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The impact of NOTCH1, FBW7 and PTEN mutations on prognosis and downstream signaling in pediatric T- cell acute lymphoblastic leukemia: A report from the Children's Oncology Group

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Abstract

We explored the impact of mutations in the NOTCH1, FBW7 and PTEN genes on prognosis and downstream signaling in a well-defined cohort of 47 pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients. In T-ALL lymphoblasts, we identified high frequency mutations in NOTCH1 (n=16), FBW7 (n=5) and PTEN (n=26). NOTCH1 mutations resulted in 1.3-3.3-fold increased transactivation of a HES1 reporter construct over wild-type NOTCH1; mutant FBW7 resulted in further augmentation of reporter gene activity. NOTCH1 and FBW7 mutations were accompanied by increased median transcripts for NOTCH1 target genes (HES1, DELTEX1, cMYC). However, none of these mutations were associated with treatment outcome. Elevated HES1, DELTEX1 and cMYC transcripts were associated with significant increases in transcript levels of several chemotherapy relevant genes, including MDR1, ABCC5, reduced folate carrier, asparagine synthetase, thiopurine methyltranserase, Bcl-2 and dihydrofolate reductase. PTEN transcripts positively correlated with HES1 and cMYC transcript levels. Our results suggest that (1) multiple factors should be considered with attempting to identify molecular-based prognostic factors for pediatric T-ALL, and (2) depending on the NOTCH1 signaling status, modifications in the types

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or dosing of standard chemotherapy drugs for T-ALL, or combinations of agents capable of targeting NOTCH1, AKT and/or mTOR with standard chemotherapy agents may be warranted.

Keywords

acute lymphoblastic leukemia; NOTCH1; FBW7; PTEN; HES1; DELTEX1; cMYC; chemotherapy; T-cell

Introduction

Acute lymphoblastic leukemia (ALL) comprises a range of diseases with diverse morphologic, immunologic, and genetic features. Immunophenotypically unique ALL subgroups exhibit characteristic biochemical, clinical, and cytogenetic features that are typically associated with different prognoses (1-4). For B-precursor (BP)-ALL in children, ∼80% of patients experience long-term survivals (>5 y) with modern therapies (1-3). The prognosis of children with T-cell ALL (T-ALL) has improved, accompanying use of aggressive multi-agent therapies, and has been reported to approach that for BP-ALL (2,3,5). However, long-term survival rates for pediatric T-ALL still lag behind those for BP-ALL by up to 20% (2,3,5).

T-ALL is associated with few unique features upon which to base risk group stratification, even though non-random translocations specific to T-ALL were identified (2,3,5). Common translocations in T-ALL involve promoter and enhancer elements of the T-cell receptor (TCR) genes and genes encoding developmentally important transcription factors such as cMYC and the HoxA cluster (5,6). Of interest is t(7;9)(q34;q34.3) in which the C-terminal sequence of the NOTCH1 gene is translocated adjacent to TCRβ (7). NOTCH1 is a single-pass heterodimeric transmembrane (TM) receptor that regulates genes important to cell differentiation, proliferation, and apoptosis (8,9). NOTCH1 is comprised of an extracellular subunit consisting of 36 N-terminal epidermal growth factor-like repeats that mediate ligand binding, 3 iterated NOTCH1/Lin12 repeats that maintain the receptor in the inactive state in the absence of ligand, and a 103 amino acid stretch that interfaces with a 65 amino acid portion of the intracellular subunit [collectively, termed the heterodimerization (HD) domain] (10-12). The intracellular subunit mediates NOTCH1 signaling and contains a RAM (RBP-j_κ-associated molecule) domain, 7 iterated ankyrin (ANK) repeats, and a C-terminal PEST (Pro-Glu-Ser-Thr) sequence implicated in protein stability.

Normally, NOTCH1 signaling activity is initiated by binding of DSL (Delta-Serrate-Lag2) ligands on an adjacent cell and results in sequential proteolytic cleavages in the TM subunit (S2 and S3, catalyzed by an ADAM-type metalloprotease and γ secretase, respectively) (10-15). S3 cleavage results in free activated intracellular NOTCH1 (ICN1) which translocates to the nucleus, and binds to the transcriptional repressor CSL [CBF1/Su(H)/Lag1], converting it to a transcriptional activator (8,9,16). Mastermind-like activator proteins then complex with CSL/ICN1, recruiting the ARC-L/MED mediator complex, histone ubiquitin ligase Bre-1, and histone acetyltransferases to assemble an active transcriptional complex on target promoters (8,9,16).

NOTCH1 is regulated by NUMB, which affects nuclear localization by targeting the transmembrane form of NOTCH1 for ubiquitination by the E3 ligase Itch, resulting in its degradation (17), and by DELTEX1, which can both inhibit and activate NOTCH1 signaling (18-20). Hyperphosphorylation of the PEST domain of ICN1 by cyclin-dependent kinase 8 facilitates ubiquitination by FBW7 (Sel10, hCDC4) ubiquitin ligase and proteosomal degradation, thus modulating duration of the NOTCH1 signal (19,21,22).

A limited number of downstream NOTCH1 targets have been reported. Transcriptional targets include HES ("Hairy and Enhancer of Split") 1 and 5, the HES-related protein ("HERP") family, and DELTEX1 (20,23,24). HES and HERP proteins negatively regulate transcription of many downstream target genes, including those involved with apoptosis and proliferation (24). Other NOTCH1-regulated genes include cMYC (25), p21 (26), CD25 (27), pre-Tα (28), cyclin D1 (29), the proapoptotic receptor NUR (30), and transcription factors of the NF-κB family such as Relb and Nfkb2 (31). PTEN (phosphatase and tensin homolog) was described as an indirect NOTCH1 target (via HES1 and cMYC), resulting in constitutively active PI3K-AKT signaling (32), whereas another report described activation of the mTOR pathway by NOTCH1 independent of PI3K-AKT (33).

t(7;9)(q23;q34.3) results in constitutive activation of NOTCH1 (TAN1) that is independent of ligand binding, however, this occurs in <1% of T-ALLs (7). The identification of *putative* gain-of-function NOTCH1 mutations involving both HD and PEST domains in T-ALL lymphoblasts from both children and adults at frequencies ranging from 38-71% (34-37) implies a more general role for constitutively active NOTCH1 in this disease. HD mutations could affect ligand-independent conformational changes to facilitate S3 cleavage and ICN1 nuclear translocation (34,38). PEST mutations should elevate ICN1 levels by increasing its half-life (34,38). For both HD and PEST mutants, constitutively active NOTCH1 signaling can be abolished with γ secretase inhibitors (GSIs). However, activating effects of clinically relevant NOTCH1 mutants have been confirmed in only a few cases and range from completely inert to significant wide-ranging stimulation of NOTCH1-responsive reporters (34,39). Findings of high frequency mutations (8.6-30%) in the substrate binding domain (e.g., Arg465, Arg479, Arg 505) of FBW7 in T-ALL cell lines and primary specimens that result in sustained NOTCH1 signaling (21,22,40) and GSI-resistance, suggest an alternate mechanism for NOTCH1 deregulation. Finally, mutations in the PTEN gene resulting in constitutive AKT signaling were also associated with GSI resistance in T-ALL (32).

The prognostic importance of NOTCH1 mutations in T-ALL remains controversial. For adult patients with T-ALL (n=24), NOTCH1 mutations were accompanied by poor event-free-survivals (EFS) and overall survivals (35). While in two studies with children (n=53 and n=70, respectively), there was no association with prognosis (35,41), a recent study of 157 pediatric T-cell ALL patients treated on the ALL-Berlin-Frankfurt-Munster protocol (82 with mutated NOTCH1) established an association between NOTCH1 mutations and an excellent prognosis (36). In up to 30% of children with T-ALL, mutations in both NOTCH1 and FBW7 were also associated with a favorable overall prognosis (21). Most recently, both NOTCH1 (n=88) and FBW7 (n=34) mutations were identified in 141 adult diagnostic T-ALL samples from patients treated on the LALA-94 or the GRAALL-2003 trials, and reported to be an independent good prognostic factor for EFS and overall survival (42).

Finally, both FBW7 (22,40) and NOTCH1 (37) mutations were associated with relapsed T-ALL.

In this report, we explore the associations between NOTCH1, FBW7 and PTEN mutations and downstream signaling in pediatric T-ALL in an attempt to better understand the relationship with disease progression and treatment response.

Materials and Methods

Patient specimens and identification of mutations

Forty seven T-cell ALL patient specimens [including 24 patients who did not fail treatment (‘not failed’) and 23 patients who failed treatment (‘failed’)] were obtained from the Children's Oncology Group (COG) ALL cell bank and used for this study. Patients were treated on Pediatric Oncology Group (POG) protocols including POG 8704 (14 failed and 14 not failed), 9086 (4 failed, 3 not failed), 9295 (1 not failed), 9296 (2 failed, 1 not failed), 9297 (2 failed, 3 not failed), and 9398 (1 failed, 2 not failed). Patients in the ‘not failed’ group were children who remained in remission for 4 or more years following diagnosis, and patients in the ‘failed’ group were children who suffered bone marrow relapses within 4 years of diagnosis. Major chemotherapy drugs used were L-asparaginase, doxorubicin, 6-mercaptopurine, methotrexate, prednisone, and vincristine. Patients who died in remission within 4 years of diagnosis were excluded from the study.

Sample handling and data analysis protocols were approved by the Committee on Investigation Involving Human Subjects at Wayne State University. Leukemic blasts were purified by standard Ficoll-Hypaque density centrifugation. Total RNAs were extracted from primary ALL lymphoblasts using the RNEasy Midiprep Kit (Qiagen, Valencia, CA). cDNAs were prepared from 1 μg RNAs using random hexamers and a RT-PCR kit (PerkinElmer, Boston, MA), and purified with the QIAquick PCR Purification Kit (Qiagen).

Mutations in NOTCH1 (HD and PEST domain) and PTEN (entire coding sequence) were identified in cDNAs by nested PCR methods. FBW7 mutations were identified either in cDNAs or in genomic DNAs by amplifying sequence including exons 11, 12 and 14, previously reported as mutation “hotspots”. Primer sequences and PCR conditions are summarized in the Supplement (Table 1S). PCR products were separated on 1% agarose gels with ethidium bromide and purified with a gel extraction kit (Marligen Biosciences, Ijamsville, MD). Alternatively, PCR products were directly purified using the QIAquick cDNA purification kit (Qiagen) and sequenced in both directions with M13 forward and reverse primers (primer sites located on the primers used for PCR) or gene-specific primers at either the Wayne State University Applied Genomics Technology Core or Genewiz, Inc. (South Plainfield, NJ). For a small number of samples, PCR products were subcloned into a TA-cloning vector [pCRII-TOPO (Invitrogen, Carlsbad, CA)] and transformed into One Shot® MACH1T1 competent cells. Plasmids were isolated using the Wizard® Plus Mini Prep DNA purification system (Promega, Madison, WI) for DNA sequencing.

Real-time RT-PCR analysis of gene expression profiles

Analyses of gene expression levels were performed in a blinded manner. Transcript levels for 22 chemotherapy-related genes, PTEN, downstream NOTCH1 targets (HES1, DELTEX1, cMYC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured with a LightCycler real-time PCR machine (Roche, Indianapolis, IN). Reactions contained 2 μl of purified cDNA or standard plasmid, 4 mM $MgCl_2$, 0.5 μM each of sense and antisense primers, and 2 μl FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche), as described (43). Specificity of the amplifications was confirmed by melting curve analysis and comparisons to standard templates. Gene-specific primers and PCR conditions are summarized in Table 2S (Supplement). For each gene of interest, external standard curves were constructed using serial dilutions of linearized templates, prepared by amplification from cDNA templates, subcloning into a TA-cloning vector, and restriction digestion. Transcript levels for genes of interest were normalized to GAPDH transcripts.

NOTCH1 and FBW7 mutagenesis

The wild type NOTCH1 expression construct in pCDNA3 was a gift from Dr. Spyros Artavanis-Tsakonas (Harvard University, Cambridge, MA). Full-length wild-type FBW7 cDNA (variant 1; NM_033632.2) was amplified from the T-ALL cell line MOLT4 (American Type Culture Collection, Rockville, MD) using primers located in the 5′ untranslated region (UTR) (TTCAC**GGTACC**CGAAGGAGGAAGGGAACCAACC; bold sequence indicates KpnI site) and 3′ UTR (TTCAC**GAATTC**AGGGGGAAGGGCAGGGAGTA; bold sequence indicates EcoRI site). Following PCR amplification and sub-cloning into pCRII-Topo (Invitrogen), FBW7 constructs were digested with KpnI and EcoRI, and purified with the QIAquick Gel Extraction Kit (Qiagen). Digested FBW7 was subcloned into pcDNA3 and transformed into JM109 competent cells (Promega). FBW7-pcDNA3 constructs were isolated with the Wizard® Plus Midiprep DNA purification system (Promega). Site-directed mutagenesis of the HD and PEST domains of wild type NOTCH1 and the WD40 domains of FBW7 used the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer's protocols with the following modifications: (a) 100 ng of wild type NOTCH1 or FBW7 (both in pcDNA3) were used as template; (b) extension time was 30 sec/kb at 68° C; and (c) Dpn I digestions were for 10 min. Mutant primer sequences are available upon request. Mutant plasmids were transformed into XL10-Gold ultracompetent cells and (d) LB-ampicillin agar plates were incubated at 24-30° C for >24 h to prevent recombination. Mutant constructs were transformed into JM109 competent cells to obtain higher copy number plasmids. Plasmids were isolated and the mutants were confirmed by DNA sequencing.

Generation of HES1 promoter reporter construct and reporter gene assays

A construct with an artificial luciferase reporter gene under the control of a HES1 promoter containing CSL/ICN1 binding sites (HES1-luc) in pGL3-Basic (Promega) was prepared as follows. The promoter region of the human HES1 gene between positions -942 and -158 from the translation start site was isolated by PCR from genomic DNA prepared from

CMK16 cells (DSMZ, Braunschweig, Germany) using forward (5′TTCAC**GCTAGC**GTCTAAGGCCCCAAATCCAAACGAG) and reverse (5′TTCAC**CTCGAG**CAGTAGCGCTGTTCCAGGACCAAG) primers (bold sequences indicate NheI and XhoI restriction sites, respectively). The amplified fragment was digested with NheI and XhoI and subcloned into pGL3-Basic vector (Promega).

Human U20S osteosarcoma cells (American Type Culture Collection) were cultured at 37° C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen), and 1% L-glutamine (200 mM) at 37°C under 5% CO_2.

NOTCH1 and FBW7 expression plasmids were transiently transfected into U2OS cells with HES1-Luc and pRL-SV40, using Lipofectamine Plus (Invitrogen). Briefly, 3.2 X 10^5 U2OS cells were seeded per well (35 mm) of a six well plate, allowed to adhere overnight, then co-transfected with 1 μg of HES1-Luc, 30 ng of pRL-SV40 (Promega), and 0.9 μg of wild type or mutant NOTCH1-pcDNA3 constructs, or ICN1-pcDNA3 (provided by Dr. Lucio Miele, Loyola University, Chicago, IL). Total DNA was maintained constant by adding empty pcDNA3 plasmid (Invitrogen). For the FBW7-NOTCH1 co-transfections, wild type and mutant FBW7-pcDNA3 constructs (0.9 μg) were co-transfected into U2OS with wild type and mutant NOTCH1-pcDNA3 constructs (0.9 μg of each) with 500 ng of HES1-Luc/30 ng of pRL-SV40. For both series, 48 h post-transfection, the cells were lysed and luciferase activities were assayed using a Dual Luciferase reporter assay system (Promega) on a Turner Designs 20/20 luminometer. Relative luciferase activities of the cell lysates were normalized to *Renilla* luciferase activity (encoded by pRL-SV40). Data are reported as mean values plus/minus SEM from replicate assays.

Statistical methods

Data analyses were performed using the SAS System (44) and R (45), or GraphPad Prism 4.0. For analyses of overall NOTCH1 signaling, transcript levels for HES1, DELTEX1, and cMYC were categorized into low and high levels, respectively, corresponding to values below and above the median values. The non-parametric Wilcoxon test was used for comparisons of transcript levels between various subgroups (cases versus controls, NOTCH1 mutant versus non-mutant, age group, WBC, high versus low HES1/DELTEX1/cMYC transcripts). The associations between high/low transcript levels of a gene and outcome or prognostic factors such as age group or WBC group were tested using Fisher's Exact test. The non-parametric Spearman's correlation coefficient was used to measure the associations between NOTCH1 target genes. The paired t test was used to make comparisons between the luciferase activities associated with the NOTCH1 mutants and with wild-type NOTCH1.

Results and Discussion

Identification of NOTCH1 mutants in primary T-ALL specimens

Although significant improvements have been documented in the treatment outcome of T-ALL in children, T-ALL remains an aggressive disease with a substantially poorer prognostic outlook than that for BP-ALL. Following reports of high frequency mutations in

the NOTCH1 receptor (34-36) involving the HD (positions 4710 to 5163) and PEST (positions 6930 to 7665) domains [position numbers are based upon NOTCH1 sequence (NM_017617.3)], we became interested in the prognostic significance of mutant NOTCH1 and possible explanations for disparate reports of both good and poor prognoses for T-ALL patients with NOTCH1 mutations (21,35,36,41,42). A well-characterized cohort of 47 pediatric T-ALLs with documented treatment outcomes was used to explore this clinically important question. The 47 children included 38 boys and 9 girls diagnosed with T-ALL, 23 of whom relapsed within 4 years of diagnosis (failed) and 24 of whom remained in remission for 4 or more years after diagnosis (not failed). Patient ages ranged from 1.8 to 19.9 y (median = 7.48 y) and white blood counts (WBC) ranged from 8.2 to 999 × 10^9 cells/L (median = 240 × 10^9 cells/L) (Table 1).

RNAs from the 47 T-ALL specimens were reverse transcribed and cDNAs were PCR amplified across the NOTCH1 HD and PEST domains. The amplicons were sequenced in both directions with M13 primers to identify potential HD and PEST mutations. In a few cases, amplicons were subcloned into a T/A cloning vector and individual plasmid clones were isolated for DNA sequencing. Twenty-five samples showed a high frequency polymorphism (C5094T) in the HD domain that was silent (GAC and GAT both encode aspartic acid). NOTCH1 mutations resulting in modified primary sequence were detected in 16 patients (9 HD, 4 PEST, 3 HD and PEST) and wild type NOTCH1 sequences were detected in 31 patients (Table 2; Supplement, Table S3). NOTCH1 mutations included single point mutations, deletions, and insertions in the HD and PEST domains (Table S3, Supplement) that variously resulted in amino acid substitutions and premature translation terminations (Table 2). With a few exceptions (V1671I, 2514 RVP*Stop, 2459*Stop, and 2503*Stop), these mutations are unique from those previously described as probable “gain-of-function” in T-ALL (34,36). The frequency (34%) of NOTCH1 mutations in our analysis is somewhat lower than that which was originally reported in pediatric T-ALL (34), and may reflect the unique features of our T-ALL cohort (i.e., ∼50% of patients relapsed). Nonetheless, similar frequencies have been reported in both pediatric and adult T-ALL patients in other studies (35-37).

For our 47 patient cohort, there were no associations between age or WBC and the presence of NOTCH1 mutations. In contrast to recent reports that the presence of NOTCH1 mutations were good prognostic factors in T-ALL (21,36), no statistically significant difference was seen in frequencies of mutations between patients who relapsed (9 of 23 patients harbored NOTCH1 mutations) and patients who did not (7 of 24 patients harbored NOTCH1 mutations; p=0.5469, by Fisher's exact test). Interestingly, for the 28 patients treated on a single (POG8704) protocol (14 failed, 14 not failed), there was a decrease in the frequency of NOTCH1 mutations in patients who relapsed (3 of 14 patients harbored NOTCH1 mutations) compared to those who responded to treatment (6 of 14 patients harbored NOTCH1 mutations). However, this difference was still not statistically significant (p=0.4197).

We reasoned that our inability to establish statistically significant associations between relapse and mutant NOTCH1 in our 47 patient cohort could be due to (i) various levels of overall signaling resulting from different activating potencies for the assorted NOTCH1

mutants. Further, other factors may also be important such as (ii) high frequency mutations in the FBW7 ubiquitin ligase that impact steady state levels of ICN1 independent of NOTCH1 mutation status (21,22), or (iii) decreased expression (due to HES1 and cMYC) and/or inactivating mutations involving the PTEN gene, resulting in increased AKT signaling (32). Finally, (iv) the T-ALL specimens were from patients treated with different protocols and aberrant NOTCH1 signaling may impact sensitivities to various chemotherapy drugs to different extents.

Activating potential of patient-derived NOTCH1 mutations

To begin to consider the above possibilities, we prepared mutant NOTCH1 constructs containing the HD and PEST domain mutations identified in the 16 patient specimens. Mutant NOTCH1 constructs in pcDNA3 were transiently transfected into U2OS cells with a HES1-Luc reporter; firefly luciferase activities (normalized to *Renilla* luciferase) were compared to those for wild type NOTCH1 and ICN1. The 16 clinically relevant NOTCH1 mutants showed increased transactivating potentials toward HES1-Luc over wild type NOTCH1 (1.3-3.3-fold), albeit consistently less than by ICN1 (Figure 1A). Interestingly, most NOTCH1 constructs with mutations in either the PEST domain alone, or in combination with HD domain mutations, showed higher levels of reporter gene activation than constructs with mutations in the HD domain alone. This result is somewhat different from that reported by Weng *et al.* (34) with a much smaller group of clinically relevant NOTCH1 mutants.

Identification of FBW7 mutations in T-ALL specimens

Since high frequency mutations in the E3-ubiqutin ligase FBW7 [NM_001013415.1] substrate binding domain were reported in up to 30% of pediatric T-ALL patients (21,22,40), the 47 T-ALL specimens were analyzed for mutations in the FBW7 gene. cDNAs from 44 specimens were amplified across exons 8-14 for direct sequencing of the amplicons, whereas for one specimen, the product was subcloned (PCRII-TOPO) and multiple plasmid clones were sequenced. For 2 samples, exons 11, 12 and 14 were individually amplified and sequenced from genomic DNAs. FBW7 mutations were detected in exon 11 for 5 patients (11%; Table 2; Supplement, Table S3, Supplement), all of which were heterozygous and one (R465C) of which was previously documented as inactivating (21,22,40). For one sample, there was an additional heterozygous insertion of 49 nucleotides in exon 8 that resulted in an early translation termination. As expected, all of the samples with FBW7 mutations were accompanied by wild type PEST sequence for NOTCH1 (Table 2). Thus, when combined with the 7 T-ALL specimens with PEST domain mutations, 12 of 47 (25%) samples exhibited disruptions of FBW7 function. Two of the samples with FBW7 mutations contained wild type NOTCH1, whereas three of the FBW7 mutations were accompanied by mutations in the HD domain of NOTCH1 (Table 2).

We found no statistically significant difference in the frequencies of NOTCH1 plus FBW7 mutations between the 23 patients who failed treatment (10 of 23) and the 24 patients who did not fail treatment (8 of 24) ($p=0.5556$).

Analysis of downstream gene targets of NOTCH1 as a measure of downstream signaling

To evaluate *overall NOTCH1 signaling* resulting from mutations in NOTCH1 and FBW7 genes as measures of possible gain of function or constitutive activity, real-time RT-PCR was used to measure transcript levels for HES1, DELTEX1, and cMYC, all documented NOTCH1 gene targets (20,23-25), in the 47 T-ALL specimens. Transcript levels for cMYC and DELTEX1 significantly correlated with HES1 transcripts [Spearman's correlation coefficient r= 0.5219 (cMYC) and 0.6829 (DELTEX1); p=0.0002 and p<0.0001, respectively (not shown)] over a 38-300-fold range of expression. Median transcript levels for HES1, DELTEX1, and cMYC were all increased in the NOTCH1/FBW7 mutant group over specimens expressing wild type NOTCH1/FBW7 (5.6-, 4-, and 1.9-fold, respectively); however, transcript levels were remarkably variable and appreciably overlapped. For HES1 and cMYC, differences between the mutant and wild type groups were statistically significant (p=0.0147 and 0.0102, respectively) (Figure 2).

For the 13 specimens with only NOTCH1 mutations, we showed similarly increased levels of HES1 (5.8-fold; p=0.0817), DELTEX1 (3.2-fold; p=0.2010), and cMYC (2.7-fold; p=0.0083) over the 29 specimens without either NOTCH1 or FBW7 mutations (data not shown). In contrast to our results for the HES1-luciferase reporter assays (Figure 1A), for samples identified as harboring NOTCH1 mutations, there was no difference in the patterns of expression for downstream target genes between samples with HD mutations alone, versus those with PEST mutations alone, or between samples with only one mutant domain versus those with mutations in both the HD and PEST domains (data not shown). Further, for individual NOTCH1 mutations, there was no consistent association between reporter activities and overall NOTCH1 signaling (as reflected in HES1/DELTEX1/cMYC transcripts). These results likely reflect contributions from other factors such as FBW7 in determining levels of overall NOTCH1 signaling, as noted above.

Since three of the samples with FBW7 mutations also contained NOTCH1 HD mutations (Table 2), it was possible to separate the impact of FBW7 mutations from that resulting from NOTCH1 mutations on overall signaling for only a very small number of samples. For the 5 samples with FBW7 mutations (with and without NOTCH1 mutations; see above), transcript levels of HES1 and DELTEX1 were increased (4.6- and 4-fold respectively) over samples with wild type FBW7, though these differences were not significant.

The impact of the clinically relevant FBW7 mutations on transactivation of a HES1 reporter (HES1-Luc) on top of that resulting from the clinically relevant NOTCH1 mutations (in samples 1, 2 and 12; Table 2) or wild type NOTCH1 (for samples 17 and 18) was further analyzed by reporter gene experiments (Figure 1B). Mutant FBW7 constructs in pcDNA3 were transiently transfected into U2OS cells with the HES1-Luc reporter, together with wild type or mutant NOTCH1 constructs, as appropriate. Whereas wild type FBW7 had minimal impact on HES1 transactivation with wild type NOTCH1, when tested in their clinically relevant contexts (Table 2) the FBW7 mutants augmented transactivation by both wild type and mutant NOTCH1 (1.4- to 1.6-fold). For FBW7 mutants 2 and 12, these increases were statistically significant (p=0.0359 and p=0.0443, respectively).

Thus, although levels of HES1/DELTEX1/cMYC transcripts in T-ALLs might be expected to be the most accurate measure of overall NOTCH1 signaling and reflect the impact of both NOTCH1 and FBW7 mutations along with other factors (e.g., NUMB), we found no significant difference in the distribution of HES1/DELTEX1/cMYC transcript levels (i.e., greater or less than the median value) between the group of patients who failed therapy and patients who did not [p=0.7683 (HES1), 0.5559 (DELTEX1), and 0.7683 (cMYC)].

Expression analysis of chemotherapy-related genes in primary T-ALLs and relationship to NOTCH1 signaling

We hypothesized that differences in prognostic value of NOTCH1 and FBW7 between studies (21,35,36,41,42) (including our own) may reflect the inclusion of T-ALL specimens from patients treated with different chemotherapy protocols and the possibility that aberrant NOTCH1 signaling may impact sensitivities to various chemotherapy drugs to different extents. We used real-time RT-PCR with our 47 patient T-ALL cohort to measure transcript levels for 22 gene targets most relevant to major drugs used to treat T-ALL, including asparaginase, doxorubicin, 6-mercaptopurine, methotrexate, corticosteroids, and vincristine. Genes of interest encoded drug transporters, drug metabolizing enzymes, drug targets, or apoptosis signaling proteins and included (a) ABCG2, (b) ABCC1, (c) ABCC2, (d) ABCC3, (e) ABCC4, (f) ABCC5, (g) asparagine synthetase, (h) B-cell leukemia/lymphoma 2 (BCL2), (i) B-cell leukemia/lymphoma X long isoform (BCL-XL), (j) dihydrofolate reductase, (k) folylpolyglutamate synthetase, (l) γ glutamyl hydrolase, (m) glucocorticoid receptor, (n) human reduced folate carrier, (o) hypoxanthine guanine phosphoribosyl transferase, (p) MDR1, (q) microtubule-associated protein 4, (r) thiopurine methyltransferase, (s) topoisomerase 2 α, (t) topoisomerase 2 β, (u) β tubulin class 1, and (v) β tubulin class 3.

For each of these genes, a broad range of transcript levels was detected, from slightly over 569-fold for ABCG2 to 6.7-fold for BCL-XL (Supplement, Tables S4-S6). When transcript levels for the individual genes were correlated with relative NOTCH1 signaling, as reflected in HES1, DELTEX1, and cMYC transcript levels, elevated transcripts for these established NOTCH1 gene targets were accompanied by consistent and statistically significant increases (1.5-3.0-fold) in transcript levels for MDR1, ABCC5, asparagine synthetase, Bcl-2, human reduced folate carrier, dihydrofolate reductase, and thiopurine methyltransferase (Figure 3; Supplement, Tables S4-S6). Folylpolyglutamate synthetase and hypoxanthine phosphoribosyltransferase were associated with elevated expression for 2 of the 3 established NOTCH1 targets (HES1 and DELTEX, and HES1 and cMYC, respectively). For the entire cohort of 47 T-ALL patients, transcript levels for none of these 22 genes were prognostic, in contrast to our previous findings in BP-ALL (43).

PTEN levels and mutations in primary T-ALL specimens

Since NOTCH1 has been reported to directly (activate, via CSL) (46) and indirectly (repress, via HES1 and cMYC) (32) regulate PTEN, we extended our analysis of our T-ALL cohort to include this important gene, given its likely relevance to clinical responses to therapy. We initially performed real-time RT-PCR analysis of PTEN transcript levels for the 47 T-ALLs. PTEN transcript levels spanned an 833-fold range. PTEN transcripts exhibited a

slight positive correlation (Spearman's analysis) with transcript levels of both HES1 (r=0.3507; p=0.0157) and cMYC (r=0.3840; p=0.0077).

For 43 samples for which there was sufficient RNA, the entire PTEN coding region was amplifed using primers in the 5′ and 3′ UTRs for direct sequencing with gene-specific primers. With a few samples, amplicons were subcloned into a T/A cloning vector and individual plasmid clones were sequenced. Altogether, PTEN mutations were detected in 27 of 43 specimens (Table S3, Supplement), 22 of which would result in truncated proteins (Table 2). Two of these contained a mutation in the ATG translation start. Eight of the 27 mutations were homozygous. The higher frequency of both heterozygous and homozygous PTEN mutations in our T-ALL cohort are different from results previously reported (32,33), but this may reflect inclusion of approximately 50% of patients who relapsed within 4 years in our study. Regardless of the NOTCH1 and FBW7 mutational status, loss of PTEN in these samples should result in increased AKT signaling and resistance to standard chemotherapy and GSIs (32). However, constitutively high AKT signaling may also occur independent of PTEN mutations due to inactivating posttranslational modifications of PTEN protein (47). Further, NOTCH1 may also activate mTOR independent of PTEN/PI3K/AKT (33).

For the 43 patients whose PTEN status was established, we found no significant difference in the frequency of PTEN mutations between the 22 patients who failed treatment (15 of 22 PTEN mutants) and 21 patients who did not fail treatment (12 of 21 PTEN mutants) (p=0.5365). With the 43 patients for whom all three genes were analyzed for mutations, there was no significant difference between the number of children who failed treatment and had any combination of PTEN, NOTCH1, and FBW7 mutations (15 of 22 patients), and those who harbored these mutations and did not relapse (12 of 21) (p=0.5365).

Conclusions

Our results suggest that multiple factors should be considered when attempting to identify molecularly-based prognostic factors for pediatric T-ALL. Our results further establish the presence of high frequency mutations in NOTCH1 and FBW7 in pediatric T-ALL. Mutant NOTCH1 was associated with a range of activating potentials, as reflected in activities from HES1 promoter-reporter gene assays that were consistently elevated over wild type NOTCH1. Although mutant NOTCH1 and mutant FBW7 were associated with increased HES1 promoter-reporter activities and increased transcript levels for the NOTCH1 target genes, HES1, DELTEX1, and cMYC, the range of transcripts was surprisingly broad and there was significant overlap between the mutant and wild type T-ALL samples. This appears to reflect the different transactivating potencies for the various NOTCH1 mutants and possibly other factors that impact overall NOTCH1 signaling [e.g., NUMB (17)]. Collectively, these results imply that, rather than simply scoring the mutant status of NOTCH1 and/or FBW7, overall signaling activity, as reflected in the cumulative transcript levels for these established NOTCH1 target genes, is likely to be far more meaningful to the biology and therapy of T-ALL.

In our analysis, neither the presence of NOTCH1 and/or FBW7 mutations nor relative HES1/DELTEX1/cMYC transcript levels were directly associated with treatment failure in

our pediatric cohort. Likewise, neither PTEN mutations alone, nor combinations of mutations in PTEN with NOTCH1 and FBW7 were prognostic.

Interestingly, elevated transcripts for the downstream NOTCH1 gene targets were accompanied by consistent and statistically significant increases in transcript levels for chemotherapy-related genes including MDR1, ABCC5, asparagine synthetase, Bcl-2, human reduced folate carrier, dihydrofolate reductase, and thiopurine methyltransferase. While the nature of these associations, including causal mechanisms, is not established, from these results, the net level of chemotherapy drug response would seem to reflect a composite phenotype, including an *increased sensitivity* to methotrexate due to increased human reduced folate carrier, and *increased resistance* for assorted chemotherapy agents due to increased Bcl-2 (multiple agents), MDR1 (doxorubicin, vincristine), ABCC5 (6-mercaptopurine, methotrexate), asparagine synthetase (L-asparaginase), and dihydrofolate reductase (methotrexate). Perhaps most importantly, the relative importance of these mechanisms would reflect the combinations of chemotherapy drugs administered, along with drug doses and schedule. Additional determinants of chemotherapy activity include PTEN levels, or the presence of inactivating PTEN mutations and potential downstream effects of NOTCH1 on PI3K-AKT and mTOR signaling pathways.

Additional studies are undoubtedly necessary to establish mechanisms (i.e., direct or indirect effects of NOTCH1 and downstream signaling) that result in altered expression of these drug resistance/sensitivity genes, along with studies to extend these results to other tumors with aberrant NOTCH1 signaling. Deregulated NOTCH signaling involving NOTCH receptors, ligands and targets has been also observed in solid tumors and high levels of NOTCH1 and Jagged1 ligand were associated with a poor prognosis in breast cancer (48) and metastasis in prostate cancer (49). Finally, an important implication of our results is that, depending on the NOTCH1 signaling status, modifications in the types or dosing of standard chemotherapy drugs for T-ALL, or combinations of agents capable of targeting NOTCH1 such as GSIs (50) or AKT and mTOR inhibitors, with standard chemotherapy agents may be warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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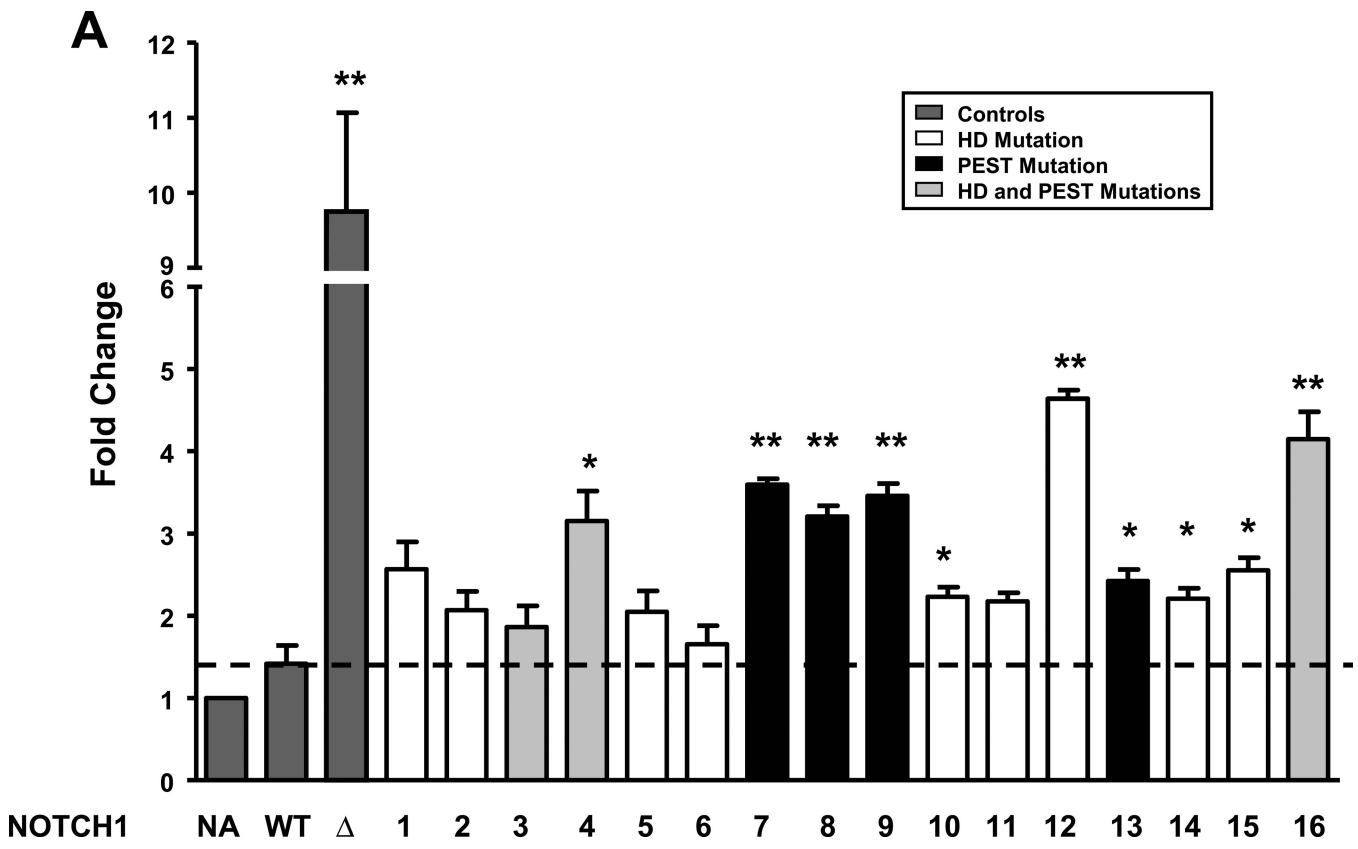

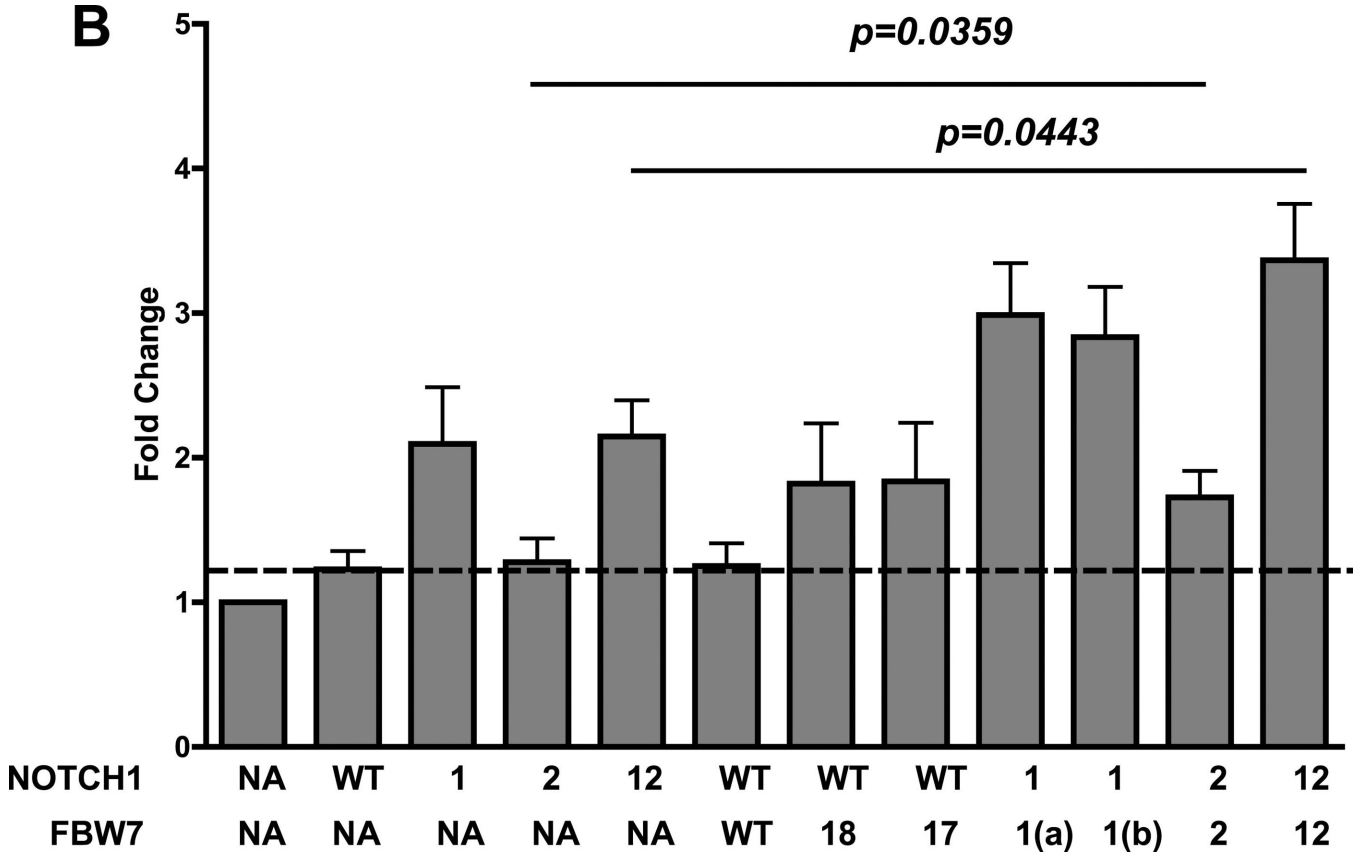

Figure 1. Potencies of clinically relevant NOTCH1 and FBW7 mutations, as measured by reporter gene assays

Human U2OS cells were transiently co-transfected in 35 mm dishes with 0.9 μg of the indicated NOTCH1 expression plasmid alone (A), or with 0.9 μg of both a NOTCH1 expression plasmid and FBW7 expression plasmid (B). For (A), 1 μg of HES1-Luc reporter gene construct and 30 ng of *Renilla* luciferase (pRL-SV40) internal control were used, whereas for (B), 500 ng HES1-Luc/30 ng pRL-SV40 were used. For all transfections, constant plasmid was maintained at 0.9 μg of pcDNA3 plasmid per well. Results represent normalized luciferase activities of whole cell lysates, relative to a control in which HES1-luc was co-transfected with 0.9 μg pcDNA3 vector in lieu of NOTCH1/FBW7 (assigned a value of 1). Results are presented as mean values ± standard errors [n=6 for (A); n=6 for (B)]. For (A), p-values were calculated using paired t tests, comparing the luciferase activities of the different NOTCH1 mutations to wild-type NOTCH1 (*, $p \leq 0.05$; **, $p \leq 0.005$). For (B), p-values were calculated using paired t-tests, comparing the clinically relevant NOTCH1 and FBW7 mutants as shown in the figure. For (A), the sample numbers designate the patient samples listed in Table 2. For (B), NOTCH1 and FBW7 forms refer to the sample numbers in Table 2. For sample 1, (a) is the early termination at position 322 and (b) is R465L. Abbreviations are: Wt, wild type; Δ, ICN (activated NOTCH1); NA, no addition.

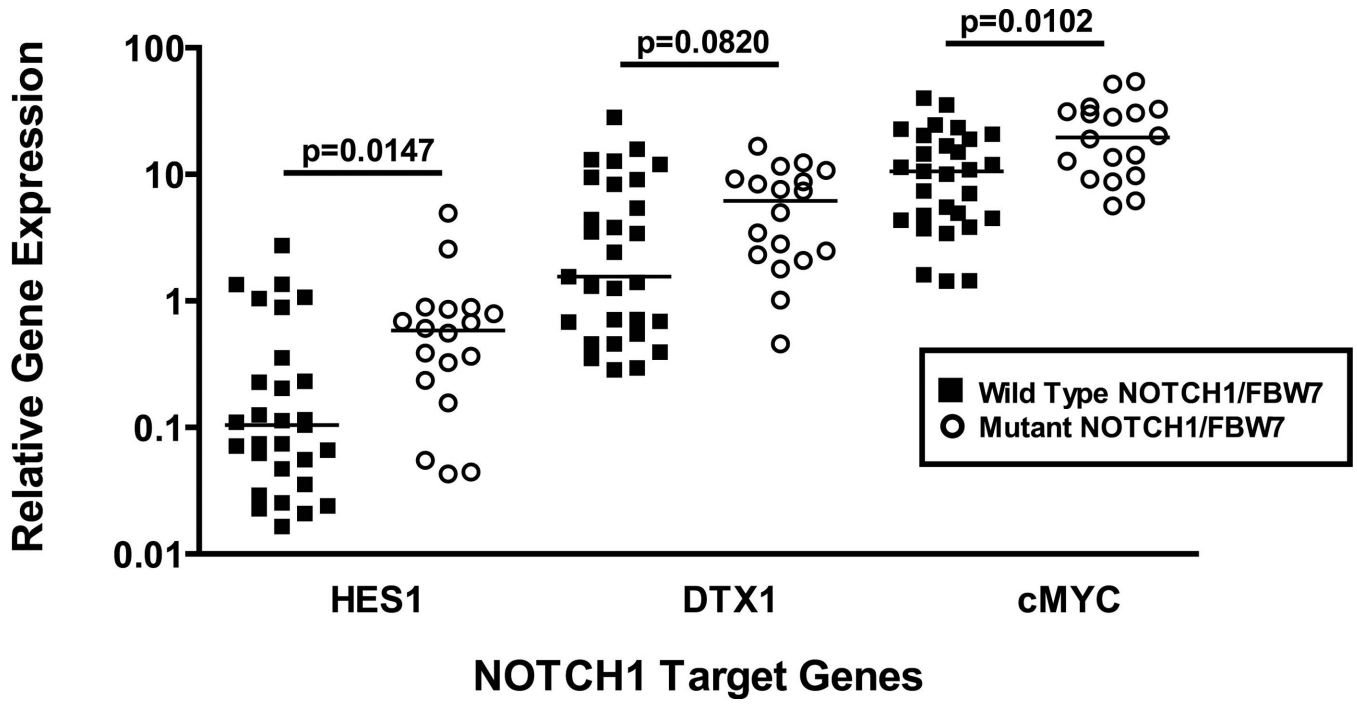

Figure 2. Expression of HES1, DELTEX1 and cMYC transcripts in patients harboring NOTCH1 and/or FBW7 mutations

Transcript levels were measured using real-time RT-PCR and normalized to those for GAPDH. Primers and PCR conditions are summarized in the Supplement (Table S2). Results are shown for HES1, DELTEX1, and cMYC transcript levels in T-ALL specimens exhibiting NOTCH1 and/or FBW7 mutations and T-ALL specimens characterized by wild type NOTCH1 and FBW7. Data were were analyzed using the non-parametric Wilcoxon test. Horizontal bars represent median values. Abbreviations: DTX, DELTEX.

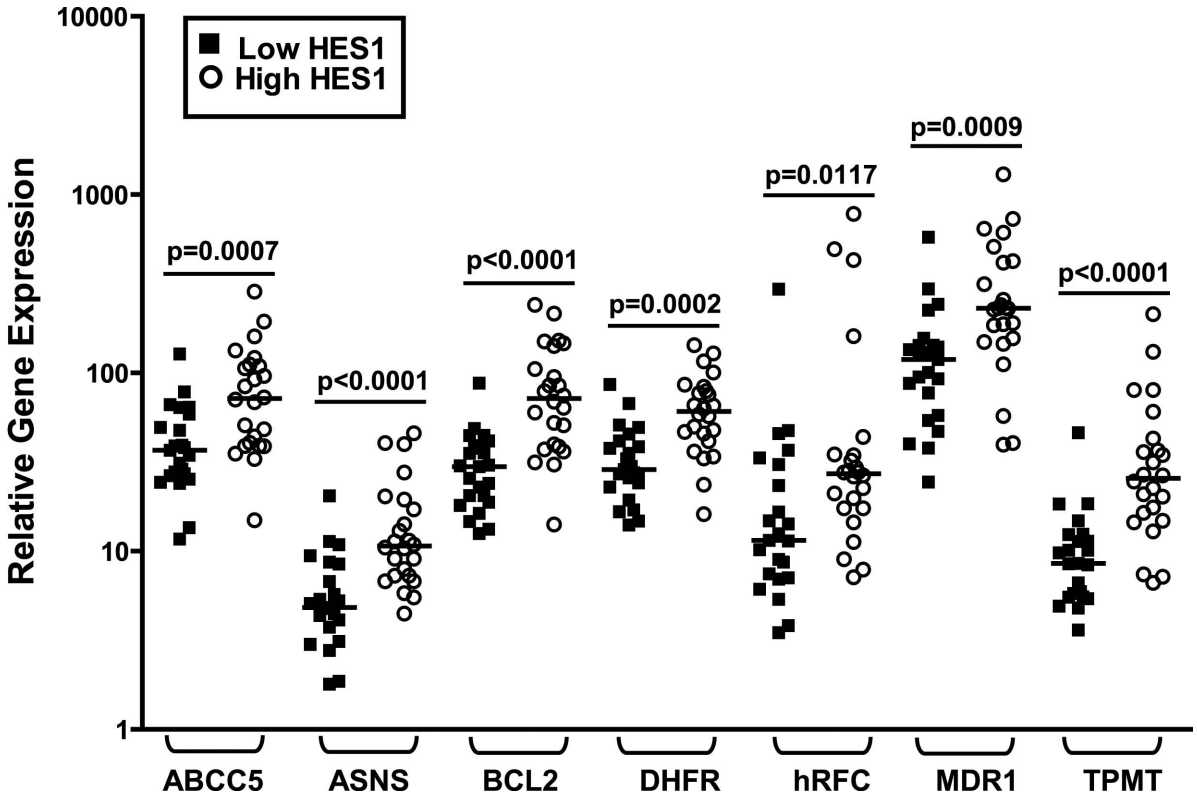

Figure 3. Expression of relevant chemotherapy genes in relation to HES1

Patients with HES1 transcript levels (Figure 2) below the median value were considered to have low HES1 expression, and those with HES1 transcript expression above HES1 median values were considered to have high HES1 expression. Relative transcript levels for 22 chemotherapy-related genes were measured by real-time RT-PCR, as described in the Materials and Methods section, and are summarized in Table S4 (Supplement). Horizontal bars represent median values. Twelve of 22 genes were significantly over-expressed in samples with high HES1 transcripts ($p<0.05$ by non-parametric Wilcoxon test) (Table S4, Supplement) and of these, the 7 gene targets in the figure also showed a statistically significant association with levels of DELTEX1 and cMYC transcripts (Tables S5 and S6, Supplement). Abbreviations: ABCC5, Multidrug resistance-associated protein 5 (MRP5); ASNS, asparagine synthetase; BCL2, B-cell leukemia/lymphoma 2; DHFR, dihydrofolate reductase; hRFC, human reduced folate carrier; MDR1, multidrug resistance 1; TPMT, thiopurine-S-methyltransferase.

Table 1

Patient characteristics for T-ALL cohort

Gender	Age (y)						WBC (× 10^9 cells/L)					
	N	Minimum	Median	Mean	SD	Maximum	*N*	Minimum	Median	Mean	SD	Maximum
Male	38	1.83	7.48	8.66	4.86	19.86	38	8.20	267.50	335.86	262.81	999.90
Female	9	4.13	6.45	6.49	2.26	11.62	9	20.00	171.80	242.77	224.12	680.00
Total	47	1.83	7.15	8.24	4.54	19.86	47	8.20	240.00	318.03	256.25	999.90

SD, Standard deviation.

Table 2

Summary of amino acid sequence changes in NOTCH1, FBW7 and PTEN resulting from mutations in primary T-ALLs

Sample	NOTCH1	FBW7	PTEN
1	INS1609(PQP); A1610T	Stop@322; R465L	N/A
2	R1633L	R465C	G39F
3	G1654S; P2514R; E2515V; S2516P; 2517*	WT	S170T; L181M
4	R1662L; Q2503*	WT	No Translational Start
5	V1671I	WT	C71W; A72G; E73I; R74*
6	A1650T	WT	Δ27-46(CIHFCGCSSLPFCHSLRTWE): N48*
7	Q2459*STOP	WT	Q245Y; P246Q; L247F; V249M; C250F; G251L; D252V; I253W; K254*
8	E2506D; H2507L; P2508L; F2509P; L2510P; T2511*	WT	Δ233-253 (EEKTSSCTLSSLSRYLCVVIS); V255*
9	2511*	WT	WT
10	V1671I	WT	R55S; DEL 56-70 (FLDSKHKNHYKIYNL); C250W; G251E; D252K; I253F
11	G1659D	WT	N82R; C83*
12	DEL 1676(V)	R505S	WT
13	A2441V; P2514R; E2515V; S2516P; P2517*	WT	N/A
14	A1634S	WT	C71W; A72G; E73I; R74*
15	INS1588(SFHFLPRLPHNS)	WT	No Translational Start
16	INS1588(SFHFLPRLPHNS); S2451*	WT	N/A
17	WT	R465L	WT
18	WT	R465C	WT
19	WT	WT	Y27C; Δ30-41 (FCGCSSLPFCHS) 43-46 (RTWE); R47*
20	WT	WT	INS 71 (SWSYQGTANHTDI); G306A; S307G; L308R; A309*
21	WT	WT	F56*
22	WT	WT	S10I; Δ165-170 (ILQEVF) 172-177 (IKALLS); Y178*
23	WT	WT	R233L; Δ235-249 (RKTSSCTLSSLSRYL) 251-252 (VV) 254-255(SK); E256*
24	WT	WT	L247E; Δ249-257 (LYLCVVISK); F258*
25	WT	WT	Δ246-249 (TGYL) 251-252 (VV) 254-255 (SK); E256*
26	WT	WT	R189T; P246Q; INS 246 (NRE)
27	WT	WT	DEL 88-327
28	WT	WT	Δ27-32 (LYLTRH); I33*
29	WT	WT	Y27C; Δ29-41 (HFCGCSSLPFCAS) 43-46 (RTWE); R47*
30	WT	WT	G165I; V166L; T167S; I168L; P169W; Q171A; R172S; R173*
31	WT	WT	Δ143-228 (QVYGKTSSCTLSSLSRYLCVVRYQSRVLPQTEQDAK) 230-239 (GQNVSLLGKY) 241-248 (LHTRTRGNLRKSRKWKSM); V249*

Sample	NOTCH1	FBW7	PTEN
32	WT	WT	Δ293-201 (ETGRQVHVL); T202*
33	WT	WT	G165I; V166L; T167S; I168L; P169W; Q171A; R172S; R173*
34	WT	WT	Δ216-222 (TLLWSAS); K223*

Nucleotide mutations were identified by PCR amplification of cDNAs or genomic DNAs across the HD and PEST domains for NOTCH1, across exons 8-14 for FBW7, or the entire PTEN coding region. PCR products were either sequenced directly or subcloned into a TA-cloning vector. Nucleotide sequence changes are shown in Table S3 (Supplement). The positions of amino acid sequence changes in NOTCH1 mutations are based on NM_017617.3, those in FBW7 were based on NM_033632.2 (isoform 1), NM_018315.4 (isoform 2) and NM_001013415.1 (isoform 3), and those in PTEN were based on NM_000314.4. Abbreviations are: HD, Heterodimerization; INS, Insertion; DEL, Deletion; *, Stop codon; N/A, Not Available; Δ, changed sequence