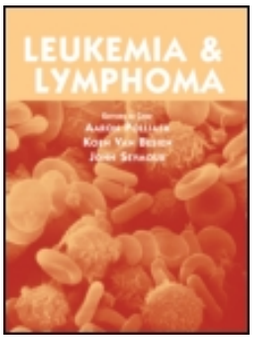

Leukemia & Lymphoma

ISSN: 1042-8194 (Print) 1029-2403 (Online) Journal homepage: https://www.tandfonline.com/loi/ilal20

Complexity of NOTCH1 juxtamembrane insertion mutations in T-cell acute lymphoblastic leukemia

Sara Colomer-Lahiguera & Sabine Strehl

To cite this article: Sara Colomer-Lahiguera & Sabine Strehl (2016) Complexity of NOTCH1 juxtamembrane insertion mutations in T-cell acute lymphoblastic leukemia, Leukemia & Lymphoma, 57:5, 1219-1222, DOI: 10.3109/10428194.2015.1080366

To link to this article: https://doi.org/10.3109/10428194.2015.1080366

LEUKEMIA & LYMPHOMA, 2016
VOL. 57, NO. 5, 1219–1222
http://dx.doi.org/10.3109/10428194.2015.1080366

Taylor & Francis
Taylor & Francis Group

LETTER TO THE EDITOR

Complexity of NOTCH1 juxtamembrane insertion mutations in T-cell acute lymphoblastic leukemia

Sara Colomer-Lahiguera & Sabine Strehl

[1]CCRI, Children's Cancer Research Institute, St. Anna Kinderkrebsforschung e.V., Vienna, Austria

In T-cell acute lymphoblastic leukemia (T-ALL) constitutively active NOTCH1 signaling triggered by activating mutations in the *NOTCH1* gene contributes to the malignant transformation of T-cell precursors and is a hallmark of this disease [1]. NOTCH1 mutations are found in more than 60% of T-ALL and affect critical protein domains responsible for preventing the spontaneous activation of the NOTCH1 receptor in the absence of ligand or for terminating NOTCH1 signaling in the nucleus [2]. The mutation hotspots within NOTCH1 are the heterodimerization domain (HD), the PEST, and the juxtamembrane domains (JMD). More specifically, *NOTCH1* mutations cluster in exons 26–27 and 34, encoding the HD and PEST domains, respectively. While PEST domain mutations result in the loss of the domain and increased intracellular NOTCH1 levels due to its impaired proteasomal degradation, HD mutations induce ligand-independent activation of NOTCH1 [2]. Most HD mutations, comprising single amino acid substitutions and small in-frame deletions and insertions (class 1 mutations), destabilize the HD-LNR (LIN-12/NOTCH1 repeats) interaction and reduce heterodimer stability [3]. In rare cases, insertions of extra amino acids between the distal part of the HD (HD-C) and the S2 cleavage site (class 2 mutations) displace the S2 away from the protective effects of the LNR-HD complex and expose it to cleavage by ADAM-type metalloproteases [2,3]. Moreover, another class of NOTCH1 mutations located in the vicinity of exon 28 encoding the extracellular juxtamembrane domain, termed juxtamembrane expansion mutations (JEMs), which distance the LNR-HD complex from the membrane and allow ligand-independent proteolytic processing of S2, has been identified [4]. Generally, both HD-C class 2 and JME mutations are generated by relatively long insertions resulting from, at least partial, internal tandem duplications (ITDs). However, in case of class 2 HD-C NOTCH1 mutations, aberrant S2 cleavage is induced by the insertion of extra amino acids immediately proximal to the S2 cleavage site, which is displaced closer to the membrane and out of reach of the protective effects of the LNR-HD complex [5]. In contrast, JME insertions are located distal to the S2 cleavage site and displace the LNR-HD complex and the S2 site away from the membrane without altering the primary structure of any of these elements [3,4,6].

While NOTCH1 HD and PEST domain mutations are found in about 40% and 20–25% of T-ALL, respectively, JMD mutations are rather rare and have been detected in roughly 3% of the cases [5,7]. However, since most studies have focused on identifying mutations in the HD and PEST domains and rarely included exon 28, the frequency of JMD mutations may have been underestimated and their complexity has not been explored in detail. Herein we describe five pediatric T-ALL patients with different types of JMD mutations and show, for the first time, their impact on *HES1* expression in primary leukemia samples. Furthermore, we summarize all mutations within this region described so far and discuss how these mutations may affect NOTCH1 signaling.

We screened 39 pediatric T-ALL patients registered in the Austrian ALL-BFM 90, 95 or 2000 clinical trials [8,9] for mutations in *NOTCH1* exons 26–28 and 34 by RT-PCR and direct sequencing of the PCR products as previously described [10]. In three cases (3/39; 7.7%) we detected insertions in exon 28, which were analyzed in detail by sequence analysis of the cloned PCR products [Fig. 1A] and resulted in in-frame insertions of 7 (06-0195), 13 (96-0030) or 14 (04-0573) amino acids in the extracellular juxtamembrane region [Fig. 2; Supplementary Table SI (see http://dx.doi.org/10.3109/10428194.2015.1080366), available online]. Of note, in cases 96-0030 and 06-0195 the mutations led to amino acid substitutions at the insertion sites but only the

Correspondence: Sabine Strehl, CCRI, Children's Cancer Research Institute, St. Anna Kinderkrebsforschung e.V., Zimmermannplatz 10, A-1090, Vienna, Austria. Tel: +43 1 40470 4020. Fax: +43 1 40470 7150. sabine.strehl@ccri.at

Supplemental data for this article are available at http://dx.doi.org/10.3109/10428194.2015.1080366

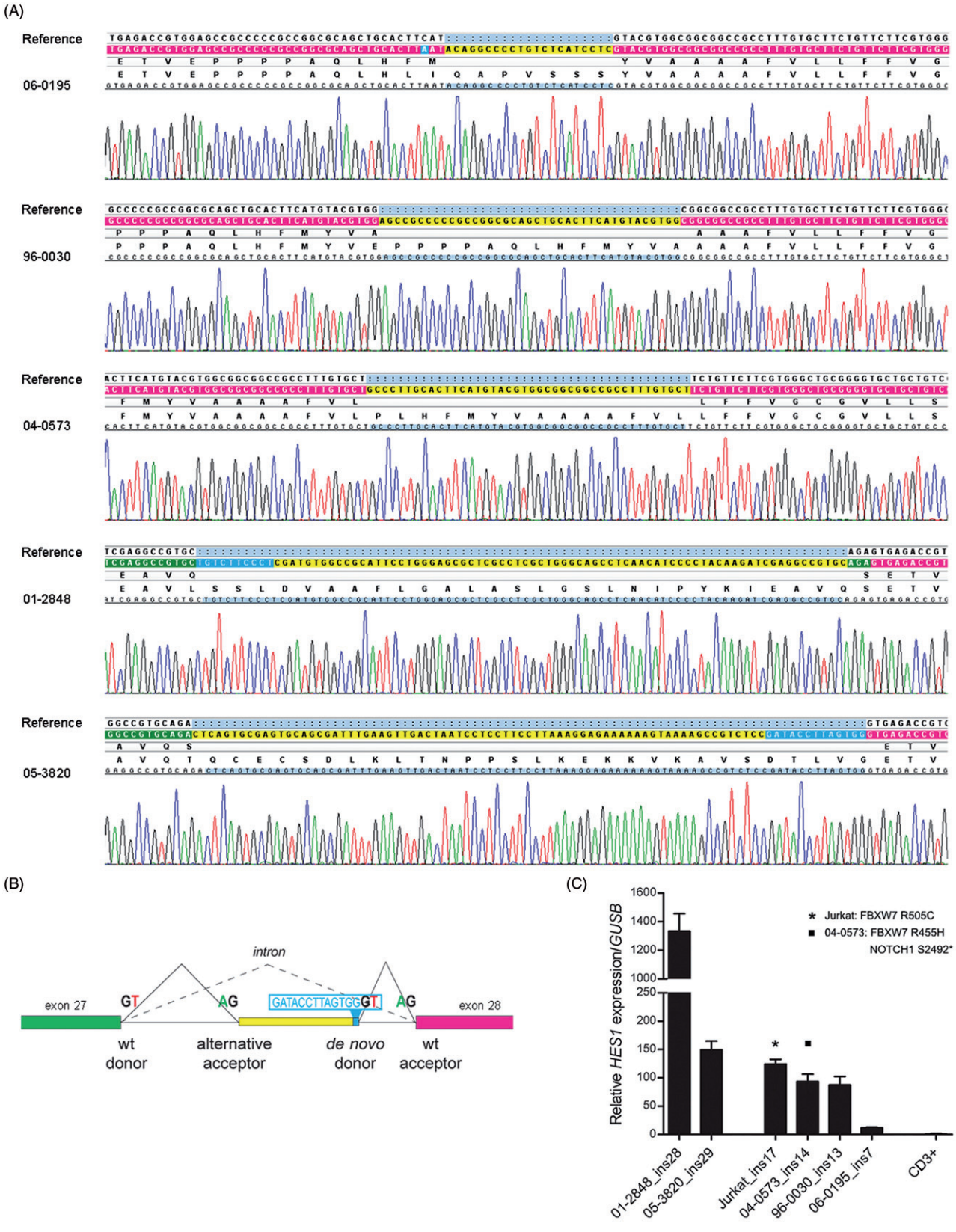

Figure 1. Analysis of NOTCH1 juxtamembrane mutations. (A) Sequence analysis of cloned RT-PCR products showing the duplications and *de novo* insertions marked in yellow and blue, respectively. (B) Schematic representation (not drawn to scale) of the partial intron retention (yellow bar) and the *de novo* insertion (blue sequence) in case 05-3820 creating a new splice donor site and resulting in alternative acceptor splice site usage. wt, wild-type. (C) *HES1* expression in cases with juxtamembrane insertion mutations. Relative expression was determined by RT-qPCR using *GUSB* as reference gene and normalization to pooled CD3+ cells isolated from peripheral blood of 3 healthy individuals. *Of note*: Jurkat and case 04-0573 harbor additional mutations in NOTCH1 and/or FBXW7.

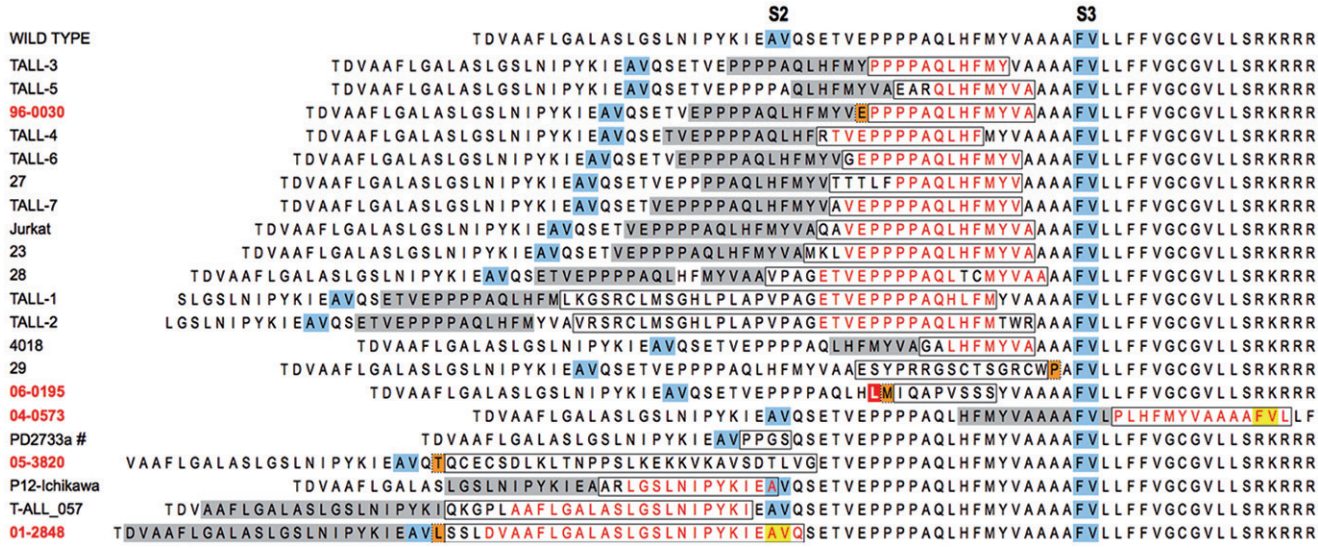

Figure 2. Prediction of the amino acid changes of NOTCH1 juxtamembrane insertion mutations detected in T-ALL cell lines and primary leukemia samples retrieved from the COSMIC database and the cases described herein (marked in bold/red) arranged based on the S3 cleavage site. S2, ADAM-type metalloproteases cleavage site; S3, γ-secretase cleavage site; boxed amino acids, insertions; red, tandem duplicated amino acids (ITDs); gray, duplicated amino acids; light blue, S2 and S3 cleavage sites; yellow, potential *de novo* cleavage sites; orange, amino acid substitutions; red square, missense mutation. [#]*Of note*: Due to differences in the description of the mutation in case PD733a in the original publication and the COSMIC database, we have reanalyzed the sequence and these data are shown [Supplementary Table SI, available online].

actual number of amino acids expanding the protein were counted [Fig. 2]. Remarkably, in case 04-0573 the ITD of 14 amino acids created a potential second S3 γ-secretase cleavage site [Fig. 2]. Furthermore, while in two of the cases (96-0030 and 04-0573) the inserted amino acids included an ITD, in the third case (06-0195) they were completely unrelated to NOTCH1 sequences [Fig. 2]. Additionally, in the latter case a heterozygous point mutation leading to an F1736L amino acid change was detected [Figs 1A, and 2].

To obtain a more comprehensive picture of the amino acid compositions of JME mutations, we examined all cases with such mutations previously reported [4,11–13] and deposited in the COSMIC database (http://cancer.sanger.ac.uk/cosmic). Our analysis revealed an additional patient (case 29) showing a *de novo* insertion rather than an ITD [Fig. 2; Supplementary Table SI]. Consequently, these two cases (06-0195 and 29) lacked the common tetrapeptide QLHF motif present in the JME mutants previously identified [4], and case 4018 displayed only a tripeptide LHF sequence [Fig. 2], supporting the notion that NOTCH1 activation by JME mutants is not dependent on this motif [4].

In addition, we detected two cases (2/39; 5.4%) harboring rare juxtamembrane insertion mutations [Fig. 1A] in the NOTCH1 HD-C domain adjacent to the S2 cleavage site [Fig. 2; Supplementary Table SI]. Similarly to the mutations described above, also these resulted in amino acid substitutions at the insertion sites and also in these cases just the actual numbers of introduced amino acids were considered as expansion. In case 01-2848 the mutation was a 28 amino acids long, almost perfect ITD creating a potential second S2 site and in 05-3820 an insertion of 29 entirely NOTCH1-unrelated amino acids [Fig. 2]. Sequence analysis of the cloned RT-PCR product of the latter case uncovered a partial retention of intron 27 followed by a small *de novo* insertion [Fig. 1A]. Genomic PCR using primers located in the mutation flanking sequences (NOTCH1_27FW 5′-AGGCCGTGCAGAGTAAGTGT-3′ and NOTCH1_28RV 5-CCACGAAGAACAGAAGCACA-3′) followed by direct sequencing of the PCR product confirmed the insertion in intron 27. Notably, *in silico* splice site prediction revealed the presence of an alternative acceptor splice site in intron 27 and the generation of a new donor splice site by the insertion, suggesting that aberrant alternative splicing is involved in the generation of the mutated NOTCH1 protein [Fig. 1B].

Although insertion mutations in this region have already been described [Supplementary Table SI] [2,14,15], it is interesting to note that – except for one case (PD2733a) with a short 4 amino acids long insertion distal to the S2 site – all mutations retrieved from the COSMIC database were located at the N-terminal rather than the C-terminal side of the S2 cleavage site [Fig. 2]. While insertions at the S2 C-terminal side may well displace the NOTCH1 negative regulatory region

and leave the S2 site unprotected, it is tempting to assume that those on its C-terminal side are more similar to JME mutations. However, in case 05-3820 the ITD of the region containing the S2 cleavage site might also displace at least one of the sites away from the protective effects of the LNR-HD complex.

To assess the activation potential of NOTCH1 juxtamembrane mutations, we analyzed the respective primary leukemia samples for *HES1* expression by RT-qPCR using primers HES1_ex3F2 (5′-ACGACACCGGATAAACCAA-3′) and HES1_ex3-4R1 (5′-TGCCGCGAGCTATCTTTCTT-3′). As previously determined by reporter gene assays [4], *HES1* expression levels increased with the number of the inserted amino acids [Fig. 1C]. Although in the two samples with ITDs of 14 (04-0573) and 13 (96-0030) amino acids these were inserted at different positions, namely p.L1746_L1747 and p.V1739_A1740, respectively, they showed similar *HES1* expression levels, suggesting that, independent of their position, these two expansions have a similar activation potential [Fig. 1C]. However, it remains to be determined whether in case 04-0573 the introduction of a second S3 site, the displacement of the S2 site or both are critical for constitutive NOTCH1 signaling levels. Of note, case 04-0573 harbors an additional mutation in the NOTCH1 PEST domain and in both the cell line Jurkat and case 04-0573 the *FBXW7* gene is mutated as well [Fig. 1C], which may confound the actual activation potential of the ITD itself.

In line with the assumption that insertions on the C-terminal side of the S2 site in the NOTCH1 HD-C domain might closely resemble classical JME mutations, case 05-3820 showed a similar *HES1* expression level compared to those with the latter type of mutations [Fig. 1C]. Remarkably, in case 01-2848 harboring the HD-C insertion mutation, creating a second S2 cleavage site, *HES1* expression was extremely elevated [Fig. 1C], indicating that the presence of two such sites may enhance the accessibility to ADAM metalloproteases.

Taken together, insertion expansion mutations in the NOTCH1 juxtamembrane region may be generated by complex mechanisms including tandem duplications, *de novo* insertions and partial intronic retention events in combination with alternative splicing. Further mutation screening in this region and functional analysis will be required to determine the entire spectrum of such mutations and to assign them to specific functional subclasses.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References

[1] Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. J Clin Invest 2012; 122: 3398–3406.

[2] Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004; 306: 269–271.

[3] Malecki MJ, Sanchez-Irizarry C, Mitchell JL, et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. Mol Cell Biol 2006; 26: 4642–4651.

[4] Sulis ML, Williams O, Palomero T, et al. NOTCH1 extracellular juxtamembrane expansion mutations in T-ALL. Blood 2008; 112: 733–740.

[5] Tzoneva G, Ferrando AA. Recent advances on NOTCH signaling in T-ALL. Curr Top Microbiol Immunol 2012; 360: 163–182.

[6] Gordon WR, Roy M, Vardar-Ulu D, et al. Structure of the Notch1-negative regulatory region: implications for normal activation and pathogenic signaling in T-ALL. Blood 2009; 113: 4381–4390.

[7] Jenkinson S, Koo K, Mansour MR, et al. Impact of NOTCH1/FBXW7 mutations on outcome in pediatric T-cell acute lymphoblastic leukemia patients treated on the MRC UKALL 2003 trial. Leukemia 2013; 27: 41–47.

[8] Moricke A, Zimmermann M, Reiter A, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia 2010; 24: 265–284.

[9] Conter V, Bartram CR, Valsecchi MG, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. Blood 2010; 115: 3206–3214.

[10] Strehl S, Nebral K, Konig M, et al. ETV6-NCOA2: a novel fusion gene in acute leukemia associated with coexpression of T-lymphoid and myeloid markers and frequent NOTCH1 mutations. Clin Cancer Res 2008; 14: 977–983.

[11] Zhu YM, Zhao WL, Fu JF, et al. NOTCH1 mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. Clin Cancer Res 2006; 12: 3043–3049.

[12] Asnafi V, Buzyn A, Le Noir S, et al. NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): a Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. Blood 2009; 113: 3918–3924.

[13] Fogelstrand L, Staffas A, Wasslavik C, et al. Prognostic implications of mutations in NOTCH1 and FBXW7 in childhood T-ALL treated according to the NOPHO ALL-1992 and ALL-2000 protocols. Pediatr Blood Cancer 2014; 61: 424–430.

[14] Maser RS, Choudhury B, Campbell PJ, et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature 2007; 447: 966–971.

[15] Breit S, Stanulla M, Flohr T, et al. Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia. Blood 2006; 108: 1151–1157.