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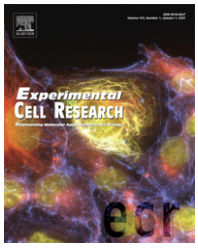

Research Article

Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines

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ABSTRACT

In T-cell acute lymphoblastic leukemia (T-ALL) NOTCH 1 receptors are frequently mutated. This leads to aberrantly high Notch signaling, but how this translates into deregulated cell cycle control and the transformed cell type is poorly understood. In this report, we analyze downstream responses resulting from the high level of NOTCH 1 signaling in T-ALL. Notch activity, measured immediately downstream of the NOTCH 1 receptor, is high, but expression of the canonical downstream Notch response genes *HES 1* and *HEY 2* is low both in primary cells from T-ALL patients and in T-ALL cell lines. This suggests that other immediate Notch downstream genes are activated, and we found that Notch signaling controls the levels of expression of the E3 ubiquitin ligase SKP2 and its target protein p27Kip1. We show that in T-ALL cell lines, recruitment of NOTCH 1 intracellular domain (ICD) to the *SKP2* promoter was accompanied by high SKP2 and low p27Kip1 protein levels. In contrast, pharmacologically blocking Notch signaling reversed this situation and led to loss of NOTCH 1 ICD occupancy of the *SKP2* promoter, decreased SKP2 and increased p27Kip1 expression. T-ALL cells show a rapid G1–S cell cycle transition, while blocked Notch signaling resulted in G0/G1 cell cycle arrest, also observed by transfection of p27Kip1 or, to a smaller extent, a dominant negative SKP2 allele. Collectively, our data suggest that the aberrantly high Notch signaling in T-ALL maintains SKP2 at a high level and reduces p27Kip1, leading to more rapid cell cycle progression.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant disease, originating in thymocytes. T-ALL constitutes a substantial fraction of ALL tumors, both in children and in adults [1]. Current treatment is based on combination chemotherapy, but long-term survival rates are reduced, particularly in older patients, emphasizing the need for improved therapy. The molecular mechanisms underpinning T-ALL are likely to be complex, as a number of genes, including *c-MYC*, *HOX 11*, *TAL1*

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and *LMO*, are involved in chromosomal translocations with the T-cell receptor locus (for review see [1]). Mutations in the Notch signaling pathway have recently emerged as an important genetic component in T-ALL. The involvement of Notch was first observed in rare t(7,9)(q34;q34.3) translocations, which brings an activated form of the NOTCH 1 receptor gene (TAN 1) under control elements from the T-cell receptor gene [2]. More recently, it was shown that more than 50% of all T-ALL patients carry *NOTCH1* gain-of-function mutations that, similar to TAN 1, generate an activated form of Notch [3].

The Notch signaling pathway is important for cell fate decisions in many different cell types, including the T-cell lineage [4]. Notch signaling is initiated when the transmembrane Notch receptor interacts with DSL ligands on juxtaposed cells. This leads to two proteolytic processing events in the receptor, first at the extracellular side and subsequently in the plasma membrane. The latter cleavage is executed by the gamma-secretase complex and leads to liberation of the Notch intracellular domain (Notch ICD), which translocates to the nucleus. In the nucleus, Notch ICD binds to the DNA-binding protein CSL, and converts CSL from a repressor to a transcriptional activator [5].

T-ALL mutations lead to aberrantly high Notch signaling and affect two different regions of the NOTCH 1 receptor [3]. One class of mutations falls in the heterodimerization domain (HD), making the receptor more prone to proteolytic processing and thus less dependent on ligand-activation. The other class of mutations occurs in the PEST domain of NOTCH 1, generating truncated versions of the NOTCH 1 ICD, which increases the stability of the normally short-lived intracellular domain [6]. Further mutational analysis of the PEST domain has revealed four serine residues that serve as a negative regulatory sequence, and deletion of these residues makes the Notch ICD more active [7]. The link between deregulated Notch signaling and T-ALL receives further support from mouse models of T-ALL, where Notch 1 mutations occur as secondary hits in mice engineered for deregulation of *c-Myc*, *TAL1*, *Ikaros* and *Pbx1* expression [8–12].

The downstream response of Notch signaling is only partially understood. There is a wealth of data supporting the notion that *Hes* and *Hey* genes, which encode bHLH negative transcriptional regulators [13,14], are immediate downstream genes, but there are situations where Notch signaling exert biological effects without affecting *Hes* and *Hey* expression [15]. It is therefore likely that other immediate downstream genes exist that are directly activated by Notch ICD via CSL in specific cellular contexts, and several such genes have recently been identified, e.g. *Nrarp*, *GFAP*, *GATA2*, *c-Myc* and $p21^{cip1}$ (see [16] for review).

In T-ALL, our understanding of which downstream genes are activated, and how this affects cell cycle regulation and leads to cellular transformation, is more limited. The finding that *HES 1* activation is not always required for Notch-induced growth promotion [17], may suggest that other downstream responses could be relevant for T-ALL. In keeping with this, *c-MYC* has recently been identified as an important new direct target gene in some T-ALL cell lines [18]. *c-MYC* was, however, not activated in all T-ALL cell lines [18], suggesting the existence of other immediate Notch downstream effectors.

In this report, we have addressed the downstream aspects of Notch signaling in T-ALL. We find that T-ALL tumors and cell lines have low levels of HES1 and HEY2 expression. Instead, increased Notch signaling results in elevated levels of SKP2, the F-box component in the SCF^{Skp2} E3 ubiquitin ligase complex [19]. The high level of SKP2 was paralleled by low levels of the CDK inhibitor (CKI) p27Kip1, a target substrate for ubiquitinylation and thus negatively regulated by the SCF–Skp2 complex [20] (for review see [19]). Blocking Notch signaling pharmacologically reversed the situation, and this "Notch off" state was associated with reduced SKP2 and increased p27Kip1 expression and subsequent G1 cell cycle arrest. In conclusion, our data suggest that a NOTCH/SKP2/p27Kip1 axis may contribute to the development of T-ALL.

Materials and methods

Cell lines, tumor samples and plasmids

Malignant T-ALL and pre B-ALL cell lines were cultured as described [21]. JM-JURKAT, MOLT4, CEM, peer, RS4 and REH6 cell lines were derived from the ATCC collection. Samples of cryopreserved lymphoblasts were collected from children with the diagnosis of T-ALL and B-precursor-cell phenotype, treated at the Karolinska Hospital, Stockholm, Sweden, and leukemic cells were purified as previously described [22]. Approval was obtained from the Institutional Review Board for these studies. Normal T- and B-cells were obtained from buffy coats of healthy blood donors and prepared by PHA and IL-2 and grown as previously described [22].Human embryonic kidney (HEK) 293T is a highly transfectable derivative of the 293 cell line and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin. CMV-β-gal, 12×CSL–EGFP and 12×CSL–luciferase (6×CBF–1-luc) plasmids have been previously described [23–25]. The dominant negative SKP2 plasmid (pcDNA3 MT-Skp2deltaF30) and the p27Kip1 plasmid (pCAG-p27Kip1), kind gifts from Drs. Wilhelm Krek and Jonas Muhr, respectively, have also been previously described [20].

RNA preparation and real-time PCR

Total RNA from cells and tumors was prepared with the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 2 μg of total RNA was reverse transcribed using Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Applied Biosystems). The mastermix (SYBR Green, nucleotides, *Taq* polymerase and buffer, as described by the manufacturer Applied Biosystems) was mixed with 0.2 μl of primers (100 μM) and 1.2 μl of cDNA in total volume of 25 μl. Real-time amplification was performed with initial denaturation at 95 °C for 10 min, followed by 40 cycles of two-step amplification (95 °C for 15 s, 60 °C for 1 min). Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Applied Biosystems) and analyzed on the ABI Prism 7500 (Applied Biosystems). All reactions were performed in triplicate. For the SYBR Green system, the following probes were used: SKP2 (forward; 5′-TCAACTACCTCCAACACC-TATCAC-3′, reverse; 5′-GACAACTGGGCTTTTGCAGT-3′), HES1 (forward; 5′-CAACACGACACCGGATAAAC-3′, reverse; 5′-TTTTCTCCATAATAGGCTTTG-3′), HEY1 (forward; 5′-GAAAAAGCCGAGATC-3′, reverse; 5′-TAACCTTTCCCTCCT-3′), HEY2 (forward; 5′-AGATGCTTCAGGCAACAGGG-3′, reverse; 5′-