NOTCH1 Signaling as a Therapeutic Target in Sézary Syndrome

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NOTCH signaling is important for development and tissue homeostasis and is activated in many human cancers. We investigated a role for NOTCH1 signaling in Sézary syndrome (SS), a cutaneous T-cell lymphoma in which CD4+ tumor cells (Sézary cells) are present in the skin, lymph nodes, and peripheral blood. We show consistent expression of activated NOTCH1 by Sézary cells isolated from peripheral blood of SS patients, as well as the SS-derived cell lines SeAx and HuT78. In addition, immunohistochemical stainings of skin biopsies from SS patients showed consistent expression of nuclear NOTCH1 and its downstream target hairy/enhancer of split-1 (HES1) by Sézary cells. We demonstrate that this persistent NOTCH1 activation is not caused by mutations in the coding regions of *NOTCH1* and *F-box and WD40 domain protein 7* (FBWX7) genes. Inhibition of NOTCH1 signaling by gamma secretase inhibitors decreased cellular viability and induced apoptosis of Sézary cells. These observations argue that NOTCH1 signaling is functionally involved in the pathogenesis of SS, and inhibition of NOTCH1 signaling represents a new therapeutic target for the treatment of SS.

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INTRODUCTION

Sézary syndrome (SS) is an aggressive lymphoma/leukemia of skin-homing, CD4+ memory T cells characterized by the presence of neoplastic T cells (SS cells) in the skin, lymph nodes, and peripheral blood. Although SS can be treated with low-dose prednisone and chlorambucil or immunomodulating therapies (extracorporeal photophoresis, IFN-α), the prognosis is poor, with a median survival between 2 and 4 years after diagnosis and a disease-specific 5-year survival around 20%. New treatments are being developed for SS, including histone deacetylase inhibitor and allogenic stem cell transplantation. However, these treatments are associated with significant acute and long-term toxicities, and therefore identification of new therapeutic targets remains an urgent medical need (Olsen *et al.*, 2011).

Activated NOTCH1 signaling has been implicated in the tumorigenesis of a growing list of hematologic malignancies including T-cell acute lymphoblastic leukemia (T-ALL), Hodgkins disease, anaplastic large-cell lymphoma, and chronic lymphocytic leukemia (Ellisen *et al.* 1991; Jundt *et al.*, 2002; Weng *et al.*, 2004; Asnafi *et al.*, 2009; Rosati *et al.*, 2009; Fabbri *et al.*, 2011). NOTCH proteins are single-pass transmembrane receptors noncovalently joined as heterodimers through a structural motif termed the heterodimerization domain (HD). In humans, four NOTCH homologs are known (NOTCH1-4) and two families of NOTCH ligands have been identified, the Jagged/Serate-like ligands (Jagged1 and Jagged2) and the Delta-like ligands (DLL1, DLL3, and DLL4). On ligand binding, NOTCH1 undergoes multiple proteolytic cleavages that will allow the intracellular domain of NOTCH (icNOTCH) to translocate to the nucleus. Nuclear icNOTCH associates with the DNA-binding factor CBF1-suppressor of hairless-Lag1, leading to recruitment of the scaffolding proteins of the Mastermind-like family and other cofactors, resulting in transactivation of target genes such as the transcriptional repressor hairy/enhancer of split (HES) (Oellers *et al.*, 1994; Heitzler *et al.*, 1996), c-MYC, CD28, and the NOTCH receptors themselves. The F-box and WD40 domain protein 7 (FBXW7) acts as a negative regulator of NOTCH signaling by binding to the phosphatase and tensin homolog (PEST) domain in the C terminus of icNOTCH, thereby marking activated NOTCH for ubiquitinylation and proteosomal degradation (Fryer *et al.*, 2004; Tetzlaff *et al.*, 2004).

A role for the *NOTCH1* gene in human leukemogenesis was originally recognized through identification of chromosomal translocation (7;9)(q34;q34.3) in T-ALL (Ellisen *et al.*

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Abbreviations: FBXW7, F-box and WD40 domain protein 7; GSI, γ-secretase inhibitor; HD, heterodimerization domain; HES, hairy/enhancer of split; icNOTCH, intracellular domain of NOTCH; SS, Sézary syndrome; STAT, signal transducer and activator of transcription; T-ALL, T-cell acute lymphoblastic leukemia

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1991). This translocation results in the juxtaposition of the 3′region of *NOTCH1* into the TCR-β locus and constitutive expression of activated icNOTCH. In addition, over 50% of T-ALL harbor mutations in *NOTCH1*, either in the HD region leading to ligand-independent NOTCH1 activation or in the PEST domain resulting in deletion of the recognition site for proteosomal degradation by the FBXW7/stem cell factor complex (Weng *et al.*, 2004; Asnafi *et al.*, 2009). In addition to these mutations in the *NOTCH1* gene, about 15% of T-ALL contain mutations in *FBXW7* that impair substrate recognition by the FBXW7/stem cell factor complex and impair the degradation of activated icNOTCH1 (O'Neil *et al.*, 2007; Thompson *et al.*, 2007). In 20% of T-ALL cases, the tumor-suppressor phosphatase and tensin homolog (*PTEN*) gene is mutated or lost, which has been suggested to be a critical event leading to resistance to NOTCH inhibition (Palomero *et al.*, 2007; Medyouf *et al.*, 2010).

Recently, expression of NOTCH1 protein by SS cells was described in two of five skin biopsies and one peripheral blood sample from SS patients (Kamstrup *et al.*, 2010). These observations prompted us to investigate the expression of NOTCH1 and hairy/enhancer of split-1 (HES1), evaluate underlying mutations, and explore the potential therapeutic effect of inhibition of NOTCH1 signaling in SS.

RESULTS

Sézary cells express activated NOTCH1 and HES1

We performed immunohistochemical studies on skin biopsies from 12 SS patients (Figure 1) using an antibody specific for the N-terminal sequence of icNOTCH1. In all 12 cases, nuclear localization of icNOTCH1 was detected in 80 to 100% of neoplastic cells, consistent with active NOTCH1 signaling (Figure 1d and e). In addition, intra-epidermal collections of neoplastic cells (Pautrier micro-absesses) stained positive for icNOTCH1 (Figure 1g). In line with the observed expression of activated NOTCH1, we found strong nuclear expression of the downstream NOTCH1 target gene HES1, the prototype human HES gene, in 80 to 100% of neoplastic cells in all SS biopsies (Figure 1f and h). Skin biopsies from psoriasis ($n=4$) and atopic dermatitis ($n=4$) showed weak nuclear staining of infiltrating T cells for icNOTCH1 in 80 to 100% and HES1 in 70 to 100% (Figure 1i and j). Expression of NOTCH1 by keratinocytes in the stratum basale and stratum spinosum served as an internal control (Nickoloff *et al.*, 2002).

Flow cytometric analysis was performed on SS cells isolated from the peripheral blood of seven SS patients using an antibody that specifically recognizes the cleaved intracellular part of NOTCH1, and possesses low affinity for the full-length NOTCH1. In SS cell line SeAx, a sub-population of cells was negative for icNOTCH1, whereas the majority of cells stained positive; however, all HuT78 cells were positive (Figure 2a). In primary SS cells isolated from all patients, consistent increased staining was detected with the icNOTCH1 antibody, when compared with the isotype control (Figure 2b, $P=0.012$, Wilcoxon test). In addition, flow cytometric analysis of CD4+ cells from the peripheral blood from patients with psoriasis ($n=2$) and eczema ($n=4$) was performed. These cells also stain positive for icNOTCH1, which is in agreement with results presented in Figure 1, as well as with recent literature that showed NOTCH1 activation on stimulation of mature T cells (Koyanagi *et al.* 2012). The amount of icNOTCH1 expression, depicted as median fluorescence intensity relative to the isotype control, was not significantly different between patients with SS, psoriasis, and eczema (Figure 2c).

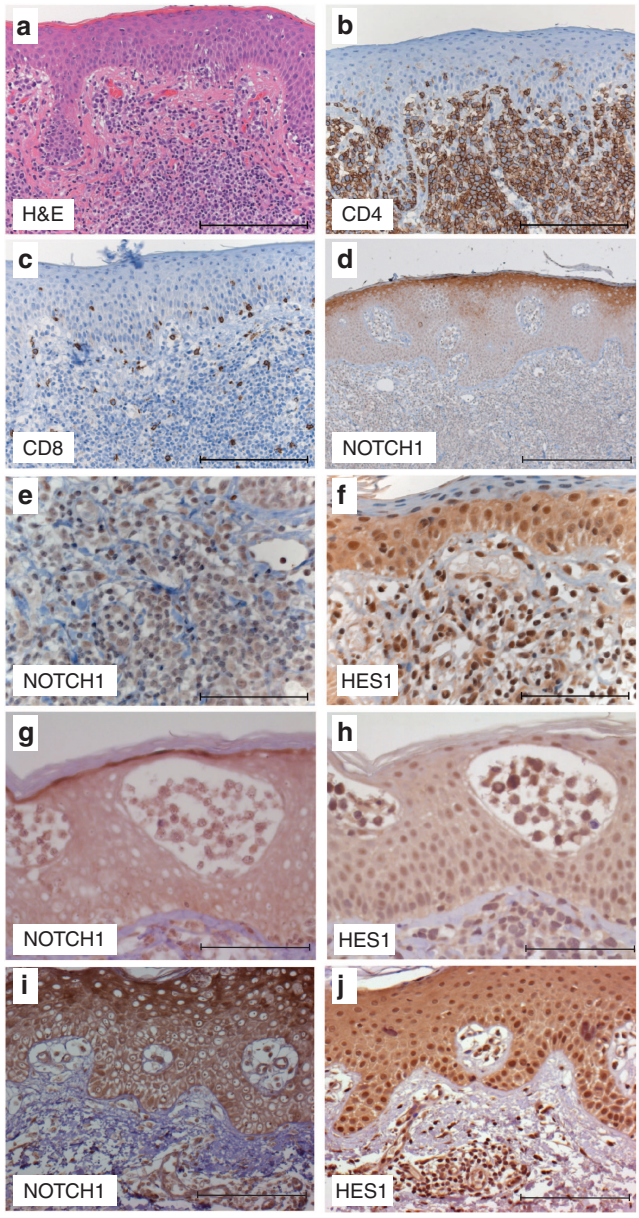

Figure 1. Sézary cells demonstrate nuclear expression of NOTCH1 and hairy/enhancer of split 1 (HES1). Two representative examples of paraffin-embedded skin biopsies from Sézary patients ($n=12$) showing dermal infiltration of large atypical T cells in hematoxylin and eosin (H&E) staining (**a**) that express CD4 (**b**) but are negative for CD8 (**c**). The majority of atypical T cells show nuclear expression of NOTCH1 (**d**, **e**, **g**) and HES1 (**f**, **h**). In addition, infiltrating lymphocytes in psoriatic skin stain positive for NOTCH1 (**i**) and HES1 (**j**). The depicted scale bars represent 200 μm (**a**, **b**, **c**, **i**, **j**), 400 μm (**d**), and 100 μm (**e**, **f**, **g**, **h**).

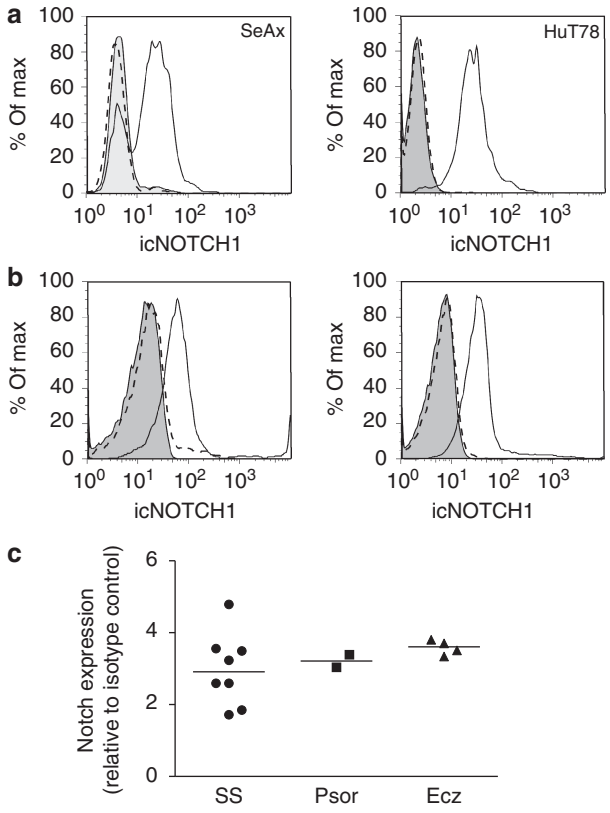

Figure 2. Sézary cells express activated NOTCH1. icNOTCH1 expression was assayed by flow cytometry in Sézary syndrome (SS) cell lines SeAx and HuT78 (**a**), and in CD4+ T cells isolated from peripheral blood of SS patients ($n=7$; two representative examples are shown, **b**). Cells remained unstained (filled graph), or were stained using a phycoerythrin (PE)-conjugated isotype control antibody (dashed line) or a PE-conjugated antibody recognizing the intracellular part of NOTCH1 (solid line). The median fluorescent intensity, relative to the isotype control, was calculated for CD4+ cells from seven SS patients, two psoriasis patients (Psor), and four patients with atopic dermatitis (Ecz) (**c**).

Sézary cells do not contain mutations in *NOTCH1*, *FBXW7*, and *PTEN* genes

Aberrant activation of NOTCH1 in T-ALL has been attributed to mutations in the HD domain and PEST domain of the *NOTCH1* gene, or to mutations in *FBXW7* that impair ubiquitinization and proteosomal degradation of NOTCH1 protein. To assay whether these type of mutations underlie the activated NOTCH1 signaling that is observed in SS, we performed sequence analysis of the HD region (exon 26) and PEST region (exon 34) of the *NOTCH1* gene and the entire coding region of the *FBXW7* gene on genomic and complementary DNA of 10 SS patients. We found no mutations in *FBXW7* and no activating mutation in the HD and PEST region of the *NOTCH1* gene. Mutation analysis of the entire coding region of *PTEN* was performed on genomic complementary DNA of 10 SS patients as well, because it has been reported that mutations in *PTEN* can lead to resistance to NOTCH1 inhibition. However, none of these 10 patients showed any mutation in *PTEN*. Careful examination of previously obtained array-based comparative genome hybridization data available for six of these SS patients (Vermeer *et al.*, 2008) showed amplification of *FBXW7* in one of six patients and heterozygous deletion of *PTEN* in four of six patients (Supplementary Table S1 online). Copy number alterations for the *NOTCH1* gene could not be assayed, as the *NOTCH1*-encoding region was not covered by BAC clones on the array.

GSI-I and GSI-XXI induce apoptosis in Sézary cells

We assayed the effects of inhibition of NOTCH signaling on the survival and apoptosis of SS cells, by treating SS cells with γ-secretase inhibitors (GSI)-I and GSI-XXI. Incubation of SeAx cells with GSI-I resulted in a consistent decrease in icNOTCH1, whereas the effects of GSI-XXI were less consistent (Figure 3a and b).

Treatment of primary SS cells with high doses of GSI-XXI ($\geqslant 5\,\mu M$) for 72 hours resulted in marginally decreased numbers of viable cells in four of six Sézary patients, whereas no decrease in the number of viable SS cells was observed in two patients (Figure 3c), as well as for SeAx and HuT78 cell lines (data not shown).

In contrast, incubation of freshly isolated CD4+ SS cells with GSI-I (0.5–5 μM) for 72 hours led to a dose-dependent decrease in cell viability for all patients tested ($n=3$, $P=0.034$, Mann–Whitney test) (Figure 3b), as well as for SeAx and HuT78 cell lines (data not shown).

We further examined whether this reduced number of viable cells after NOTCH inhibition is due to the induction of apoptosis. Freshly isolated CD4+ T cells from three Sézary patients were incubated with 5 μM GSI-I for 16 to 24 hours, and flow cytometric analysis was performed with Annexin-V and propidium iodide staining. A low percentage of apoptotic cells were detected after culturing in medium alone (1–3%), and this percentage did not change on addition with a volume equivalent of DMSO. However, after incubation with 5 μM GSI-I, the number of apoptotic cells was significantly increased to 5–15%. In addition, the number of dead cells increased on GSI-I induction (Figure 4, $P=0.034$, Mann–Whitney test).

NOTCH1 signaling does not potentiate STAT3 signaling in SS

Previous studies showed that the NOTCH1 target gene HES1 can facilitate JAK2/signal transducer and activator of transcription (STAT)-3 complex formation and promote STAT3 phosphorylation and activation (Kamakura *et al.*, 2004). As a functional role for activated STAT3 signaling in SS has been demonstrated previously (Eriksen *et al.*, 2001; van Kester *et al.*, 2008), we assessed whether cross talk between NOTCH1 and STAT3 signaling is operative in SS. NOTCH1 signaling was activated in freshly isolated SS cells and SeAx cells by incubation with NOTCH1 ligand (JAG1 peptide) for various time points. Incubation of SeAx cells with JAG1 peptide resulted in a slight but consistent increase in icNOTCH1 expression, demonstrating the effectiveness of the peptide (Figure 5a and b). In contrast, JAG1 peptide did not have any effect on activation of STAT3, as assessed by flow cytometry using an antibody recognizing phosphorylated STAT3 (Figure 5c). In addition, pre-incubation of freshly isolated SS cells or SeAx cells with JAG1 peptide for 16 hours

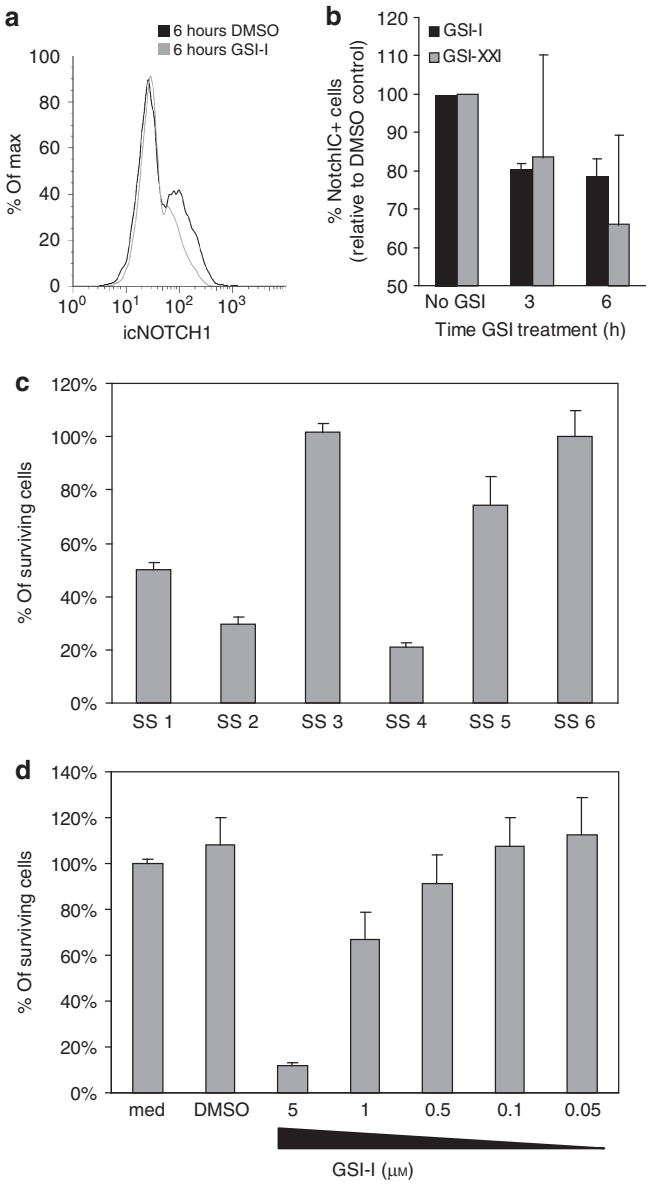

Figure 3. Inhibition of Notch signaling by the γ-secretase inhibitors (GSI)-I and GSI-XXI leads to reduced viability of Sézary cells. SeAx cells were treated with GSI-I or GSI-XXI, and intracellular domain of NOTCH1 (icNOTCH1) expression was assayed by flow cytometry. A representative example of a flow cytometric experiment is shown (**a**). The percentage of icNOTCH-positive cells was calculated relative to the percentage in DMSO-treated cells for each individual experiment (3 hours, $n=2$; 6 hours, $n=3$), and is depicted in panel **b**. Freshly isolated Sézary cells were incubated with 10 μM GSI-XXI (**c**) or GSI-I at the concentration indicated (**d**). Cell viability was assayed after 72 hours using a WST test. Bars represent the percentage of viable cells, relative to the number of nontreated cells. A representative example of three patients is shown in panel **b**.

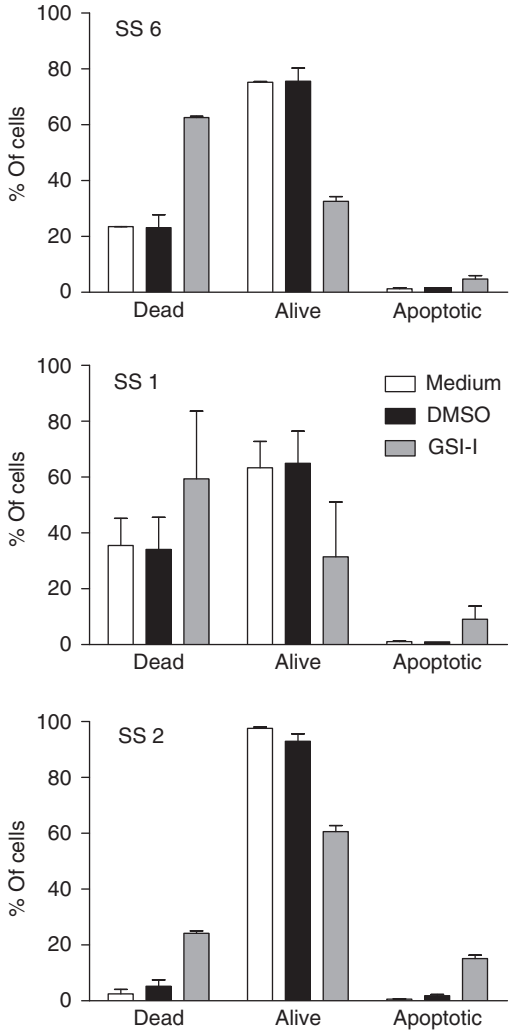

Figure 4. Inhibition of Notch1 signaling by the γ-secretase inhibitor (GSI)-I in Sézary cells results in increased apoptosis. Freshly isolated CD4+ T cells of three different Sézary syndrome (SS) patients were cultured with or without 5 μM GSI-I or with a volume equivalent of DMSO for 16 to 24 hours. Cells were subjected to propidium/Annexin-V labeling, and apoptotic cells were quantified by flow cytometric analysis. The number of dead, living, and apoptotic cells was calculated as percentage of the total number of cells. Bars denote mean ± SD of duplicate measurements.

did not lead to potentiation or elongation of the STAT3 response after subsequent stimulation of these cells with IL-21, a known activator of STAT3 signaling (Figure 5d).

DISCUSSION

In this paper, we demonstrate that NOTCH1 signaling and expression of HES1, a canonical NOTCH1 target gene, is consistently found in the large majority of SS cells in peripheral blood and skin. We found that the activated NOTCH1 signaling does not result from *NOTCH1* or *FBWX7* mutations and show that inhibition of NOTCH1 signaling results in apoptosis of Sézary cells. These results suggest that NOTCH1 signaling is a promising therapeutic target in SS.

In this study, staining studies of skin biopsies from SS patients using an antibody specific for the N-terminal sequence of icNOTCH1 showed nuclear staining in the majority of tumor cells in all cases. In line with this finding, FACS analysis of peripheral blood from SS patients demonstrated consistent expression of icNOTCH1 in the majority of tumor cells. Analysis of skin biopsies and peripheral blood from psoriasis and atopic eczema patients showed expression of icNOTCH1 and HES1 by the majority of reactive cells as

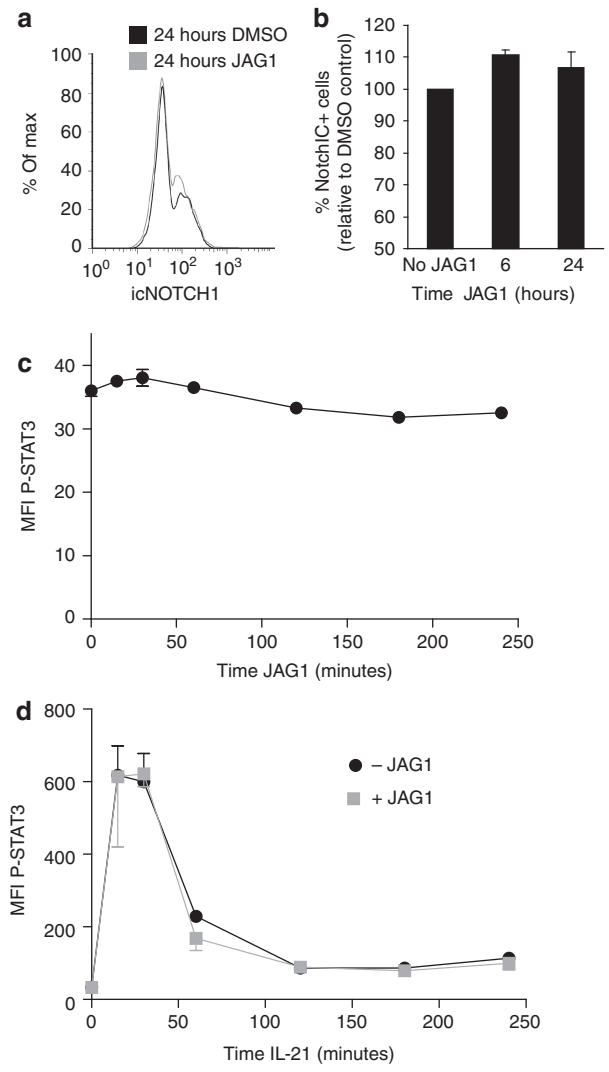

Figure 5. Stimulation of NOTCH signaling does not influence signal transducer and activator of transcription (STAT)-3 activation in Sézary syndrome (SS) cells. SeAx cell were incubated for the indicated time points with 40 μM JAG1 peptide, and intracellular domain of NOTCH1 (icNOTCH1) expression was determined by flow cytometry to assay the effectiveness of the peptide preparation. A representative example is shown in panel **a**. In addition, the percentage of icNOTCH-positive cells was calculated relative to the percentage in DMSO-treated cells for each individual experiment ($n = 3$), and is depicted in panel **b**. Freshly isolated CD4+ T cells from an SS patient were stimulated with 40 μM JAG1 peptide for the indicated time points (**c**), or overnight, and subsequently activated by IL-21 for various time points (**d**). STAT3 activation was assayed by flow cytometric analysis using an antibody specifically recognizing phosphorylated STAT3, and is depicted as mean fluorescent intensity (MFI) of duplicated measurements (mean ± SD).

well. These results are in line with a recent publication demonstrating the rapidly increased expression of NOTCH1 and NOTCH3 in mature T cells on activation (Koyanagi *et al.*, 2012). A previous study using a different antibody directed against full-length and truncated NOTCH1 showed mainly cytoplasmic and occasionally nuclear staining of NOTCH1 in two of five skin biopsies (Kamstrup *et al.*, 2010). The more homogeneous expression pattern of icNOTCH1 described by us compared with these previous findings most probably results from differences in staining characteristics of the antibodies that were used in the different studies. The consistent expression of icNOTCH1 found in the large majority of SS cells in skin, as well as peripheral blood detected in this study, strongly suggests that activated NOTCH1 signaling is operative in SS cells.

Various mechanisms can attribute to constitutive NOTCH1 signaling in tumor cells, including translocation and mutation of the *NOTCH1* gene. We show that in SS no mutations are present in the HD and PEST domains of the *NOTCH1* and *FBXW7* gene, indicating that other regulatory mechanisms are operative in SS.

Alternative explanations for increased NOTCH1 signaling in cancers that do not contain NOTCH1 mutations include decreased expression of miR-200 members that target NOTCH pathway components (Brabletz *et al.*, 2011), high expression of Mastermind-like 2 (Köchert *et al.*, 2011), and deletion of the 5′ *NOTCH1* promoter that allows generation of leukemogenic NOTCH1 protein from cryptic internal promoters in this region (Gomez del Arco *et al.*, 2010). In addition, studies in Hodgkin and anaplastic large-cell lymphoma showed Jagged1 expression in malignant and bystander cells colocalizing with NOTCH1-positive tumor cells, suggesting that NOTCH1 signaling may be activated through homotypic or heterotypic cell–cell interactions (Jundt *et al.*, 2002). If similar or yet other mechanisms are responsible for NOTCH1 expression in SS is clearly of interest.

Studies in human T-ALL cell lines reported that loss of *PTEN* correlates with resistance to NOTCH inhibition and loss of PTEN accelerated disease onset in a murine T-ALL model (Palomero *et al.*, 2007; Medyouf *et al.*, 2010). These observations raised the concern that therapeutic intervention inhibiting NOTCH signaling will be less effective in PTEN insufficient tumors. We did not detect PTEN mutations in the patients included in this study, but examination of previously published array-based comparative genome hybridization data of a subset of patients included in this study identified a heterozygous deletion of *PTEN* in four of six SS patients. Whether the loss of one *PTEN* allele leads to resistance to inhibition of NOTCH1 signaling in SS is currently unknown and should be addressed in future studies.

Important direct target genes of NOTCH1 signaling include c-MYC and HES1. In line with the activated NOTCH1 signaling, we found consistent expression of HES1 protein by the majority of SS cells. In T-ALL, NOTCH1 signaling induces expression of HES1 that downregulates the deubiquitinase CYLD, a negative IKB kinase complex regulator, leading to sustained NF-kB activation (Espinosa *et al.*, 2010; D'Altri *et al.*, 2011). In line with these findings, studies in SeAx cell line showed that downregulation of NOTCH1 resulted in downregulation of NF-kB in these cells as well (Kamstrup *et al.*, 2010). On the basis of these findings, it will be of interest to investigate whether NOTCH1-induced HES1 protein has a role in the nuclear expression of NF-kB that was reported in SS patients (Sors *et al.*, 2006).

Gene expression studies and chromatin immune precipitation (ChIP) analysis showed that *c-MYC* is a direct target of

NOTCH1, and ''ChIP on ChIP'' analysis revealed that c-MYC and NOTCH1 share common targets regulating growth, metabolism, and proliferation (Palomero *et al.*, 2006; Weng *et al.*, 2006). As a high proportion of SS cells contain copy number alterations leading to increased expression of c-MYC (Vermeer *et al.*, 2008), it will be of interest to investigate whether NOTCH1 and c-MYC are involved in a feed-forward loop that reinforces the expression of genes required for growth and proliferation in SS. Moreover, NOTCH1 signaling can induce resistance to apoptosis in T cells by upregulating the expression of inhibitor of apoptosis, B-cell lymphoma 2 (BCL-2), and FLICE-like inhibitor protein (Sade *et al.*, 2004). Given the resistance to apoptosis and expression of FLICE-like inhibitor protein typically observed in SS cells, it will be of interest to investigate whether these genes are regulated by NOTCH signaling in SS as well (Contassot *et al.*, 2008).

Among the STAT3 target genes that were described previously, several gene targets including the cell–cycle regulators (e.g., CYCLIN-D1 and c-MYC) and inhibitors of apoptosis (BCL2) are shared with NOTCH1. Recent studies demonstrated in mouse neuroepithelial cells and HeLa cervix cancer cell line showed that NOTCH1-induced expression of HES-1 proteins can associate with JAK2 and STAT3, thereby facilitating JAK2/STAT3 complex formation and promoting STAT3 phosphorylation and activation (Kamakura *et al.*, 2004; Lee *et al.*, 2009). As understanding of these interactions enhances the ability to design rational combination regimens, we investigated whether NOTCH1 signaling could support the expression of activated STAT3 that is observed in SS. However, using short-term culturing systems, we could not demonstrate cooperation of NOTCH1 and STAT3 signaling in SS.

In addition to T-ALL, dysregulated expression of NOTCH-1 has been found in myeloma, colon cancer, breast cancer, pancreas cancer, and melanoma (Ranganathan *et al.*, 2011), and various treatments are being developed to inhibit NOTCH signaling. The most extensively studied target is gamma-secretase, which can be inhibited effectively by GSI treatment. Clinical trials with GSI are now performed in T-cell leukemias, multiple myeloma, melanoma, pancreas carcinoma, breast carcinoma, lung carcinoma, prostate carcinoma, and glioblastoma. To investigate whether inhibition of NOTCH induces apoptosis of SS cells and consequently might serve as a therapeutic target in this disease, we performed short-term cultures of SS cells with GSI-I and GSI-XXI. We found that inhibition of NOTCH1 induces apoptosis of Sézary cells, in a manner similar to that demonstrated for other hematological malignancies such as T-ALL, myeloma, and solid cancers including glioblastoma, breast cancer, and colon carcinoma cells. The GSIs included in this study (GSI-I and GSI-XXI) show considerable differences in efficacy in the inhibition of NOTCH signaling and induction of apoptosis (Figure 3). These results are in line with previous reported differential effects of GSIs in other malignancies including Kaposi's sarcoma, cutaneous lymphomas, and breast cancer (Curry *et al.*, 2005; Rizzo *et al.*, 2008; Kamstrup *et al.*, 2010). Although we clearly demonstrate that treatment of Sézary cells with GSI-I results in reduced icNOTCH1 expression and subsequent increased apoptosis, off-target effects of GSI-I on Sézary cells cannot be excluded and remain to be elucidated.

In summary, we demonstrate that NOTCH1 and its canonical target gene HES-1 are expressed by Sézary cells. We found no mutations in the NOTCH1 and the FBWX7 gene that could underpin the icNOTCH1 expression and demonstrate that inhibition of NOTCH1 signaling leads to induction of apoptosis of Sézary cells. Together, these data indicate that inhibition of NOTCH1 signaling might offer a promising therapeutic target for SS.

MATERIALS AND METHODS

Patient selection

Sixteen patients with SS (nine men and seven women; median age of 64 years) were diagnosed based on criteria of the WHO/EORTC 2005 classification (Willemze *et al.*, 2005). All patients presented with erythroderma, and showed T-cell clonality in the peripheral blood, increased CD4 counts with a CD4/CD8 ratio of >10 (mean 30, range 11–245). Follow-up data revealed that seven patients had died of SS after a median follow-up of 44 months (Supplementary Table S2 online). As controls, patients with psoriasis ($n=6$) and atopic dermatitis ($n=8$) were included.

Approval for these studies was obtained from the Leiden University Medical Center review board, and written informed consent was provided according to the Declaration of Helsinki Principles.

Immunohistochemistry

Serial sections of 4 μm were blocked for endogenous peroxidase with 1% H_2O_2 in 100% methanol for 30 minutes. Antigen retrieval was performed with 10 mM monocitric acid (pH 6.0) at 100 °C for 15 minutes. The slides were blocked with nonimmune serum for 20 minutes at room temperature. The sections were stained using the primary antibodies against cleaved NOTCH1 (anti-NOTCH1, 1:75; Cell Signaling Technology, Boston MA) and HES1 (anti-HES1, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Binding of the primary antibody was visualized by the addition of Envision (horseradish peroxidase-labeled mouse antibody, undiluted; DAKO, Glostrup, Denmark). Normal, healthy human colon was used as control.

Cell culturing and stimulation

SeAx, a cell line derived from the peripheral blood of a patient with SS (kindly provided by Dr K Kaltoft) (Kaltoft *et al.*, 1987), was cultured in RPMI-1640 (Invitrogen, Breda, The Netherlands), supplemented with 10% fetal calf serum (HyClone/Greiner, Nürtingen, Germany), 2 mM L-glutamine (Invitrogen), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Invitrogen), and 200 U ml^{-1} IL-2 (PeproTech, Rocky Hill, NJ). HuT78, a cell line derived from peripheral blood of a patient with SS (ATCC number TIB-161), was cultured in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Heparinized blood was drawn from patients and peripheral blood mononuclear cells were isolated by Ficoll density centrifugation. Subsequently, CD4+ T cells were isolated by negative selection with magnetic beads (CD4+ T-cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cell population was determined by flow cytometry for CD3, CD4, and CD8 expression, and was generally $>85\%$. In addition, earlier

experiments demonstrated that >90% of the CD4+ cells comprise malignant cells characterized by expression of a clonally rearranged T-cell receptor. The freshly isolated CD4+ T cells were maintained in RPMI 1640 medium supplemented with 10% human AB serum (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 200 U ml^{-1} IL-2, and 5 ng ml^{-1} IL-7 (PeproTech).

Cells were incubated with GSI-XXI (Compound E, Alexa Biochemicals, San Diego, CA), GSI-I (Calbiochem, San Diego, CA), in the concentrations indicated, 40 µM JAG1 peptide (AnaSpec, Freemont, CA), or 100 ng ml^{-1} IL-21 (ImmunoTools, Friesoythe, Germany).

Analysis of cell survival and apoptosis

To measure cell survival after inhibition of NOTCH1 signaling, primary SS cells or SeAx were incubated with GSI in the concentrations indicated in a 96-wells plate (six wells per condition). After 72 hours, cell proliferation reagent WST-1 (Roche, Mannheim, Germany) was added, and absorbance at 450 nm and 650 nm (reference wavelength) was measured after 8 hours. The number of viable cells was calculated relative to the numbers of nontreated cells.

Apoptosis was evaluated by staining cells with FITC-conjugated antibodies against Annexin-V (ImmnunoTools, Friesoythe, Germany) and propidium iodide according to the manufacturer's recommendation. Samples were acquired on a flow cytometer (FacsCalibur, BD Biosciences, Franklin Lakes, NY) and analyzed using the CellQuest software (BD Biosciences).

Flow cytometry

STAT3 activation was measured by flow cytometry. Cells were activated, fixed, and stained using Alexa647-conjugated antibodies against phosphorylated STAT3 (BD Bioscience), as described previously (van der Fits *et al.* 2011).

To measure the levels of icNOTCH1, cells were permeabilized using BD lyse solution (BD Biosciences) and stained with phycoerythrin-conjugated anti-icNOTCH1 (Abcam, Cambridge, UK) or phycoerythrin-conjugated mouse IgG (Abcam) for 30 minutes at 4 °C in the dark. Samples were acquired on a flow cytometer (FacsCalibur, BD Biosciences) and analyzed using the CellQuest software (BD Biosciences).

Mutation analysis

Genomic DNA and total RNA were obtained from the CD4+ T cells isolated from peripheral blood of 10 SS patients using DNeasy and RNeasy (Qiagen, Hilden, Germany), respectively. Two µg isolated RNA from each patient was subjected to DNAse treatment (RQ1, Bio-Rad, Hemel Hempstead, UK) before complementary DNA synthesis (IScript Kit, Bio-Rad). The coding region of each gene was amplified by PCR using a series of overlapping primers. The PCR primer sets (Beacon Designer, premier Biosoft, Palo Alto, CA) typically generate PCR products between 500 and 1,000 bp, under typical 40 thermal cycles (95 °C/20 s; 60 °C/15 s; 72 °C/50 s). The specificity of PCR products was verified by melting curve analysis (My IQ, Bio-Rad) before purification (MinElute 96UF, Qiagen). The purified PCR fragments were sequenced both with forward and reverse primers (ABI 3700, Applied Biosystems, Foster City, CA). The sequenced PCR fragments of each gene were aligned and then referred to the corresponding normal sequences (Ensembl database). The nucleotide alterations of each gene were analyzed using a software (Mutation Explorer, Softgenetic, State College, PA) based on the consensus of sequencing results of forward and reverse sequencing. Finally, the identified nucleotide alterations were blasted against NCBI SNP database.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Asnafi V, Buzyn A, Le Noir S *et al.* (2009) 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* 113:3918–24

Brabletz S, Bajdak K, Meidhof S *et al.* (2011) The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells. *EMBO J* 30: 770–82

Contassot E, Kerl K, Roques S *et al.* (2008) Resistance to FasL and tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in Sezary syndrome T-cells associated with impaired death receptor and FLICE-inhibitory protein expression. *Blood* 111:4780–7

Curry CL, Reed LL, Golde TE *et al.* (2005) Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. *Oncogene* 24:6333–44

D'Altri T, Gonzalez J, Aifantis I *et al.* (2011) Hes1 expression and CYLD repression are essential events downstream of Notch1 in T-cell leukemia. *Cell Cycle* 10:1031–6

Ellisen LW, Bird J, West DC *et al.* (1991) TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66:649–61

Eriksen KW, Kaltoft K, Mikkelsen G *et al.* (2001) Constitutive STAT3-activation in Sezary syndrome: tyrphostin AG490 inhibits STAT3-activation, interleukin-2 receptor expression and growth of leukemic Sezary cells. *Leukemia* 15:787–93

Espinosa L, Cathelin S, D'Altri T *et al.* (2010) The Notch/Hes1 pathway sustains NF-κB activation through CYLD repression in T cell leukemia. *Cancer Cell* 18:268–81

Fabbri G, Rasi S, Rossi D *et al.* (2011) Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. *J Exp Med* 208:1389–401

Fryer CJ, White JB, Jones KA (2004) Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell* 16:509–20

Gómez-del Arco P, Kashiwagi M, Jackson AF *et al.* (2010) Alternative promoter usage at the Notch1 locus supports ligand-independent signaling in T cell development and leukemogenesis. *Immunity* 33:685–98

Heitzler P, Bourouis M, Ruel L *et al.* (1996) Genes of the enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* 122:161–71

Jundt F, Anagnostopoulos I, Förster R *et al.* (2002) Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood* 99:3398–403

Kaltoft K, Bisballe S, Rasmussen HF *et al.* (1987) A continuous T-cell line from a patient with Sézary syndrome. *Arch Dermatol Res* 279:293–8

Kamakura S, Oishi K, Yoshimatsu T *et al.* (2004) Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat Cell Biol* 6:547–54

Kamstrup MR, Gjerdrum LM, Biskup E *et al.* (2010) Notch1 as a potential therapeutic target in cutaneous T-cell lymphoma. *Blood* 116:2504–12

van Kester MS, Out-Luiting JJ, von dem Borne PA *et al.* (2008) Cucurbitacin I inhibits STAT3 and induces apoptosis in Sézary cells. *J Invest Dermatol* 128:1691–5

Köchert K, Ullrich K, Kreher S *et al.* (2011) High-level expression of Mastermind-like 2 contributes to aberrant activation of the NOTCH signaling pathway in human lymphomas. *Oncogene* 201130:1831–40

Koyanagi A, Sekine C, Yagita H (2012) Expression of Notch receptors and ligands on immature and mature T cells. *Biochem Biophys Res Commun* 418:799–805

Lee JH, Suk J, Park J *et al.* (2009) Notch signal activates hypoxia pathway through HES1-dependent SRC/signal transducers and activators of transcription 3 pathway. *Mol Cancer Res* 7:1663–71

Medyouf H, Gao X, Armstrong F *et al.* (2010) Acute T-cell leukemias remain dependent on Notch signaling despite PTEN and INK4A/ARF loss. *Blood* 115:1175–84

Nickoloff BJ, Qin JZ, Chaturvedi V *et al.* (2002) Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. *Cell Death Differ* 9:842–55

Oellers N, Dehio M, Knust E (1994) bHLH proteins encoded by the enhancer of split complex of drosophila negatively interfere with transcriptional activation mediated by proneural genes. *Mol Gen Genet* 244:465–73

O'Neil J, Grim J, Strack P *et al.* (2007) FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* 204:1813–24

Olsen EA, Rook AH, Zic J *et al.* (2011) Sézary syndrome: immunopathogenesis, literature review of therapeutic options, and recommendations for therapy by the United States Cutaneous Lymphoma Consortium (USCLC). *J Am Acad Dermatol* 64:352–404

Palomero T, Lim WK, Odom DT *et al.* (2006) NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci USA* 103:18261–6

Palomero T, Sulis ML, Cortina M *et al.* (2007) Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med* 13:1203–10

Ranganathan P, Weaver KL, Capobianco AJ (2011) Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer* 11:338–51

Rizzo P, Miao H, D'Souza G *et al.* (2008) Cross-talk between notch and the estrogen receptor in breast cancer suggests novel therapeutic approaches. *Cancer Res* 68:5226–35

Rosati E, Sabatini R, Rampino G *et al.* (2009) Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. *Blood* 113:856–65

Sade H, Krishna S, Sarin A (2004) The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem* 279:2937–44

Sors A, Jean-Louis F, Pellet C *et al.* (2006) Down-regulating constitutive activation of the NF-kappaB canonical pathway overcomes the resistance of cutaneous T-cell lymphoma to apoptosis. *Blood* 107:2354–63

Tetzlaff MT, Yu W, Li M *et al.* (2004) Defective cardiovascular development and elevated cyclin E and Notch proteins in mice lacking the Fbw7 F-box protein. *Proc Natl Acad Sci USA* 101:3338–45

Thompson BJ, Buonamici S, Sulis ML *et al.* (2007) The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J Exp Med* 204:1825–35

van der Fits L, van Kester MS, Qin Y *et al.* (2011) MicroRNA-21 expression in CD4+ T cells is regulated by STAT3 and is pathologically involved in Sézary syndrome. *J Invest Dermatol* 131:762–8

Vermeer MH, van Doorn R, Dijkman R *et al.* (2008) Novel and highly recurrent chromosomal alterations in Sézary syndrome. *Cancer Res* 68:2689–98

Weng AP, Ferrando AA, Lee W *et al.* (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306:269–71

Weng AP, Millholland JM, Yashiro-Ohtani Y (2006) c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* 20:2096–109

Willemze R, Jaffe ES, Burg G *et al.* (2005) WHO-EORTC classification for cutaneous lymphomas. *Blood* 105:3768–85