RLTPR Q575E: A novel recurrent gain-of-function mutation in patients with adult T-cell leukemia/lymphoma

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Abstract

Objectives: Adult T-cell leukemia/lymphoma (ATL) is an intractable T-cell malignancy caused by long-term infection with human T-cell leukemia virus type-1 (HTLV-1). While ATL pathogenesis has been associated with HTLV-1-derived oncogenic proteins, including Tax and HBZ, the contribution of genomic aberrations remains poorly defined.

Methods: To elucidate the genomic basis of ATL, whole exome sequencing was performed on cells from 47 patients with aggressive ATL.

Results: We discovered the novel mutation *RLTPR* Q575E in four patients (8.5 %) with a median variant allele frequency of 0.52 (range 0.11–0.68). Despite being reported in cutaneous T-cell lymphoma, three ATL patients carrying *RLTPR* Q575E lacked skin involvement. Patients carrying *RLTPR* Q575E also harbored *CARD11* (75 %), *PLCG1* (25 %), *PRKCB* (25 %), or *IKBKB* (25 %) mutations related to TCR/NF-κB signaling. Jurkat cells transfected with *RLTPR* Q575E cDNA displayed increased NF-κB activity, and significantly increased IL-2 mRNA levels under stimulation. RLTPR Q575E increased the interaction between RLTPR and CARD11, while RLTPR directly interacted with Tax.

Conclusions: We identified, and functionally validated, a novel gain-of-function mutation in patients with aggressive ATL. During TCR activation by Tax or gain-of-function mutations, *RLTPR* Q575E selectively upregulates NF-κB signaling and may exert oncogenic effects on ATL pathogenesis.

Keywords: adult T-cell leukemia/lymphoma; gain-of-function mutation; NF-κB signaling; *RLTPR* Q575E; T-cell receptor signaling.

1. INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) is a highly intractable hematological malignancy caused by long-term human T-cell leukemia virus type-1 (HTLV-1) infection [1]. Tax is an HTLV-1 derived oncoprotein that exerts oncogenic effects by activating the NF-κB pathway [2]. However, although more than half of all ATL patients lack functional Tax protein expression, due to *Tax* deletion or methylation [3], NF-κB remains activated. Recent comprehensive genomic analysis has revealed that ATL is characterized by frequent gain-of-function alterations in T-cell receptor (TCR) signaling-related genes which constitutively activate NF-κB, most of which are also known to interact with Tax [4]. Thus, gain-of-function mutations in TCR pathway-related genes may maintain oncogenic function in ATL, with or without Tax expression.

RLTPR is a lymphocyte-specific actin-uncapping protein that is essential for TCR co-stimulation via CD28 and the development of regulatory T cells [5]. Specifically, RLTPR acts as a scaffold protein that bridges CD28 and CARD11 to the NF-κB signaling pathway [6], while its deletion prevents Th1 and Th17 $CD4^+$ T-cell differentiation. A previous comprehensive genomic analysis of 81 ATL samples (38

acute, 12 lymphomatous, 26 chronic, and 5 smoldering) identified 88 candidate driver genes, which were validated by targeted sequencing in over 400 ATL samples. Of these genes, 50 were significant and 13 were affected in more than 10 % of ATL cases. Lawrence *et al.* reported that large-scale genomic analyses can identify nearly all known cancer genes and that near-saturation may be achieved with 600–5,000 samples per tumor type [7]. Therefore, we collected mutational data from ATL patients to identify novel driver mutations and elucidate ATL pathogenesis in more detail to promote the development of novel molecular-targeted drugs.

Herein, we performed whole exome sequencing (WES) on samples from 47 patients with aggressive ATL and found a similar mutation profile to that reported previously. We also identified a novel recurrent gain-of-function mutation in the TCR co-stimulatory pathway, *RLTPR* Q575E, previously reported in cutaneous T-cell lymphoma (CTCL) [8]. We then characterized the clinical manifestations of ATL with *RLTPR* Q575E and assessed its effects *in vitro* on other genes in the TCR signaling pathway and Tax.

2. MATERIALS AND METHODS

2.1 Patients and samples

Written informed consent was obtained from the patients or their families according to the Declaration of Helsinki and with approval from the Ethics Committee for Life Sciences and Genetic Analysis of Kagoshima University Graduate School of Medical and Dental Sciences. Genomic analysis was performed on peripheral blood-derived genomic DNA from ATL patients. The analyzed ATL cells were enriched by > 70 % using CD4/CD25 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) if

there were < 20 % white blood cell counts at collection. Jurkat cells, 293T cells, HeLa cells (ATCC, Manassas, VA, USA), HTLV-1 infected cell-derived cell line MT2[9], and the ATL patient-derived Tax-non-expressing cell line Su9T01[10] were used for *in vitro* assays.

2.2 Sequencing

WES was performed on samples from patients with ATL using libraries designed and constructed with Ion AmpliSeq™ Exome technology (Thermo Fisher Scientific, Waltham, MA, USA). Generated amplicons were genotyped using an Ion Proton™ platform (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequences were aligned against the reference genome (GRCh37/hg19) using Torrent Mapping Alignment Program Alignment (Thermo Fisher Scientific). The variants obtained from WES genotyping of RLTPR were confirmed by Sanger sequencing. Candidate mutations with a sequencing depth > 40 and a variant allele frequency > 0.10 were filtered further to exclude synonymous single nucleotide variants, known variants listed in the 1000 Genomes Project (allele frequency > 0.01), and known Japanese variants listed in the 1000 Genomes Project (allele frequency > 0.01) and ToGoVAR (https://togovar.biosciencedbc.jp).

2.3 RLTPR expression plasmids and retroviral vector constructs

RLTPR (CARMIL2, NM 001013838) isoform 1 cDNA (#RC217662) was obtained from OriGene (Rockville, MD, USA) and subcloned into pcDNA3.1 (Thermo Fisher Scientific). A 3xFLAG tag was introduced at the N-terminus of RLTPR cDNA using an In-Fusion HD cloning kit (Takara Bio, Mountain View, CA, USA). RLTPR isoform 3 [8],

was constructed by removing part of exon 14 with an In-Fusion HD cloning kit and the following primers: RLTPR-⊿ex14-F, 5′-TGG ACA CTC TCT TCG CAG CGG TAT CCC G-3′ and RLTPR-⊿ex14-R, 5′-CGA AGA GAG TGT CCA GGG CAG TGT CGG-3′. The RLTPR-Q575E mutation was introduced into pcDNA3.1-RLTPR-wild-type (WT)-isoform 1/3 by site-directed mutagenesis using an In-Fusion HD cloning kit to produce pcDNA3.1-RLTPR-Q575E-isoform 1/3.

Retroviral vectors expressing human RLTPR-WT-isoform 1/3 and RLTPR-Q575E-isoform 1/3 were constructed by substituting GFP with RLTPR-WT-isoform 1/3 or RLTPR-Q575E-isoform 1/3 cDNA in pMX-IRES-GFP plasmid [11]. Jurkat cells, or Su9T01 cells were retrovirally transduced with RLPTR-WT-isoform 1/3 or RLTPR-Q575E-isoform 1/3 as described previously [11]. pHR'EF-cTax-WPRE-SIN was used as a Tax cDNA expression plasmid by replacing a-galA cDNA with Tax cDNA [12].

2.4 Immunoblotting and immunoprecipitation

Anti-RLTPR antibody (ab122717) was purchased from Abcam (Cambridge, UK). CARD11 rabbit antibody (4440), NF-κB p65 (C22B4) rabbit mAbs, phospho-NF-κB p65 (Ser468) rabbit polyclonal antibody, GAPDH (D16H11) XP(R) rabbit mAbs, and anti-rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Technologies (CST, Danvers, MA, USA). Mouse TrueBlot® ULTRA: anti-mouse Ig HRP (18-8817-31) and rabbit TrueBlot®: anti-rabbit IgG HRP (18-8816-31) were purchased from Rockland Immunochemicals (Limerick, Ireland). Anti-Tax monoclonal antibody used was clone Lt-4 [13]. Anti-FLAG M2 mouse mAbs (F1804; Sigma-Aldrich, St. Louis, MI, USA) and Protein G Magnetic Beads (70024S; CST) or Protein G Mag

Sepharose (GE Healthcare Life Sciences, Little Chalfont, UK) were used for immunoprecipitation. FLAG-tagged RLTPR-WT-isoform 1/3 or RLTPR-Q575E-isoform 1/3-transduced Jurkat cells (1 x 10^7 cells) were cultured and lysed with lysis buffer (0.1 % NP-40, 150 mM NaCl, 10 % glycerol, 0.05 M Tris pH 8.0). Tax and RLTPR-WT-isoform 1/3 or RLTPR-Q575E-isoform 1/3-transfected 293T cells were cultured (1 x 10^6 cells) and lysed with lysis buffer (0.05 % NP-40, 120 mM NaCl, 10 % glycerol, 500mM Tris-HCl pH 8.0). Cellar debris was removed by centrifugation at 14,000 × *g* for 10 min. Protein from cell lysates were mixed with 2 μg anti-FLAG antibodies and 8 μL Protein G Magnetic Beads or 6 μL Protein G Mag Sepharose per 300 μL volume cell lysates, respectively. The immune complex was washed three times with lysis buffer.

2.5 Flow cytometric analysis

Primary ATL cell surface and intracellular molecules were detected by flow cytometry using anti-CD4, CD7, CD8, CD25, CCR4, FOXP3, and CD45RA antibodies.

2.6 Luciferase assay

RLTPR-WT-isoform 1/3, RLTPR-Q575E-isoform 1/3-transduced Jurkat cells (3 x 10^5), or Su9T01 cells (2.5 x 10^5) were transfected with firefly luciferase vector pGL4.30[luc2P/NFAT-RE/Hygro], pGL4.32[luc2P/NF-κB-RE/Hygro], or pGL4.44 [luc2P/AP1 RE/Hygro] (Promega, Madison, WI, USA) to measure NF-κB, AP-1, or NFAT promoter activity respectively. Cells were transfected using Lipofectamine LTX Plus Reagent (Thermo Fisher Scientific) with 300 ng/well of each reporter plasmid or the empty expression vector as a control. After 20 h, cells were stimulated with 50

ng/mL of Phorbol 12-myristate 13-acetate (PMA)/300 ng/mL of ionomycin with or without 1μg/ml of CD86/Fc chimera (R&D systems, Minneapolis, MN, USA) for 6 h. NF-κB, AP-1, or NFAT promoter activity were measured by luciferase activity 26 h post-transfection using a Dual-Luciferase® Reporter Assay System (Promega). The pGL4.74 [*hRluc*/TK] (Progema) vector containing the *hRluc* luciferase reporter gene under an HSV-TK promoter was used as a control to normalize firefly luciferase activity. Luciferase activity was assayed using a TriStar LB941 (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). Results represent the average relative luciferase activity of five independent experiments.

2.7 Quantitative Reverse Transcription PCR (qRT-PCR)

IL2 mRNA (Hs00174114) was quantitated using Taqman Gene Expression Assays (Thermo Fisher Scientific). The house-keeping gene beta-actin (ACTB) (Hs01060665) was used as a control. Each experiment was performed at least three times. To identify RLTPR isotype 3, pre-made primer and Taqman probe sets were purchased from Applied Biosystems to detect the exon 14 splicing variant (forward primer, 5′-CCT GAG CCG TCC TAA CGT ACT G-3′; probe, 5′-TGG ACA CTC TCT TCG CA-3′; reverse primer, 5′-AGC GTC GAG GTG GGT AAG G-3′). Primers and Taqman probes to detect the exon 19/20 boundary (Hs01009136) were used as control.

2.8 Statistical analysis

Student's *t* tests were performed using the Excel statistical software package. *P* values of < 0.05 were considered significant: $*P < 0.05$; $**P < 0.01$.

3. RESULTS

3.1 Genome sequencing analysis of aggressive ATL samples

The demographics of the 47 patients with aggressive ATL included in this study are summarized in Table 1. There were 17 male and 30 female patients, with a median age of 59.5 (range 27–77) and the following ATL prognostic index (ATL-PI): low, 17; intermediate, 20; high, 7; not available, 3[14]. WES was performed on samples from all 47 patients, looking for 224 mutations reported in ATL [4,15] and other T-cell lymphomas, including peripheral T-cell lymphoma not otherwise specified [16], CTCL [8], Sezary syndrome [17], Mycosis fungoides [18], angioimmunoblastic T-cell lymphoma [19], ALK-negative anaplastic large cell lymphoma [20], and natural killer/T-cell lymphoma [21]. Mutations detected in more than 2 % of samples are shown in Table 2, revealing a similar mutational profile to that reported previously [4]. Possible ATL driver mutations beyond those reported previously were extracted using a SHIFT value of < 0.05 and PolyPhen value of > 0.908 in ToGoVAR. One putative driver gene (*RLTPR*) was identified as a recurrent mutation not previously reported in ATL (Table 2).

3.2 Identification of recurrent RLTPR mutation in ATL

RLTPR encodes a scaffolding protein that has been implicated in TCR co-stimulation via CD28 [5,6] and the *RLTPR* Q575E mutation was recently detected in 3.1 % of CTCL cases [8]. In our cohort, four of the 47 (8.5 %) patients with acute ATL harbored *RLTPR* Q575E, as confirmed by Sanger sequencing, whose clinical and genetic characteristics are summarized in Table 3. All four patients harbored co-mutations in *CARD11, TP53, IKBKB, HLA-B,* or *PLCG1*; however, only one had skin involvement, unlike CTCL patients. Further analysis of ATL cells with the *RLTPR* Q575E mutation revealed a

typical $CD4^+/CD25^+/CCR4^+/CD45RA^-/FOXP3^{bright}$ positive ATL surface phenotype, corresponding to the phenotype of effector Treg cells (Table 3).

3.3 In vitro function of RLTPR Q575E

Three RLTPR isoforms have been reported and isoform 3, which splices out a portion of exon 14, defined as the most abundant in CD4+ T cells and CTCL [8]. To examine the effects of *RLTPR* Q575E, we used *RLTPR* isoforms 1 and 3, with or without *RLTPR* Q575E, and analyzed the RLTPR isoform profile and protein expression in lysates from primary ATL cells (Fig 1A,B). RLTPR isoform 3 was abundant in primary ATL cells at the mRNA level (Fig 1A) and was expressed in all primary ATL cells ($n = 5$), an HTLV-1-infected cell-derived cell line (MT2), Jurkat cells, and CD4+ peripheral blood mononuclear cells from healthy donors (Fig 1B). Phospho-NF-κB (S468) was also detected in primary ATL cells (Fig 1B); however, *RLTPR* Q575E ($n = 2$) did not correlate with phospho-NF-κB protein expression.

To assess the importance of *RLTPR* Q575E in T-cell activation, we measured NF-κB promoter activity in Jurkat or Su9T01 cells retrovirally transduced with WT or mutant *RLTPR* (isoform 1/3), transfected with luciferase cDNA under a NF-κB responsive element, and cultured with or without PMA/ionomycin stimulation. Without stimulation, Jurkat cells harboring *RLTPR* Q575E displayed no difference in luciferase activity to those transduced with WT *RLTPR* (Fig 2A); however, *RLTPR* Q575E did increase luciferase activity under PMA/ionomycin stimulation (mean increase 9.46-fold; $P < 0.0005$; two-sided *t*-test; Fig 2B), particularly in the presence of the CD28 ligand, CD86 (mean increase 3.52-fold; $P < 0.005$). A similar, but slightly lower, response was also observed in studies with Su9T01 cells, a line derived from ATL patient cells (Fig

2C).

To determine the effect of *RLTPR* Q575E on NF-κB activation, we measured *IL2* mRNA expression by quantitative RT-PCR. *RLTPR* mutation did not affect *IL2* mRNA production in unstimulated Jurkat cells (Fig 2D), but increased IL-2 production by 3.3 fold in response to PMA/ionomycin and CD86 stimulation ($P = 0.0005$; two-sided paired ratio *t*-test; Fig 2E). Since TCR signaling also activates NFAT and AP1 signaling, we assessed whether mutated *RLTPR* affected these pathways using AP1 and NFAT promoter assays. The AP1 promoter was marginally activated by RLTPR isoform 3 regardless of its mutation status (Fig 2F,G), whereas the NFAT promoter was unaffected by either isoform (data not shown).

A recent comprehensive genomic analysis of ATL [4] revealed that mutated genes were enriched in the TCR signaling pathway, including *PLCG1*, *PRKCB,* and *CARD11,* and resulted in NF-κB activation. CARD11 forms a CBM complex with BCL10 and MALT1 to activate NF-κB; therefore, we immunoprecipitated FLAG-tagged RLTPR-WT or RLTPR-pQ575E and immunoblotted for CARD11. We found that the p.Q575E alteration dramatically increased the interaction between RLTPR and CARD11, whereas neither RLTPR-WT-isoform 3 or RLTPR-Q575E-isoform 1 increased this interaction (Fig 3A). The same study also found that many mutated genes had been reported to interact with Tax [4]. To determine whether RLTPR was a component of the Tax interactome, we immunoprecipitated FLAG-tagged RLTPR-WT or RLTPR-p.Q575E and blotted for Tax or immunoprecipitated Tax and blotted for RLTPR, confirming a direct interaction between RLTPR and Tax (Fig 3B).

4. DISCUSSION

In this study, we performed WES on samples from 47 patients with aggressive ATL to investigate the driving gene mutations underlying ATL pathogenesis. Unfortunately, we were only able to identify one novel putative driver gene mutation, possibly due to a lack of paired germ line information and a higher cut off for sequencing depth (> 40) and allele frequency (> 0.10), which may fail to detect mutations with a low variant allele frequency. Previously, Shah *et al.* reported that North American and Japanese ATL have distinct mutational profiles, although only 30 cases were examined [15]. Here, we observed cases with mutations in various TCR/NF-κB pathway genes (*PRKCB*, *CARD11*, *PLCG1*), and JAK/STAT pathway genes (*STAT3*), consistent with the Japanese report, but with fewer mutations in *TP53* or *EP300*, which were predominant in the North American cohort. However, the North American study did not test for CCR4 mutations, which were prevalent in both Japanese ATL and our cohort, and have been proposed as a biomarker for Mogamulizumab treatment [22]. In addition, the North American study did not test for *PRKCB, VAV1, CSNK1A1, CSNK2B*, or *IRF4*, which were detected in more than 10 % of analyzed cases in the Japanese cohort. Thus, the distinct mutational profile observed for the North American ATL cohort may not be truly representative. Another report from North America suggested a high frequency of *FBXW7* mutations (8/32 ATL patients) which were not detected in our cohort. Although we were able to reproduce the findings from previous Japanese ATL WES, possible ethnic differences in mutational profiles must be taken into consideration when developing new treatment strategies for ATL targeting gene mutations.

We identified a novel recurrent *RLTPR* mutation in patients with ATL, which has recently been reported as a lymphoid cell-specific, actin-uncapping protein essential for co-stimulation via CD28 and the development of Treg cells [5,6]. Patients with

loss-of-function *RLTPR* mutations have been shown to present with allergy, infection, and reduced numbers of Tregs and memory $CD4^+$ T cells [23,24]. The majority of ATL cells reportedly express the Treg phenotype and several genes frequently mutated in ATL have been shown to play critical roles in Treg development and maintenance [25,26]. Therefore, we speculate that this *RLPTR* gain-of-function mutation may help to maintain this Treg phenotype in ATL.

RLTPR Q575E was first reported in patients with CTCL[8] where it selectively activated the NF-κB pathway. In this study, we report a recurrent RLTPR Q575E mutation in 8.2 % (4/47) of aggressive ATL cases. Previously, Kataoka *et al.* detected the RLTPR Q575E mutation in an exome analysis; however, this finding was not considered significant as it was only identified in one case[4]. One aim of the present study was to highlight the possibility the possibility that this was an oversight, which we achieved. Indeed, our *in vitro* analysis showed that the mutation increased NF-κB promoter activity, as determined by luciferase promoter assays, and increased *IL-2* mRNA levels, as demonstrated previously [8]. The TCR signaling and co-stimulatory pathway including CD28 ligation not only activates NF-κB, but also AP-1 and NFAT signaling; however, RLTPR Q575E only marginally activated AP-1 and NFAT promoter activities. Since RLTPR is known to link CD28 to CARD11, yet only mutated RLTPR isoform 3 directly interacted with CARD11 without TCR or CD28 stimulation, RLTPR Q575E may solely activate the NF-κB pathway by linking CD28 and CARD11. We speculate that the mutated form of RLTPR stabilizes binding between CARD11 and CD28, thus constitutively activating CD28 co-stimulatory signaling; however, the exact mechanisms must be elucidated further.

Many molecules have been reported in the RLTPR interactome, including CARD11, CD28, VAV1, FYB, and GRB2 [6], none of which are foreign in origin. Similarly, the interactome of the HTLV-1-derived protein, Tax, reportedly contains more than 100 proteins [27]. In this study, we found that RLTPR directly interacts with Tax, yet it remains unclear whether this finding is functionally meaningful *in vitro*. Since Tax was previously reported to be rarely expressed in ATL cells, it is unclear how the mutant RLPTR might act through Tax in ATL cells. However, Mahgoub *et al.* reported that, in a minor fraction of primary ATL cells, Tax is expressed at any given time, and its expression spontaneously switches between on and off states[28]. The precise effect induced of mutant RLTPR on Tax in primary ATL cells requires further investigation.

Indeed, CARD11 mutation or Tax increase NF-κB activation to a much greater degree than the additive or synergistic effects of RLTPR mutation (data not shown). During TCR activation by PMA/Ionomycin, RLTPR Q575E was found to upregulate NF-κB signaling, particularly when the CD28 receptor was engaged by CD86. CD28 fusion/mutations and VAV1 mutations have been the main abnormalities in CD28 TCR co-stimulatory pathway genes reported in patients with ATL. Thus, our findings suggest that both TCR signaling and the CD28 co-stimulatory pathway may be involved in ATL pathogenesis. Since RLTPR Q575E was originally reported CTCL, we investigated whether this mutation increased tumor dermatotropism; however, three of the four ATL cases with the *RLTPR Q575E* mutation showed no cutaneous ATL invasion. As such, further comparative genetic analyses of cutaneous and systemic ATL samples would help to determine which factors promote cutaneous lymphoma.

In conclusion, we identified a novel recurrent gain-of-function mutation, RLTPR Q575E, in patients with ATL. Further comprehensive genomic analysis could identify

unknown mutations to elucidate ATL pathogenesis and identify candidate "druggable" target molecules in the future.

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Authorship

Contribution: All authors contributed substantially to the content of this paper and agreed to submission in its current format. M.Y. and Y.U. designed and performed the study, analyzed data, and wrote the manuscript; N.A., M.Hachiman, and S.K. performed the research; S.K., M.Hayashida, Y.K., D.N., and A.A. collected patient samples and wrote the manuscript; H.H., Y.T., and K.I designed the study, interpreted data, supervised the project, and revised the manuscript.

Conflict of interest

All authors declare that they have no competing financial interests to disclose.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Clinical and laboratory characteristics of patients with ATL

Characteristic	***n* = 47**
Median age (range)	59.5 (27-77)
Sex (male/female)	17/30
Shimoyama classification at analysis	
Acute	43
Lymphoma	1
Chronic with unfavorable prognostic factor	3
Laboratory data	
WBC(/μL), (range), NA = 1	15690 (4120-150550)
Abnormal lymphocytes (%), NA = 3	40.25 (0-85.5)
LDH (IU/L), (range), NA = 3	455 (195-4257)
Soluble IL2 receptor (U/L), (range), *N* = 3	14183 (4115-148452)
ATL-PI	
Low	17
Intermediate	20
High	7
Not evaluable	3
Median observation period (range)	425 days (32-5121)
Median observation period for survivor (range)	1156.5 days (32-5121)
Patients receiving allogeneic hematopoietic stem cell transplantation	16
Alive/Dead/Not evaluable	12/34/1

Table 2. Somatic mutations detected by whole exome sequencing and mutational profiles from previous studies

Gene	This study *n* = 47 (%)	Kataoka *et al.* *n* = 370	Shah *et al.* *n* = 30
CARD11	21.3	24	6.6
PLCG1	19.1	36	0
PRKCB	19.1	33	NT
CCR4	17.0	29	NT
VAV1	17.0	18	NT
STAT3	12.8	21	0
TP53	10.6	18	23.3
POT1	8.5	10	6.6
RLTPR	**8.5**	**NT**	**NT**
CSNK1A1	4.3	5	NT
CSNK2B	4.3	5	NT
EP300	4.3	6	20
GATA3	4.3	15	6.6
HLA-B	4.3	6	NT
IDH2	4.3	1	0
TBL1XR1	4.3	17	13.3
IKBKB	2.1	1	0
IRF2BP2	2.1	8	NT
IRF4	2.1	14	NT
RELA	2.1	1	NT

1 NT, not tested

Table 3. Clinical characteristics of ATL patients with *RLTPR* Q575E mutation

	UPN#1	UPN#2	UPN#3	UPN#4
Age/sex	44/M	55/M	39/F	43/F
Disease type at diagnosis	Acute	Acute	Acute	Acute
ATL-PI	Intermediate	Low	Low	Intermediate
Skin lesions	No	No	No	Yes
Organ infiltration	Brain, lung, PB	PB	PB	Stomach, PB
Other mutations	CARD11, TP53	TP53, IKBKB, HLA-B	PLCG1	CARD11, TP53
Initial chemotherapy	mLSG15	Not available	mLSG15	mLSG15
Initial chemotherapy response	PD	Not evaluable	CR	CR
Allogeneic transplantation	No	No	Yes	Yes
Outcome	Death due to interstitial pneumonia	Death due to ATL	Alive	Death due to allogeneic transplantation-related lung injury
CD4	+	+	+	+

CD7	-	-	-	-
CD8	-	-	-	-
CD25	+	+	+	+
CCR4	+	+	+	+
Foxp3	bright	bright	NA	bright
CD45RA	-	-	-	-

UPN, unique patient number; ATL-PI, ATL prognostic index; PB, peripheral blood; mLSG15, modified LSG15; NA, not available; CR, complete remission; PD, progressive disease.

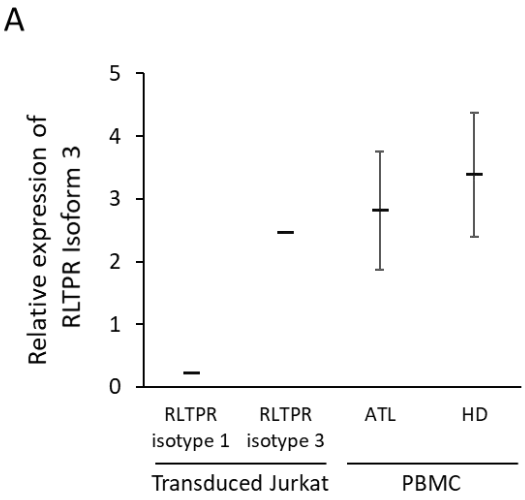
FIGURE LEGENDS

Figure 1. RLTPR isoform 3 expression in primary ATL cells. (**A**) **RLTPR isoform 3 mRNA expression in primary ATL cells.** RLTPR isoform 3-transfected Jurkat cells, or peripheral blood mononuclear cells from healthy volunteers were used as controls. (HD; healthy donor). (**B**) **RLTPR protein expression in primary ATL cells**. HTLV-1 infected cell line (MT-2), Jurkat, 293T, Hela, or peripheral blood mononuclear cells from healthy volunteers were used as controls. CARD11, phospho-NK-kB, and NF-κB were also measured with RLTPR protein expression (UPN; unique patient number). + indicates having RLTPR Q575E mutation.

Figure 2. NF-κB promoter activity, IL2 mRNA expression and AP-1 promoter activity by mutated RLTPR. (A) NF-κB promoter activity in wild-type (WT) or mutant RLTPR cDNA-transduced Jurkat cells without stimulation. NF-κB promoter activity was measured by luciferase reporter assay 20 h after transfection without PMA/ionomycin stimulation (*$P < 0.05$). **(B) NF-κB promoter activity in WT or mutant RLTPR cDNA-transduced Jurkat cells with PMA/ionomycin and CD86/Fc stimulation.** NF-κB promoter activity was measured by luciferase reporter assay 20 h after transfection with PMA/ionomycin ± CD86/Fc stimulation (*$P < 0.05$; **$P < 0.01$)**. (C) NF-κB promoter activity in WT or mutant RLTPR cDNA-transduced Su9T01 cells with PMA/ionomycin and CD86/Fc stimulation.** NF-κB promoter activity was measured by luciferase reporter assay 20 h after transfection, with PMA /ionomycin and CD86/Fc stimulation (*$P < 0.05$)**. (D) IL-2 mRNA levels in WT or mutant RLTPR cDNA-transduced Jurkat cells without stimulation.** IL-2 mRNA levels were measured by quantitative PCR using WT or

mutant RLTPR cDNA-transduced Jurkat cells without PMA/ionomycin stimulation. **(E) IL-2 mRNA levels in WT or mutant RLTPR cDNA-transduced Jurkat cells with stimulation.** IL-2 mRNA levels were measured by quantitative PCR using WT or mutant RLTPR cDNA-transduced Jurkat cells without PMA/ionomycin and CD86/Fc stimulation. **(F) AP-1 promoter activity in WT or mutant RLTPR cDNA-transduced Jurkat cells without stimulation.** AP-1 promoter activity was measured by luciferase reporter assay 20 h after transfection without PMA/ionomycin stimulation (*$P < 0.05$). **(G) AP-1 promoter activity in WT or mutant RLTPR cDNA-transduced Jurkat cells with stimulation.** AP-1 promoter activity was measured by luciferase reporter assay 20 h after transfection with PMA/ionomycin stimulation with CD86/Fc (*$P < 0.05$). (A-G) Data represent mean ± SD in five independent experiments.

Figure 3. RLTPR Q575E directly interacts with CARD11 and Tax. (A) Immunoprecipitation of FLAG-tagged mutated RLTPR with CARD11. RLTPR-WT-isoform 1/3 or RLTPR-Q575E-isoform 1/3 transduced Jurkat cells lysates were mixed and incubated overnight with anti-FLAG antibody and Protein G Magnetic Beads to precipitate RLTPR and CARD11. (B) **Immunoprecipitation of FLAG-tagged RLTPR with Tax.** RLTPR-WT-isoform 1/3 or RLTPR-Q575E-isoform 1/3 transfected 293T cells lysates were mixed and incubated 3 h with anti-FLAG antibody and Protein G Mag Sepharose to precipitate RLTPR and Tax.


A
Relative expression of RLTPR Isoform 3
5
4
3
2
1
0
RLTPR isotype 1
RLTPR isotype 3
ATL
HD
Transduced Jurkat
PBMC

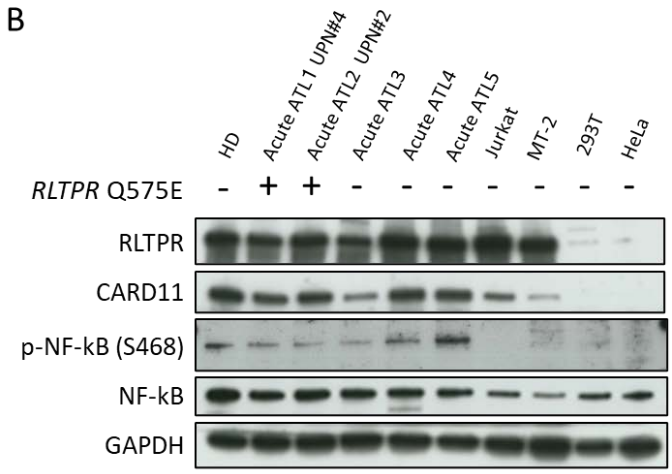
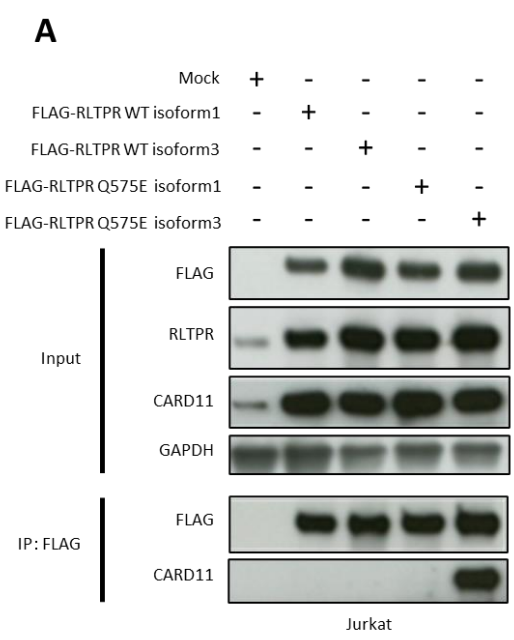
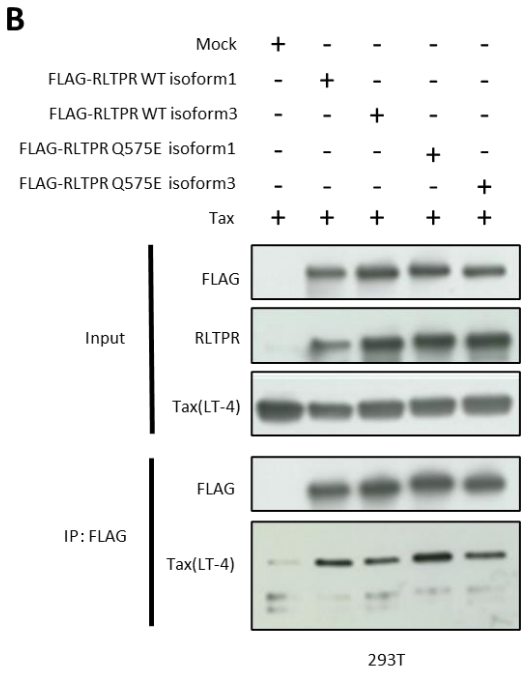

B
HD
Acute ATL 1 UPN#4
Acute ATL2 UPN#2
Acute ATL3
Acute ATL4
Acute ATL5
Jurkat
MT-2
293T
HeLa
RLTPR Q575E - + + - - - - - - -
RLTPR
CARD11
p-NF-kB (S468)
NF-kB
GAPDH

Figure1

A
B
NF-kB luciferase activity
0.1
0.08
0.06
0.04
0.02
0
50
40
30
20
10
0
*
**
Mock
WT isoform1
WT isoform3
Q575E isoform1
Q575E isoform3
Unstimulated
PMA stimulation without CD86
PMA stimulation with CD86
C
D
E
NF-kB luciferase activity
IL-2 mRNA expression
2
1.6
1.2
0.8
0.4
0
10^2
10
1
10^4
10^3
Mock
WT isoform3
Q575E isoform3
PMA stimulation with CD86
Unstimulated
PMA stimulated with CD86
F
G
AP-1 luciferase activity
0.12
0.08
0.04
0
8
6
4
2
0
Unstimulated
PMA stimulation without CD86
PMA stimulation with CD86


A
Mock + - - - -
FLAG-RLTPR WT isoform1 - + - - -
FLAG-RLTPR WT isoform3 - - + - -
FLAG-RLTPR Q575E isoform1 - - - + -
FLAG-RLTPR Q575E isoform3 - - - - +
Input
FLAG
RLTPR
CARD11
GAPDH
IP: FLAG
FLAG
CARD11
Jurkat


B
Mock + - - - -
FLAG-RLTPR WT isoform1 - + - - -
FLAG-RLTPR WT isoform3 - - + - -
FLAG-RLTPR Q575E isoform1 - - - + -
FLAG-RLTPR Q575E isoform3 - - - - +
Tax + + + + +
Input
FLAG
RLTPR
Tax(LT-4)
IP: FLAG
FLAG
Tax(LT-4)
293T