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Haematologica 2015 [Epub ahead of print]

Citation: by Lee SH, Kim JS, Kim J, Kim SJ, Kim WS, Lee S, Ko YH, and Yoo HY.
doi:10.3324/haematol.2015.133074

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A highly recurrent novel missense mutation in CD28 among angioimmunoblastic T-cell lymphoma patients

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Running title: A novel CD28 mutation in AITL

Keyword: CD28, somatic mutation, angioimmunoblastic T-cell lymphoma
Angioimmunoblastic T-cell lymphoma (AITL) is a rare, aggressive type of T-cell lymphoma (TCL) that accounts for 18.5% of mature T-cell or natural killer (NK)-cell lymphomas\(^1\). Mutations in the *IDH2*, *TET2*, and *DNMT3A* genes, frequently seen in various types of cancer, have also been linked to AITL\(^2,3\). However, the molecular mechanisms governing AITL development are largely unknown.

CD28 provides a costimulatory signal required for full T cell activation\(^4\). Although many studies have demonstrated the importance of CD28 costimulation in T cell function, the molecular mechanism underlying intracellular signal transduction triggered by CD28 ligation is poorly understood. We and other group have previously reported identification of a somatic mutation of *CD28* gene, T195P, in AITL samples\(^5,6\). Mutations in *CD28* gene have been rarely reported in other types of tumor and may play important roles in tumorigenesis and progression of AITL considering the importance of CD28 in T-cell activation.

T195P substitution was found in 3 out of 9 AITL patients we initially examined. The mutation is located in the cytoplasmic region which indicated potential alterations in downstream T-cell receptor signaling as a consequence (Figure 1A and B). To better estimate the mutation rate and determine subtype specificity among various categories of TCL, we expanded the patient cohort to include additional 40 AITL, 39 peripheral TCL not otherwise specified (PTCL-NOS) and 37 NK/T cell lymphoma (NK/T) cases (Figure 1C). Sanger sequencing of PCR products demonstrated a mutation rate of 10.2% (5 of 49) in the case of AITL (Supplementary Figure 1). All of mutation-positive cases were heterozygotes for this mutation. Importantly, the *CD28* T195P mutation was not found in PTCL-NOS or in NK/T cases indicating that this mutation is specific to AITL subtype. We also analyzed the F51V mutation in CD28\(^5\), but the CD28 F51V mutation was not found in 50 AITL cases.

Clinically, CD28 T195P mutation in AITL patients has no correlation with age, sex, international prognostic index, stage, performance status, LDH level, bone marrow
involvement, and survival. Overall survival was 45.2±31.6 months for mutation-negative group (44 patients) and 19.2 ± 2.7 months for mutation-positive group (5 patients) (p=0.553 by Log Rank) (Supplementary Figure 2).

We next explored the functional significance of T195P mutation in CD28. This mutation is located in the cytoplasmic region, next to the YMNM motif which associates with signaling proteins such as phosphatidyl inositol 3 kinase (PI3K), GRB2 and GRAP2\(^7,9\) (Figure 1B). PI3K regulates a number of signaling cascades through the phosphorylation of inositol lipids. GRB2 and GRAP2 are involved in CD28-mediated IL-2 promoter activation and NF-κB activation\(^10\). CD28 becomes tyrosine-phosphorylated by Src family kinases such as Lck and Fyn and by Tec family kinases such as Itk\(^7,11\). Phosphorylation of a tyrosine (Y191) within an YMNM motif and an PYAP motif in the cytoplasmic domain of CD28 permits recruitment of the signaling proteins, which bind to the receptor via their SH2 domains\(^7,12-15\). We first analyzed the binding of signaling proteins to the cytoplasmic domain of CD28 (Figure 2A). GST pull-down assay showed that binding of PI3K (p85/p110) to the cytoplasmic domain with T195P mutation decreased compared to the level seen with the wild type CD28. However, the binding of GRB2 and GRAP2 to the cytoplasmic domain containing T195P mutation increased either with or without phosphorylation of tyrosine (Y191). These results indicate that the substitution of 195\(^{th}\) amino acid from threonine to proline in CD28 changes the binding of multiple signaling proteins and thus likely the signaling mediated by CD28.

We next analyzed the effect of the CD28 T195P mutation on cell proliferation. Jurkat (human T cell acute lymphoblastic leukemia) cells transfected with the construct expressing CD28 T195P showed an approximately 15% higher proliferation rate than cells expressing wild-type CD28 either with or without costimulation (Figure 2B and Supplementary Figure
3). This result indicates that the T195P mutant is likely constitutively active in T-cell stimulation and promotes cell proliferation.

Next, we examined the phosphorylation of AKT, mTOR and ERK. These proteins are involved in intracellular signaling pathways required for CD28-mediated costimulation of T cells. CD28-mediated costimulation led to increased AKT, mTOR and ERK phosphorylation (Figure 2C). Importantly, cells expressing CD28 T195P showed increased AKT, mTOR and ERK phosphorylation relative to cells expressing wild-type CD28 even without stimulation. These results are consistent with that mutant CD28-mediated signaling is constitutive. We also analyzed the effect of CD28 T195P mutation on cytokine production of T cells (Figure 2D). Expression of CD28 T195P mutant in Jurkat cells enhanced production of interleukin 2 (IL-2), a key cytokine for T-cell proliferation, compared to wild-type CD28 after costimulation with anti CD3/CD28 antibodies indicating that CD28 T195P mutant promote proliferation via increasing the expression of IL-2. Because NF-κB pathway plays a prominent role in CD28-mediated T cell signaling, we analyzed the effect of CD28 T195P mutation on NF-κB signaling (Figure 2E). Expression of CD28 T195P mutant in Jurkat cells enhanced luciferase activity about 20% compared to wild-type CD28 after stimulation with PMA/ionomycin. We also examined the effect of mutation on NF-κB activation after stimulation by anti-CD3 and CD28 antibodies and found that activation was also obtained with antibody treatment albeit at slightly lower levels (data not shown). This result indicates that the T195P mutant enhances NF-κB signaling.

In sum, we have isolated a recurrent mutation of CD28 and explored mechanistic implications in T cell lymphoma development. The CD28 T195P mutation was unique to AITL and led to up-regulation of TCR signaling. Clearly, further detailed mechanistic analyses are required to fully decipher the significance. Still, our preliminary functional
characterization of CD28 T195P mutation represents an important step toward understanding the molecular mechanism underlying AITL carcinogenesis. T195P mutation in CD28 gene is oncogenic in nature and thus likely will be a genetic marker for AITL and a potential target for cancer immunotherapies.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by grants from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI14C3414 and HI14C2331).
REFERENCES


Figure Legends

Figure 1. Mutation of T195P on CD28. (A) Representative sequencing traces of wild-type and T195P mutant CD28. Threonine (ACT) was changed to proline (CCT) at the 195th amino acid of CD28. Top panel shows sequence from wild type genomic DNA. Bottom panel shows the sequence from an AITL sample. Arrows and red letters note the location of the base change. The mutant sequence trace shows heterozygosity. (B) Mutation position and functional domains of the CD28 protein. PI3-K (p110δ/p85), GRB2 and GRAP2 bind to phosphorylated YMNM motif in cytoplasmic region of CD28. The red letter denotes the mutated threonine. TM, transmembrane domain. (C) The genomic DNA of CD28 from lymphoma patients was analyzed by PCR amplification and Sanger sequencing. The mutation of CD28 T195P was detected in 5 (10.2%) of 49 AITL patients, in 0 (0%) of 39 PTCL patients and 0 (0%) of 37 NK/T patients.

Figure 2. Effect of the T195P mutation on CD28 activity. (A) GST beads containing GST, GST-CD28-CD (cytoplasmic domain), and GST-CD28 T195P-CD were incubated with Jurkat cell lysate. For tyrosine phosphorylation of CD28 cytoplasmic domain, GST-fusion proteins were expressed in E.coli strain expressing tyrosine kinase (see Methods). Bound proteins were retrieved and examined by immunoblotting with anti-PI3-K (p85/p110δ), anti-GRB2 and anti-GRAP2 antibodies. GST, GST-CD28-CD, and GST-CD28 T195P-CD on immunoblots were stained with Coomassie Brilliant Blue (CBB). (B) Jurkat cells expressing CD28 T195P mutant enhances cell proliferation (P < 0.001 compared with cells expressing wild-type CD28) with or without costimulation by anti CD3/CD28 beads. Each condition with five replicates was repeated three times, and data are expressed as mean ± standard deviation. OD, optical density; EV, empty vector. (C) Expression of CD28 T195P enhanced
phosphorylation of AKT, mTOR, and ERK without stimulation. (D) Expression of CD28 T195P mutant enhances interleukin 2 (IL-2) production after costimulation with anti-CD3/CD28 beads (P < 0.001 compared with cells expressing wild-type CD28). IL-2 production was measured three times, and data are expressed as mean ± standard deviation. (E) Expression of CD28 T195P mutant activates NF-κB pathway with or without PMA/ionomycin (P/I) stimulation (P < 0.001 compared with cells expressing wild-type CD28). Luciferase activity was measured three times, and data are expressed as mean ± standard deviation.
A) Ch2: 204599555 A to C

Wild type CD28

5’ AAC ATG ACT CCC CGC 3’
N M A

Mutant CD28 T195P

5’ AAC ATG CCT CCC CGC 3’
N M C

B) CD28

Extracellular region
TM
Cytoplasmic domain
1 18 153 179 202

CD28

P110
P85

GRB2
GRAP2

R S-S RSKRSSLHSDY MNMT PPRRPGPTKHYQP YAPP RDF AAYRS

C) Lymphoma subtype Mutation frequency

<table>
<thead>
<tr>
<th>Lymphoma subtype</th>
<th>Mutation frequency</th>
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<tbody>
<tr>
<td>AITL</td>
<td>5/49 (10.2%)</td>
</tr>
<tr>
<td>PTCL</td>
<td>0/39 (0%)</td>
</tr>
<tr>
<td>NK/T</td>
<td>0/37 (0%)</td>
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