

# Impact of promoter polymorphisms in key regulators of the intrinsic apoptosis pathway on the outcome of childhood acute lymphoblastic leukemia

Rocio Sanchez,<sup>1</sup> Janick St-Cyr,<sup>1</sup> Marie-Eve Lalonde,<sup>1</sup> Jasmine Healy,<sup>1</sup> Chantal Richer,<sup>1</sup> Vincent Gagné,<sup>1</sup> Caroline Laverdière,<sup>1,2</sup> Lewis B. Silverman,<sup>3,4</sup> Stephen E. Sallan,<sup>3,4</sup> Donna Neuberg,<sup>5</sup> Jeffery L. Kutok,<sup>6</sup> Ekaterini A. Kritikou,<sup>1</sup> Maja Krajcinovic,<sup>1,2</sup> and Daniel Sinnett<sup>1,2</sup>

<sup>1</sup>Division of Hematology-Oncology, Research Center, CHU Sainte-Justine, Montreal, Canada; <sup>2</sup>Department of Pediatrics, Faculty of Medicine, Université de Montréal, Canada; <sup>3</sup>Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, USA; <sup>4</sup>Division of Hematology/Oncology, Children's Hospital, Boston, MA, USA; <sup>5</sup>Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA, USA; and <sup>6</sup>Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

## ABSTRACT

The introduction of multiagent treatment protocols has led to a remarkable increase in survival rates for children diagnosed with acute lymphoblastic leukemia, yet for a subpopulation of patients, resistance to chemotherapeutics remains an obstacle to successful treatment. Here we investigate the role of the mitochondrial (or intrinsic) apoptosis pathway in modulating the onset and outcomes of childhood acute lymphoblastic leukemia. Cell death is a highly regulated process that plays an essential role in regulating cell homeostasis, particularly in tissues with high intrinsic proliferating capacity such as the hematopoietic system. Following the underlying paradigm that *cis*-acting genetic variation can influence disease risk and outcomes by modulating gene expression, we performed a systematic analysis of the proximal promoter regions of 21 genes involved in apoptosis. Using gene reporter assays, we show that promoter variations in 11 intrinsic apoptosis genes, including *ADPRT*, *APAF1*, *BCL2*, *BAD*, *BID*, *MCL1*, *BIRC4*, *BCL2L1*, *ENDOG*, *YWHAB*, and *YWHAQ*, influence promoter activity in an allele-specific manner. We also show that correlated promoter variation and increased expression of *MCL1* is associated with reduced overall survival among high-risk patients receiving higher doses of corticosteroid, suggesting that increased expression of this anti-apoptosis gene could lead to reduced cell death and influence treatment response in a disease- and dose-responsive manner.

## Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer among children and is likely caused by multiple genetic and environmental factors. However, few established genetic risk factors for ALL have been identified. Following our previous work and the hypothesis that variation in genes involved in core biological processes such as oxidative stress response,<sup>1,2</sup> DNA repair,<sup>3,4</sup> and cell-cycle control<sup>5,6</sup> contribute to increased susceptibility to childhood ALL, we set out to investigate whether deregulation of the tightly regulated apoptosis process could also influence disease risk and/or outcome. Apoptosis plays an essential role in the regulation of cell homeostasis, particularly in tissues with high intrinsic proliferating capacity and high cell turnover rate such as the hematopoietic system.<sup>7,8</sup> Multiple distinct signaling pathways regulate apoptosis; however, two pathways have been mostly implicated in hematologic malignancies: the mitochondrial (or intrinsic) pathway and the death receptor (or extrinsic) pathway.<sup>8</sup> Briefly, activation of the intrinsic pathway is initiated by mitochondrial damage that leads to release of apoptogenic factors, such as cytochrome c, Smac/Diablo, apopto-

sis-inducing factor (AIF), Omi/HtrA2, endonuclease G, caspase-2, and caspase-9 from the mitochondrial intermembrane space.<sup>9</sup> Release of these factors is mediated by Bcl-2 family proteins that either suppress or promote changes in mitochondrial membrane permeability.<sup>10</sup> Twenty-four Bcl-2 family members have been identified as either pro-apoptotic (e.g. Bax, Bak, BCL2L1/Bcl-XS, Bid, and Bad) or anti-apoptotic (e.g. Bcl-2 and BCL2L1/Bcl-X<sub>L</sub>).<sup>11</sup> Upon release, cytochrome c forms the apoptosome complex with the adaptor molecule APAF1, ATP, and caspase-9,<sup>12</sup> which in turn, activates the caspase-3 effector caspase cascade.<sup>13</sup> Initiation of the extrinsic apoptosis pathway involves ligand-induced aggregation of death receptors and activation of procaspase-8 or procaspase-10 within the death-inducing signaling complex (DISC). Activated procaspase-8 or procaspase-10 is released into the cytoplasm, where it induces activation of downstream effector caspases. Both the intrinsic and extrinsic apoptotic pathways converge at the level of caspase-3 activation, which functions as a common death effector molecule.<sup>14</sup>

Differential expression of a number of key apoptosis genes has been observed in children with ALL<sup>15</sup> and sequence variation in apoptosis gene promoters has been linked to various

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Correspondence: daniel.sinnett@umontreal.ca

types of cancers,<sup>16-19</sup> including leukemia,<sup>15,20</sup> suggesting that increased growth and/or survival advantage of tumor cells caused by inherited polymorphisms in these genes could serve as a common mechanism for carcinogenesis. Variation in regulatory DNA sequences has been studied less intensively, despite evidence that *cis*-acting variation may explain up to 25-35% of inter-individual differences in gene expression.<sup>21</sup> Given that deregulated expression of key apoptosis genes might contribute to cancer onset and progression as well as affect treatment responses and disease outcome, we postulated that functional *cis*-acting regulatory DNA variants in genes encoding components of the intrinsic/mitochondrial apoptosis pathway might be determinants of risk and/or disease outcome of childhood ALL.

Here we present a systematic analysis of the proximal promoter regions of 21 genes involved in apoptosis and their putative association with childhood ALL. Using gene reporter assays, we show that promoter single nucleotide polymorphisms (pSNP) and/or promoter haplotypes in 11 intrinsic apoptosis genes, including *ADPRT*, *APAF1*, *BCL2*, *BAD*, *BID*, *MCL1*, *BIRC4*, *BCL2L1*, *ENDOG*, *YWHAB*, and *YWHAQ*, influence promoter activity in an allele-specific manner. Through a case-control association study on a cohort of children with ALL, we also show that, although none of these SNP is associated with ALL susceptibility,

the anti-apoptotic gene *MCL1* was significantly associated with overall survival in this cohort.

## Methods

### Study subjects

Our cohort consisted of 321 childhood pre-B ALL patients and 329 healthy controls. Parental DNA was also collected and was available for 203 of the probands. Study subjects were all French-Canadians of European descent from the established Quebec Childhood ALL (QcALL) cohort.<sup>22</sup> Incident cases were diagnosed in the Division of Hematology-Oncology at the CHU Sainte-Justine Hospital, Montreal, Canada, between October 1985 and November 2006. The study sample comprised 190 males and 131 females with a median age of 4.3 years. The healthy controls, 182 males and 146 females, were a group of French-Canadian neonates and adults recruited at the CHU Sainte-Justine Hospital.

The polymorphism significantly associated with ALL outcome was subsequently analyzed in an extended cohort consisting of 342 childhood QcALL patients (Table 1). Events occurred in 65 children (19% of patients) including six cases in whom induction failed, two patients who died with no prior relapse, 25 patients who relapsed during treatment (or a few months following treatment) and 34 patients whose relapse occurred at a later period. In 23 cases relapse occurred more than once. The analyses were also performed in an independent replication cohort from the Dana-Farber Cancer Institute composed of 272 Caucasians.<sup>23</sup> The Institutional Review Boards approved the research protocol and informed consent was obtained from all participating individuals and/or their parents.

### Identification of promoter single nucleotide polymorphisms in apoptosis genes

To identify pSNP in candidate genes selected based on their involvement in the mitochondrial apoptosis pathway, we directly sequenced the promoters of 21 intrinsic apoptosis genes (*ADPRT*, *APAF1*, *BAD*, *BAG1*, *BAG3*, *BAG4*, *BAX*, *BCL2*, *BCL2A1*, *BCL2L1*, *BCL2L11*, *BCL2L10*, *BID*, *BIRC4*, *BIRC5*, *CASP3*, *CASP6*, *ENDOG*, *MCL1*, *YWHAB*, and *YWHAQ*) in a SNP discovery cohort consisting of 20 randomly selected unrelated healthy individuals and 20 randomly selected pre-B ALL patients from the QcALL cohort.

### Gene reporter assays

For each of the 21 intrinsic apoptosis genes, haplotype-specific fragments corresponding to ~2.0 kb of the proximal promoter region were amplified by polymerase chain reaction from genomic DNA of known genotype/haplotype to generate reporter constructs representative of the major haplotypic alleles (Table 2 and *Online Supplementary Table S2*). Constructs were generated by cloning the promoter haplotypes of interest into the pGL3-basic promoterless firefly luciferase reporter vector (Promega) as previously described.<sup>24</sup> We sequenced the chosen clones to confirm the identity and integrity of each genotype/haplotype prior to transfection. Three clones for each construct were pooled and used to transfect HeLa, JEG-3, and HepG2 cells together with the pRL-SV40 plasmid containing the *Renilla* luciferase gene, used as a positive control of transfection. The promoterless pGL3-basic plasmid was used as the negative control. Reporter activities were normalized to the activity of the internal pRL-SV40 control and expressed as relative luciferase expression (mean±SD in 3 replicates). For a subset of selected genes for which haplotype-specific constructs showed positive promoter activity, Jurkat cells were used to further confirm the observed effect in a human leukemia cell line.

**Table 1. Characteristics of ALL patients in the discovery and validation cohorts.**

Characteristic	QcALL-initial	Frequency (%) QcALL-extended	DFCI
Sex			
Female	42.1	46.2	46.0
Male	57.9	53.8	54.0
Age (years)			
<10	82.0	79.2	77.9
≥10	18.0	20.8	22.1
WBC, ×10 <sup>9</sup> /L			
<50	86.7	82.1	81.6
>50	13.3	17.9	18.4
Cell type			
Pre-B*	100.0	91.4	91.2
T		8.6	8.1
Hyperdiploidy			
Yes	21.4	19.3	n.a.
No	78.6	80.7	
Risk groups			
Standard	52.3	47.4	61.8
High	47.7	52.6	38.2
Treatment protocol			
87-01	4.1	36.3	
91-01	17.7	36.5	
95-01	41.4	18.4	34.6
00-01	36.8	8.8	65.4
Event			
Yes	18.8	19	19.5
No	82.2	81	80.5
Death			
Yes	8.3	8.5	8.1
No	91.7	91.5	91.9
<b>Total N.</b>	<b>266</b>	<b>342</b>	<b>272</b>

\*Initial discovery cohort included patients with B-cell leukemia only; n.a.: not available. QcALL: Quebec childhood ALL; DFCI: Dana-Farber Cancer Institute.

### Genotyping and quality control checks of promoter single nucleotide polymorphisms

pSNP with differential allele-specific promoter activity in the gene reporter assays were further genotyped among the 321 childhood pre-B ALL patients, 329 healthy controls, and 203 parental pairs from the QcALL cohort, using the LuminexMAP/Autoplex Analyzer CS1000 system (Perkin Elmer, Waltham, MA, USA).

## Results

### Reporter assays reveal allele-specific promoter activity in ADPRT, APAF1, BAD, BCL2, BCL2L1, BID, BIRC4, ENDOG, MCL1, YWHAB, and YWHAQ

By directly re-sequencing genomic DNA of 20 unrelated healthy individuals and 20 pre-B ALL patients (n=80 chromosomes) of European descent, we identified a total of 88 SNP (*Online Supplementary Table S1*), including 85 that were known (dbSNP build 135), located in the proximal promoter of 21 genes involved in the intrinsic/mitochondrial apoptosis pathway: *ADPRT*, *APAF1*, *BAD*, *BAG1*, *BAG3-4*, *BAX*, *BCL2*, *BCL2A1*, *BCL2L1*, *BCL2L10*, *BCL2L11*, *BID*, *BIRC4-5*, *CASP3*, *CASP6*, *ENDOG*, *MCL1*, *YWHAQ* and *YWHAB*. All pSNP were in Hardy-Weinberg equilibrium and, after eliminating those that had minor allele frequencies <5% (n= 2), 86 pSNP were retained for further investigation. Promoter haplotype phase and corresponding frequencies (based on 80 chromosomes from the SNP discovery cohort) are given in Table 2.

To assess the functional significance of these 86 pSNP on promoter activity, 2.0 kb promoter haplotypes, corresponding to the major haplotypes (frequency > 5%) of each gene, were subcloned into the promoterless pGL3-basic vector. Transient transfection experiments were carried out for each of the resulting 61 haplotype-specific constructs (Table 3 and *Online Supplementary Table S2*). Assuming a conservative definition of promoter activity as a 1.5-fold increase in the activity of the 'highly expressed allele' over the expression of the pGL3-basic vector,<sup>25</sup> most constructs showed promoter activity in at least one of the cell lines tested (Table 3). Significant fold difference ( $P < 0.05$ ) in either the HeLa, Jeg-3 or HepG2 cell line was considered as a criterion for functionality, at least *in vitro*. We observed significant differences in promoter activity associated with specific promoter haplotypes in 11 apoptosis genes (*ADPRT*, *APAF1*, *BAD*, *BCL2*, *BCL2L1*, *BID*, *BIRC4*, *ENDOG*, *MCL1*, *YWHAB*, and *YWHAQ*), suggesting that these promoter haplotypes and corresponding pSNP could function to modulate expression of these genes. However, for several constructs (e.g. *BCL2A1*, *BCL2L10*, *BIRC5*, and *CASP3*) we did not observe any promoter activity in the three cell lines tested (*Online Supplementary Table S2*). Furthermore, although we did detect promoter activity for genes *BAG1*, *BAG3*, *BAG4*, *BAX*, *BCL2L11*, and *CASP6*, there were no allele-specific differences for any of the haplotypes tested (*Online Supplementary Table S2*).

For the four pro-apoptosis genes (*ADPRT*, *APAF1*, *BAD*, and *BID*), luciferase gene expression controlled by *BID* promoter haplotypes BID-H1, BID-H2 and BID-H3 showed differential expression when transfected into HepG2, HeLa and JEG-3 cells (Table 2), with BID-H3 showing the highest promoter activity (BID-H3/Basic 2.6, 7.1, and 4.5-fold difference, respectively). We found signif-

Table 2. Promoter haplotypes studied in the gene reporter assays.

Gene	Haplotype and pSNP	Haplotype frequencies
<i>BCL2</i>	H1: -1534G, -225G	37.8 %
	H2: -1534G, -225T	16.2 %
	H3: -1534A, -225T	46 %
<i>BCL2A1</i>	H1: -1086G, -800G, -683G	50 %
	H2: -1086G, -800G, -683A	35 %
	H3: -1086C, -800A, -683G	15 %
<i>BCL2L1</i>	H1: -741A, -603G	78.3 %
	H2: -741C, -603C	21.6 %
<i>BCL2L10</i>	H1: -1687C, -954A, -692C, -493T, -330A	64.5 %
	H2: -1687C, -954G, -692C, -493T, -330A	5.3 %
	H3: -1687T, -954A, -692C, -493C, -330G	14.5 %
	H4: -1687T, -954A, -692T, -493T, -330A	10.5 %
<i>BCL2L11</i>	H1: -1728A, -1550C, -1387T, -1194C, -1140insG, -274G	29.3 %
	H2: -1728A, -1550C, -1387T, -1194C, -1140insG, -274A	13.8 %
	H3: -1728G, -1550A, -1387C, -1194T, -1140delG, -274G	55.2 %
<i>BAX</i>	H1: -1916T, -1837A, -1820G, -179G	86 %
	H2: -1916G, -1837T, -1820A, -179A	8 %
<i>BAD</i>	H1: -1685insA, -993A, -283G	44 %
	H2: -1685insA, -993C, -283A	26 %
	H3: -1685delA, -993A, -283G	29 %
<i>BID</i>	H1: -1124T, -477G	62.5 %
	H2: -1124C, -477G	30 %
	H3: -1124C, -477A	7.5 %
<i>BAG1</i>	H1: -1725G, -1710A, -1023C, -685C, -453C	23.7 %
	H2: -1725G, -1710A, -1023A, -685G, -453C	6.2 %
	H3: -1725G, -1710G, -1023C, -685C, -453C	68.7 %
<i>BAG3</i>	H1: -1724A, -856G, -788insT, -787insA, -786insA, -212C	55 %
	H2: -1724A, -856A, -788delT, -787delA, -786delA, -212T	33.3 %
	H3: -1724T, -856G, -788insT, -787insA, -786insA, -212C	10 %
<i>BAG4</i>	H1: -1103A, -118G	79.5 %
	H2: -1103C, -118C	17.9 %
<i>BIRC4</i>	H1: -1352C, -1226T	41.4%
	H2: -1352G, -1226T	7.1%
	H3: -1352G, -1226C	42.8%
<i>BIRC5</i>	H1: -1517C, -1425A, -1223T, -1123G, -1001A, -522T, -503G, -121C	35 %
	H2: -1517A, -1425G, -1223T, -1123G, -1001G, -522T, -503G, -121C	25 %
	H3: -1517C, -1425A, -1223C, -1123A, -1001G, -522C, -503C, -121C	20 %
<i>MCL1</i>	H1: -486G, -256G, -194T, -142delA	65 %
	H2: -486T, -256C, -194G, -142delA	28 %
	H3: -486T, -256C, -194G, -142insa	5 %
<i>APAF1</i>	H1: -528G, -370A, -353delb, -196T	84 %
	H2: -528G, -370A, -353insb, -196T	10.5 %
	H3: -528G, -370C, -353delb, -196A	5 %
<i>CASP3</i>	H1: -1309G, -1147C, -928C, -494C	58 %
	H2: -1309G, -1147T, -928T, -494T	17 %
	H3: -1309C, -1147C, -928T, -494C	13 %
<i>CASP6</i>	H1: -1877C, -1850A, -292C	57 %
	H2: -1877T, -1850A, -292G	5 %
	H3: -1877T, -1850G, -292C	38 %
<i>YWHAQ</i>	H1: -515T, -95C	62 %
	H2: -515T, -95G	10 %
	H3: -515C, -95G	23 %
<i>YWHAB</i>	H1: -913G, -730G, -531C, -141C, -134T, -7G, +70delCGC	22.5 %
	H2: -913C, -730T, -531C, -141C, -134T, -7G, +70delCGC	41 %
	H3: -913C, -730T, -531C, -141T, -134C, -7T, +70insCGC	21 %
<i>ENDOG</i>	H1: -1598T, -1201A, -469A, -378G, -293G, -210C	38.8 %
	H2: -1598C, -1201G, -469T, -378A, -293A, -210T	21.3 %
	H3: -1598C, -1201G, -469T, -378A, -293G, -210T	26.3 %
<i>ADPRT</i>	H1: -1491A, -886T, -589A, -279C, -239C, -219A	59.7 %
	H2: -1491G, -886T, -589G, -279T, -239T, -219T	16.1 %
	H3: -1491G, -886A, -589G, -279C, -239C, -219A	17.7 %

DNA variants were numbered with respect to the first nucleotide of the first exon as +1 and the nucleotide immediately upstream as -1 (based on Ref Seq mRNA).<sup>a</sup>18 bp deletion/insertion variation in the promoter region of MCL1, -142-/GGGGCCCTGAGCCGGGCC.<sup>b</sup>12 bp deletion/insertion variation in the promoter region of APAF1, -353-/AGCGCCTTCAC.

icant allele-specific promoter activity differences between BID-H3 and BID-H1 haplotypes in all three cell lines tested (BID-H3/BID-H1 1.4, 1.7, and 1.5-fold difference, respectively) (Table 2). Interestingly, for *BAD* we found that promoter activity was cell-specific, which is indicative of different modes of regulation in the cell lines tested. For example, *BAD*-H3 had the highest promoter activity (5.7-fold) in HeLa cells, whereas haplotype *BAD*-H2 had the highest activity in JEG-3 (4.8-fold). Similar results were observed with the *APAF1* haplotypes, with different allele-specific promoter activity in HeLa and JEG-3 cells. In HeLa cells, the *APAF1*-H2 haplotype showed highest promoter activity (15-fold) and an allele-specific promoter activity between *APAF1*-H2/*APAF1*-H1 of 3.1-fold; however, in JEG-3 cells, haplotype *APAF1*-H3 had the strongest activity with an *APAF1*-H3/*APAF1*-H1 difference of 4.9-fold. *ADPRT* showed significant increases in promoter activity for haplotype *ADPRT*-H3 compared to *ADPRT*-H1 in both HeLa (1.7-fold) and JEG-3 (2.4-fold) cells; however, in HepG2 cells *ADPRT*-H2 had significantly higher promoter activity (*ADPRT*-H2/*ADPRT*-H1 1.8-fold difference).

We also observed allele-specific promoter activity for all anti-apoptotic genes (*BCL2*, *BCL2L1*, *BIRC4*, *ENDOG*, *MCL1*, *YWHAB*, and *YWHAQ*) (Table 3). *BCL2*, *BCL2L1*, and *ENDOG* showed uniform allele-specific differences in HeLa, JEG-3 and HepG2 cells, whereas *BIRC4*, *MCL1*, *YWHAB*, and *YWHAQ* showed more cell-type specific promoter activity. For example, the luciferase levels driven by the *BCL2*-H3 haplotype were higher than the levels produced by *BCL2*-H2 in HeLa, JEG-3, and HepG2 cells (*BCL2*-H3/*BCL2*-H2 2.3, 1.2, and 1.7-fold differences, respectively) (Table 3). For *MCL1*, *MCL1*-H2 had highest promoter activity in HeLa cells (*MCL1*-H2/*MCL1*-H3 1.4-fold difference), while *MCL1*-H1 had the highest activity in JEG-3 cells (*MCL1*-H1/*MCL1*-H2 1.8-fold). Using the Jurkat leukemia cell line, we further confirmed that *MCL1*-H2 has the highest promoter activity in this cell type (*MCL1*-H2/*MCL1*-H3 1.6-fold difference) (Figure 1). The *BCL2L1* haplotype *BCL2L1*-H1 had the highest luciferase activity across all constructs tested (*BCL2L1*-H1/*BCL2L1*-H2 1.5, 1.7, and 2.2-fold in difference in HeLa, JEG-3 and HepG2 cells, respectively). Thus, we found significant variability in allele-specific promoter activity *in vitro* for the intrinsic apoptosis genes *ADPRT*, *APAF1*, *BCL2*, *BAD*, *BID*, *MCL1*, *BIRC4*, *BCL2L1*, *ENDOG*, *YWHAB*, and *YWHAQ*, which could suggest functional consequences of promoter variation on gene expression and gene function, leading to deregulation of the intrinsic apoptosis pathway and potentially modifying disease risk and outcome.

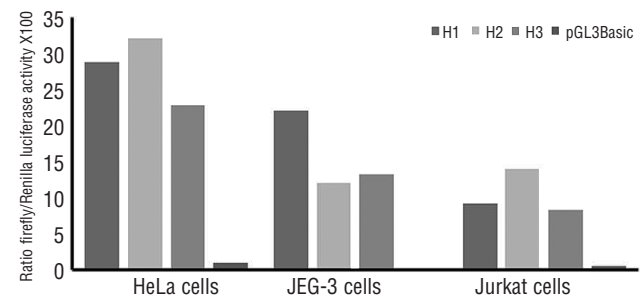
#### Promoter single nucleotide polymorphisms and haplotypes and the risk of childhood pre-B-cell acute lymphoblastic leukemia

To assess the impact of the pSNP/promoter haplotypes with allele-specific promoter activity on disease risk, we performed an association study using a case-control design to test for genotypic as well as haplotypic associations with childhood pre-B ALL. A total of 18 pSNP, in nine genes identified to be associated with changes in promoter activity, were tested for associations. The estimated odds ratios and 95% confidence intervals (95% CI) for the corresponding alleles and genotypes at these positions are given in *Online Supplementary Tables S3* and *S4*. The allele and genotype frequencies for the variant positions in

genes *ADPRT*, *APAF1*, *BAD*, *BCL2*, *BID*, *BIRC4*, *MCL1*, *YWHAB*, and *YWHAQ* did not differ significantly between patients and controls (*Online Supplementary Tables S3* and *S4*), indicating that these pSNP alone do not modify the risk of childhood pre-B ALL, at least in our cohort. To further capture the genetic variability within these promoter regions, haplotypes were reconstructed in the case-control cohort (*Online Supplementary Table S5*) and tested for association with childhood ALL. However, despite their effects on promoter activity, we did not find any evidence of association between the individual haplotypes tested and the risk of developing pre-B ALL.

#### *MCL1* genotypes influence overall survival in high-risk pre-B-cell acute lymphoblastic leukemia patients

Given that none of the pSNP/promoter haplotypes was found to be associated with disease risk, we assessed the impact of the promoter variants on disease outcome. Survival analysis between the 18 pSNP and childhood ALL revealed no significant associations with either event-free or overall survival when all patients (266 patients; for whom genotype and outcome data were available) were analyzed. No association with prognostic factors or treatment protocol was seen. Because disease severity and progression, and treatment intensity differ between risk groups, we performed the analysis with ALL outcome following stratification of patients based on risk (high or standard) and found significant associations of *MCL1* genotypes with overall survival in high-risk patients (Figure 2A). Patients with *MCL1* -486TT genotype had reduced overall survival compared with individuals carrying the G allele (hazard ratio,  $HR_{TT}=4.0$ , 95% CI=1.3-12.5;  $P=0.01$ ). Decreased overall survival was also observed in individuals who were homozygous for *MCL1* -256C ( $HR_{CC}=4.4$ , 95% CI=1.4-13.8;  $P=0.006$ ) and *MCL1* -194G ( $HR_{GG}=3.9$ , 95% CI=1.2-12.4;  $P=0.01$ ) alleles. Haplotype analysis further demonstrated that the associations observed at the three individual *MCL1* loci are not independent and reflect a single association signal deriving from haplotype *MCL1*-H2. All associations remained significant with the inclusion of typical prognostic factors in the multivariate Cox regression analysis (Table 4). We further analyzed the *MCL1* -486 polymorphism in the extended QcALL discovery cohort consisting of 342 child-



**Figure 1.** Gene reporter assays to evaluate the functional impact of the common *MCL1* promoter haplotypes. Relative luciferase activity of *MCL1* promoter haplotype constructs was measured following transient transfection in HeLa, JEG-3 and Jurkat cells. The empty promoterless pGL3-Basic vector was used as a negative control. Results are expressed as a ratio of Firefly/Renilla activity multiplied by 100. *P* values are calculated from four replicates with unpaired Student's *t*-test. Statistically significant differences: H2/H3 for HeLa ( $P<0.0005$ ) and H1/H2 for JEG-3 ( $P<0.005$ ) (see also Table 3).

hood ALL patients. A similar reduction in overall survival was observed for TT individuals (HR<sub>TT</sub> = 4.1, 95% CI=1.6-10.3; *P*=0.001) (Figure 2B) and remained significant in the multivariate Cox regression model (Table 4) and following

multiple testing corrections (false discovery rate = 11%). The *MCL1* -486 variation was subsequently analyzed in the Dana-Farber Cancer Institute replication cohort, showing reductions in both event-free and overall survival

**Table 3.** Results of the gene reporter assays for the haplotypes showing promoter-specific allelic activity.

Gene	Haplotype studied	Ratio of highest allele activity on pGL3-basic			Ratio of highest allele activity on lowest allele activity		
		HeLa	Jeg-3	HepG2	HeLa	Jeg-3	HepG2
<i>BCL2</i>	H1: G-G	2.2	9.1	2.76			
	H2: G-T	1.3	7.4	1.8			
	H3: A-T	3	9.5	3.2	H3/H2 2.3***	H3/H2 1.2*	H3/H2 1.7***
<i>BCL2L1</i>	H1: A-G	41.2	33.7	27.7	H1/H2 1.5*	H1/H2 1.7***	H1/H2 2.2***
	H2: C-C	26.9	15	16.3			
<i>BAD</i>	H1: insA-A-G	1.8	1.6				
	H2: insA-C-A	4.36	4.83	NA		H2/H1 2.9***	
	H3: delA-A-G	5.72	4.58		H3/H1 3.1***		
<i>BID</i>	H1: G-T	4	2.86	1.85			
	H2: G-C	7.09	4.22	2.12			
	H3: A-C	7.09	4.45	2.61	H3/H1 1.8***	H3/H1 1.5***	
<i>BIRC4</i>	H1: C-T	20.8	89.4	33.9	H1/H2 1.8**	H1/H2 2.8***	H1/H2 1.2*
	H2: G-T	11.8	31.3	27.2			
	H3: G-C	15.2	60.2	30			
<i>MCL1</i>	H1: G-G-T-del	27.4	67.6			H1/H2 1.8**	
	H2: T-C-G-del	30.4	37.2	NA	H2/H3 1.4***		
	H3: T-C-G-ins	21.7	40.7				
<i>APAF1</i>	H1: G-A-del-T	4.84	20.7				
	H2: G-A-ins-T	15	51	NA	H2/H1 3.1***		
	H3: G-C-del-A	12.5	102			H3/H1 4.9***	
<i>YWHAQ</i>	H1: T-C	182	58		H1/H3 1.4***	H1/H2 1.5***	
	H2: T-G	134	37.8	NA			
	H3: C-G	127	40.6				
<i>YWHAH</i>	H1: G-G-C-C-T-G-delCGC	69.7	595				
	H2: C-T-C-C-T-G-delCGC	93	1416	NA	H2/H1 1.3***	H2/H1 2.3***	
	H3: C-T-C-T-C-T-insCGC	78.7	933				
<i>ENDOG</i>	H1: T-A-A-G-G-C	13.7	14.6	13	H1/H3 1.8**	H1/H3 1.6***	H1/H3 1.3**
	H2: C-G-T-A-A-T	11.1	11.9	14.6			
	H3: C-G-T-A-G-T	7.4	8.7	9.8			
<i>ADPRT</i>	H1: A-T-A-C-C-A	17.5	4.25	7.1			
	H2: G-T-G-T-T-T	23.4	9.68	13.3			H2/H1 1.8***
	H3: G-A-G-C-C-A	29.2	10.4	12.6	H3/H1 1.7***	H3/H1 2.4***	

NA: not available. \**P*<0.05; \*\**P*< 0.005; \*\*\**P*< 0.0005.

**Table 4.** Survival analysis in ALL patients in relation to *MCL1* G to T variations - multivariate models.

Cohort	QcALL-initial group			QcALL-extended set			DFCI-replication cohort		
	HR**	95% CI	<i>P</i>	HR**	95% CI	<i>P</i>	HR**	95% CI	<i>P</i>
Sex	1.6	0.5-5.7	0.4	1.2	0.4-3.2	0.8	0.7	0.3-1.9	0.5
Age	1.9	0.7-5.2	0.2	2.6	1.1-5.9	0.02	2.4	1.3-4.5	0.008
WBC, ×10 <sup>9</sup> /L	2.9	0.8-10.1	0.1	2.0	0.7-5.8	0.2	2.1	0.9-5.0	0.1
Cell type	*			0.6	0.2-2.4	0.5	1.2	0.4-4.1	0.7
DNA index	0.6	0.1-2.9	0.5	0.7	0.2-3.5	0.7	*		
Treatment protocol	0.7	0.3-1.6	0.4	1.4	0.4-1.4	0.3	2.5	1.1-5.7	0.04
<i>MCL1</i>	4.6	1.3-15.9	0.02	5.0	1.8-14.2	0.002	8.0	1.1-60.2	0.04

A Cox regression model was built to estimate the risk of *MCL1* genotype in high-risk patients in the presence of the prognostic factors. Hazard ratios (HR) and 95% confidence intervals (CI) are indicated. The absence of TT genotype or absence of T486 allele (for the replication cohort) and categories typically associated with lower risk of relapse were considered as references (age ≥1 and <10, WBC count lower than 50×10<sup>9</sup>/L, B-cell type, female gender, hyperdiploidy, DNA index ≥1.16 or 50 chromosomes and most recent treatment protocol). There were four treatment protocols in the Quebec childhood ALL (QcALL) cohort and two in the Dana-Farber Cancer Institute (DFCI) groups (see methods). \*QcALL discovery group included B-cell leukemia only and DNA index was not available for the replication group. \*\*HR refers to overall survival analysis in the QcALL discovery cohort (discovery and extended sets) and to event-free survival in the DFCI replication cohort since the *MCL1*-related HR estimates are not applicable for overall survival (see number of events for overall survival in Figure 2C).

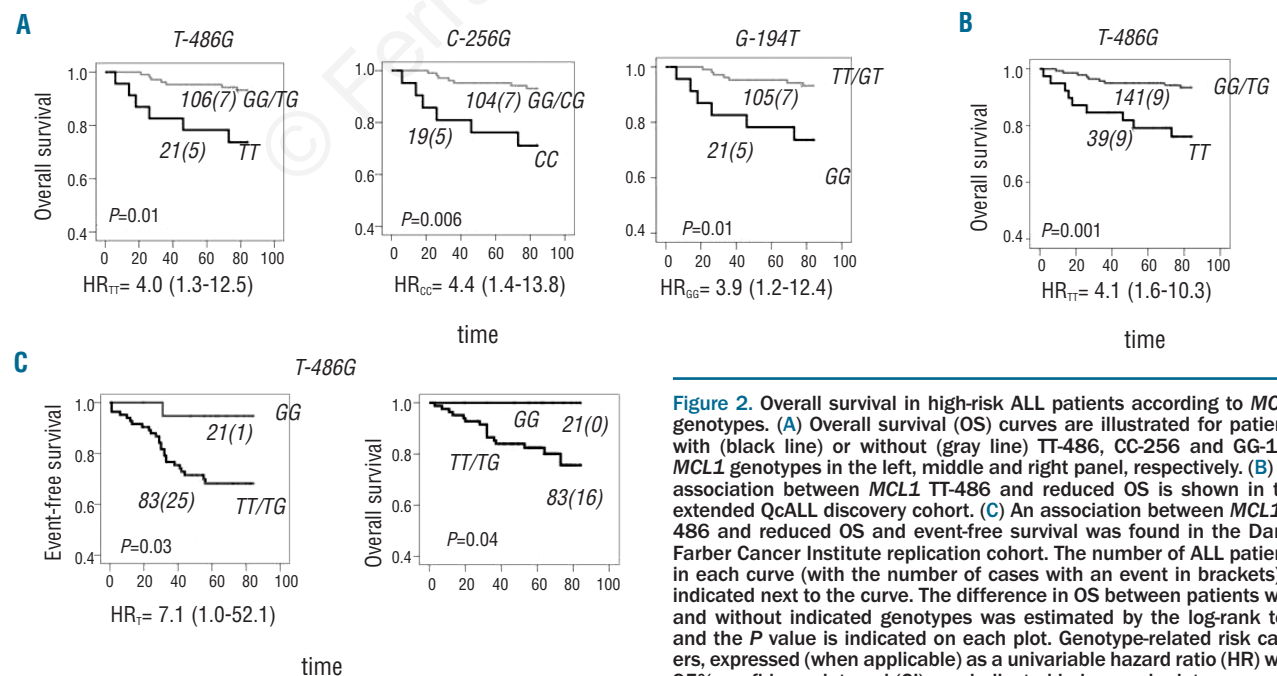
in patients with the T-486 allele ( $P=0.03$  and  $P=0.04$ , respectively; Figure 2C and Table 4).

## Discussion

In this study, we investigated whether deregulation of the highly orchestrated intrinsic apoptosis pathway could influence childhood ALL risk and/or outcome. Using gene reporter assays, we found that 32 promoter haplotypes, and 40 corresponding pSNP, in *ADPRT*, *APAF1*, *BCL2*, *BAD*, *BID*, *MCL1*, *BIRC4*, *BCL2L1*, *ENDOG*, *YWHAQ*, and *YWHAQ* influenced promoter activity in an allele-specific manner. The observed up- or down-regulation of these anti-apoptosis and pro-apoptosis genes, associated with allele-specific promoter variation, could correlate with increased survival and proliferation potential of leukemia cells in patients and affect disease onset and/or outcome. For example, decreased levels of expression of the pro-apoptosis gene *ADPRT* have been shown to decrease drug-induced apoptosis, conferring resistance in human leukemia cells.<sup>26-28</sup> In patients with chemoresistant acute myeloid leukemia, up-regulation of anti-apoptotic *BIRC4* genes and down-regulation of pro-apoptotic *APAF1* have been detected and were associated with dysregulation of the apoptosis machinery;<sup>29</sup> furthermore, leukemia patients expressing high levels of the anti-apoptotic gene *BCL2L12* have been shown to be three times more likely to relapse or die than patients with low levels.<sup>30</sup> The relation between the expression of apoptosis genes and *in vitro*<sup>31,32</sup> or *in vivo* response<sup>33-35</sup> has been extensively studied in ALL. However, to the best of our knowledge, this is the largest systematic analysis of the role of regulatory variation in modulating expression of apoptosis genes and influencing childhood ALL onset and progression. The limitations of the study include the lack of analysis in the promoter region beyond 2 kb and the large number of polymor-

phisms and genes analyzed implying the necessity for multiple testing correction.

Although we did observe gene expression changes linked to promoter variation in a number of key apoptosis genes, we did not find any of the corresponding pSNP/promoter haplotypes to be significantly associated with susceptibility to ALL. This may be due to the limited statistical power of our sample size, or because the variants may not act alone but rather in combination with other variants, in this or other related pathways, through epistatic interactions to influence disease risk. We did, however, identify a statistically significant association between the anti-apoptotic gene *MCL1* and overall survival in our childhood ALL cohort. Homozygosity for the variant alleles at positions *MCL1* -486T>G, -256G>C, -194T>G was predictive of decreased overall survival among high-risk patients. Our reporter assays clearly highlight that the same *MCL1*-H2, carrying variant alleles -486T, -256C, -194G, has higher promoter activity in HeLa and Jurkat cells, compared to the major H1 haplotype that carries the common -486G, -256G, -194T alleles. The association of -486T T>G (used as a tag SNP in further analysis) was confirmed in the extended QcALL discovery and Dana-Farber Cancer Institute replication cohorts. The association with event-free survival was also observed in the replication cohort. These results suggest that promoter variation in *MCL1* could result in higher *MCL1* gene expression and subsequently increased survival of cancer cells thereby influencing treatment response and overall survival. The absence of an association with event-free survival in the discovery cohort and the difference in gene dosage effect between the discovery and replication cohorts might be due to differences in cumulative corticosteroid doses, type of corticosteroid or heterogeneity of the event, since not all patients with an event are necessarily comparable in terms of response to treatment or disease progression. Indeed, the distribution of events between the *MCL1*-positive and -negative groups



**Figure 2.** Overall survival in high-risk ALL patients according to *MCL1* genotypes. (A) Overall survival (OS) curves are illustrated for patients with (black line) or without (gray line) TT-486, CC-256 and GG-194 *MCL1* genotypes in the left, middle and right panel, respectively. (B) An association between *MCL1* TT-486 and reduced OS is shown in the extended QcALL discovery cohort. (C) An association between *MCL1* T-486 and reduced OS and event-free survival was found in the Dana-Farber Cancer Institute replication cohort. The number of ALL patients in each curve (with the number of cases with an event in brackets) is indicated next to the curve. The difference in OS between patients with and without indicated genotypes was estimated by the log-rank test and the  $P$  value is indicated on each plot. Genotype-related risk carriers, expressed (when applicable) as a univariable hazard ratio (HR) with 95% confidence interval (CI), are indicated below each plot.

was more comparable for overall survival than for event-free survival. The number of events in the event-free and overall survival analyses was similar in the *MCL1*-positive group, but different in the *MCL1*-negative group: the overall survival analysis showed a higher frequency of cases with multiple relapses (55% *versus* 36%) and no cases with late relapse (*versus* 27% in event-free survival). Larger cohorts of patients are needed to address differences related to subtypes of events.

*MCL1* is gaining attention as a potential therapeutic target in cancer as it has been implicated in both tumorigenesis and chemoresistance, and in particular *MCL1*-dependent leukemias have been shown to be more sensitive to a wide range of chemotherapeutic agents.<sup>36</sup> There is also evidence that *MCL1* is important for the survival of myeloma cells,<sup>37</sup> and in murine bone marrow viral infection models, *MCL1* was found to accelerate both myeloid and lymphoid malignancies.<sup>38,39</sup> Furthermore, the presence of an *MCL1* promoter insertion was found to correlate with increased RNA and protein levels in leukemia and was associated with poor survival in a cohort of patients with chronic lymphocytic leukemia,<sup>40</sup> further suggesting that regulatory variants in *MCL1* can influence disease outcome. Importantly increased expression of *MCL1* has also been associated with prednisolone resistance in B-lineage ALL cells.<sup>41</sup> Differential expression of this Bcl-2 family member may contribute to the apoptotic blockage that is observed upstream of the mitochondria in prednisolone-resistant ALL cells.<sup>41</sup> Furthermore, the fact that the mTOR inhibitor rapamycin reverses glucocorticoid resistance via *MCL1* modulation confirms that *MCL1* overexpression is essential for corticosteroid-related resistance in leukemia.<sup>42</sup> We observed an association between high-activity *MCL1* haplotypes and reduced overall survival in high-risk patients who received higher doses of corticosteroid compared to the standard-risk group, suggesting that this association could be corticosteroid related. Failure to achieve the required effect of corticosteroid therapy in patients presenting with less favorable disease characteristics could lead to disease progression and dismal outcomes. Indeed, six of nine *MCL1*-H2 homozygous patients with lower overall survival in the extended cohort had resistant dis-

ease as evidenced by multiple relapses or induction failure: six patients died of disease progression, two patients died from bone marrow transplant complications following relapse and one patient died from treatment complications with no prior relapse. This study provides the first evidence that pSNP in *MCL1* could lead to dysregulation of the intrinsic apoptosis process in ALL cells, and influence patients' response to treatment, suggesting that *MCL1* is an important factor for the clinical response in childhood ALL. Furthermore, given the complex action of corticosteroids, it is possible that other genetic factors may also play a role, concomitantly or through gene-gene interactions, in modulating disease outcome.

The fact that we identified a significant association of *MCL1* promoter variants with overall survival following risk-based stratification may be explained by the presence of modifying effects of corticosteroids acting in conjunction with more aggressive presenting disease. Our results provide evidence that *MCL1* plays a role in disease progression, drug resistance and/or corticosteroid-induced apoptosis. The observed functional effects of the promoter polymorphisms/haplotypes on *ADPRT*, *APAF1*, *BCL2*, *BAD*, *BID*, *MCL1*, *BIRC4*, *BCL2L1*, *ENDOG*, *YWHAQ* and *YWHAQ* expression warrant further investigation in larger cohorts of children with ALL.

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#### Authorship and Disclosures

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