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Frequent *CTLA4-CD28* gene fusion in diverse types of T-cell lymphoma

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ABSTRACT

CTLA4 and CD28 are co-regulatory receptors with opposite roles in T-cell signaling. By RNA sequencing, we identified a fusion between the two genes from partial gene duplication in a case of angioimmunoblastic T-cell lymphoma. The fusion gene, which codes for the extracellular domain of CTLA4 and the cytoplasmic region of CD28, is likely capable of transforming inhibitory signals into stimulatory signals for T-cell activation. Ectopic expression of the fusion transcript in Jurkat and H9 cells resulted in enhanced proliferation and AKT and ERK phosphorylation, indicating activation of downstream oncogenic pathways. To estimate the frequency of this gene fusion in mature T-cell lymphomas, we examined 115 T-cell lymphoma samples of diverse subtypes using reverse transcriptase polymerase chain reaction analysis and Sanger sequencing. We identified the fusion in 26 of 45 cases of angioimmunoblastic T-cell lymphomas (58%), nine of 39 peripheral T-cell lymphomas, not otherwise specified (23%), and nine of 31 extranodal NK/T cell lymphomas (29%). We further investigated the mutation status of 70 lymphoma-associated genes using ultra-deep targeted resequencing for 74 mature T-cell lymphoma samples. The mutational landscape we obtained suggests that T-cell lymphoma results from diverse combinations of multiple gene mutations. The *CTLA4-CD28* gene fusion is likely a major contributor to the pathogenesis of T-cell lymphomas and represents a potential target for anti-CTLA4 cancer immunotherapy.

Introduction

Peripheral T-cell lymphoma is a malignant neoplasm of mature T cells. Recent genomic studies have identified highly recurrent somatic mutations in *TET2*, *DNMT3A*, *IDH2*, and *RHOA* in diverse subtypes of mature T-cell lymphoma (TCL).[1-5] However, the roles of these mutations in the regulation of T-cell signaling and oncogenesis have yet to be elucidated. Furthermore, none of the mutant genes has been clearly demonstrated to be a dominant oncogenic driver. It is, therefore, still necessary to identify additional driver mutations and dissect the interplay with T-cell signaling components.

CTLA4 and CD28, members of the immunoglobulin superfamily that are expressed on the surfaces of T cells, are regulatory co-receptors of T-cell signaling. They play critical and opposite roles in maintaining balanced T-cell signaling and,

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thus, the proper level of immune activation.[6,7] Perturbing this balance can result in a number of undesirable consequences such as autoimmunity, transplant rejection, or even malignant TCL.[6] Accordingly, controlling T-cell signaling through these two co-receptors has been a key strategy for recent cancer immunotherapies including anti-CTLA4 antibody therapy[8,9] and chimeric antigen receptor T-cell therapy utilizing the intracellular signaling domain of CD28.[10] In this study, we identified a fusion between *CTLA4* and *CD28* in a case of angioimmunoblastic TCL by whole transcriptome sequencing and analyzed the frequency of gene fusion in 117 cases of TCL. A functional study of the fusion gene indicated that fusion between CTLA4 and CD28 results in the activation of downstream oncogenic pathways. The mutational status of 70 lymphoma-associated genes was analyzed using ultra-deep targeted resequencing in 74 samples of mature TCL.

Methods

Sample description

The patients' clinical information is summarized in *Online Supplementary Table S2*. Formalin-fixed, paraffin-embedded tumor tissue was used for Sanger sequencing of 115 TCL tumor samples and targeted deep sequencing of 74 TCL tumor samples. The QIAamp DNA Mini Kit (Qiagen) and RNeasy Mini Kit (Qiagen) were used for DNA and RNA extraction, respectively. All patients' samples were obtained with informed consent in Samsung Medical Center, Seoul, Korea, and the study was approved by the Institutional Review Board in accordance with the Declaration of Helsinki.

Detection of the CTLA4-CD28 mutation

For detection of the *CTLA4-CD28* fusion transcript, we carried out reverse transcription (RT) with random hexamers and total RNA isolated from formalin-fixed, paraffin-embedded samples followed by polymerase chain reaction (PCR) amplification with the following oligonucleotide primers; Fusion_cRT1 F, Fusion_cRT1 R, Fusion_cRT2 F, Fusion_cRT2 R and Fusion_cRT3 F. For characterization of the genomic rearrangement, the *CTLA4-CD28* fusion gene was amplified by PCR using genomic DNA of the clinical specimen as the template. Amplification was performed with Herculase 2 Fusion DNA Polymerase (Agilent Technologies) and the primers; Fusion_G1 F, Fusion_G1 R and Fusion_G2 R. The resulting PCR products were analyzed by agarose gel electrophoresis and sequenced using two independent primer pairs.

Cell proliferation and cytokine assays

Jurkat (human T-cell acute lymphoblastic leukemia) and H9 (human cutaneous T lymphocyte lymphoma) cells were transfected with a construct expressing the CTLA4-CD28 fusion protein. Cells expressing CTLA4 and the CTLA4-CD28 fusion were seeded in 96-well plates in triplicate at a density of 5×10^3 cells/well in 100 μL of RPMI-1640 medium containing 10% fetal bovine serum and antibiotics. For stimulation, a 96-well plate was coated with 5 μg/mL goat anti-mouse IgG (AbFrontier) or with 2 μg/mL anti-CD3 (HIT3a, BD Pharmingen) or with the combination of 2 μg/mL anti-CD3 (HIT3a, BD Pharmingen), 2 μg/mL anti-CD28 (CD28.2 BD Pharmingen) or 5 μg/mL anti-CTLA4 (BNI3, BD Pharmingen) overnight or for 2 h at 37°C. After 48 h, cell proliferation was evaluated using Cell Counting Kit-8 (Dojindo), according to the manufacturer's instructions, and the absorbance value for each well was measured at 450 nm using a microplate reader (Spectra Max 180, Molecular Devices). Each experiment was repeated three times. For the cytokine assay, cells were stimulated with 15 ng/mL phorbol myristate acetate and 290 ng/mL ionomycin (eBioscience) for 3 h. Cells were plated on 24-well plates coated with 5 μg/mL goat anti-mouse IgG (AbFrontier) or with 2 μg/mL anti-CD3 (HIT3a, BD Pharmingen) or with the combination of 2 μg/mL anti-CD3 (HIT3a, BD Pharmingen), 2 μg/mL anti-CD28 (CD28.2 BD Pharmingen) or 5 μg/mL anti-CTLA4 (BNI3, BD Pharmingen). After 24 h, the supernatants were examined using Human IL-2 ELISA kits (Thermo Scientific) according to the manufacturer's instructions. Each experiment was repeated three times.

Selection of 70 frequently mutated genes in lymphoma and bioinformatic analysis for targeted sequencing

Through ten genomics studies on B- and T-cell lymphomas, we have identified 62 genes that are frequently mutated in these neoplasms.[1,3,5,11-18] We added three p53-related genes and five JAK-STAT signaling genes for targeted deep sequencing. The list of 70 target genes is provided in *Online Supplementary Table S1*. We further compiled somatic mutations in the target genes from the original references which collectively yielded 832 mutations in 62 genes (*Online Supplementary File S1*). Targeted sequencing was performed using 74 TCL samples. The bioinformatics analysis of the targeted deep sequencing data is described in the *Online Supplementary Methods*.

Results

Identification and validation of the CTLA4-CD28 fusion gene

The fusion transcript was initially predicted from analyses of RNA-Seq data from previously described TCL patients[5] using the FusionScan program. Multiple reads that mapped to exon 3 of the *CTLA4* gene in tandem with exon 4 of the *CD28* gene were identified (Figure 1). The depth profile of the RNA-Seq data, reflecting the expression level of each exon, shows abrupt changes at the break points in both genes, which is consistent with the presence of fusion transcripts (Figure 1B). We verified the fusion *via* RT-PCR using two different primer sets in fusion-positive samples from patients and seven TCL cell lines (Figure 2A; *Online Supplementary Figure S1*). *CTLA4-CD28* fusion cDNA was successfully amplified from the patients' samples and two T lymphoblastoid cell lines, CEMC1-15 and CEMC7-14. Subsequent Sanger sequencing confirmed that the PCR products contained the fusion transcripts between exon 3 of the *CTLA4* gene and exon 4 of the *CD28* gene.

To estimate the frequency of this gene fusion in TCL patients, we examined 115 TCL patients' samples of diverse subtypes using RT-PCR and Sanger sequencing (*Online Supplementary Table S2*). We found that the *CTLA4-CD28* fusion occurred in all the tested subtypes of TCL with an overall frequency of 38%. The fusion was observed more frequently in TCL of follicular helper T cell phenotype (TFH) than in non-TFH TCL. The fusion was identified in 26 of 45 (58%) patients with angioimmunoblastic TCL (AITL), nine of 39 (23%) patients with peripheral TCL not otherwise specified (PTCL-NOS), and nine of 31 (29%) patients with NK/T cell lymphoma (*Online Supplementary Table S2*; *Online Supplementary Figure S2*). Among PTCL-NOS, five of nine PTCL-NOS with a TFH phenotype (56%) and five of 16 non-TFH PTCL-NOS

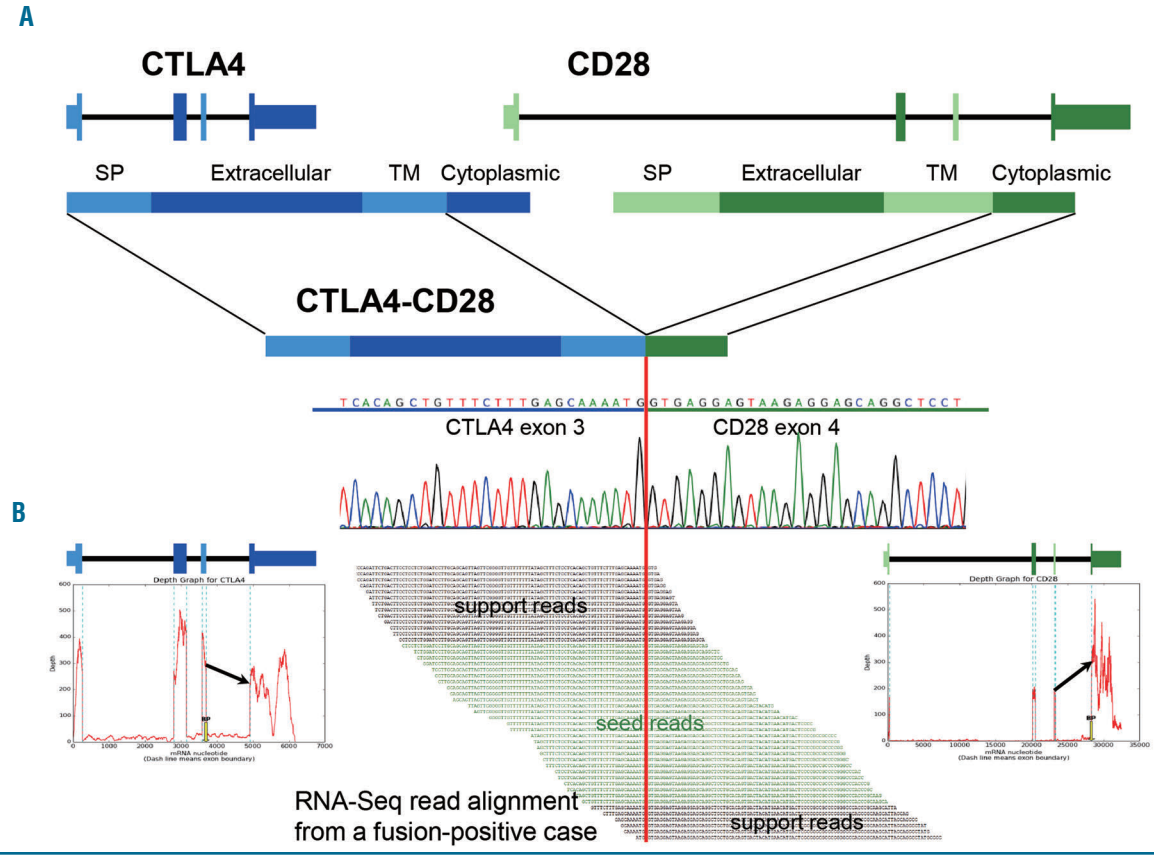

Figure 1. Identification of the *CTLA4-CD28* gene fusion. (A) Top, schematic diagram of the gene fusion; bottom, sequencing chromatogram. Numbers on the transcript indicate the nucleotide position of exons. (B) Alignment of RNA-Seq data from a fusion-positive patient. Read-depth plots indicate the depth coverage of aligned RNA-Seq reads. SP: signal peptide; TM: transmembrane region.

(31%) showed the *CTLA4-CD28* fusion. The *CTLA4-CD28* fusion was not observed in blood samples from 50 healthy individuals.

Functional analyses of the CTLA4-CD28 *fusion gene*

Notably, the predicted protein generated from this fusion gene features the extracellular and transmembrane domains of CTLA4 and the cytosolic signaling domain of CD28 (Figure 1A). A possible outcome is inappropriate activation of T-cell signaling. We, therefore, proceeded to analyze the effect of the *CTLA4-CD28* fusion on cell proliferation and cytokine production in T cells. Jurkat (human T-cell acute lymphoblastic leukemia) and H9 (human cutaneous T lymphocyte lymphoma) cells transfected with a construct expressing the CTLA4-CD28 fusion protein proliferated at a rate approximately 30% higher than that of cells transfected with only the vector or the wild-type CTLA4 expression construct after stimulation with anti-CTLA4 antibody (Figure 2B; *Online Supplementary Figure S3A*). The surface expression levels of CTLA4 and the CTLA4-CD28 fusion were comparable in the Jurkat cell line (*Online Supplementary Figure S4*). Furthermore, the production of interleukin 2 (IL-2), the definitive marker of T-cell activation, was 6-fold greater (Figure 2C; *Online Supplementary Figure S3B*). These results indicate that the CTLA4-CD28 fusion protein likely mediates activating signals upon T-cell stimulation.

Next, we examined the phosphorylation of AKT and ERK1/2, which represents the activation of two critical pathways downstream of T-cell receptor signaling. As expected, CD28-mediated co-stimulation of T cells led to increased AKT and ERK phosphorylation (Figure 2D). Importantly, cells expressing the CTLA4-CD28 fusion also showed increased AKT and ERK phosphorylation relative to cells expressing wild-type CTLA4 upon stimulation with an anti-CTLA4 antibody. These results strongly suggest that the CTLA4-CD28 fusion could lead to constitutive T-cell activation by converting inhibitory signals into activating signals. Of note, Shin *et al.* developed the *CTLA4-CD28* chimera for adoptive T-cell therapy of cancer, and studied its biological roles and therapeutic efficacy using mouse T cells.[19] Importantly, they also demonstrated that the fusion gene delivered activating rather than inhibitory T-cell signals.

Genomic structure of the CTLA4-CD28 fusion gene

The two genes are located in tandem 129 kbp apart on chromosome 2, with *CD28* preceding *CTLA4* on the same strand. The gene order is reversed in the *CTLA4-CD28* fusion, raising a strong possibility that partial duplications have occurred (Figure 3A). Extrachromosomal amplification by episome formation, which was observed for the *NUP214-ABL1* fusion in T-cell acute lymphoblastic leukemia,[20] could not be ruled out in all cases, but was

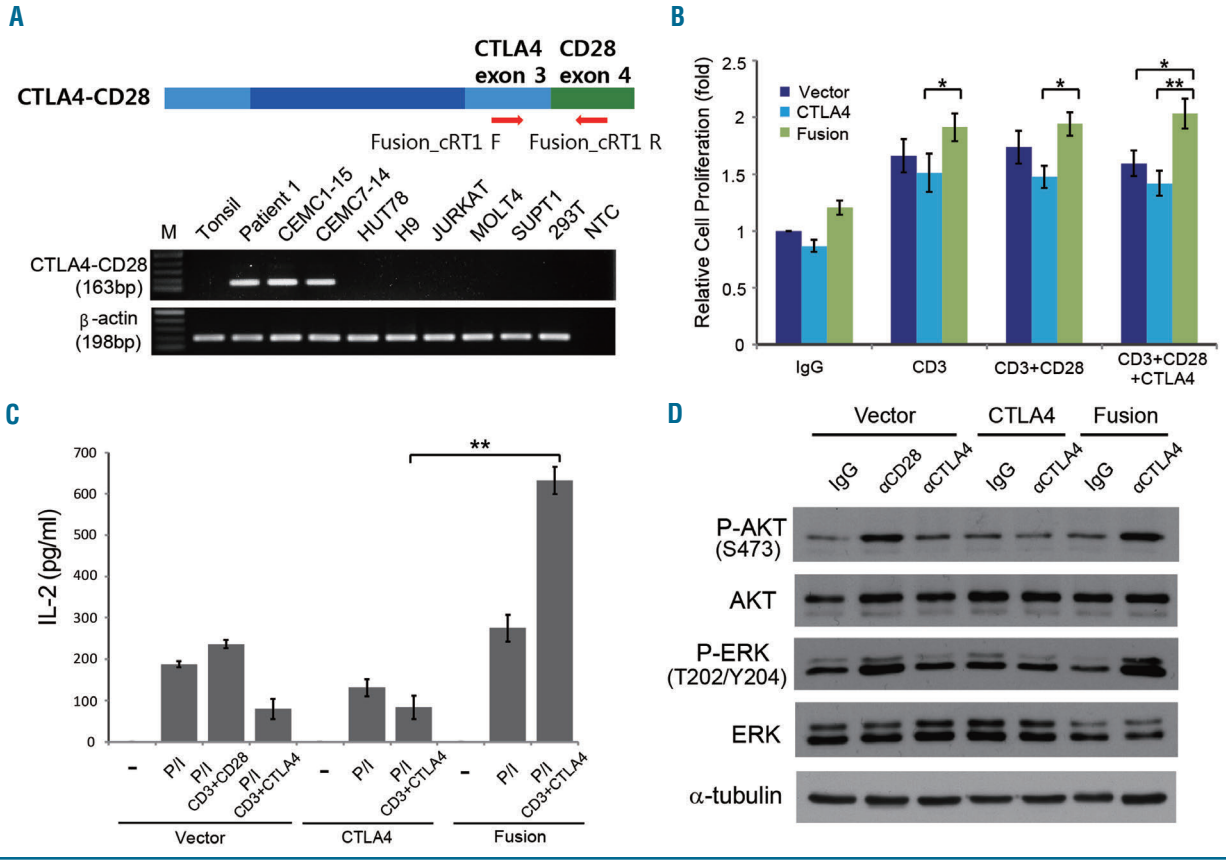

Figure 2. Validation and functional analyses of the *CTLA4-CD28* fusion gene. (A) Validation of the *CTLA4-CD28* fusion transcript by RT-PCR using samples from patients and cell lines. Arrows indicate the approximate positions of oligonucleotide primers on the *CTLA4-CD28* fusion transcript. PCR products were amplified from patient 1, CEMC1-15 cells and CEMC7-14 cells and validated by Sanger sequencing. β-actin was used as an internal control, and NTC indicates the no template control. Normal tonsil tissue and 293T cells were used as negative controls. (B) Jurkat cells expressing the CTLA4-CD28 fusion showed enhanced cell proliferation (*P<0.05 and **P<0.01 compared with cells expressing wild-type CTLA4) after co-stimulation with anti-CD3/CTLA4 antibodies. Each experiment was repeated three times with five replicates, and the data are expressed as the mean ± standard deviation. (C) Expression of the CTLA4-CD28 fusion enhanced interleukin 2 (IL-2) production after CTLA4 activation (**P<0.01 compared with cells expressing wild-type CTLA4). Jurkat cells were stimulated with phorbol myristate acetate/Ionomycin (P/I) without or with anti-CD3/CTLA4 antibodies to activate CTLA4. IL-2 was measured at three independent times, and the data are expressed as the mean ± standard deviation. (D) Expression of the CTLA4-CD28 fusion enhanced phosphorylation of AKT and ERK1/2 after CTLA4 activation with an anti-CTLA4 antibody.

shown not to have occurred in multiple cases studied by fluorescence *in situ* hybridization (*Online Supplementary Figure S5*). Consistent with our hypothesis, quantitative PCR analyses of genomic DNA demonstrated that the copy number gains for portions of the *CD28* and *CTLA4* genes represented in the fusion were significantly higher in the fusion-positive patients than those in the fusion-negative lymphoma patients (Figure 3B; *Online Supplementary Figure S6*).

Next, we mapped the exact positions of the break points in the genomic DNA of fusion-positive patients. For the fusion-positive patient shown in Figures 1 and 2, we amplified a 2.5 kb genomic DNA fragment (Figure 3D). Subsequent Sanger sequencing revealed that the fragment contained 696 bp of *CTLA4* intron 3, 1457 bp of *CD28* intron 3, and 47 bp of intervening sequence from a LINE retrotransposon element that is normally located 104 kb downstream of the *CTLA4* gene on chromosome 2 (Figure 3E; *Online Supplementary Figure S7*). We characterized another patient whose genome 426 bp of *CTLA4* intron 3 was joined to 3185 bp of *CD28* intron 3. Surprisingly, the most frequent arrangement was direct fusion of two exons with no intron sequences included. We observed six other patients and two cell lines with such fusions. Despite the diversity in their genomic structures, all the genome arrangements we observed in patients' samples would generate identical fusion transcripts and proteins.

We also analyzed the mRNA expression levels of the *CD28*, *CTLA4*, and *CTLA4-CD28* fusion genes in samples from patients with AITL using quantitative RT-PCR (Figure 3C). Fusion mRNA was expressed only in fusion-positive patients, and the expression level was comparable to that of *CTLA4*. The levels of *CD28* and *CTLA4* expression were approximately three and seven times higher in fusion-positive patients, respectively, which suggests the presence of a feed-forward circuit that amplifies the signaling from the *CTLA4-CD28* fusion receptor. A stronger binding affinity of CTLA4, compared to CD28, to CD80 and CD86 ligands may have a role in amplifying signals in the *CTLA4-CD28* fusion receptor.[6] In brief, patients with the *CTLA4-CD28* fusion not only have a copy number gain at the genomic level but also show elevated *CD28* and *CTLA4* expression, which is consistent with abnormal activation of the T-cell population.

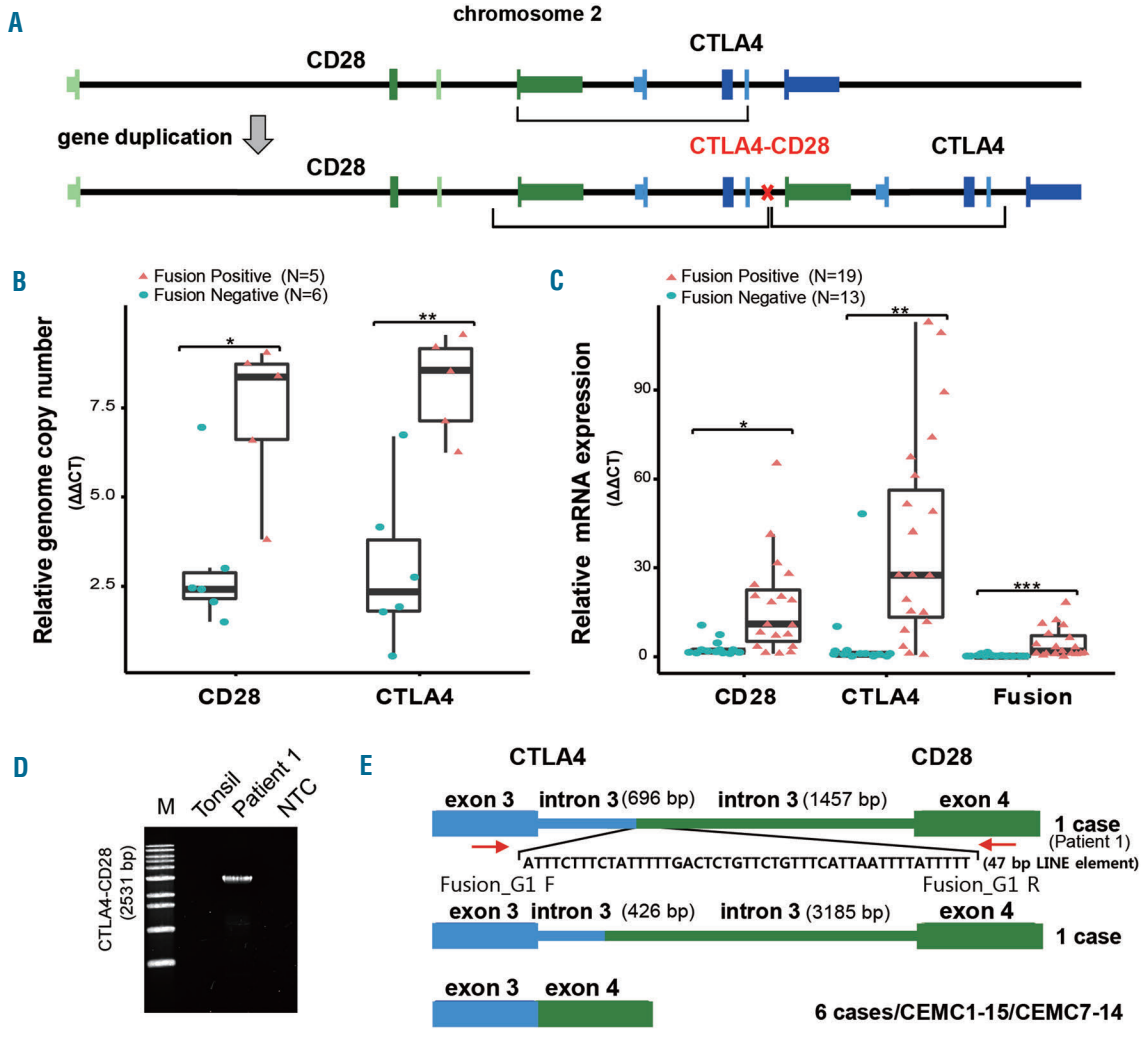

Figure 3. Genomic structure of the *CTLA4-CD28* fusion gene. (A) Schematic diagram of the gene duplication producing the fusion gene. (B) Copy-number analysis of *CD28* and *CTLA4* genes in samples from patients with AITL using quantitative -PCR (**P*=0.009, ***P*=0.001). Copy number changes, estimated relative to that in peripheral blood cells from a normal individual, are shown in the box plot. The values are from two independent experiments. (C) Expression levels of *CD28*, *CTLA4*, and *CTLA4-CD28* fusion transcripts in samples from patients with AITL using quantitative-PCR (**P*=0.001, ***P*=0.0001, ****P*=0.002). The values are from three independent experiments. (D) Structural analysis of genomic loci for a patient with the *CTLA4-CD28* fusion. The PCR product (2.5 kb) amplified from genomic DNA of patient 1 was subsequently validated by Sanger sequencing. No product was amplified from the control normal tonsil cells and the no template control (NTC). (E) Schematic diagrams of the genomic structure of the *CTLA4-CD28* fusion from eight patients and two cell lines. The arrows indicate the position of primers (Fusion_G1 F and Fusion_G1 R).

Mutational landscape of lymphoma-related genes from targeted deep sequencing

Several somatic mutations have already been implicated as driver mutations in TCL, including the point mutations *IDH2* R172K, *DNMT3A* R882H, *RHOA* G17V, and *CD28* T195P and loss-of-function (stop-gain or frameshift) mutations in the *TET2* gene.[3-5] Determining the functional relationship between these mutations and the *CTLA4-CD28* fusion would be of the utmost importance to understanding the molecular mechanisms underlying TCL. We selected 70 genes that had been reported to be frequently mutated in various types of T- and B-cell lymphoma (*Online Supplementary Table S1*).[1,3-5,11-17] Targeted deep sequencing data for all exons of the 70 genes were produced with an average sequencing depth of 1,204X and 98.9% of target coverage using 2,965 primer pairs. The ultra-high depth of the sequencing was expected to reveal variants of low frequency but with important functional roles. In total, 74 samples of tumor tissue were examined: these samples came from 29 patients with AITL, 15 with PTCL-NOS, 26 with extranodal NK/T cell lymphoma (ENKL), two with enteropathy-associated T-cell lymphoma, and two with anaplastic large cell lymphoma.

The sequencing data were analyzed using our own computational pipeline, which was designed to identify somatic mutations in tumor cells without the normal control (*Online Supplementary Figure S8*). We identified 11 missense mutations in ten different genes and 24 *TET2* loss-of-function mutations, including eight novel mutations (Figure 4; *Online Supplementary Figure S8*). Loss-of-function mutations in the *TET2* gene occurred most frequently in all subtypes (47 patients, 64%). Excluding *TET2* mutations with low allele frequency (<5%), which were observed mostly for R544X and R1404X (*Online*

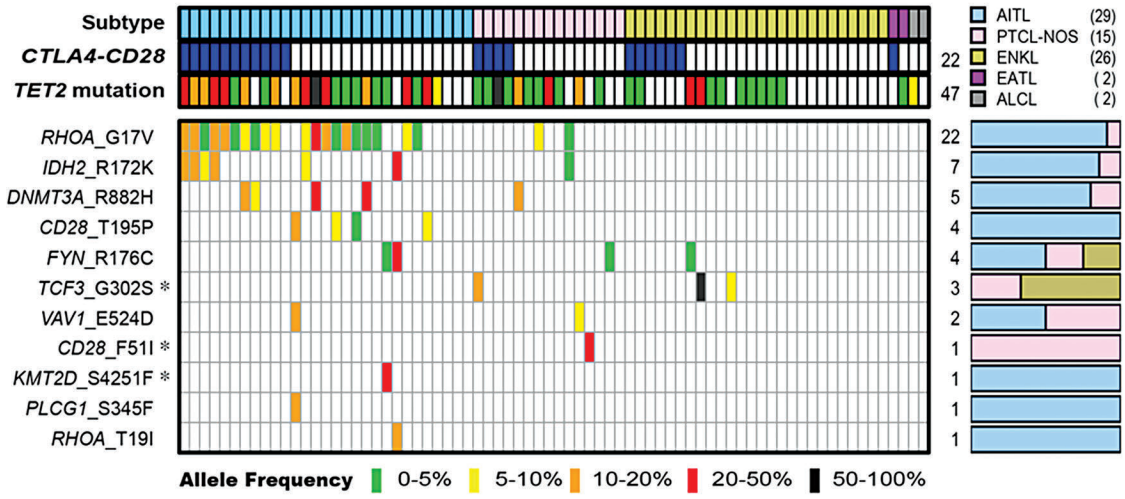

Figure 4. Mutational landscape of driver genes from targeted resequencing. Each column represents an independent patient. Novel mutations are indicated with asterisks. TCL subtypes are color-coded above the main window, and bars on the right side show the relative proportions of the indicated mutation among TCL subtypes. The *CTLA4-CD28* row indicates the presence of fusion gene based on RT-PCR and Sanger sequencing. *TET2* mutations of all locations are shown in top rows, with the color of the highest allele frequency for patients with multiple *TET2* mutations (position-wise mutation plot provided in *Online Supplementary Figure* S9). The cumulative numbers of patients positive for each mutation are indicated on the right.

Supplementary Figure S9), the *TET2* mutation rate was only 30% (22 patients), with a significant concentration in the AITL subtype (15 patients, P=0.008). Many mutations were observed at low frequencies, below 20%, demonstrating the power of ultra-deep sequencing. These low frequency mutations are likely due to the heterogeneity of the tumor cells as well as the presence of normal or stromal cells.

The *RHOA* mutation was mostly observed in the AITL subtype (21 of 29 AITL patients; 72%), with only two mutations observed in the 15 PTCL-NOS patients (13%). We identified eight low frequency mutations (allele frequency <5%) in addition to 12 (41% of AITL patients) high frequency mutations, which agrees well with previous reports on AITL based on Sanger sequencing.[3-5] Among other recurrent mutations, *IDH2* R172K and *DNMT3A* R882H mutations were found in seven and five patients, respectively. These latter two mutations have been described as ageing-related initiating mutations that are associated with the clonal expansion of pre-lymphoma stem cells and can be detected in the blood of elderly individuals without apparent hematologic malignancies.[21-23]

The frequency of the *CTLA4-CD28* fusion was 30% in this targeted deep-sequencing analysis (22 out of 74 patients), appearing in all subtypes tested. Sixteen of the 22 fusion-positive patients had additional mutations. In 11 AITL patients with the *CTLA4-CD28* fusion, nine (82%) and ten (91%) patients also harbored the *TET2* mutation and *RHOA* mutation, respectively. The *CTLA4-CD28* fusion was not found in four patients with a *CD28* T195P mutation which also led to up-regulation of T-cell receptor signaling.[24] indicating the relevance of CD28 in the process of lymphomagenesis.

In ENKL, most of the fusion cases were devoid of additional mutations. In fact, a substantially high proportion of cases did not have any mutations among the tested genes, and even *TET2* mutation-positive cases mostly did not have other mutations. It is in this regard that a novel recurrent *TCF3* G302S is notable, especially for the ENKL subtype. The transcription factor *TCF3* (E2A) is required for B and T lymphocyte development, and the significance of mutations in *TCF3* and its negative regulator *ID3* has recently been highlighted in Burkitt lymphoma.[14,15,17] Genomic examination of additional ENKL patients should be carried out to substantiate the *TCF3* G302S mutation as a marker and a potential driver of the ENKL subtype.

Discussion

Here, we report that the *CTLA4-CD28* fusion gene is a novel, high-frequency mutation for diverse types of TCL. Two other groups have recently reported the identification of the *CTLA4-CD28* fusion gene in Sézary syndrome, an aggressive rare variant of cutaneous T-cell lymphoma.[25,26] That this mutation is not limited to Sézary syndrome but is found in a broad range of TCL types with an overall frequency of 30% and typically in combination with other mutations should be of significance.

We also provide the overall mutational landscape for TCL which indicates that the *CTLA4-CD28* fusion represents one of recurrent genetic events for full blown neoplastic transformation. Targeted deep sequencing analyses for 70 genes implicated in TCL showed that a large majority of patients had more than one mutated gene. Although not all of the mutations have been mechanistically demonstrated to be oncogenic, we suggest that multiple mutational events, including those described in this study, are required for the full development of TCL. It has been proposed and partly demonstrated that TCL and myeloid leukemia feature the age-related accumulation of premalignant mutations in *DNMT3A, TET2, JAK2,* and *GNAS* which are associated with subsequent clonal hematopoietic expansion.[21-23] Similarly in B-cell lymphoma, it has been shown that circulating B cells bearing

BCL2 translocations do not cause follicular lymphoma *per se* but can evolve into overt follicular lymphoma with additional mutations.[27]

Our data indicate that the *CTLA4-CD28* fusion disrupts cellular homeostasis *via* inappropriate activation of T-cell signaling. Given that such dysregulation likely lies at the core of oncogenesis, it is possible that the CTLA4-CD28 fusion provides a target for potential immunotherapy. In fact, Sekulic *et al.* reported a one-patient trial in a female who had suffered from Sézary syndrome for 8 years.[25] Administration of the CTLA4-blocking antibody ipilimumab[8] produced dramatic initial responses for first 2 months, but the disease subsequently progressed rapidly resulting in death 3 months after the last dose. Notwithstanding the tragic outcome, it was shown that an immunotherapy targeting the fusion gene is viable in principle, and with improved dosage and timing, the response may be more durable. Targeted therapy and immunotherapy as parts of a combination therapy regimen is an emerging paradigm of cancer treatment. Thus, the identification of the frequent *CTLA4-CD28* fusion gene will provide a new therapeutic opportunity for TCL patients with this fusion, and elucidation of the exact mechanism by which the *CTLA4-CD28* fusion interacts with other mutations should provide further insights into the molecular nature of TCL development, as well as new strategies for curbing this disease.

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