Gamma-secretase inhibitor does not induce cytotoxicity in adult T cell leukemia cell lines despite NOTCH1 overexpression

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Abstract

Background: Activated mutations in Notch homolog 1, translocation-associated (Drosophila; NOTCH1) are driver oncogenes of T-cell type acute lymphoblastic leukemia/lymphoma. The γ-secretase inhibitor (GSI), which suppresses the function of NOTCH1, is expected to be a molecular-targeted agent. NOTCH1 is also expressed in other malignant neoplasms. We aimed to determine the function of NOTCH1 expression and the effects of GSI on adult T-cell leukemia/lymphoma (ATL) caused by long-term human T-cell leukemia virus type I (HTLV-1) infection.

Methods: We analyzed active NOTCH1 in six ATL and HTLV-1-infected cell lines and investigated the influence of activated NOTCH1 together with GSI on cell proliferation.

Results: Activated NOTCH1 found in ATL and HTLV-1-infected cell lines was undetectable after incubation with GSI, regardless of whether the contactin gene Tax, which encodes HTLV-1 protein, was expressed. Whole exome sequencing revealed that activated NOTCH1 mutations were undetectable in six cell lines infected with ATL and HTLV-1 regardless of abundant NOTCH1 expression. Moreover, GSI did not suppress the growth of ATL cell lines.

Conclusions: These findings suggested that NOTCH1 protein is constitutively activated, but is likely a passenger during NOTCH1 mutation negative ATL cell proliferation.

Background

NOTCH1 was discovered by analyzing the DNA flanking the breakpoints of a recurrent t(7;9)(q34;q34.3) chromosomal translocation in < 1% T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) [1]. Over 50% of human T-ALL has activated mutations that involve the extracellular heterodimerization domain (HD) and/or C-terminal glutamic acid, serine, and threonine (PEST) domain of NOTCH1 [2]. These findings have expanded the role of activated NOTCH1 in the molecular pathogenesis of T-ALL and provide justification for targeted therapies that affect with NOTCH1 signaling. NOTCH1 normally undergo proteolytic processing at a site termed S1 into an extracellular N-terminal subunit (NEC) and a transmembrane subunit (NTM). NOTCH ligand binding to the epidermal growth factor-like repeat region of NEC stimulates the metalloprotease cleavage at a second protease-cleavage site termed S2, to create membrane-bound NTM*I monomers. These are subsequently cleaved at several sites within the HD that bind extracellular and transmembrane subunits *via* the γ-secretase (GS) protease complex. This results in the release of intracellular domain 1 (ICN1), which forms a complex that stimulates effector transcription [3]. The ICN1 contains regulators of amino acid metabolism, ankyrin repeat and transcriptional activation domains, and a C-terminal polypeptide domain enriched with proline, glutamate, serine, and threonine (PEST). Ankyrin and transcriptional activation domains are critical for T-ALL induction in mice [4]. Gamma secretase inhibitors (GSIs) might be effective against T-ALL subsets [2].

Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy of mature peripheral T lymphocytes that is acquired after long-term infection with human T-cell leukemia virus type I (HTLV-1). The regulatory

protein Tax encoded by HTLV-1 plays a central role in the early stages of ATL. Molecular targeted approaches, unlike standard cytotoxic chemotherapy treatments, should lead to the eradication of ATL [5]. Kogure et al. found that 15% of ATL has activating mutations in NOTCH1 [6]. Unlike exclusive activated frameshift mutations, those in ATL are single-substitution mutations in the PEST domain [6]. We therefore investigated NOTCH1 protein expression and the effects of GSI in ATL cell lines to determine the function of NOTCH1 in ATL.

Materials And Methods

Cell lines

The human ATL cell lines, S1T and Su9T01, and the HTLV-1-infected T-cell lines, Oh13T, K3T, F6T, and MT-2, were maintained in Gibco RPMI 1640 supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (all from Thermo Fisher Scientific Inc., Waltham, MA, USA). All cell lines except MT-2, were established from patients in our laboratory [7]. We purchased MT-2 cells from the Japanese Cancer Research Resources Bank (JCRB1210; Osaka, Japan). We analyzed Tax protein-positive and-negative cell lines to determine the influence of Tax on NOTCH1 protein expression and function, as well as on cell growth. We also examined NOTCH1 protein expression in clones K3T and F6T that produce Tax, and the S1T and Su9T01 clones that do not. The T-ALL cell lines, JURKAT without a NOTCH1 mutation, SUP-T1, with t(7;9)(q34;q34.3), resulting in aberrant expression of the NOTCH transmembrane subunit (NTM), absence of full-length NOTCH1, and refractoriness to GSI, and HD-Mar, with t(9;14)(q34.3;q11.2) resulting in high GSI sensitivity despite NOTCH1 overexpression [8]. We used the human Burkitt lymphoma cell line Namalwa and peripheral blood lymphocytes (PBLs) from healthy individuals with or without activation. The SUP-T1, HD-Mar, and Namalwa cell lines were obtained from DSMZ (Department of Human and Animal Cell Cultures, Braunschweig, Germany). Activated PBLs were incubated with 10 U/mL of recombinant human IL-2 (Amgen Biologicals, Thousand Oaks, CA, USA) for 6 days at 37°C in 95% humidity under a 5% CO_2 atmosphere. The S1TcTax clones consisted of S1T cells stably transfected with the Tax expression vector pcTax WT that harbors wild-type tax cDNA and the neomycin-resistance gene. Control S1TcNeo cells harbored a plasmid containing the neomycin resistance gene. The S1TcTax05 and S1TcTax10 clones express abundant Tax mRNA [7]. The Ethics Committee and Institutional Review Board of Kagoshima University approved the study, in which healthy persons provided written informed consent to participate.

Protein analyses

Proteins were analyzed by western blotting as follows. Cells (4×10^6) were lysed with 50 µL of RIPA buffer comprising 50 µL 2x SDS buffer, 1 µL aprotinin (1 mg/mL), and 5 µL of phenylmethylsulfonyl fluoride (20 ng/mL). Lysates (20 µL) were loaded onto 6% SDS-PAGE gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) and blotted onto nitrocellulose membranes (Schleicher Schuell, Dassel/Germany) using a semi-dry technique. Loading was checked using Ponceau dye and an anti-human β-

actin antibody (Ab) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). We obtained the following NOTCH1 Abs from the respective suppliers: ANK domain Ab against mN1A (BD Biosciences, San Diego, CA, USA) and TM/RAM domain Ab against Ab8925 (Abcam, Cambridge, UK). The Ab8925 epitopes require prior exposure to GSI according to the manufacturer.

Reagents

N-[N-(3,5-difluorophenyl)-L-alanyl]-S-phenyl-glycine t-butyl ester (DAPT; Peptides International Inc., Louisville, KY, USA) was dissolved in 10 mM dimethyl sulfoxide (DMSO) stock solution.

MTT reduction assays

Cell lines were cultured for 96 h with the indicated concentrations of DAPT or DMSO. Cell viability was then determined using MTT assays (Sigma-Aldrich Corp., St Louis, MO, USA).

Statistical analysis

Data are shown as means ± standard error (SE). Statistical significance was determined using Student t-tests. Values with $p < 0.05$ were considered statistically significant

Sequencing

We created libraries using Ion AmpliSeq^{TM} Exome technology (Thermo Fisher Scientific Inc.) then genotyped amplicons by whole exome sequencing (WES) using an Ion Proton^{TM} platform (Life Technologies Corp., Carlsbad, CA, USA) as described by the manufacturer. Sequences were aligned against a reference genome (GRCh37/hg19) using TMAP (Thermo Fisher Scientific Inc.), then genotyped variants were confirmed by Sanger sequencing.

Results

Expression of NOTCH1 protein is excessive in ATL and HTLV-1-infected T-cell lines

Figure 1 shows western blots of the minimal transforming regions and ankyrin domains in ATL and HTLV-1-infected T cell lines. The control was the JURKAT T-ALL cell line without a NOTCH1 mutation. This cell line expresses moderate amounts of full-length NOTCH1 (~300 kDa) and NTM (~120 kDa) proteins. The ~300 kDa band was absent, and aberrant protein expression undercut the 117 kDa wild-type polypeptide in SUP-T1 and NOTCH1 translocation cell lines, indicating translocation-driven

expression (Figure 1). Except for the SU9T01 cell line, all ATL and HTLV-1-infected cell lines expressed abundant NTM protein and moderate amounts of full-length NOTCH1 protein. In contrast to SUP-T1, aberrant protein expression undercut the 117 kDa wild-type polypeptide that was undetected in all ATL and HTLV-1-infected T cell lines. Neither full-length NOTCH1 nor NTM was detected in Namalwa, a human Burkitt B cell lymphoma cell line. Faint NTM was detected in peripheral blood mononuclear cells. In contrast, activated T cells expressed abundant normal-sized NTM, indicating that NOTCH1 alleles are overexpressed not only in tumors, but also in activated normal cells (Figure 1).

HTLV-1-Tax protein did not affect NOTCH1 protein expression in ATL and HTLV-1- infected T cell lines

We analyzed NOTCH1 protein expression in the Tax-negative S1T cell line and a clone expressing tax mRNA, S1TcTax05, and S1TcTax10 [7]. The control was S1TcNeo cells. Full-length NOTCH1 was slightly overexpressed in S1TcNeo, S1TcTax05, and S1TcTax10 compared with the wild type S1T cell line. In contrast, the expression of full-length NOTCH1 and NTM did not significantly differ among S1TcNeo, S1TcTax05, and S1TcTax10 (Figure 2). Moreover, we analyzed Tax clones of JURKAT cells stably transfected with the expression vector pcTax WT, which contains wild-type Tax cDNA and a neomycin-resistance gene. The HTLV-1-Tax protein did not affect NOTCH1 protein expression in JURKAT, compared with S1T cell lines (data not shown).

Activated NOTCH1 dissipation was affected by GSI

Activated NOTCH1 (ICN1) generated *via* GS-cleavage and labeled using a TM/RAM domain antibody was detected in ATL and HTLV-1-infected T cell lines (Figure 3). Activated NOTCH1 expression was more abundant in the T-ALL cell line HD-Mar with the NOTCH1 rearrangement t(9;14)(q34.3;q11.2) [8], than in ATL and HTLV-1-infected T cell lines. Activated NOTCH1 was undetectable in cells incubated with GSI, regardless of Tax expression. These findings emphasized the dependence of TM/RAM expression on GSI activity in T-ALL, HD-Mar, ATL and HTLV-1-infected T cell lines.

Growth of ATL and HTLV-1-infected T cell lines was not suppressed by GSI

We assessed the effects of the potent GSI, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) on the growth of ATL and HTLV-1-infected T cell lines. After a 96-h incubation, DAPT significantly and dose-dependently suppressed the growth of HD-Mar, but not the ATL and the HTLV-1-infected T cell lines (Figure 4). These findings suggested that NOTCH1 was constitutively activated but probably acted as a passenger in the proliferation of ATL and HTLV-1-infected T cell lines.

Activated NOTCH1 mutations were undetectable in the ATL and HTLV-1-infected cell lines.

Six ATL and HTLV-1-infected cell lines, including ATLs with single-substitution mutations in the PEST domain were assessed by whole exome sequencing (WES) [9]. Activated NOTCH1mutations, especially in the PEST domain, were undetectable in six ATL and HTLV-1-infected cell lines (data not shown). However, NOTCH1 p.Arg879Gln was detected in the Su9T01 translocated cell line with low NOTCH1 protein expression (Figure 1).

Discussion

Gamma secretase inhibitors are useful to screen cell lines for evidence of ongoing NOTCH processing based on the accumulation of GS-cleaved NOTCH1 and its dependence on NOTCH nuclear access for growth and survival. Moderate and abundant expression of full-length NOTCH1 protein and NTM, respectively, was detected in five of six ATL, and HTLV-1-infected cell lines. Although GSI reduced the amount of GS-cleaved NOTCH1, it did not suppress the growth of these cell lines.

Unlike SUP-T1 translocated cells with aberrant NTM expression, the absence of full-length NOTCH1 implied translocation-driven expression [8]. We did not detect aberrant NOTCH1 expression in any of the ATL and HTLV-1-infected cell lines studied (Figure 1). As a driver oncogene mutation, translocation has a more prominent effect than gene amplification on carcinogenesis. To date, NOTCH1 translocation in ATL has not been reported.

Progression might be less dependent on the NOTCH1 signaling pathway in ATL than in T-ALL.

We found that forced expression of the Tax protein did not affect full-length NOTCH1 or NTM expression in ATL cells without Tax expression, or in the S1T cell line (Figure 2). Because Tax plays a central role in the early stages of ATL pathogenesis, we considered that NOTCH1 overexpression is a late-stage event in leukemogenesis in ATL, unlike T-ALL with NOTCH1 alterations.

Pancewicz et el. reported that 30% of ATLs have PEST domain single-substitution mutations and showed that the GSI inhibition of NOTCH1 signaling reduces tumor cell proliferation and tumor formation in mice engrafted with ATL. They concluded that NOTCH1 signaling could result in cellular proliferation in ATL [9]. Although GSI affected the dissipation of the activated form of NOTCH1 (Figure 3), it did not suppress ATL and HTLV-1-infected T cell lines (Figure 4). The reasons for this discrepancy at the point of NOTCH1 signaling dependence on ATL proliferation are as follows. We did not find any mutations that activated the PEST domain in any of the cell lines used in this study (data not shown). We previously described a NOTCH1 rearrangement, t(9;14)(q34.3;q11.2), in HD-MAR and HT-1 T-ALL cell lines, both of which are dependent on the NOTCH1 signaling pathway for proliferation. Gamma secretase inhibitors reduced proliferation. t(9;14) in HD-MAR and HT-1 cells, where NOTCH1 is truncated inside the HD under transcriptional control of the T cell receptor alpha variable [8]. The weaker TM/RAM epitope expression of

the three ATL and HTLV-1-infected T cell lines compared with HD-Mar before incubation with GSI (Figure 3) suggests a protein structural basis, and NOTCH1 signaling is much more important for the progression of T-ALL with t(9;14) than ATL without a PEST domain mutation. Another reason for the discrepancy is that we could not discount the possibility that additional mutations that occurred during culture for several years might have resulted in NOTCH1 signaling becoming dispensable for growth *in vitro*.

We detected NOTCH1 p.Arg879Gln in Su9T01 cells (data not shown) that weakly express the NTM (Figure 1). This variant has a rare missense change located in the epidermal growth factor-like repeat region of NEC which was predicted not to affect protein function. It has been classified as a variant with uncertain significance (https://www.ncbi.nlm.nih.gov/clinvar/). Our data suggested that NOTCH1 p.Arg879Gln could be a loss-of-function mutation, and the entity described herein provides a model cell line for topics of clinical and scientific interest.

Conclusions

Progression might be less dependent on the NOTCH1 signaling pathway in ATL cells than in T-ALL.

Declarations

Statement

all methods were carried out in accordance with relevant guidelines and regulations.

Ethics approval and consent to participate

The Ethics Committee and Institutional Review Board of Kagoshima University approved the study (H22-5), in which healthy persons and patients provided written informed consent to participate.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analysed during the current study are available in the DNA Data Bank of Japan repository, https://ddbj.nig.ac.jp/resource/biosample/SAMD00453566.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

S.S. designed and performed the experiments, analysed the data, and wrote and revised the manuscript. S.H. and Y.U. performed experiments. K.U., M.Y., N.A., S.U. and K.I. analysed the data, and wrote and revised the manuscript.

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Authors' information (optional)

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Figures

Figure 1

NOTCH1 protein expression of ATL and HTLV-1-infected T cell lines.

Western blots of NOTCH1-ANK domain antibody (Ab) to mN1A in ATL and HTLV-1-infected T cell lines. Normal protein expression of full-length NOTCH1 (~300 kDa) and NTM (~120 kDa) was detected in NOTCH1 non-translocated Jurkat cells. High protein expression of NTM (~120 kDa) was found in ATL and HTLV-1-infected T cell lines, regardless of Tax expression except SU9T01. Absence of full-length NOTCH1 bands and aberrant protein expression undercut 117 kDa wild-type polypeptide, implying NOTCH1 translocation-driven expression in SUP-T1. Red band is non-specific.

Figure 2

NOTCH1 protein expression in S1T cells with forced Tax expression.

Western blots of mN1A in S1T cells with forced Tax expression. Control S1TcNeo cells harbor plasmids containing neomycin resistance gene. S1TcTax05 and S1TcTax10 clones expressed abundant Tax mRNA.

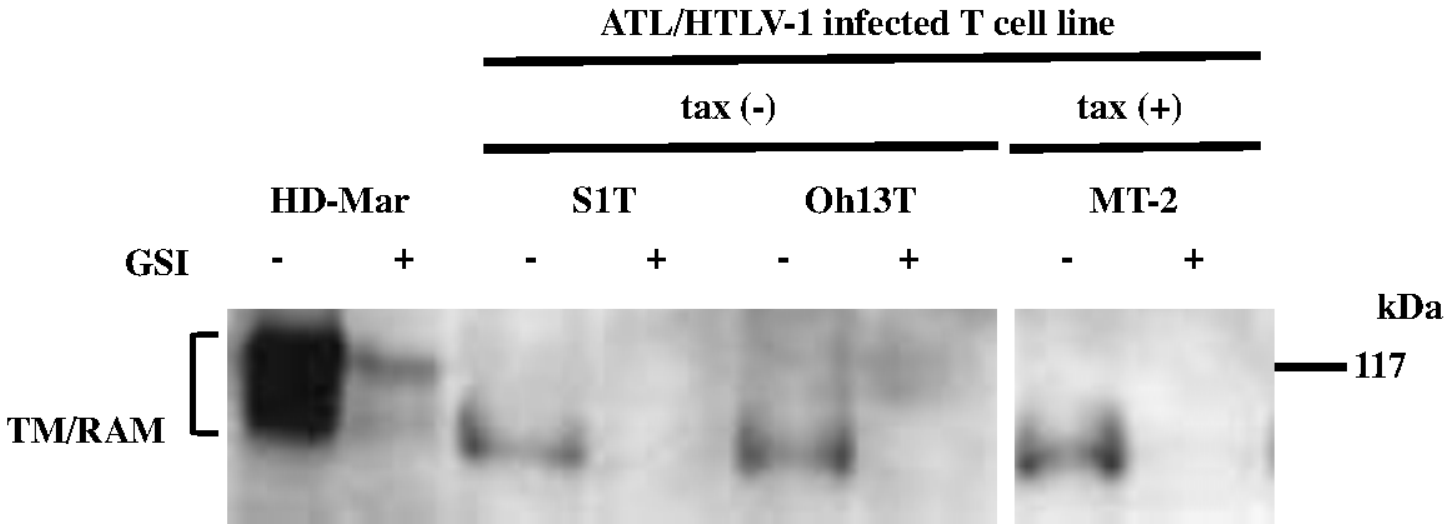

Figure 3

Active form of NOTCH1 following GSI treatment.

NOTCH1 polypeptides were immunoprecipitated from whole-cell extracts using antibodies against GS-cleaved active NOTCH1 (ab8925). TM/RAM species (ICN1) were lost after incubation with GSI. Brackets show aberrant accumulation of TM/RAM species of HD-Mar with NOTCH1 rearrangement.

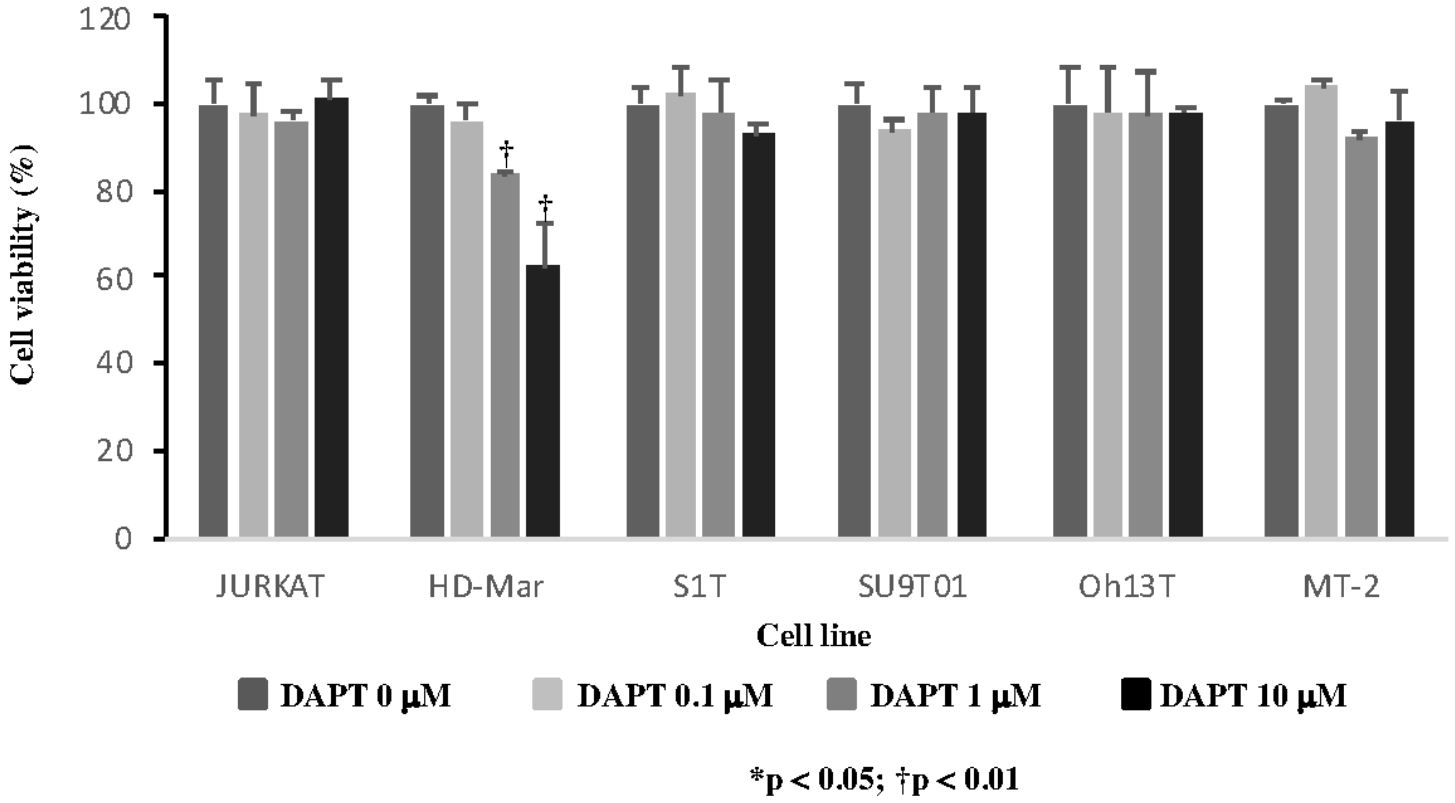

Figure 4

Effects of progression in ATL and HTLV-1-infected T cell lines after incubation with GSI.

T-ALL, ATL and HTLV-1-infected T cell lines were cultured for 96 h with indicated concentrations of DAPT or dimethyl sulfoxide (DMSO).