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Recurrent *NR3C1* Aberrations at First Diagnosis Relate to Steroid Resistance in Pediatric T-Cell Acute Lymphoblastic Leukemia Patients

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Abstract

The glucocorticoid receptor NR3C1 is essential for steroid-induced apoptosis, and deletions of this gene have been recurrently identified at disease relapse for acute lymphoblastic leukemia (ALL) patients. Here, we demonstrate that recurrent NR3C1 inactivating aberrations—including deletions, missense, and nonsense mutations—are identified in 7% of pediatric T-cell ALL patients at diagnosis. These aberrations are frequently present in early thymic progenitor-ALL patients and relate to steroid resistance. Functional modeling of NR3C1 aberrations in pre-B ALL and T-cell ALL cell lines demonstrate that aberrations decreasing NR3C1 expression are important contributors to steroid resistance at disease diagnosis. Relative *NR3C1* messenger RNA expression in primary diagnostic patient samples, however, does not correlate with steroid response.

Introduction

Synthetic steroids, also denoted as glucocorticoids, remain cornerstone chemotherapeutic drugs in the treatment of acute lymphoblastic leukemia (ALL). Insufficient response to glucocorticoids during induction therapy remains an important predictor for inferior outcome in pediatric ALL, particularly for T-cell ALL (T-ALL).[1,2] Activation of the glucocorticoid receptor (NR3C1) leads to its cytoplasmic-to-nuclear translocation, facilitating its function as a transcription factor resulting in cell death.[3] Steroid treatment may therefore provide selective pressure of leukemic cells to acquire genetic events that reduce a functional steroid response resulting in therapy failure and relapse, as was recently demonstrated in an elegant T-ALL mouse model.[4] An alternative underlying mechanism may include inhibitory phosphorylation of NR3C1 that impairs its nuclear localization and transactivation potential to activate important downstream target genes including *BIM* and *NR3C1* itself.[5,6] Low NR3C1 levels, the failure to upregulate *NR3C1* as a positive feedback loop following steroid exposure or epigenetic silencing of *BIM* have all been linked to steroid resistance.[7,8] Inactivation of NR3C1 by mutations or entire gene deletions have also been identified in leukemic cell lines[9] and are reported in about 6% of ALL patients at relapse.[10-12] In particular, over 16% of ETV6-RUNX1-positive pre-B ALL patients have acquired *NR3C1* deletions at relapse.[13] Point mutations or small insertion/deletion mutations in *Nr3c1* were also identified in 18% of dexamethasone-resistant relapsed samples in the previously mentioned T-ALL mouse model.[4]

In our previous integrated study on pediatric T-ALL patients, where we combined genetic data with clinical data including outcome and therapy response of diagnostic biopsies, activating interleukin-7 receptor (IL7R) signaling mutations were identified as an important contributor to steroid resistance.[14] In addition, physiological IL7R signaling can also raise cellular steroid resistance.[15] Here, we observe that *NR3C1* aberrations are already recurrently present in pediatric T-ALL as early as at first diagnosis. Moreover, we confirm that NR3C1 abnormalities relate to steroid resistance and may facilitate disease selection under first-line induction therapy resulting in relapse.

Materials and methods

Patient samples

Primary diagnostic bone marrow or peripheral blood samples were used from a total of 146 primary pediatric T-ALL patients in this study: 72 enrolled on the Dutch Childhood Oncology Group (DCOG) protocols ALL-7/8 (n = 30)[16,17] or ALL-9 (n = 42)[18] and 74 patients enrolled on the German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-97, n = 74)[19] with a median follow up of 67 and 52 months, respectively. The patients' parents or legal guardians provided informed consent to use leftover diagnostic material for research in accordance with the Institutional Review Board of the Erasmus MC Rotterdam and the Declaration of Helsinki. Leukemia cells were harvested from blood or bone marrow samples and enriched to a purity of at least 90%.[16-19] Screening for *NR3C1* mutations and loss of heterozygosity (LOH) was performed by Ion Torrent sequencing, array-based comparative genomic hybridization, and/or multiplex ligation-dependent probe amplification analyses as previously described.[14] Transcriptional *NR3C1* expression of primary patient material was determined using U133plus 2.0 arrays. Prednisolone LC50 was determined by performing a 4-day MTT-assay, with the prednisolone concentration ranging from 0.008 to 250 µg/mL.

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Constructs and transduction

For REH cells, gateway multisite recombination (Invitrogen) was used for gateway cloning of lentiviral expression vectors by using a gateway-adapted lentiviral pLEGO-iC2 destination vector (Addgene) as previously described.[14] The entry vectors existed of (1) *attL1/attR5*-flanked doxycycline-inducible promoter (third generation; Clontech); (2) *attL5/attL4*-NR3C1 (wild-type or mutant) complementary DNA sequence; (3) *attR4/attR3*-flanked DDK-tag followed by a stop codon, Woodchuck hepatitis virus Posttranscriptional Regulatory Element sequence, and a constitutive spleen focus forming virus promotor promoter; and (4) Tetracycline (doxycycline)-induced transcriptional activator protein-Thosea asigna virus 2A-truncated Nerve Growth Factor Receptor reporter. For the short hairpin RNA (shRNA) experiments in REH and SUP-T1 cells, lentiviral transduction was performed with PLKO.1-puro lentiviral shRNA constructs directed against the human *NR3C1* gene that were selected from the MISSION T shRNA Library (Sigma-Aldrich).

Western blot

Protein extraction and subsequent blotting procedure on REH or SUP-T1 cells were performed as previously described.[14] Primary antibodies used for western blotting: DKDDDDDK Tag (Cell Signaling Technologies, number 2368) and β-actin (number ab6276).

Real-time quantitative polymerase chain reaction

RNA was isolated with TRIzol reagent (Thermo Fisher Scientific). Real-time quantitative polymerase chain reaction were performed by using the DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific) under 1X conditions, supplemented with 4 mM magnesium chloride using the CFX384 Touch-Time polymerase chain reaction Detection System (Bio-Rad). Expression levels were calculated relative to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) household gene. Primers used are *GAPDH* Fw primer 5′-GTCGGAGTCAACGGATT-3′, *GAPDH* Rev primer 5′-AAGCTTCCCGTTCTCAG-3′, *GILZ* Fw primer 5′-TGGCCATAGACAACAAGAT-3′, *GILZ* Rev primer 5′-TTGCCAGGGTCTTCAA-3′, *FKBP5* Fw primer 5′-GAATGGTGAGGAAACGC-3′, *FKPB5* Rev primer 5′-ATGCCTCCATCTTCAAATAA-3′, *SGK1* Fw primer 5′-GGAGCCTGAGCTTATGAAT-3′, and *SGK1* Rev primer 5′-TTCCGCTCCGACATAATA-3′.

Statistical tests

Associations between NR3C1 mutational/deletion status and transcriptional *NR3C1* expression or in vitro prednisolone LC50 were calculated by using the Mann-Whitney *U* test. Survival analysis for relapse-free survival in our patient cohort was calculated using the log-rank (Mantel-Cox) test. For all tests, $P < 0.05$ was used as significance level.

Results

Recurrent *NR3C1* aberrations in T-ALL patients at diagnosis

In our initial patient cohort for which we combined results from sequencing and LOH analyses,[14] we identified 6 out of 69 (9%) diagnostic patient biopsies that harbor *NR3C1* deletions (n = 4) or carried heterozygous missense mutations (n = 2; G323V and K777N). To substantiate these findings, we screened an additional cohort of 77 diagnostic pediatric T-ALL patient samples (totaling 146 patients) for *NR3C1* mutations and/or LOH using Ion Torrent sequencing, array-based comparative genomic hybridization, and/or multiplex ligation-dependent probe amplification analyses. We identified 2 additional patients with *NR3C1* deletions and 3 patients who harbored *NR3C1* mutations. A heterozygous single nucleotide insertion resulting in a premature frameshift in the protein coding domain (E116fs) was identified in 1 patient, whereas a second patient had a heterozygous G568W missense mutation. The third patient had acquired 3 mutations including 2 missense mutations (N130D and R386L) and a nonsense mutation (G371X) that truncates the NR3C1 protein before the DNA binding and ligand-binding domains. Thus, we identified 6 patients having *NR3C1* deletions (4%) and 5 other patients with newly identified missense or nonsense *NR3C1* mutations (3%) in a total of 146 diagnostic T-ALL patient samples (Figure 1 and Supplemental Table 1, http://links.lww.com/HS/A123).

Four out of 6 *NR3C1*-deleted patients were classified as early thymic progenitor acute lymphoblastic leukemia (ETP-ALL) based on unsupervised cluster analysis of gene expression data.[20] These *NR3C1* deletions occurred in the context of larger 5q deletion (Figure 2A), which is consistent with the observation that ETP-ALL patients frequently harbor interstitial or terminal 5q deletions.[21] One patient (number 1944) had a *TLX1* translocation and co-clustered with the TLX cluster, and in contrast to ETP-ALL patients had a relative small deletion on 5q that includes the *NR3C1* locus. For 1 patient, its cluster had not been determined.

In relation to outcome, none of the *NR3C1* deleted patients relapsed, while 3 out of 5 patients with a *NR3C1* mutation relapsed. In addition to small patient numbers, no significant difference in survival was observed compared to patients that lack *NR3C1* aberrations (Figure 2B). As a consequence of *NR3C1* deletions, *NR3C1* expression levels were significantly lower for *NR3C1* deleted patients compared to wild-type

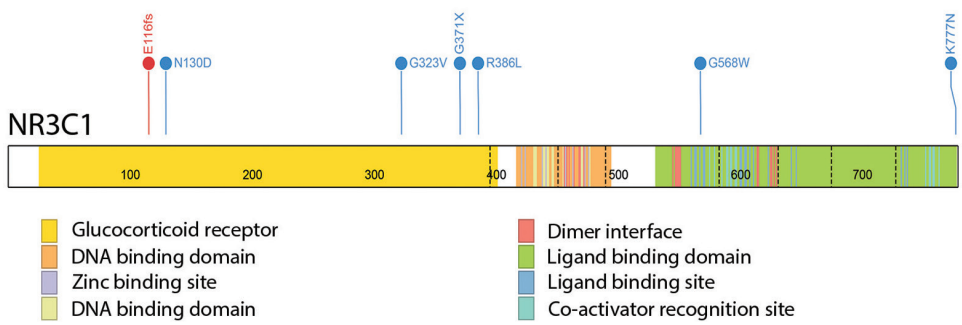

Figure 1. Schematic overview of the NR3C1 protein. Missense mutations (blue) and frameshift (red) mutations, as found in our cohort of 146 T-ALL patients, have been indicated. T-ALL = T-cell acute lymphoblastic leukemia.

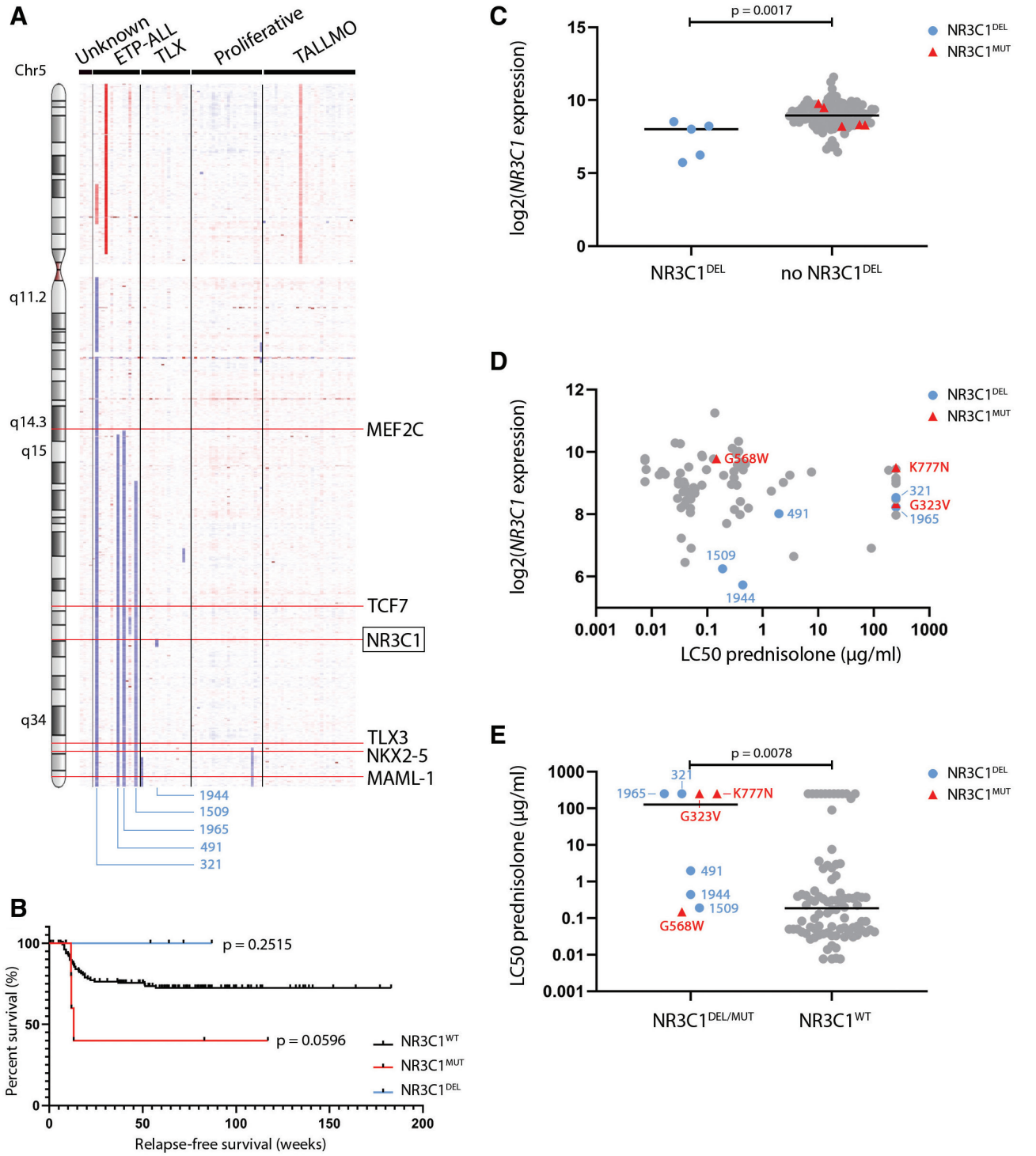

Figure 2. Characteristics and steroid response of NR3C1 aberrations in primary T-ALL patient samples. (A), Array-CGH data of 92 diagnostic biopsies from pediatric T-ALL patients. Patients are horizontally orientated and T-ALL subtypes are indicated as determined by gene expression profiling. Deleted regions of the 5 NR3C1-deleted patients detected by Array-CGH are characterized by the larger blue lesions around the 5q31 locus. (B), Kaplan-Meier survival analysis of 146 T-ALL patients who have been treated with different treatment protocols (Supplemental Table 1, http://links.lww.com/HS/A123). The survival for either *NR3C1* deleted or *NR3C1* mutated patients was compared to patients that lack *NR3C1* aberrations and analyzed using the log-rank (Mantel-Cox) test. (C), Median *NR3C1* expression of 116 diagnostic biopsies from pediatric T-ALL patients determined by U133plus 2 arrays. NR3C1 deleted cases (blue dots) had significant lower *NR3C1* expression levels compared to non-NR3C1 deleted cases (including NR3C1 mutated patients highlighted by red triangles) (Mann-Whitney *U* test, $P = 0.0017$). (D), Matched *NR3C1* expression data to in vitro prednisolone response of 83 diagnostic biopsies from pediatric T-ALL patients. (E), In vitro prednisolone response of 96 diagnostic biopsies from pediatric T-ALL patients. Patients that harbor NR3C1 aberrations (deletion [blue dots] or mutation [red triangles]) were significantly more resistant to prednisolone compared to NR3C1 wild-type patients (Mann-Whitney *U* test, $P = 0.0078$). CGH = array-based comparative genomic hybridization; ETP-ALL = early thymic progenitor acute lymphoblastic leukemia; T-ALL = T-cell acute lymphoblastic leukemia.

patients (Figure 2C, $P = 0.0017$). Interestingly, no correlation was found between the relative *NR3C1* expression and in vitro steroid response as measured for 83 treatment-naïve patient samples (Figure 2D). This indicates that relative basal *NR3C1* messenger RNA (mRNA) expression levels in patient biopsies are not predictive for steroid responsiveness of primary T-ALL patients at diagnosis. This is in line with previous observations that steroid responsiveness is largely determined by the ability to upregulate steroid response genes including *NR3C1* itself and pro-apoptotic *BIM* following steroid exposure.[22,23] Therefore, steroid responsiveness seems independent of a certain *NR3C1* expression threshold. Upon measuring actual steroid-induced cytotoxicity, we found that patients with *NR3C1* aberrations overall had a significantly inferior in vitro steroid response compared to *NR3C1* wild-type patients (Figure 2E, $P = 0.0078$). Four *NR3C1*-aberrant patients were completely resistant in vitro, and 4 had an intermediate in vitro response to prednisolone.

NR3C1 levels determine steroid response levels

To further investigate the relationship between NR3C1 expression and steroid responsiveness, we used the REH cell line that lacks expression of a functional glucocorticoid receptor.[24] For this, we stably transduced REH cells with a doxycycline-inducible *NR3C1* expression construct (denoted as REH^{NR3C1}). Restoration of wild-type NR3C1 expression following doxycycline induction effectuated a highly sensitive prednisolone response in REH^{NR3C1} cells (Figure 3A). To explore whether reductions in NR3C1 levels would proportionally diminish the steroid response, we knocked-down NR3C1 expression in REH^{NR3C1} cells by introducing 4 isopropyl β- d-1-thiogalactopyranoside (IPTG)-inducible *NR3C1*-directed shRNA lentiviral constructs. Partial knockdown of NR3C1 upon IPTG treatment in doxycycline-induced REH^{NR3C1} cells reduced the sensitivity to prednisolone treatment. For these derivative REH^{NR3C1} lines, knockdown by short hairpin constructs led to pronounced NR3C1 knockdown and steroid insensitivity (Figure 3, B and C). As a control, no reduction in steroid cytotoxicity was observed for REH^{NR3C1} cells that were transduced with shRNA construct (sh5) directed against the 3′UTR of the normal *NR3C1* gene, which was absent in the lentiviral *NR3C1* expression construct.

To further visualize the relationship between NR3C1 protein level and steroid sensitivity in a T-ALL context, we transduced steroid-sensitive SUP-T1 T-ALL cells with *NR3C1*-directed

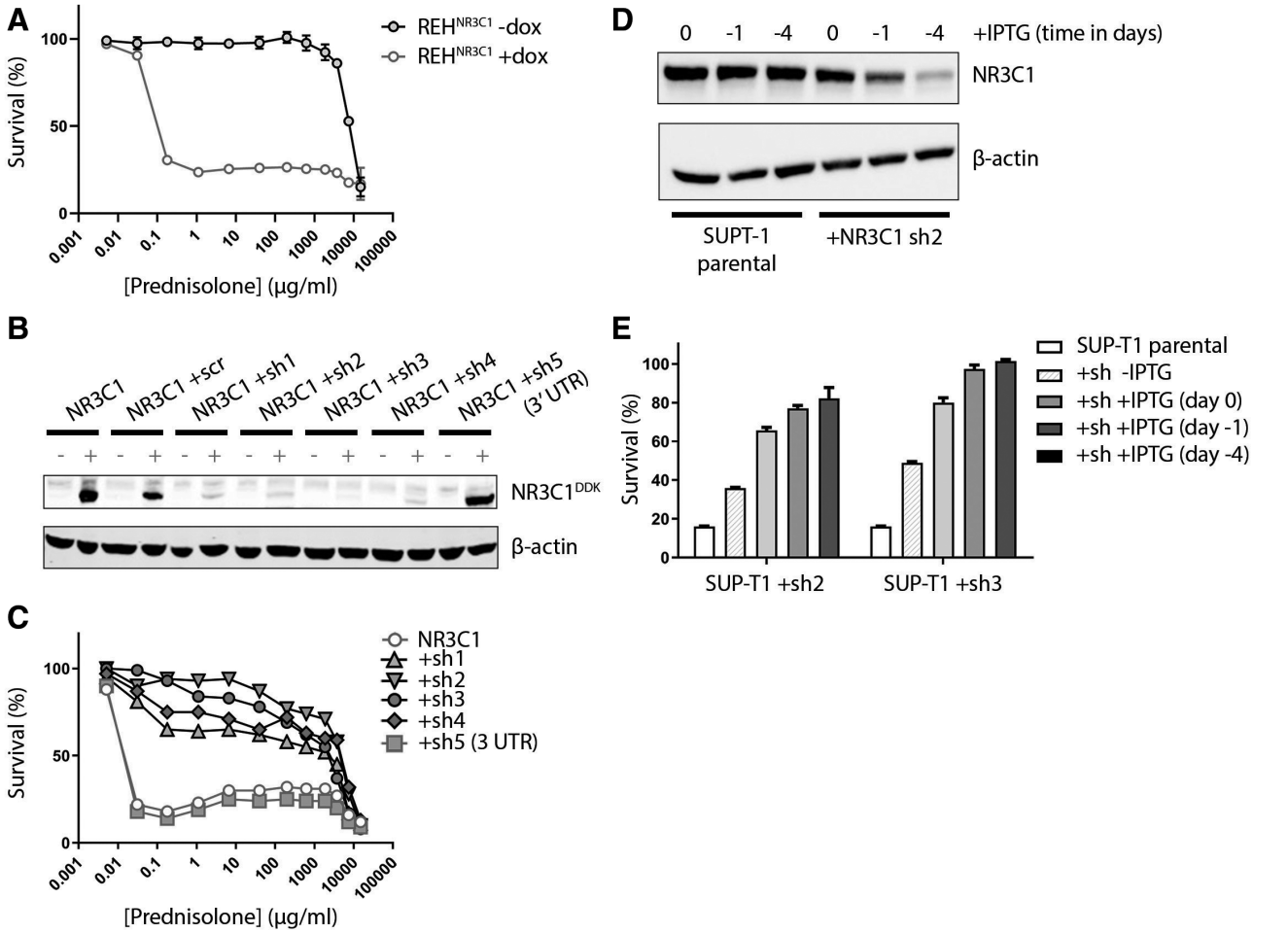

Figure 3. NR3C1 expression levels predict steroid response in REH cells. (A), Steroid response curves for doxycycline-induced (+dox) or noninduced (–dox) steroid-resistant REH cells following 96 h exposure to serial dilutions of prednisolone. Cell survival was determined by flow cytometry measured cell counts. REH cells were transfected with a doxycycline-inducible *NR3C1* wild-type construct. (B) and (C), Steroid response curves and NR3C1 protein levels and steroid response curves of NR3C1-REH cells (eg, doxycycline-induced REH NR3C1 wild-type cells) that have been transduced with *NR3C1*-directed lentiviral shRNA constructs (sh1-4). Control shRNA construct sh5 is directed against the 3′UTR of *NR3C1*, which is not included in the *NR3C1* expression construct. (D) and (E), Steroid response curves and (endogenous or short hairpin reduced) NR3C1 protein levels of steroid-sensitive SUP-T1 cells. shRNA constructs were induced by IPTG 4 d or 1 d prior to—or at the start (day 0) of steroid treatment. Steroid response was determined after 96 h by flow cytometry measured cell counts. IPTG = isopropyl β- d-1-thiogalactopyranoside; shRNA = short hairpin RNA.

shRNA lentiviral constructs sh2 or sh3. Timed activation of these shRNA constructs by addition of IPTG (eg, 4 or 1 d prior or at the start of prednisolone treatment) demonstrated that a gradual increase in NR3C1 knockdown augmented steroid resistance (Figure 3, D and E). These results highlight an interdependency between NR3C1 expression and steroid response within a specific cellular context.

NR3C1 mutations and steroid response

We then used the same REH model to study the functional consequences of the identified *NR3C1* missense (K777N, G323V, or N130D) or G371X nonsense mutation on steroid responsiveness. REH cells were stably transduced with either of these mutant *NR3C1* expression constructs, resulting in expression of mutant NR3C1 receptors following doxycycline induction (Figure 4A). Using these bulk transduced lines, expression of K777N, G323V, or N130D mutant molecules elicited an efficient transcriptional steroid response, as demonstrated by the upregulation of specific NR3C1 target genes such as *GILZ*, *FKBP5*, and *SGK1* after steroid treatment (Figure 4B). Moreover, the functionally of these mutations was reflected by their steroid responsiveness since these mutations were equally sensitive steroid response compared to REH^{NR3C1} cells (Figure 4C). As expected, expression of the truncating G371X mutant was totally ineffective to restore a steroid response (Figure 4, B and C).

Discussion

In this study, we found that relative *NR3C1* mRNA expression levels in primary diagnostic patient samples do not correlate with steroid response. Interindividual differences in other factors that contribute to steroid resistance may influence the steroid response at diagnosis. For example, IL7R signaling mutations, epigenetic silencing of the *BIM* locus, or overexpression of BCL2 have all been associated with steroid resistance.[8,14,25-27] Thus, the relative *NR3C1* mRNA level is not useful as a predictive biomarker for clinical steroid responsiveness for patients at diagnosis per se. However, for experiments performed within a specific and controlled cellular context such as REH or SUP-T1 cells, we observed a strong interdependency between NR3C1 levels and steroid response levels, in line with previous findings by others.[4,28]

Loss of NR3C1 expression due to mutations, deletions, or other mechanisms that occur under the pressure of steroid treatment[4] explain the relative increased incidence of *NR3C1* deletions and mutations at disease relapse.[10,11,13] However, our study demonstrates that heterozygous *NR3C1* deletions or truncating mutations are already present in diagnostic biopsies of approximately 5% of pediatric T-ALL patients and associate with steroid resistance. This may suggest that natural steroid hormones that normally shape the immune system[29,30] already elicit a strong selection pressure on (pre)leukemic cells before actual diagnosis of disease.

We identified *NR3C1* missense mutations in 4 T-ALL patients (including 1 patient that also harbored a truncating mutation). The missense mutations tested in REH cells demonstrated an efficient transcriptional steroid response and seemed equally effective as the wild-type NR3C1 molecule in effectuating steroid-induced apoptosis. This is in contrast to a recent functional screening using a similar experimental approach in REH cells, demonstrating that (distinct) relapse-enriched *NR3C1* mutations in ALL did confer steroid resistance.[31] This indicates that *NR3C1* missense mutations found at relapse are selected during therapy and cause resistance towards synthetic steroids, while mutations at diagnosis do not necessarily drive steroid resistance per se. The N130D mutation co-occurred with the truncating G371X mutation in one of our patients and may

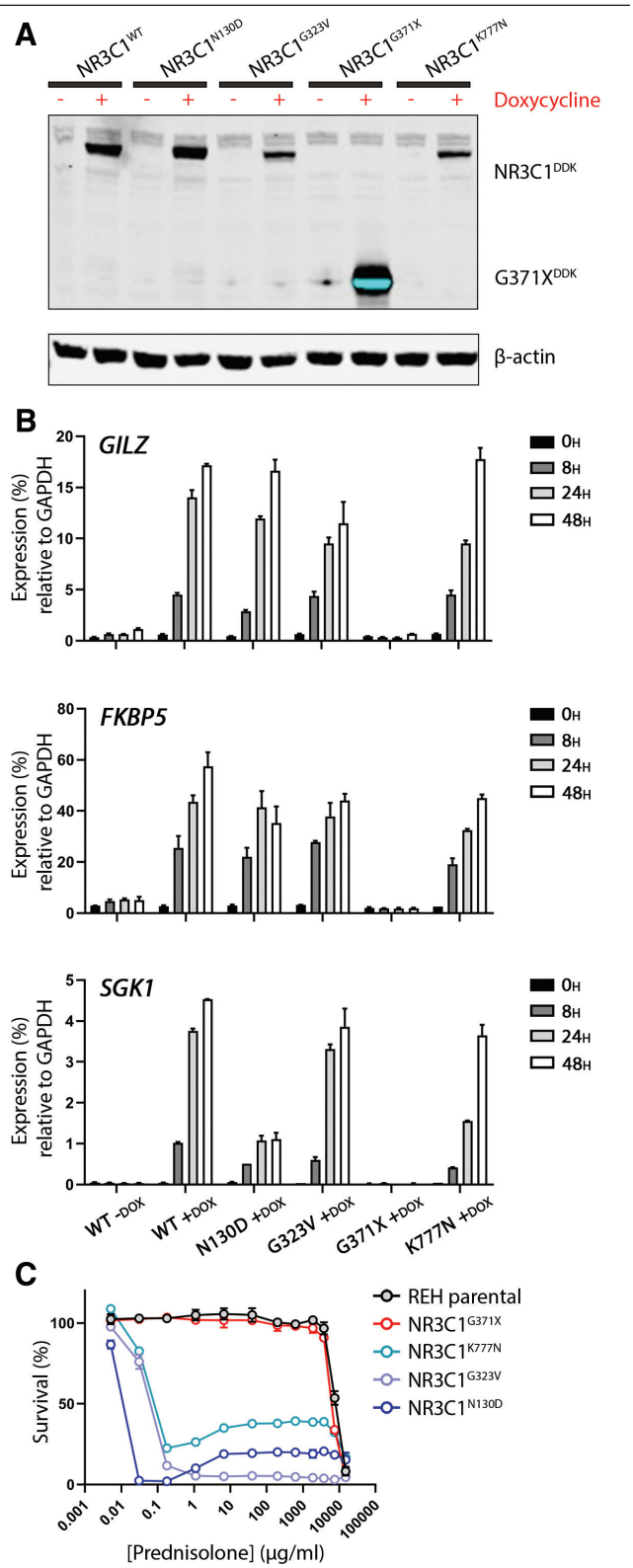

Figure 4. Nontruncated NR3C1 missense mutations induce efficient steroid-induced cell death. (A), Western blot results of total NR3C1 levels for REH cell lines transfected with doxycycline-inducible *NR3C1* or mutant *NR3C1*-constructs as indicated. (B), Transcriptional steroid response of REH cells that have been transfected with doxycycline-inducible WT or mutant *NR3C1* constructs. Doxycycline-induced cells were treated with 250 µg/mL prednisolone. Expression of glucocorticoid receptor target genes *GILZ*, *FKBP5*, and *SGK1* was determined at 8, 24, and 48 h following prednisolone treatment. (C), Steroid response curves of REH parental and REH cells transfected with doxycycline-inducible mutant *NR3C1* constructs. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; WT = wild-type.

therefore represent a passenger mutation rather than reflecting a steroid resistance driving mutation.

The steroid-sensitive phenotype of our mutant NR3C1 overexpressing cells somewhat contradict the intermediate or steroid-resistant phenotype of corresponding primary patient blasts. Since induced expression of mutant NR3C1 molecules exceeds the physiological level as normally expressed in ALL blasts, we cannot exclude the possibility that subtle effects of the studied NR3C1 mutations on steroid responsiveness are masked. This may be exemplified by the T-ALL patient harboring the K777N mutation since this mutation was also preserved at relapse. In contrast to the controlled context of REH cells, many other factors are influential in the steroid sensitivity of leukemic blasts. The presence of mutant NR3C1 may therefore synergize with other (epi)genetic aberrations at disease diagnosis. Regardless of a supporting or causal role, the presence of *NR3C1* mutations related to steroid resistance in our cohort, and 3 out of 4 patients with missense *NR3C1* mutations relapsed during therapy.

In conclusion, approximately 7% of patients that are diagnosed with T-ALL already harbor NR3C1 inactivating events that influence their leukemic response to steroid treatment.

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