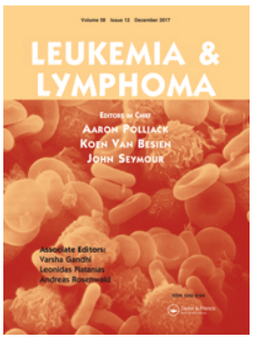

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MEF2C-dysregulated pediatric T-cell acute lymphoblastic leukemia is associated with *CDKN1B* deletions and a poor response to glucocorticoid therapy

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ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disease in which multiple genetic abnormalities cooperate in the malignant transformation of T-lymphoid progenitors. Although in pediatric T-ALL, *CDKN1B* deletions occur in about 12% of the cases and represent one of the most frequent copy number alterations, neither their association with other genetic alterations nor the clinical characteristics of these patients have been determined yet. In this study, we show that loss of *CDKN1B* increased the prevalence of cell cycle regulator defects in immature T-ALL, usually only ascribed to *CDKN2A/B* deletions, and that *CDKN1B* deletions frequently coincide with expression of *MEF2C*, considered as one of the driving oncogenes in immature early T-cell precursor (ETP) ALL. However, *MEF2C*-dysregulation was only partially associated with the immunophenotypic characteristics used to define ETP-ALL. Furthermore, *MEF2C* expression levels were significantly associated with or may even be predictive of the response to glucocorticoid treatment.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic disorder resulting from the malignant transformation of T-lymphoid progenitors and accounts for 10–15% of pediatric ALL [1]. Originally associated with a poor prognosis, the currently employed intensive treatment regimens have remarkably improved the outcome of pediatric T-ALL patients and nowadays about 80% of them remain in long-term remission [2,3]. However, 15–20% of the patients still relapse within the first two years following diagnosis and their prognosis remains dismal, emphasizing the need to scrutinize the molecular genetic mechanisms responsible for disease development, progression, and therapy resistance.

In T-ALL, leukemic transformation is a multistep process during which numerous genetic lesions cooperate in altering self-renewal, cell cycle control, proliferation, survival, and differentiation during thymocyte development [1,4,5]. Constitutive activation of NOTCH1 signaling, which provides T-cell precursors with self-renewal capacity, and impairment of cell cycle control through deletion of *CDKN2A/B*, constitute the core of the oncogenic program in the development of T-ALL [1,6,7]. Although loss of *CDKN2A/B* plays a key role in the pathogenesis of the disease, cell proliferation is tightly controlled by a number of other cyclin-dependent kinases [8]. Notably, genome-wide studies of pediatric T-ALL have shown that heterozygous deletion of *CDKN1B*, encoding the haploinsufficient tumor suppressor p27kip1, is one of the most frequent copy number alterations, occurring in about 12% of the cases [9–11].

In contrast to the diversity of genetic abnormalities, gene expression profiling studies have revealed a

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Supplemental data for this article can be accessed here.

limited number of T-ALL subtypes with unique gene expression signatures, reflecting distinct stages of thymocyte development [12]. These expression profiles include the *TAL/LMO*, *TLX1*, *TLX3*, and *HOXA* clusters [1,13,14] as well as the recently described proliferative and immature T-ALL subtypes characterized by overexpression of *NKX2-1* or *NKX2-2* and *MEF2C*, respectively [15]. MEF2C appears to function as a transcriptional regulator, which elicits a comprehensive transcriptional program characteristic for early T-cell precursor (ETP) ALL [15,16]. Furthermore, immunophenotypically characterized ETP-ALL overlaps with the immature *MEF2C* expressing cluster, indicating that both may constitute a single disease entity [17].

In this study, we have for the first time characterized *CDKN1B*-deleted pediatric T-ALL in more detail. Our data show that *CDKN1B* deletions frequently occur in the *MEF2C*-dysregulated and *HOXA* subtypes of T-ALL and suggest that *MEF2C* expression is associated with a poor response to glucocorticoid therapy.

Methods

Primary leukemia samples and cell lines

All T-ALL patients analyzed in this study were enrolled in the Austrian ALL-BFM 86, 90, 95, 2000 or 2009 clinical trials. Written informed consent for treatment as well as for tissue banking and research studies was obtained from the patients, their parents or legal guardians in accordance with the Declaration of Helsinki. All samples analyzed were obtained from the study center upon institutional review board approval. In a retrospective study, 102 consecutive T-ALL samples with available genomic DNA were subjected to array comparative genomic hybridization (aCGH). Cases with *CDKN1B* deletions and based on material availability, a randomly selected *CDKN1B* wild-type cohort ($n = 28$; $n = 19$ from the aCGH cohort and 9 additional ones) were subjected to further genetic analysis (Supplementary Figure S1). There was no significant difference regarding their clinical characteristics between the 28 *CDKN1B* wild-type patients and those 71 of the aCGH cohort not analyzed: age, median 8.5 (range 1.5–17.5) vs 9.3 (2.0–18.0); sex, 71% vs 75% males; WBC $\times 10^9$/L, median 94.3 (range 3.8–697.9) vs 74.8 (2.1–460.0); and poor prednisone response, 37% vs 34%.

The human T-ALL cell lines Loucy, RPMI-8402, CCRF-CEM, MOLT-16, and HSB-2 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany); TALL-1 and DND-41 were a kind gift from F. Speleman (Ghent University, Belgium). Loucy has an ETP-like immunophenotype and, like RPMI-8402, expresses *MEF2C* due to a del(5)(q14q35) [18]. The cell line CCRF-CEM harbors a *BCL11B-NKX2-5* rearrangement, resulting in the ectopic expression of *NKX2-5*, which in turn induces *MEF2C* expression [15,18,19].

Detection of copy number alterations and analysis of genetic subtypes

Array comparative genomic hybridization (aCGH) of 102 pediatric T-ALL samples was performed using a whole genome tiling-path bacterial artificial chromosome (BAC) DNA array consisting of ~36,000 BAC clones as described elsewhere [20,21]. In addition, some patients were analyzed using the CytoScan HD Array platform (Affymetrix) and the Chromosome Analysis Suite (ChAS 3.1) software (Affymetrix). Patients were assigned to specific molecular cytogenetic T-ALL subgroups by cytogenetic analysis and fluorescence in situ hybridization (FISH) and/or RT-PCR results for *TRA/D*, *TRB*, *TLX1*, *TLX3*, *KMT2A*, *NUP214*, *BCL11B*, and *SIL-TAL1* rearrangements using standard protocols.

Mutation screening

Mutations were assessed by PCR amplification of genomic DNA or cDNA with primer sets encompassing the mutation hot spots of *NOTCH1*: heterodimerization (HD), transmembrane (TM), and PEST domains encoded by exons 26–27, 28, and 34, respectively; *FBXW7*: WD40 domains encoded by exons 8–12, *IL7R*: exon 6, and *PTEN*: C2-domain encoded by exon 7; and direct sequencing of the PCR products as described [22–24]. Further details are provided in the Supplementary file.

Immunophenotyping

Immunophenotyping and classification into pro- (T-I), pre- (T-II), cortical (T-III), and mature (T-IV) T-ALL was conducted for diagnostic purposes according to the guidelines of the European Group for the Immunological Characterization of Leukemias (EGIL) [25], using the following markers: CD1a, cytoplasmic CD3 (cyCD3) and surface CD3 (sCD3), CD5, CD7, CD4, CD8, TdT, and HLA-DR in virtually all of the cases. In addition, for the majority of the cases the presence/absence of the stem cell and myeloid-specific markers CD34 and CD13 as well as CD10 was determined; and at least for some of the cases, expression data for CD33, CD117, and CD79a were available. Of note, due to the historic nature of the immunological data, ETP-ALL was not routinely assessed and was retrospectively

analyzed based on the available marker profiles. Unsupervised hierarchical clustering of flow cytometry data was conducted using the 'pheatmap' library in R statistical environment with 'euclidean distance' and 'complete linkage'.

MEF2C expression analysis

Total RNA was isolated from bone marrow samples obtained at diagnosis using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative expression analysis of *MEF2C* transcript levels was carried out using the IQ™ SYBR Green Supermix (Biorad, Vienna, Austria) and an ABI 7500-fast Real-Time PCR system (Applied Biosystems, Vienna, Austria). Assays were run in triplicates and transcript values were analyzed with the 7500 System Software (Applied Biosystems, Foster City, CA) and normalized using the ΔΔCt method [26,27] relative to the expression of the reference gene *GUSB* and to the cell line Loucy. Primer sequences and PCR conditions are provided in the Supplementary file.

Statistical analysis

Statistical significances between patient groups were determined by the two-tailed Fisher's exact test. Differences in gene expression were calculated using the Mann–Whitney test in the GraphPad Prism software v.5 (La Jolla, CA). Cut-off values for *MEF2C* dysregulation were defined based on dichotomization of the continuous expression levels using the Cutoff Finder software [28] http://molpath.charite.de/cutoff.

Results

Detection of CDKN1B deletions

Deletions of *CDKN1B* located at chromosome region 12p13 were detected in 12 cases (12%; 12/102) of pediatric T-ALL (Supplementary Figure S1), confirming their previously reported frequency [9–11]. The size of the deletions was highly variable, ranging from 1.2 to 27.8 Mb, but the minimal region of deletion always included *CDKN1B* (Supplementary Table S1), indicating that this haploinsufficient tumor suppressor represents a key target of these deletions. Two further cases with *CDKN1B* deletions were detected by routine cytogenetics, which showed a loss of 12p13. In all cases, the presence of the *CDKN1B* deletion was verified by quantitative genomic PCR: in thirteen of them, we observed a heterozygous and in one (02-3018) even a homozygous deletion (Supplementary Figure S2; Table S2).

CDKN1B deletions and coinciding genetic alterations

Since impairment of cell cycle control via deletion of the cell cycle regulators *CDKN2A/B* constitutes a component of the core oncogenic program in the development of T-ALL [1,29], we compared the frequencies of *CDKN2A/B* deletions in *CDKN1B*-deleted and wild-type cases in the 102 samples analyzed by aCGH. This analysis revealed that patients with *CDKN1B* deletions show a lower incidence of *CDKN2A/B* deletions than *CDKN1B* wild-type ones: 50% (6/12) vs 79% (71/90) (two-tailed Fisher's exact test, $p = .067$). Although not statistically significant, these data nevertheless support the notion that in T-ALL loss of *CDKN1B* may represent an additional mechanism to impair cell cycle regulation and to enhance proliferation.

To assess whether mutations of *NOTCH1, FBXW7, IL7R* or *PTEN,* frequently present in T-ALL [1,7,29], are associated with the *CDKN1B* deletion status, we conducted a mutation screening of these genes in *CDKN1B*-deleted ($n = 14$) and *CDKN1B* wild-type ($n = 28$) samples (Supplementary Figure S1). However, the incidences of mutations in *CDKN1B*-deleted cases were highly similar to those observed in unselected T-ALL cohorts [4,15,29], indicating that none of these mutations positively or negatively correlates with the presence of *CDKN1B* deletions (Table 1).

Molecular cytogenetic subtype classification of CDKN1B-deleted T-ALL

Employing FISH and/or RT-PCR we next determined to which of the major molecular cytogenetically defined T-ALL subtypes, reflecting the *TAL/LMO*, *TLX1*, *TLX3*, and *HOXA* clusters, *CDKN1B*-deleted cases belong. This approach revealed that one case each was positive for *TLX1*, *TLX3*, *TRA-MYC, KMT2A-MLLT4* (*MLL-AF6*), *PICALM-MLLT10* (*CALM-AF10*), and *TRB-HOXA*; and one harbored a cryptic *LMO2*-activating deletion [30] detected by aCGH and confirmed by FISH (Table 1; Supplementary Table S2). Notably, 21% (3/14) of the *CDKN1B*-deleted cases belonged to the *HOXA* subtype, which generally accounts for roughly 10% of T-ALL and is characterized by specific genetic alterations including *PICALM-MLLT10*, *KMT2A* and *HOXA* rearrangements [1,13,31]. However, this association was not statistically significant (3/14 *CDKN1B*-deleted vs 1/28 *CDKN1B* wild-type; two-tailed Fisher's exact test, $p = .1$), which may be due to the rather small number of patients analyzed.

Table 1. Frequencies of genetic alterations in *CDKN1B*-deleted and *MEF2C*-dysregulated patients compared to unselected T-ALL cohorts.

Genetic alterations	*CDKN1B*-deleted	*CDKN1B* wild-type	*MEF2C*-dysregulated	Unselected T-ALL cohorts[a]
Mutations				
NOTCH1	54% (7/13)[b]	68% (19/28)	50% (5/10)	>60%
HD	15% (2/13)	39% (11/28)	–	20%
PEST	8% (1/13)	0% (0/28)	–	5%
PEST + HD	31% (4/13)	18% (5/28)	–	20%
JME	0% (0/13)	10% (3/28)	–	3%
FBXW7	0% (0/13)[b]	18% (5/28)	10% (1/10)	8–30%
PTEN	14% (2/14)	7% (2/28)	10% (1/10)	10%
IL7R	14% (2/14)	0% (0/28)	10% (1/10)	10%
Copy number alterations				
CDKN2A/B deletion	43% (6/14)	75% (21/28)	40% (4/10)	70%
CDKN1B deletion	–	–	60% (6/10)	12%
Molecular cytogenetic subtype				
TAL/LMO	7% (1/14)	18% (5/28)	–	20–35%
TLX3	7% (1/14)	29% (8/28)	–	20–25%
TLX1	7% (1/14)	7% (2/28)	–	5–10%
HOXA	21% (3/14)	4% (1/28)	–	5–10%
MYC	7% (1/14)	0% (0/28)	–	6%
MEF2C expression				
MEF2C	54% (6/11)[c]	14% (4/28)[d]	–	10%

[a]These data have been retrieved from the literature [1,4,10,15,29,34]. HD: heterodimerization domain; JME: juxtamembrane expansion.
[b]From one of the *CDKN1B*-deleted samples insufficient material for complete mutation analysis was available.
[c]Including patient 99-3248 (*LMO2*-activating deletion) and patient 10-0338 (*TRA-MYC* translocation).
[d]Including patient 01-2231 (*TRA-TLX1*).

In the remaining *CDKN1B*-deleted patients, our approach failed to detect any of the sentinel T-ALL-specific chromosomal translocations.

MEF2C dysregulation in CDKN1B-deleted T-ALL

Since several of the *CDKN1B*-deleted cases did not fall into any of the major T-ALL subgroups, we aimed to determine whether they belong to the immature T-ALL subtype harboring a number of different molecular cytogenetic alterations, which converge on the activation of *MEF2C* expression, which in turn activates genes typically expressed in ETP-like ALL [15]. *CDKN1B*-deleted ($n = 11$) and wild-type ($n = 28$) samples, of which material was available (Supplementary Figure S1), were subjected to quantitative gene expression analysis for *MEF2C*. The expression values were normalized to that of the T-ALL cell line Loucy, known to have an ETP-like phenotype and to express *MEF2C* (Figure 1; Supplementary Figure S3) [15,32]. As additional controls, we included the cell lines RPMI-8402, CCRF-CEM, and MOLT-16 also expressing *MEF2C* as well as the *MEF2C*-negative cell lines HSB-2, TALL-1, and DND-41 [18].

Intriguingly, *CDKN1B*-deleted T-ALLs showed a significantly higher frequency of *MEF2C* dysregulation as compared to *CDKN1B* wild-type cases (6/11 (54%) vs 4/28 (14%); two-tailed Fisher's exact test, $p = .017$), suggesting that they might belong to the immature/*MEF2C*-dysregulated T-ALL subtype, which overall accounts for roughly 10% of T-ALL (Table 1) [15]. Worth noting, the case with the *TRA-MYC* translocation (10-0338) and the cell line MOLT-16 harboring the same genetic alteration [33,34] showed comparable, but only moderate *MEF2C* expression levels (Figure 1), probably reflecting a later stage of differentiation arrest [15]. Interestingly, MEF2C is able to synergize with MYC in driving cellular transformation [15].

Since in particular *BCL11B* enhancer-mediated activation of *NKX2-5* or other partner genes results in the overexpression of *MEF2C* [15,19], we analyzed the *MEF2C*-positive cases with unknown subtype-defining alterations for rearrangements of the *BCL11B* locus. In the *CDKN1B*-deleted case 02-3977, employing FISH and subsequently RT-PCR for *NKX2-5* expression, we verified the presence of a *BCL11B-NKX2-5* rearrangement (Supplementary Figure S4). In addition, the *CDKN1B* wild-type case 10-2023 showed a FISH pattern indicative of a *BCL11B* fusion gene with an unknown partner at 6q27 (Supplementary Figure S4). These data confirm that a number of different genetic alterations, several of which remain to be determined, result in the induction of *MEF2C* expression [15,18].

The frequencies of mutations in *NOTCH1*, *FBXW7*, *IL7R*, and *PTEN* did not differ between *MEF2C* expressing and non-expressing cases (Table 1; Supplementary Table S2). However, as shown for *CDKN1B*-deleted samples, also the *MEF2C*-dysregulated ones, which in part overlap with the former, showed a lower percentage of *CDKN2A/B* deletions (Table 1; Figure 1).

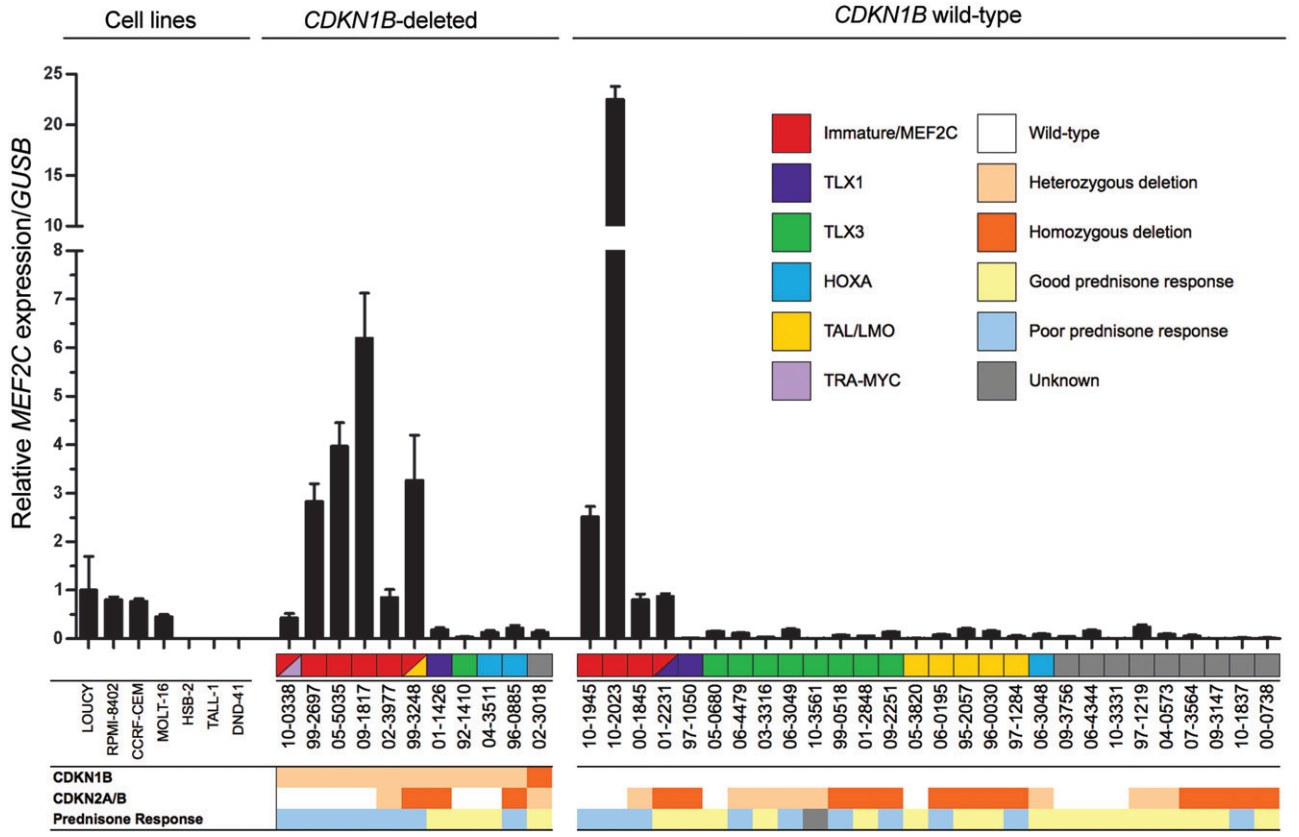

Figure 1. *MEF2C* expression levels in T-ALL patients and cell lines. Relative *MEF2C* expression was determined by RT-qPCR and normalized to the reference gene *GUSB* and the cell line Loucy. Patients are ordered based on *CDKN1B* status, and clustered according to the main T-ALL subtypes; and the *CDKN2A/B* deletion status is provided. Poor prednisone response corresponds to the presence of $\geq$1000/μl blasts in the peripheral blood on day 8 of glucocorticoid monotherapy.

Immunophenotypic characteristics of CDKN1B-deleted and MEF2C-dysregulated T-ALL

The association of *CDKN1B* deletions with the expression of the oncogenic driver *MEF2C*, which results in a block at an early stage of T-cell differentiation and whose dysregulation is linked to an expression signature closely resembling that of ETP-like ALL [12,13,15,35], prompted us to examine the immunophenotypic characteristics of the blast cells of both *CDKN1B*-deleted and *MEF2C*-dysregulated T-ALL.

For this purpose, we used the available immunological data of all cases analyzed in this study and added two typical ETP-ALLs (12-1462 and 15-4984) displaying the original ETP phenotype: absence of CD1a and CD8 ($<$5% of blast cells), CD5 present on $<$75% of leukemic cells, and coexpression of at least one of the myeloid/stem cell antigens CD117, CD34, HLA-DR, CD13, CD33, CD11b, or CD65 on $\geq$25% of blast cells [35,36]. Noteworthy, both of these cases expressed *MEF2C* and one of them carried a heterozygous *CDKN1B* deletion, further supporting the association between these two genetic lesions (Supplementary Table S2).

Unsupervised hierarchical clustering of the immunological data showed that overall *CDKN1B*-deleted patients did not cluster, indicating that they do not display any particular marker profile (Figure 2). In contrast, 11 samples formed a cluster that was enriched for *MEF2C* positivity (8/11, 73%). The main immunophenotypic characteristics of this cluster were absence of CD1a, CD4/CD8 double negativity and expression of at least one stem cell or myeloid marker (CD34, CD13, CD33, CD117, CD65 or HLA-DR) (Table 2; Figure 2; Supplementary Table S3). The blast cells of the three cases in this cluster lacking *MEF2C* expression were either CD4$^+$ on $>$50% (99-0518 and 09-2251) or CD5$^+$ on 97% (06-4479) of the cells (Supplementary Table S3). However, also the *MEF2C*-positive cases 99-2697 and 05-5035 expressed CD5 on 84% and 93% of the cells, respectively, confirming that *MEF2C* dysregulation is not consistently associated with low CD5 ($<$75% of the blast cells) expression [15,17].

On the other hand, of the remaining *MEF2C* high expressing cases that did not cluster with the other *MEF2C*-positive samples, 99-3248 (*LMO2*) was CD1a$^+$ and CD8$^+$ and 01-2231 (*TLX1*) CD1a$^+$ and both lacked

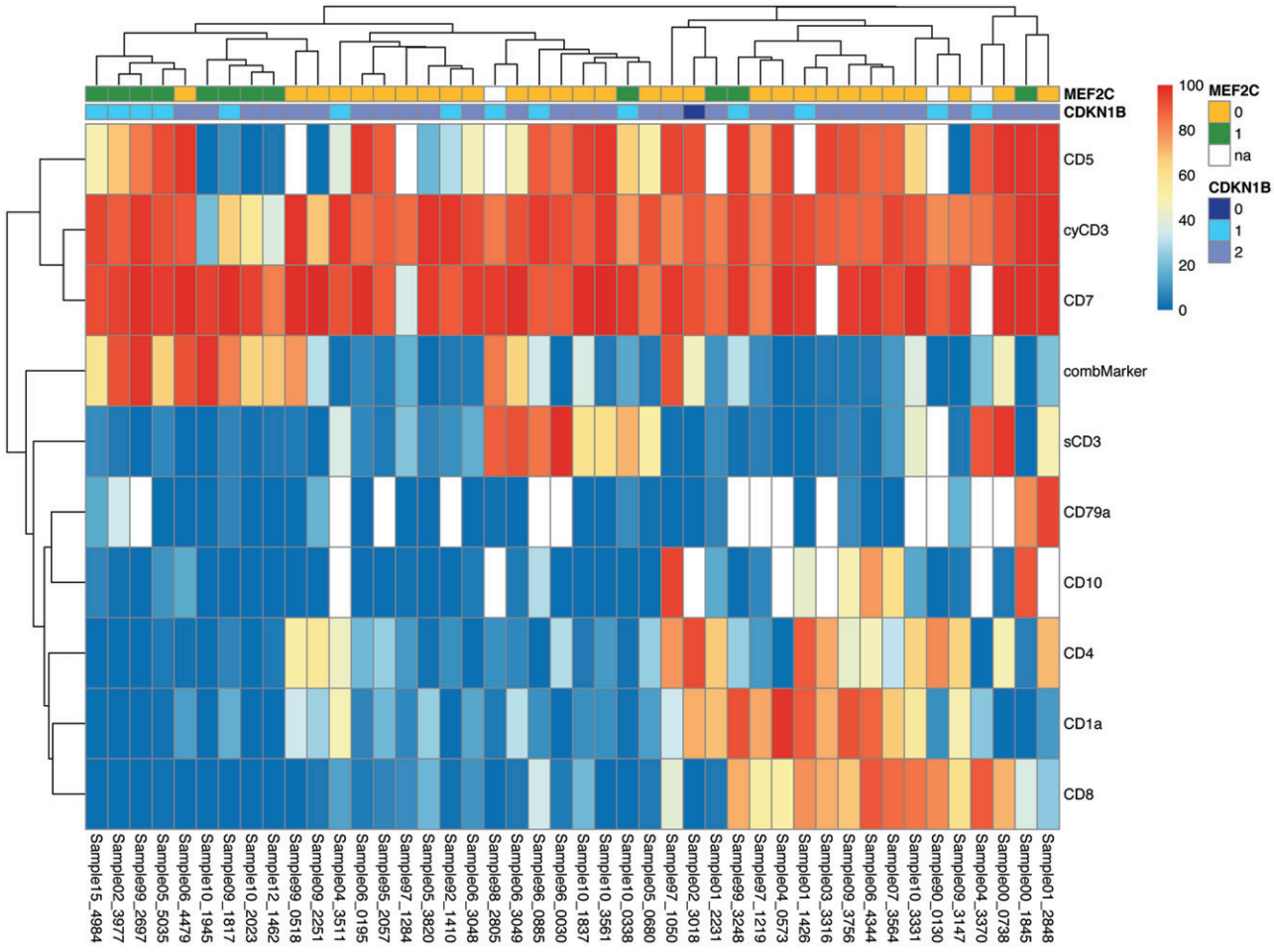

Figure 2. Unsupervised hierarchical clustering of immunophenotypic data. The heatmap and dendrogram depict the immunophenotypic markers available for each sample; columns represent each of the 44 T-ALLs and rows the percentage (0–100%) of positive blast cells for each marker. CombMarker indicates the highest value for any of the following antigens: CD34, CD13, CD33, CD117, CD65, or HLA-DR. For each sample, the respective *MEF2C* expression levels (0: not expressed; 1: expressed; na: not available) and the *CDKN1B* status (0: homozygous deletion; 1: heterozygous deletion; 2: wild-type) are shown. White fields indicate missing data.

expression of myeloid and stem cell markers (Table 2; Figure 2), thus not fulfilling the proposed ETP/immature T-ALL criteria [35,37]. Notably, the blast cells of case 00-1845, though CD1a$^-$ and CD4$^-$, expressed CD8 and were positive for CD10 and the B-cell-specific marker CD79a (Table 2; Figure 2), a pattern not seen in any other of the *MEF2C* expressing samples. Together, *MEF2C* dysregulation is not necessarily associated with an immature/ETP-like cell surface marker profile.

MEF2C expression predicts a poor response to glucocorticoid therapy

For T-ALL patients with an ETP-like phenotype treated according to ALL-BFM protocols, resistance to first line treatment has been reported [35,37]. Remarkably, virtually all *MEF2C*-dysregulated patients (90% (9/10), or 92% (11/12) when including the two additional ETP-ALL cases selected for immunological profiling) showed a poor response to glucocorticoid (GC) therapy (Figure 1; Supplementary Table S3) and *MEF2C* expression levels were significantly associated with GC response (Figure 3). In contrast, only 25% (3/12) of the patients failed to respond to induction therapy, as defined by $\geq$5% blast cells morphologically on day 33 of treatment [3]. Of the patients with available minimal residual disease (MRD) data ($n = 7$), four showed high, one intermediate, and two standard risk MRD levels; one of the latter being the only good prednisone responder. Considering that generally only one-third of T-ALL patients fails to respond to GC treatment [38], these data indicate that *MEF2C* expression may be predictive of a poor response to glucocorticoids. However, it remains to be determined whether *MEF2C* expression may be directly linked to GC-response.

Table 2. Immunophenotypic characteristics of *CDKN1B*-deleted and *MEF2C*-dysregulated pediatric T-ALL cases.

	Case	EGIL	MEF2C	cyCD3	sCD3	CD7	CD2	CD5	CD1a	CD4	CD8	Tα/β	Tγ/δ	CD34	CD117	HLA-DR	CD13	CD33	CD65	CD79a	CD10
CDKN1B-deleted	98-2805	T-IV	nd	84	89	96	97		5	10	1	0	81	84		5		3		3	
	04-3370	T-IV	nd	85	91		15	91	24	1	90	4	94	22	2	15					
	90-0130	T-II	nd	80		90			10	80	80					0	0	0	0		0
	92-1410	T-II	–	97	10	90		30	0	10	3			4		0	0	0	0		0
	02-3018	T-III	–	90	2	92	9	92	73	93	0			11		46	0			1	
	04-3511	T-III	–	97	37	92	1	39	48	45	14			0	3	1					
	01-1426	T-III	–	93	2	97	97		90	90	79	1	3	2		3				2	43
	96-0885	T-IV	–	96	85	90		90	10	0	35	1	77	0		0	0	0	35		30
	10-0338	T-IV	+	78	72	97	88	67	3	3	1	4	70	8	4	15	0	1	1	9	2
	02-3977	T-II	+	90	4	95	4	69	0	2	2	3	1	0	0	3	92	0		34	3
	99-2697	T-II	++	97	0	99	14	84	0	0	0	0	0	98		7	0	9	23		0
	99-3248	T-III	++	95	3	97	98	96	92	26	73			0	1	2	0	0	31		0
	05-5035	T-II	++	92	8	96	95	93	3	5	3	8	1	67	0	19	58	4		2	11
	09-1817	T-II	++	67	7	99	97	10	17	7	0	7	1	83	0	24	17	24		7	0
CDKN1B wt	00-1845	T-II	+	98	0	99	99	98	0	6	37			0		2	1	1	1	80	91
	01-2231	T-III	+	84	9	87	91		70	68	4	8	1	1		11	4	2		7	16
	10-1945	T-II	++	21	0	95	81	2	1	1	1	2	0	54	88	98	21	4		0	1
	10-2023	T-II	+++	57	3	95	99	0	0	2	1	1	0	3	0	65	22	1		1	0

Numbers represent percentages of positive blast cells; empty fields, no data available; EGIL: European Group for the Immunological Characterization of Leukemias; cy: cytoplasmatic; s: surface; *MEF2C* expression: nd: not determined; – negative, + moderate, ++ high, +++ very high; wt: wild-type.

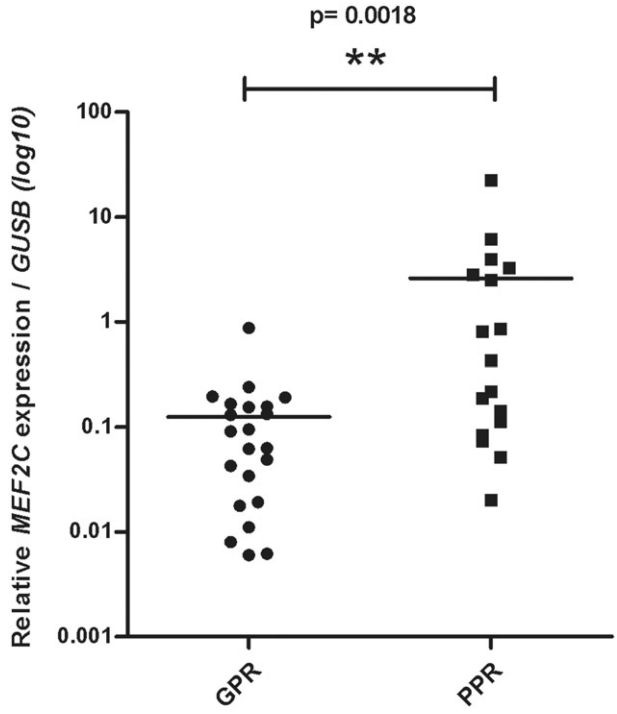

Figure 3. *MEF2C* dysregulation and prednisone response. Increased levels of *MEF2C* are associated with a poor prednisone response. Lines indicate the median, each symbol represents an individual patient, and the significance level was calculated using the Mann–Whitney test. p value $<.05$ is significant; GPR: good prednisone response; PPR: poor prednisone response.

Notably, inhibition of BCL2 appears to sensitize MEF2C expressing cells to GC treatment and BCL2 inhibitors synergize with GCs in apoptosis induction [39,40].

After a median follow-up time of 4.4 years (range 0.2–15.9 years), 75% (9/12) of the *MEF2C*-dysregulated T-ALL patients remain in complete first remission, while one relapsed, one died of infectious complications following bone marrow transplantation and another developed a secondary tumor and was lost for follow-up. Of the surviving patients, four, including the relapsed patient, were transplanted in first remission and one patient has a transplantation indication, but has not been transplanted yet.

Discussion

In this study, we have for the first time characterized *CDKN1B*-deleted pediatric T-ALL in more detail and our most salient findings are: First, *CDKN1B*-deleted cases have a lower incidence of *CDKN2A/B* deletions, indicating that haploinsufficiency of p27kip1 may represent another mechanism to impair cell cycle control. Second, *CDKN1B* deletions are frequently present in the immature *MEF2C*-dysregulated and *HOXA* subtypes

of T-ALL. And third, *MEF2C* dysregulation correlates with a poor response of the patients to glucocorticoid therapy.

Remarkably, about half of the *CDKN1B*-deleted patients displayed *MEF2C* dysregulation (Table 1), which is considered to elicit the transcriptional program characteristic for ETP-ALL [15,17,35,37]. Together with the observation, that 20% of *CDKN1B*-deleted cases belong to the *HOXA* cluster characterized by specific genetic alterations and a distinct gene expression signature [13], *CDKN1B* deletions appear to be strongly associated with immature T-ALL.

Generally, ETP-like or early immature T-ALL shows lower frequencies of prototypical T-ALL-specific genetic lesions such as *CDKN2A/B* deletions and NOTCH1 pathway activating mutations but higher incidences of mutations typically found in acute myeloid leukemia [7,41,42]. In our cohort, we did not find lower mutation frequencies in *NOTCH1*, *FBXW7*, and *PTEN* in either *CDKN1B*-deleted or *MEF2C*-dysregulated T-ALL (Table 1). In the latter subtype, this finding is in line with the data of Zuurbier et al., who observed no differences in the occurrence of NOTCH1- or PI3K/AKT-activating events between immature/ETP-like and other T-ALL patients defined by gene expression profiling [17]. These data, however, contradict other studies [41,43], which may be due either to the different selection criteria or the different numbers of patients analyzed.

However, the high incidence of *MEF2C*-dysregulated cases within the *CDKN1B*-deleted cohort (54%) or, *vice versa,* the high frequency of about 60% *CDKN1B* deletions in *MEF2C*-dysregulated T-ALL, strongly suggests an association between these genetic events. Notably, in *MEF2C*-dysregulated cases, either *CDKN1B* and/or *CDKN2A/B* may be affected by deletions (Figure 1; Supplementary Table S2), increasing the prevalence of cell cycle regulator defects in this ETP-like T-ALL subtype to the 70%, resembling the incidence of loss of *CDKN2A/B* alone in unselected T-ALL cohorts [1].

We can certainly not rule out that also other genes such as *ETV6* are the primary targets of the 12p13 deletions. However, in one of the cases, *ETV6* was not deleted (01-1426; Supplementary Table S1) and heterozygous loss of *ETV6* appears to be functionally different from the *ETV6* mutations found in immature T-ALL [32]. Therefore, it is highly conceivable that *CDKN1B* is the main target of 12p13 deletions and that in T-ALL haploinsufficiency of p27kip1 may play a role in cell cycle progression. Notably, haploinsufficiency of p27kip1 also contributes to the development of T-cell prolymphocytic leukemia, in which about 50% of the cases harbor heterozygous *CDKN1B* deletions [44,45].

Even though *MEF2C* is considered as one of the driving oncogenes in ETP-like ALL [15], in this study only two thirds of the cases expressing increased levels of *MEF2C* showed the typical immunophenotypic characteristics of this immature T-ALL subtype independent of the proposed marker profile [17,35]. Notably, however, the majority of the *MEF2C*-dysregulated cases expressed one or the other stem cell, myeloid or B-lineage-specific marker (Table 2; Figure 2), suggesting a certain lineage plasticity of the leukemic blasts. Together, it appears that *MEF2C* dysregulation defines a T-ALL subtype, which may not be predictable by immunophenotyping, at least not with the currently used marker combinations.

Since in childhood ALL glucocorticoids (GCs) are essential components of therapy and a poor response to GC treatment is a strong predictor of adverse outcome [46,47], it is important to note that *MEF2C* expression in the primary leukemic cells was associated with a significantly higher rate of patients' resistance to GC monotherapy (Figure 3). However, despite their initially poor response to GC therapy, patients with *MEF2C*-dysregulated T-ALL had a reasonable outcome with 75% of the patients remaining in long-term remission. This observation is in line with the recent results of an AIEOP-BFM study, which showed that 55% of ETP-like ALL patients failed to respond to prednisone while their outcome (78% of patients in continuous complete remission) was similar to that of non-ETP-like cases [37]. The rather good overall survival rates of the patients may be due to the risk stratification used in the BFM protocols, in which patients with poor GC response are allocated to the high-risk group and receive intensified therapy [46,47]. Nevertheless, patients with ETP-like ALL might in the future benefit from targeted therapies [39,40,48].

Conclusions

In summary, we show that *CDKN1B* deletions frequently occur in immature, mainly *MEF2C*-dysregulated, T-ALL and that *MEF2C* overexpression, though associated with, is not exclusively found in early T-cell precursor-like leukemia as defined by current immunological criteria. Furthermore, our data indicate that *MEF2C* expression is associated with or may even be predictive of a poor response to glucocorticoid treatment. Hence, in prospective studies, analysis of *MEF2C* expression in combination with highly standardized immunophenotyping may help to refine the classification of T-ALL subtypes.

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