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Everolimus-Induced Remission of Classic Kaposi's Sarcoma Secondary to Cryptic Splicing Mediated *CTLA4* Haploinsufficiency

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To the Editor:

CTLA4 haploinsufficiency causes an autosomal dominant inborn error of immunity characterized by immune dysregulation, lymphoproliferation, and autoimmunity due to

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Compliance with Ethical Standards

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defective regulatory T cell (Treg) function, effector T cell hyperactivation, and lymphocytic organ infiltration (1, 2). Penetrance is incomplete (~ 60–70%), and genotype-phenotype correlations are variable, thus making diagnosis and treatment difficult (3). Deficiency of CTLA4 may be caused by microdeletions in chromosome 2, missense, nonsense, frameshift, and splice variants (3) and pseudo-CTLA4 deficiencies such as LRBA (4) and DEF6 deficiency (5). Patients with CTLA4 haploinsufficiency are unable to control infection with some herpes viruses, including Epstein-Barr virus (EBV) and cytomegalovirus (CMV), evidenced by chronic viremia, EBV-induced hemophagocytic lymphohistiocytosis, and reactivation of CMV infection. Furthermore, ~ 12% of these patients develop secondary malignancies, notably, EBV-associated lymphomas and gastric carcinomas (6). Classic Kaposi's sarcoma (cKS) is a rare cancer of lymphatic endothelial cells affecting the skin, lymph nodes, and visceral organs caused by infection with human herpes virus-8 (HHV8), a latent γ-herpes virus similar to EBV (7). However, there have been no reports to date of cKS in patients with CTLA4 haploinsufficiency. Here, we describe a cryptic splice site mutation in *CTLA4* presenting with cKS and the clinical response to targeted therapy with everolimus.

A 52-year-old HIV-negative male of Italian ancestry (P1) with a long-standing history of excessive fatigue, hypersomnolence, poor libido, cognitive dysfunction, chronic diarrhea (with lymphocytic infiltrate on colonic biopsy), and polyarthralgia presented with pain and swelling in his right groin (Fig. 1a and Table 1). He was diagnosed with cutaneous and nodal cKS secondary to HHV8 infection on biopsy of his right inguinal lymph node. He was initially successfully treated with liposomal doxorubicin. However, his systemic symptoms persisted, and he was referred for investigation of a possible underlying immunod eficiency disorder. Immunophenotyping revealed an expanded oligoclonal population of $CD4^{hi}CD8^{dim}$ double-positive (DP) T cells, decreased naïve $CD4^+$ and $CD8^+$ single-positive (SP) T cells, increased SP T cells with a $CCR7^{neg}CD45RA^+$ effector memory RA (T_{EMRA}) phenotype, and expanded $CD21^{lo}$ memory B cells and NK cell lymphopenia (Fig. 1b and Table 1). The DP T cells did not express CD8β, indicating that they expressed the CD8αα homodimer instead of the conventional CD8αβ heterodimer (Fig. 1c), suggesting they are terminally differentiated $CD4^+$ T cells that have upregulated CD8αα in response to chronic antigenic stimulation (8, 9) consistent with their exhausted T_{EMRA} phenotype (Fig. 1b). Bone marrow examination did not reveal any evidence for a hematological malignancy. Serum immunoglobulin levels were normal, and autoimmune serology was negative. Whole genome sequencing identified a heterozygous chr2 (GRCh37) : g.204735656G > A; NM_005214.49(*CTLA4*):c.457G>A (p.Asp153Asn) variant of unknown significance at the 3′ end of exon 2 of the *CTLA4* gene which was confirmed by Sanger sequencing (Fig. 1d). There were no pathogenic variants in the *IFNGR1*, *WAS*, *STIM1*, or *TNFRSF4* genes which have previously been associated with cKS (10). He subsequently had a biopsy-proven relapse in his right groin.

Further characterization revealed normal Treg numbers with normal FOXP3 expression. However, there was a decreased expression of CTLA4 on resting and stimulated on $CD25^{hi}CD127^{lo}$ $CD45RA^{neg}CD45RO^+$ memory Tregs (Fig. 1e) (11). RNA sequencing of peripheral blood mononuclear cells showed a 50% drop-off in the number of sequence reads starting 67 base pairs (bp) upstream of the 3′ end of exon 2 (Fig. 1f). Analysis of

the mutated gene sequence revealed the presence of a cryptic splice site at this location. The CTLA variant was originally observed from split-read alignments and verified with LeafCutter (12) and Whippet (13). Notably, the patient's variant is predicted to delete the MYPPPY hexapeptide motif at the 3′ end of exon 2 that is required for binding to the B7 family of costimulatory molecules and CTLA4 function (14). This variant also causes a frameshift that results in a premature stop codon and terminates transcription 8 bp from the 3′ end of exon 3 (Fig. 1f). RT-PCR across the exon 2-exon 3 junction confirmed the presence of both a wildtype and truncated PCR product in P1 (Fig. 1g). These data confirm that the c.457G>A variant was responsible for cryptic splicing and haploinsufficiency of CTLA4 in P1. We therefore initiated mechanism-based treatment with sirolimus 2 mg daily (15) in order to suppress unrestrained activation of conventional T cells and restore Treg function (16). Furthermore, sirolimus has been reported to have direct anti-tumor effects and be effective in treating cKS (17). However, this was poorly tolerated and ceased. After a 6-month drug holiday, he was re-treated with everolimus 2 mg daily, which has been reported to be effective in some (18, 19) but not all cases of KS (20). This resulted in resolution of the pain and swelling in his groin, and repeat PET/CT scan showed complete metabolic remission of his cKS. Interestingly, he also reported improvement in his energy levels, short-term memory, and attention span and resolution of his diarrhea. Immune phenotyping showed possible small improvements with an increase in total B cells, a decrease in $CD21^{lo}$ memory B cells, and a decrease in total T cells. We also noted increased expression of CD56 on NK cells and decreased expression of CD69 on SP and DP T cells and decreased CD57 on DP TEMRA.

From the Clinical Immunogenomics Research Consortium Australasia (CIRCA) cohort, we also identified a 15-year-old female patient (P2) and her 46-year-old mother (P3) of Sri Lankan ancestry with more typical clinical features of CTLA4 haploinsufficiency (Fig. 1a and Table 1). P2 has recurrent suppurative ear and chest infections and bronchiectasis secondary to a specific antibody deficiency and autoimmune enteropathy. P3 has bilateral uveitis and developed stage IV intraabdominal Hodgkin's lymphoma (EBV-negative) at age 41, which was successfully treated with cyclophosphamide, doxorubicin, etoposide, vincristine, and bleomycin, and WHO grade 3 anaplastic oligodendroglioma in her right parietal lobe (also EBV-negative) at age 46 which was surgically resected. Whole exome sequencing revealed an intronic c.457+ 1G>A variant in *CTLA4*. RNA sequencing and RT-PCR confirmed that this variant, similar to the c.457G>A variant in P1, disrupted the splice donor site at the 3′ end of exon2 and resulted in aberrant splicing to the same upstream cryptic splice site (Fig. 1f, g). We also identified a 21-year-old cousin, P4, in the extended family with enteropathy who also carried the c.457+1G>A variant (Fig. 1a and Table 1). These 3 additional patients demonstrate unequivocally that the c.457G>A variant is pathogenic, and this has been corroborated by a recent independent study (21).

CTLA4 haploinsufficiency has been associated with defective NK cell function (22), and this may contribute to the impaired anti-viral and anti-cancer immunity in P1 and P3, who both had NK cell lymphopenia and cancer. P1 (but not P3) had evidence of chronic antigenic stimulation with expanded oligoclonal populations of exhausted DP T cells, T_{EMRA}, and T cell. Low-dose sirolimus and everolimus, while considered immunosuppressive drugs, may have paradoxical anti-tumor effects by metabolic reprogramming of exhausted T cells

and recalibrating anti-viral and anti-cancer immunity. It will be interesting to determine if long-term mTOR inhibition reduces the risk of secondary malignancy in patients with CTLA4 haploinsufficiency.

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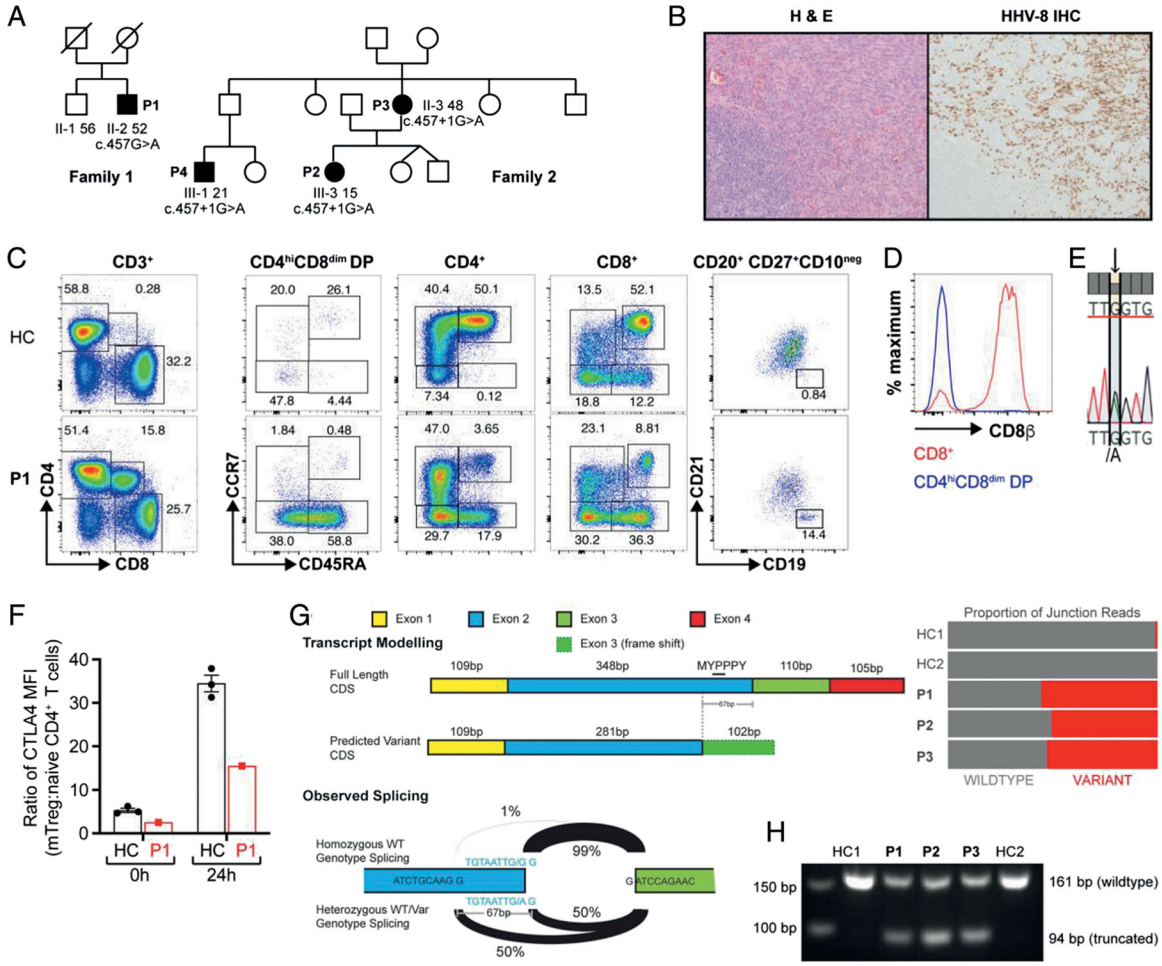

Fig. 1.

CTLA4 haploinsufficiency due to cryptic splicing of exon 2. **a** Pedigrees and genotypes of 4 patients from 2 families. **b** Hematoxylin and eosin (H & E) and immunohistochemistry (IHC) of inguinal lymph node showing HHV8 staining (brown). **c** Expan d ed T $_{EMRA}$ ($CCR7^{neg}CD45RA^{+}$) in peripheral blood $CD4^{+}$, $CD8^{+}$, and DP T cells, and elevated $CD19^{hi}CD21^{lo}$ memory B ($CD20^{+}$ $CD27^{+}CD10^{neg}$) cells in P1. **d** Expression of CD8β by $CD8^{+}$ and $CD4^{hi}CD8^{dim}$ DP T cells in P1. **e** Sanger sequencing confirms heterozygous c.457G>A mutation in P1. **f** Relative CTLA4 expression summarized as the ratio of mean fluorescence intensity (MFI) of resting memory Tregs (mTregs, $CD4^{+}$ $CD25^{hi}CD127^{lo}$ $CD45RA^{neg}CD45RO^{+}$) or stimulated mTregs to resting conventional (conv) naïve $CD4^{+}$ ($CD4^{+}$ $CD25^{lo}CD127^{int/hi}$ $CD45RA^{+}CD45RO^{neg}$) T cells in P1. Graphs are a representative of 3 experiments. **g** RNA sequencing reveals cryptic CTLA4 splicing, resulting in the deletion of MYPPPY motif, a ligand binding motif. Full-length CDS: CTLA4 transcript variant 1 mRNA (NM_005214.4); exon 2-exon 3 splice sequence: TGTAATTG/ATCCAGAAC. Predicted variant CDS: Longest open reading frame (ORF) from in silico translation of aberrantly spliced transcript sequence; exon2-exon 3 splice sequence: ATCTGCAAG/ATCCAGAAC. **h** RT-PCR across the exon 2-exon 3 junction reveals WT and truncated band

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Table 1

Novel clinical and laboratory features of CTLA4 haploinsufficiency due to cryptic splicing due to the c.457G>A variant

	CTLA4 haploinsufficiency	P1	P2	P3	P4
Mutation					
CTLA4 gene mutation	Yes	c.457G>A	c.457+1G>A	c.457+1G>A	c.457+1G>A
Typical Clinical Manifestations of CTLA4 haploinsufficiency based on 165 reported cases					
Enteropathy	58%	+	+	Absent	+
Splenomegaly	45%	Absent	Absent	Absent	Absent
Lymphadenopathy	38%	Absent	Absent	Absent	Absent
AIHA	38%	Absent	Absent	Absent	Absent
ITP	36%	Absent	Absent	Absent	Absent
Interstitial lung disease	34%	Absent	Absent	Absent	Absent
Brain lesions on MRI	21%	Absent	N.D.	Absent*	N.D.
Psoriasis	16%	Absent	Absent	Absent	Absent
Arthritis	15%	+	Absent	Absent	Absent
Autoimmune thyroiditis	12%	Absent	Absent	Absent	Absent
Lymphoma	7%	Absent	Absent	+	Absent
Novel Clinical Manifestations					
Kaposi's sarcoma	Not reported	+	Absent	Absent	Absent
Chronic fatigue	Not reported	+	Absent	Absent	Absent
Oligodendroglioma	Not reported	Absent	Absent	+	Absent
Typical Laboratory findings					
Hypogammaglobulinemia or specific antibody deficiency	72%	Absent	+	Absent	Absent
$CD4^+$ lymphopenia	22%	+	+	+	Absent
Decrease $CD45RA^+CD62L^+$ naïve T cells	74%	+	+	N.D.	Absent
Increased $CD4^+$ $FOXP3^+$ Tregs	% unknown	Absent	+	N.D.	N.D.

	CTLA4 haploinsufficiency	P1	P2	P3	P4
Decreased CTLA4 expression on stimulated Tregs	100%	+	+	N.D.	N.D.
Increased $CD19^{hi}CD21^{lo}$ memory B cells	83%	+	+	Absent	Absent
NK cell lymphopenia	56%	+	Absent	+	Absent
Novel Laboratory findings					
Increased $CD4^{+}$ TEMRA	Not reported	+	Absent	Absent	Absent
Increased $CD8^{+}$ TEMRA	Not reported	+	Absent	Absent	Absent
Increased DP TEMRA	Not reported	+	Absent	Absent	Absent
HHV8 testing of PBMCs					
HHV8 ELISA	Not reported	+	N.D.	N.D.	N.D.
HHV8 ELISPOT	Not reported	+	N.D.	N.D.	N.D.
HHV8 PCR	Not reported	Negative	N.D.	N.D.	N.D.

N.D. not done

*P3 had oligodendroglioma; yellow cells indicate the presence of known clinical or laboratory findings; green cells indicate the presence of novel clinical or laboratory findings