.1%; CD3+CD4+ T cells, 38.5%; CD3+CD8+ T cells, 28.1%; CD56+ NK cells, 12.7%; CD19+ B cells, 15.2%). However, the serum immunoglobulin levels further decreased (IgG 320 mg/dL, IgA 10 mg/dL, IgM 40 mg/dL). The serum EBV-DNA was 190 copies/µg of DNA when evaluated after 1 cycle of ABVD and became negative after the next cycle of chemotherapy. However, it became positive again half a year before the appearance of lymphadenopathy and remained positive at low levels thereafter (20–60 copies/µg of DNA).

Persisting lymphadenopathy with low immunoglobulin levels and serum EBV-DNA positivity led us to consider the possibility of congenital immune dysfunction. After receiving written informed consent from the patient and her parents, the patient's peripheral blood DNA was screened for germline mutations (Kazusa DNA Research Institute, Chiba, Japan). A missense mutation in exon 2 of the *CTLA4* gene (c.251T>C, p.V84A) was found and the mutation was confirmed by Sanger sequencing of the patient's buccal cell DNA (Figure 3A). This variant was not listed in public databases (Clinvar, ExAC and HGMD), and evaluated as probably damaging by PolyPhen2 (0.987) but tolerable by the

SIFT score (0.26). We evaluated CTLA4 expression upon stimulation of peripheral regulatory T cells, which was markedly reduced according to flow cytometry[6] (Figure 3B), and the patient was diagnosed with CTLA4 haploinsufficiency. Neither of the parents had this mutant allele of *CTLA4* (Figure 3A) and the case was considered to be sporadic.

Flow cytometric analysis of the peripheral blood at rest revealed a decrease in CD45RA+CCR7+ naïve T cells and an increase in CD45RA- memory CD4+ T cells. CTLA4 and PD-1-positive T cell subsets also increased (Figure 3C), possibly to compensate for the defective CTLA4 function. Consistent these results, *FOXP3, CTLA4,* and *PDCD1* gene expression levels increased in the patient's T cells as a whole (Figure 3D). Detailed histological analysis of the cervical lymph node revealed CTLA4-positive cells in both the interfollicular and intrafollicular areas, and FOXP3-positive cells were mainly present in the interfollicular areas (Figure 2B, f-h). This suggested that CTLA4 was predominantly expressed by regulatory T cells and activated non-regulatory T cells in the interfollicular and intrafollicular areas, respectively.

The patient was screened for autoimmune diseases, but there were no findings suggestive of autoimmune diseases on blood testing. The patient received regular intravenous immunoglobulin replacement therapy and her lymphadenopathy has been stable for two years.

DISCUSSION

CTLA4 haploinsufficiency has a broad clinical spectrum and a variety of clinical manifestations. In patients with CTLA4 haploinsufficiency, CTLA4 ligand binding and transendocytosis of CD80 are impaired, resulting in the hyperactivation of effector T cells.[3] However, the penetrance of CTLA4 haploinsufficiency is estimated to be around 70%[5,7] and some carriers are asymptomatic. Although low immunoglobulin levels were a clue for the diagnosis of CTLA4 haploinsufficiency in this patient, it was unclear whether they were specific findings because immunoglobulin levels are often low in patients with lymphoma due to the disease itself.[8] Another clue was her medical history of atopic dermatitis, which is sometimes observed in CTLA4 haploinsufficiency patients,[9] albeit a less specific finding. Therefore, CTLA4 haploinsufficiency in patients who lack typical immunological abnormalities and solely develop lymphomas may remain largely undiagnosed.

In a previous study by Garcia-Perez *et al.*,[10] CTLA4 gene expression levels, but not those of *PDCD1* and *FOXP3,* were reported to increase in CD4+ T cells of patients with CTLA4 haploinsufficiency compared with those of healthy controls. *FOXP3* and *PDCD1* gene expression levels may have been upregulated under chronic inflammation in our patient considering the persistence of lymphadenopathy.

Asymptomatic EBV viremia is also a supportive finding of CTLA4 haploinsufficiency.[11,12] In a previous report by Egg *et al.*, 17 malignancies were documented among the 131 affected *CTLA4* mutation carriers, including 10 lymphomas
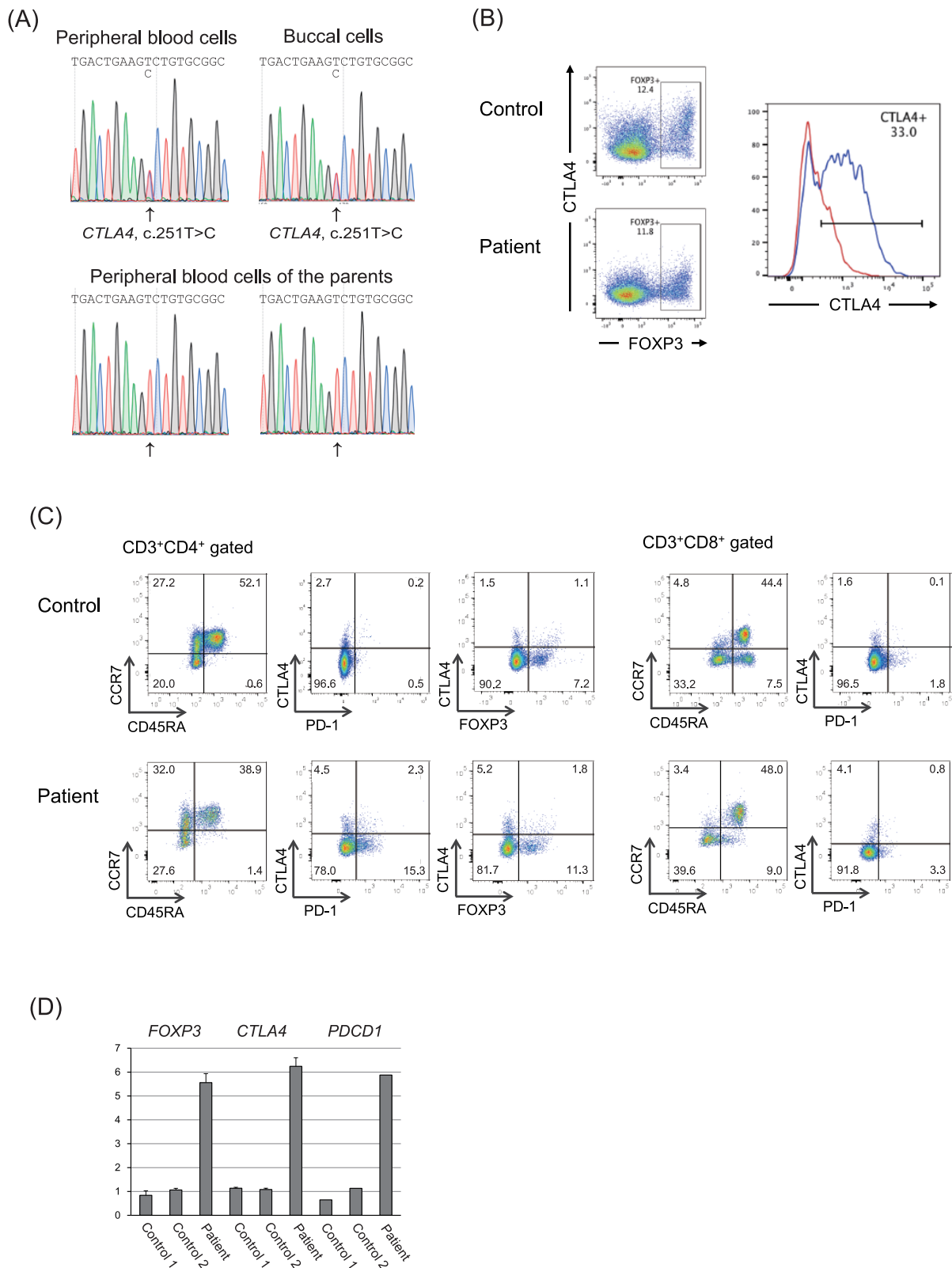
Fig. 3.
(*A*) Results of Sanger sequencing of the *CTLA4* gene. A monoallelic germline *CTLA4* mutation was detected in the patient (upper panels), but not in her parents (lower panels). (*B*) The CTLA4 expression level in the activated regulatory T cells was lower in the patient (red line) than that in a healthy control (blue line). Peripheral blood mononuclear cells were stimulated with CD3/CD28 beads and intracellular CTLA4 expression levels of CD4+FOXP3+ T cells were measured. Cells were gated for CD4+. (*C*) Phenotypic analysis of peripheral T cells at rest of the patient and a healthy control. (*D*) Comparison of *FOXP3, CTLA4,* and *PDCD1* gene expression levels in peripheral T cells of the patient and healthy controls. The expression levels were normalized to that of *ACTB*. Means and standard errors of two technical replicates are shown.

consisting of six cHL, three DLBCL, and one Burkitt lymphoma.[13] Of note, seven lymphoma and three gastric adenocarcinoma cases, i.e., 10 of the 17 malignancies, were EBV-associated. Therefore, it may be helpful to monitor EBV-DNA load in *CTLA4* mutation carriers for the early detection of EBV-associated malignancies.

EBV-positive DLBCL generally develops in elderly patients, probably in the background of immunosenescence, and is associated with a poor prognosis.[14] On the other hand, EBV-positive DLBCL is reported to have another smaller peak in the third decade and the patients in this younger subgroup are not associated with unfavorable outcomes.[15,16] Although there are no notable immunological defects in this younger subgroup, some of these patients are expected to have innate immune abnormality, similar to our patient.

This case suggests that some adult lymphoma patients with no remarkable medical history have background primary immune disorder. As immune-targeted therapies are now widely used for the treatment of malignancies, including lymphomas, it is increasingly important to recognize the underlying primary immune disorders to properly manage the disease and avoid unexpected complications of immunotherapies.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

H.Y., Y.O., and T.K. analyzed the patient samples, M.N. and C.U. treated the patient and collected clinical data. T.Y. and H.K. made the diagnosis of CTLA4 haploinsufficiency, and provided clinical and experimental advice. M.F., M.H., and H.H. performed pathological examination, A.T-K. supervised the study. M.N. designed and wrote the manuscript, and all authors reviewed and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest related to this work.

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