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# Wilms' tumor gene (*WT1*) is strongly expressed in high-risk subsets of pediatric acute lymphoblastic leukemia

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## Abstract

**Aim:** The purpose of the present study was to perform a comprehensive analysis of *WT1* gene expression in high-risk pediatric acute lymphoblastic leukemia (ALL).

**Methods:** We performed a meta-analysis of *WT1* gene expression for normal hematopoietic cells *vs.* primary leukemia cells from 801 pediatric ALL samples deposited in the Oncomine database combined with an in-depth gene expression analysis using our in-house database of gene expression profiles of primary leukemia cells from 1416 pediatric ALL cases. We also examined the expression of *WT1* in primary leukemic cells from 299 T-lineage ALL patients in the Oncomine database and 189 T-lineage ALL patients in the archived datasets GSE13159, GSE13351, and GSE13159.

**Results:** Our data provide unprecedented evidence that primary leukemia cells from patients with *MLL* gene rearrangements (*MLL-R*) express highest levels of *WT1* expression within the high-risk subsets of pediatric B-lineage ALL. Notably, *MLL-R*<sup>+</sup> patients exhibited > 6-fold higher expression levels of the *WT1* gene compared to the other B-lineage ALL subtypes combined (*P* < 0.0001). Our findings in 97 *MLL-R*<sup>+</sup> infant B-lineage ALL cases uniquely demonstrated that *WT1* is expressed at 1.5-4.2-fold higher levels in *MLL-R*<sup>+</sup> infant leukemia cells than in normal hematopoietic cells and revealed that *WT1* expression level was substantially higher in steroid-resistant infant leukemia cells when compared to non-leukemic healthy bone marrow cells. Furthermore, our study demonstrates for the first time that the *WT1*-regulated *EWSR1*, *TP53*, *U2AF2*, and *WTAP* genes (i.e., *WT1* interactome) were differentially upregulated in *MLL-R*<sup>+</sup> leukemia cells illustrating that the *MLL*-regulatory pathway is aberrantly upregulated in *MLL-R*<sup>+</sup> pediatric B-lineage ALL. These novel insights provide a compelling rationale for targeting *WT1* in second line treatment of *MLL-R*<sup>+</sup>



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pediatric B-lineage ALL, including *MLL-R*<sup>+</sup> infant ALL. Furthermore, our study is the first to demonstrate that leukemia cells from 370 Ph-like patients had significantly higher *WT1* expression when compared to normal hematopoietic cells. Finally, our findings demonstrate for the first time that chemotherapy-resistant primarily leukemic cells from relapsed B-lineage ALL patients exhibit higher expression levels of *WT1* than primary leukemia cells from newly diagnosed B-lineage ALL patients ( $P = 0.001$ ).

**Conclusion:** Our findings indicate that the *WT1* gene product may serve as a target for immunotherapy in high risk/poor prognosis subsets of newly diagnosed as well as relapsed pediatric B-lineage ALL. Our findings also significantly expand the current knowledge of *WT1* expression in T-lineage ALL and provide new evidence that *WT1* gene and its interactome are expressed in T-lineage ALL cells at significantly higher levels than in normal hematopoietic cells. This previously unknown differential expression profile uniquely indicates that the protein product of *WT1* would be an attractive molecular target for treatment of T-lineage ALL as well.

**Keywords:** Wilms' tumor gene, *WT1* gene, leukemia, chemotherapy resistance, immunotherapy

## INTRODUCTION

The Wilms' tumor gene (*WT1*) is a tumor-suppressor gene associated with Wilms' tumor, a pediatric kidney cancer<sup>[1-3]</sup>. The protein product of the *WT1* gene is a zinc-finger transcription factor and a tumor-associated antigen. WT1 protein is abundantly expressed in numerous hematological malignancies, including acute myeloid leukemia (AML)<sup>[4-18]</sup>. While WT1 is an intracellular protein, WT1-derived peptides are expressed on the surface of cancer cells in the context of human leukocyte antigen (HLA) Class I antigens. As such they can be targeted by bispecific T-cell engagers, chimeric antigen receptor (CAR)-T cells and WT1-specific CTL that expand after WT1 peptide vaccine treatments<sup>[19-26]</sup>. Several WT1-specific targeted cancer immunotherapy modalities, including therapeutic vaccines, WT1-specific CTL or CAR-T cell platforms, are currently being evaluated in clinical trials for multiple therapeutic indications<sup>[19-26]</sup>. Recently, a T-cell receptor (TCR) that specifically reacts with the WT1 peptide in the context of HLA-A\*24:02 has been identified<sup>[27]</sup>. This WT1-specific TCR-gene was transduced to autologous T-cells using a retroviral vector encoding small interfering RNAs for endogenous TCR genes to generate WT1-specific TCR-T cells capable of executing an immune reaction to WT1-expressing AML cells. The early results of the first-in-human Phase 1 clinical trial (www.umin.ac.jp as #UMIN000011519) of this WT1 TCR transduced T-cell therapy (also known as WT1 antigen-specific RetroNectin TCR gene therapy - Takara Bio) in therapy-refractory AML patients have been reported<sup>[24]</sup>. Shah *et al.*<sup>[19]</sup> reported the safety and feasibility of a WT1 peptide-loaded donor-derived dendritic cell (DC) vaccine given with donor lymphocyte infusions designed to enhance and direct the graft-versus-leukemia effect. Preliminary results indicate the DC-based vaccination is safe and feasible after allogeneic hematopoietic stem cell transplantation (HSCT), and suggest that this strategy could potentially be used to sensitize the repopulated allogeneic-donor immune system to WT1+ leukemia cells. Currently, there are several active clinical studies evaluating the safety and clinical potential of different WT1 targeting treatment platforms in pediatric (4 studies registered at clinicaltrials.gov: NCT0289541, NCT0277082, NCT0220390, NCT0316405) or adult (11 studies registered at clinicaltrials.gov: NCT02405338, NCT02550535, NCT02895412, NCT03083054, NCT01734304, NCT02770820, NCT02750995, NCT02203903, NCT03164057, NCT0313998, NCT02498665) AML patients.

In contrast to AML, there is a paucity of knowledge regarding the expression of WT1 antigen in acute lymphoblastic leukemia (ALL). Only a few studies with relatively small patient numbers have evaluated WT1 expression in ALL<sup>[8-10,12]</sup> and there is no active interventional clinical trial evaluating the clinical potential of a WT1-targeting therapeutic platform in ALL patients. The purpose of the present study was to perform a comprehensive analysis of *WT1* gene expression in high-risk pediatric ALL. Our results provide new evidence that *WT1* is expressed at very high levels in pediatric ALL subsets commonly associated with chemotherapy resistance, including B-lineage ALL with *MLL* gene rearrangements, Ph-like ALL, and T-lineage ALL.

## METHODS

### Gene expression analyses

We utilized an online data mining tool, Oncomine™ research edition, harboring 715 datasets with 86,733 samples for cancer patients and cell lines to assess expression of *WT1* across major cancer subtypes (<https://www.oncomine.org/resource/main.html>). We focused our analysis on mRNA expression comparing normal vs. ALL samples ( $\log_2$  median centered expression values for each data set) and filtered expression signals that were greater than 1.5-fold difference and *P*-value from two sample *t*-tests less than 0.001. In addition, we compiled 6 archived gene expression profiling datasets that measured expression from B-lineage ALL patients hybridized to Human Genome U133 Plus 2.0 Array, including GSE11877, *n* = 207; GSE13159, *n* = 823; GSE13351, *n* = 107; GSE18497, *n* = 82; GSE28460, *n* = 98; GSE7440, *n* = 99 (Total *n* = 1416), as described<sup>[28-31]</sup>.

To enable comparison of samples across studies, a normalization procedure was performed that merged the raw data from the 6 datasets (CEL files). Perfect Match (PM) signal values for probesets were extracted utilizing raw CEL files matched with probe identifiers obtained from the Affymetrix provided CDF file (HG-U133\_Plus\_2.cdf) implemented by Aroma Affymetrix statistical packages<sup>[32]</sup> ran in R-studio environment (Version 0.97.551, R-studio Inc., running with R 3.01). The PM signals were quantified using Robust Multiarray Analysis in a 3-step process including robust multi-array analysis (RMA) background correction, quantile normalization, and summarization by Median Polish of probes in a probeset across 1,416 samples (RMA method adapted in Aroma Affymetrix). RMA background correction estimates the background by a mixture model whereby the background signals are assumed to be normally distributed and the true signals are exponentially distributed. Normalization across all 6 studies and 1,416 samples was achieved using a two-pass procedure. First the empirical target distribution was estimated by averaging the (ordered) signals over all arrays, followed by normalization of each array toward this target distribution. Expression for specific probe sets was compared for a subset of newly diagnosed B-lineage ALL [GSE1187 (*n* = 207), GSE13159 (*n* = 575), GSE13351 (*n* = 92)], newly diagnosed T-cell ALL [GSE13159 (*n* = 174), GSE13351 (*n* = 15)] and normal control cells [GSE13159 (*n* = 74); 73 control samples contained normal nonleukemic bone marrow cells, and one sample was a non-leukemic peripheral blood sample].

A more targeted comparison was also performed comparing fusion-transcript positive high-risk ALL subsets {*MLL-R*<sup>+</sup> [GSE11877 (*n* = 21), GSE13159 (*n* = 70), GSE13351 (*n* = 4)], *E2A-PBX1*<sup>+</sup> [GSE11877 (*n* = 23), GSE13159 (*n* = 36), GSE13351 (*n* = 2)], *BCR-ABL/Ph*<sup>+</sup> [GSE13159 (*n* = 122), GSE13351 (*n* = 1)] and normal cells [GSE13159 (*n* = 74)]}. The in-house database was further updated utilizing the RMA normalization procedure that included the 6 datasets plus the addition of 2 datasets that examined infant ALL cases [GSE32962, *n* = 43; GSE68720, *n* = 97 (total in the updated database *n* = 1556 samples)] for comparison of infant ALL cases with other high-risk ALL subsets.

Ph-like B-lineage ALL cases were previously identified using a set of 110 gene probeset gene expression signature developed utilizing the Affymetrix U133 plus 2.0 microarray platform<sup>[33,34]</sup>. In our study, expression values for each of the 110 probesets were averaged across 123 *BCR-ABL*<sup>+</sup> cases from the normalized database of 1416 patients to provide an expression template for subsequent correlation analysis. Three studies (GSE11877, GSE13159, GSE13351) referenced normal samples (*n* = 74), T-lineage ALL (*n* = 189), *BCR-ABL*<sup>+</sup> ALL (*n* = 123), *E2A-PBX1*<sup>+</sup> ALL (*n* = 61), *MLL-R*<sup>+</sup> ALL (*n* = 95) and other (*n* = 595) subtypes of primary samples. Gene expression of each sample was correlated against the 110 probeset expression template resulting in a distribution of correlation coefficient values (*r*) for each subtype of primary samples. Ph-like ALL samples were defined from a cut off “*r*” value such that the sample from the “*r*” value was greater than the 99% percentile of the “*r*” values in normal samples and greater than the minimum “*r*” value for the known *BCR-ABL*<sup>+</sup> samples. The “Other” group was further partitioned into “Ph-like” and “Other” groups based on the “*r*” value cut off. This procedure identified 370 Ph-like samples used in subset comparisons.

We also examined the archived gene expression profiling data on primary leukemia cells from studies examining matched-pair B-lineage ALL samples taken at diagnosis and at relapse [GSE18497 (27 pairs) and GSE28460 (49 pairs)]. A mixed ANOVA model (probeset level and sample level) was utilized to determine significant differences in expression between the matched diagnostic and relapse samples. A two-way agglomerative hierarchical clustering technique was used to organize expression patterns using the average distance linkage method such that probesets having similar matched pair differences across patients and samples having similar matched pair differences levels were grouped together. Archived gene expression datasets from the Microarray Innovations in Leukemia (MILE) Study, that were generated utilizing the Affymetrix Human Genome U133 Plus 2.0 Array and deposited in GEO (GSEA13159), were also used. The samples consisted of 2,096 blood or bone marrow samples of acute and chronic leukemia patients. Pre-processed data reported as DQN3 values were directly downloaded for analysis. The expression measure included a summarization and quantile normalization step to generate probe set level signal intensities for each microarray experiment (DQN3 values calculated using a published algorithm<sup>[35]</sup>). One way ANOVA was performed for 18 subclasses of ALL and AML<sup>[36]</sup> and significant differences were assessed using Dunnett's *post hoc* tests of each class of leukemic vs. normal samples (R packages multcomp 1.4-6 and Stats 3.3.1).

### Statistical comparisons

Statistical significance was assessed for pairwise comparisons of leukemic samples with non-leukemic control samples using analysis of variance methods. Expression profiles of *WT1*, *STAT3*, *CD19*, *CD7* and the *WT1* interactome from the HPRD interaction set were defined in the Oncomine database [*EWSR1*, *PAWR*, *SRC*, *TP53*, *U2AF2*, *UBE21*, *WTAP* (<https://www.oncomine.org/resource/ui/component/conceptInformation.html?component=gs:130465>)] were utilized to determine differences in expression and error distributions for calculation of *P*-values. *STAT3*, *CD19* and *CD7* were included as control genes. The inclusion of the *WT1* interactome in the analysis results in a more robust determination of model errors for *P*-value calculations being members of the same pathway. We constructed a 3 factor Mixed Model Analysis of Variance analysis to compare the expression of the *WT1* interactome and control genes in high-risk cancer patients extracted from the RMA normalized database. Two fixed factors were considered in this model: One factor was the expression of probesets (34 human Affymetrix and 17 Illumina probesets representing the 8 *WT1* HPRD interaction set), the second factor was comparison of patient sub-groups (Cancer subgroup vs. Normal samples) and one interaction term for probeset x patient sub-group. We included a random factor for each sample to correct for multiple measurements taken from each patient and accounting for variation between patients partitioned as a separate variance component. Least squares method was used fit the parameters for these Mixed ANOVA models (R packages lme4 v1.1-12 and lmerTest v2.1-32). The Root Mean Square Error term calculated from the deviation of data points from the model was utilized to determine significant changes in probeset expression in the planned linear contrasts. These contrasts compared the effect sizes (expressed as difference in log<sub>2</sub> transformed RMA normalized values converted to fold change relative to normal samples) such that the combination of linear parameters to be jointly tested sum to zero for each level of the contrast. Least square mean values were used to construct contrasts between patient sub-groups (set to -1) with the comparison group (set to 1) for the overall increase in the probesets representing the *WT1* interactome by taking the effect sizes and standard error of mean calculated for the "patient-subgroup" factor, and for each individual probeset from the effect sizes and standard error of mean calculated using the interaction terms. Two-tailed tests for differences between the Least Square Means with *P*-values less than 0.05 were deemed statistically significant after controlling for False Discovery Rate.

## RESULTS

### Expression of *WT1* in ALL

Oncomine database survey of *WT1* mRNA expression in ALL vs. non-leukemic healthy bone marrow samples showed 1.5- to 9.0-fold higher expression in T-lineage ALL patients (*n* = 299) and 2.4-fold to 4.9-fold higher expression in B-lineage ALL patients (*n* = 502) with highly significant *P*-values for all studies deposited in

**Table 1. Oncomine survey of *WT1* mRNA expression in leukemic bone marrow samples vs. non-leukemic bone marrow specimens from healthy individuals**

WT1 expression comparing leukemic vs. normal specimens					Sample size		Primary database accession <sup>#</sup>
ALL subtype	P-value	T-statistic	Fold change	Oncomine reference	Normal	Cancer	
T-cell childhood ALL	$6.35 \times 10^{-4}$	5.5	9.0	Coustan-Smith Leukemia	4	46	GSE28497
Pro-B ALL	$9.88 \times 10^{-29}$	18.4	4.9	Haerlach Leukemia	74	70	GSE13159
Pro-B ALL	$4.87 \times 10^{-12}$	12.8	3.0	Haerlach Leukemia 2	58	23	GSE13164
B-cell ALL	$1.70 \times 10^{-4}$	5.5	2.4	Andersson Leukemia	5	50	GSE7186
T-cell ALL	$1.17 \times 10^{-24}$	11.9	1.9	Haerlach Leukemia	74	174	GSE13159
B-cell childhood ALL	$7.14 \times 10^{-42}$	15.4	1.8	Haerlach Leukemia	74	359	GSE13159
T-cell ALL	$1.33 \times 10^{-9}$	6.7	1.5	Haerlach Leukemia 2	58	79	GSE13164

We utilized an online Oncomine<sup>TM</sup> data mining tool harboring mRNA expression profiles for cancer patients and cell lines to assess expression of *WT1* across major cancer subtypes (<https://www.oncomine.org/resource/main.html>). We focused our analysis on mRNA expression comparing non-leukemic normal control samples vs. leukemic samples from acute lymphoblastic leukemia (ALL) patients (5 independent comparisons, log<sub>2</sub> median centered expression values for each data set) and filtered expression signals that were greater than 1.5-fold difference and *P*-value from two sample *t*-tests less than 0.001. *WT1* expression was ranked according to fold change to identify significantly different expression values. The table depicts the *T*-statistic, *P*-value, sample sizes for non-leukemic normal and ALL samples for each study that were also referenced to the Gene Expression Omnibus identifier (GSE<sup>#</sup>). All 5 studies in the Oncomine database exhibited significant increases in *WT1* expression for ALL samples (fold change ranged from 1.5 to 9.0 relative to non-leukemic bone marrow samples)

the Oncomine database [Table 1].

### Amplified Expression of *WT1* in T-lineage ALL

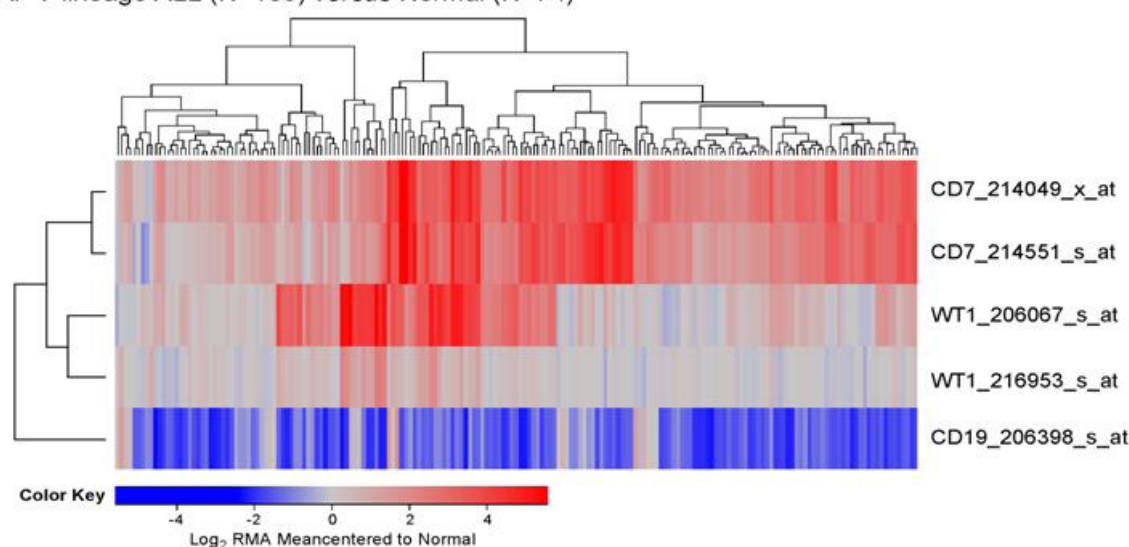
We used the archived datasets GSE 13351 and GSE13159 to examine the expression of *WT1* in primary leukemic cells from 189 T-lineage ALL patients vs. normal hematopoietic cells in 74 control samples. In T-lineage ALL cells, the gene for the pan-T lineage marker antigen CD7 that served as a positive control exhibited significantly higher expression levels (Figure 1A and B; Expression > 4-fold change for both CD7 probesets,  $P < 1 \times 10^{-16}$ ), whereas the negative control gene *CD19*, a pan-B lineage marker, was expressed at exhibited significantly lower expression levels than in normal hematopoietic cells (Figure 1; Fold change for *CD19* expression relative to normal = 0.4,  $P < 1 \times 10^{-16}$ ). Notably, one of the 2 *WT1* probesets, 206067\_s\_at detected a 2.7-fold increased expression level in T-lineage ALL cells with a highly significant linear contrast *P*-value of  $< 1 \times 10^{-16}$ . A significantly increased > 1.2-fold higher expression level was also detected with the *WT1* probeset WT1\_216953\_s\_at [Figure 1B]. We next used a 3-factor mixed model analysis of variance (ANOVA) to compare the expression of the *WT1*-directed interactome from the Oncomine HPRD interaction set defined by 36 probesets using the RMA-normalized combined database. As shown in Figure 1, the *WT1*-regulated genes *EWSR1* (4 of 4 probesets), *TP53* (2 of 2 probesets), *PAWR* (3 of 6 probesets) showed upregulated expression in T-lineage ALL cells (Linear Contrast,  $P < 0.01$  for all comparisons). Further, 1 of the 4 probesets for the *WT1*-regulated *U2AF2* gene and 1 of the 6 probesets of the *WT1*-regulated *WTAP* gene also showed significantly amplified expression in T-lineage ALL cells. Taken together, these results provide new evidence that *WT1* gene and its interactome are expressed in T-lineage ALL cells at higher levels than in normal hematopoietic cells.

### Expression of *WT1* in distinct molecular subsets of B-lineage ALL

We next interrogated the GSE13159 database of gene expression data that were generated utilizing the Affymetrix Human Genome U133 Plus 2.0 Array from the MILE Study for *WT1* mRNA expression in distinct molecular subsets of B-lineage ALL patients (i.e., t(9;22)/Ph<sup>+</sup>, Hyperdiploid, t(1;19)<sup>+</sup>, 11q23/MLL-R<sup>+</sup>, t(8;14)<sup>+</sup>, t(12;21)<sup>+</sup>;  $n = 339$ ). The MILE study compared gene expression profiles for subsets of leukemia defined by gold standard laboratory techniques, and controlled for statistical batch effects from hybridizations measured at different clinics utilizing pre-processing methods including DQN3 normalization of the detected signals. Leukemic cells from B-lineage ALL patients with *MLL-R*<sup>+</sup>, corresponding the most immature immunophenotype (Pro-B) frequently associated with chemotherapy resistance, exhibited the highest



## A. T-lineage ALL (N=189) versus Normal (N=74)



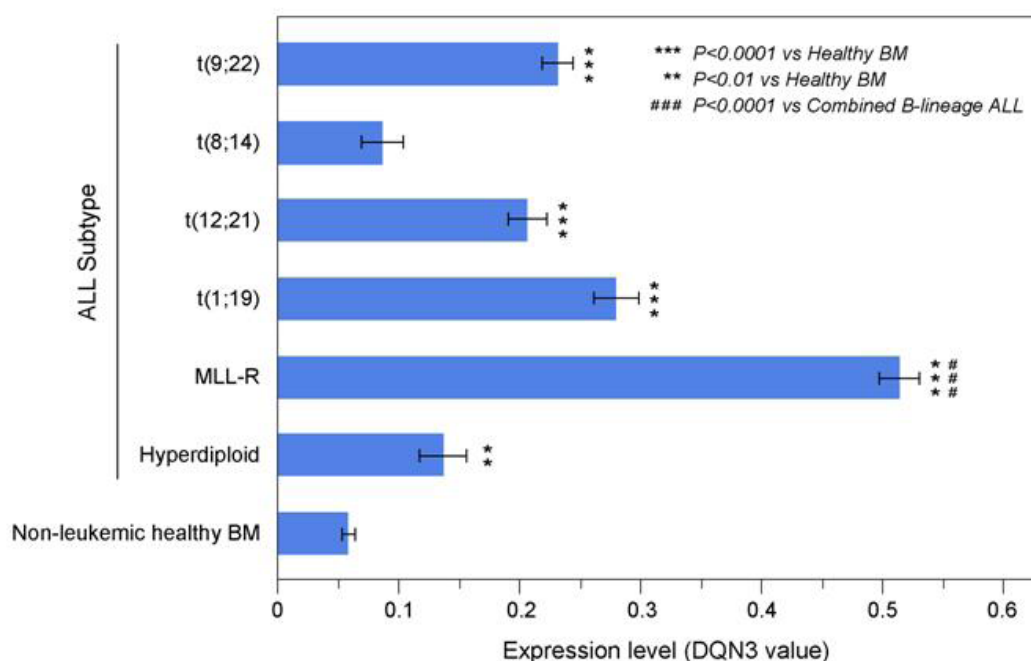
## B. Representation of the WT1 Interactome in T-lineage ALL Transcriptome

Gene_AffylID	Fold Difference (vs Healthy BM)	Linear Contrast (P-value)
CD7_214049_x_at	6.2	<1.0×10 <sup>-16</sup>
CD7_214551_s_at	4.9	<1.0×10 <sup>-16</sup>
WT1_206067_s_at	2.7	<1.0×10 <sup>-16</sup>
EWSR1_229966_at	2.4	<1.0×10 <sup>-16</sup>
EWSR1_210012_s_at	1.8	<1.0×10 <sup>-16</sup>
PAWR_226223_at	1.7	2.8×10 <sup>-13</sup>
PAWR_204005_s_at	1.6	2.8×10 <sup>-10</sup>
TP53_201746_at	1.5	1.2×10 <sup>-9</sup>
PAWR_204004_at	1.5	2.7×10 <sup>-9</sup>
U2AF2_218381_s_at	1.4	6.9×10 <sup>-6</sup>
PAWR_229515_at	1.4	2.0×10 <sup>-5</sup>
TP53_211300_s_at	1.3	7.0×10 <sup>-5</sup>
WTAP_1560274_at	1.3	4.4×10 <sup>-4</sup>
EWSR1_209214_s_at	1.3	5.4×10 <sup>-4</sup>
EWSR1_210011_s_at	1.2	3.0×10 <sup>-3</sup>
WT1_216953_s_at	1.2	4.2×10 <sup>-3</sup>
CD19_206398_s_at	0.4	<1.0×10 <sup>-16</sup>

**Figure 1.** Augmented expression of *WT1* in Pediatric T-lineage acute lymphoblastic leukemia (ALL). Expression of log<sub>2</sub> transformed, robust multi-array analysis (RMA) normalized values for primary leukemia cells from 189 newly diagnosed pediatric T-lineage ALL patients were mean centered to the mean expression of 74 normal samples and clustered using a two-way algorithm (average distance metric) to determine co-regulation of *WT1* and the control genes. A: The heat map ranging from blue to red represents expression lower than normal to greater than normal in T-lineage ALL samples respectively for the transformed expression values. *CD7* and *WT1* were upregulated and *CD19* was downregulated in T cell ALL samples; B: expression levels were also examined for the WT1 interactome identified from the HPRD database. A 3 factor Mixed Model Analysis of Variance analysis was utilized to compare the expression of the WT1 interactome plus the control genes in T lineage ALL patients with normal samples extracted from the in-house RMA normalized database (36 probesets analyzed to determine least square error of the expression measurements used to calculate *P*-values) and the most significantly affected expression levels are shown. Fold change and linear contrast *P*-values are depicted for the *WT1*, WT1 interactome, *CD19* and *CD7* probesets

expression levels for *WT1* (~8.8-fold higher levels than in normal hematopoietic cells, *P* < 0.00001) [Figure 2]. By contrast, leukemia cells from Burkitt's/B-cell leukemia patients with t(8;14) corresponding to the most mature immunophenotype had the lowest *WT1* expression levels that were not significantly different from the *WT1* expression levels of normal hematopoietic cells. Leukemic cells from all other molecular subsets

## A. MILE database WT1\_206067\_s\_at expression



## B.

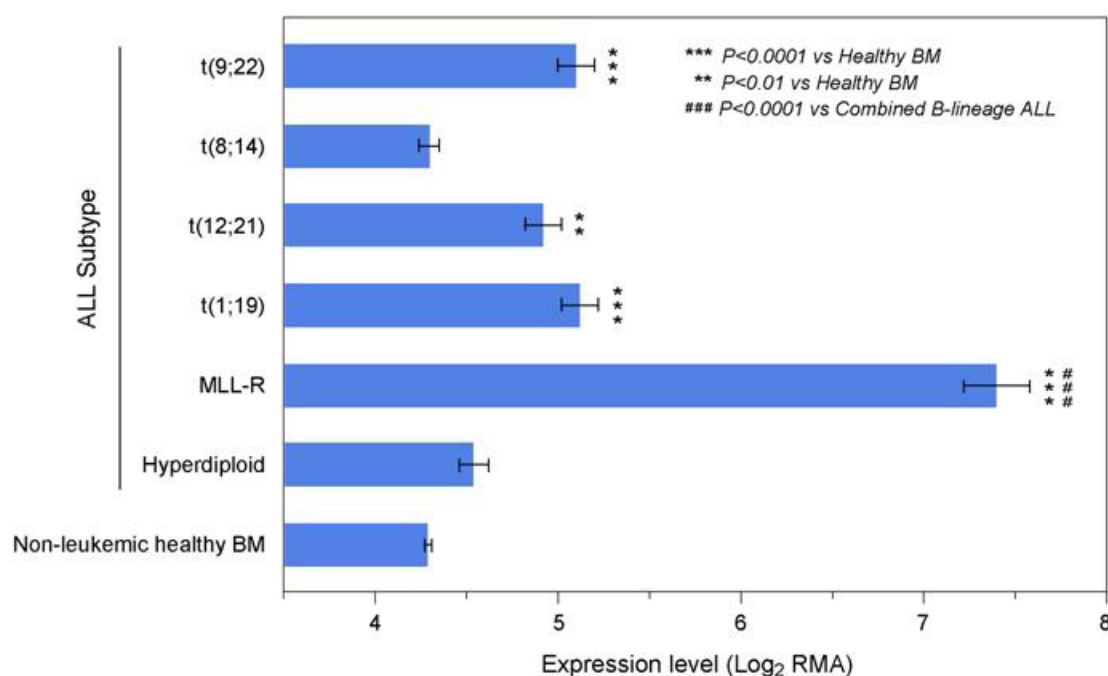
ALL subtype	Sample Size (N)	Expression level (DQN3)	SEM	Fold Difference (vs Healthy BM)	Linear Contrast (P-value)	Fold Difference (vs Combined B-lineage ALL)	Linear Contrast (P-value)
Non-leukemic healthy BM	74	0.059	0.006				
Hyperdiploid	40	0.137	0.019	2.3	0.001		
MLL-R	70	0.514	0.017	8.8	<0.0001	6.6	<0.0001
t(1;19)	36	0.280	0.019	4.8	<0.0001		
t(12;21)	58	0.206	0.016	3.5	<0.0001		
t(8;14)	13	0.087	0.018	1.5	0.4		
t(9;22)	122	0.232	0.013	3.9	<0.0001		

**Figure 2.** Augmented expression of *WT1* in cytogenetically distinct B-lineage acute lymphoblastic leukemia (ALL) subsets. Increased levels of *WT1* expression in ALL subsets compared to normal samples from the Microarray Innovations in Leukemia (MILE) Study. Gene expression profiling data from the MILE Study were interrogated for differences in *WT1* expression levels. A: DQN3-normalized expression levels for the *WT1* probeset, 206067\_s\_at in ALL subsets are depicted using mean and standard error values presented in the bar chart ( $\pm$  SEM). Primary leukemia cells from *MLL-R*<sup>+</sup> B-lineage ALL patients exhibited the highest expression levels for *WT1*. Sample sizes for t(9;22), t(8;14), t(12;21), t(1;19), *MLL-R*, Hyperdiploid, Non-leukemic healthy BM were 122, 13, 58, 36, 70, 40 and 74 respectively; B: one way ANOVA was performed across 7 subgroups of B-lineage ALL cases in the MILE study and statistical significance was assessed utilizing linear contrasts of each subgroup vs. nonleukemic control samples, and for *MLL-R* subgroup vs. the other B-lineage ALL subgroups combined. Depicted are the mean values fold differences in expression and the corresponding *P*-values

had *WT1* expression levels that were higher than those of normal hematopoietic cells [Figure 2].

We then used our in-house database containing the combined dataset compiled from the archived datasets GSE11877, GSE13159, GSE13351 to examine the expression of *WT1* in primary leukemic cells from 95 newly diagnosed pediatric B-lineage ALL patients vs. normal hematopoietic cells in 74 control samples (73 non-leukemic bone marrow samples and one non-leukemic blood sample). In this compiled dataset, *MLL-R*<sup>+</sup> patients exhibited the highest level of expression of *WT1*, 206067\_s\_at probeset compared to the other B-

## A. Inhouse database WT1\_206067\_s\_at expression



## B.

ALL subtype	Sample Size (N)	Expression level (Log <sub>2</sub> RMA)	SEM	Fold Difference (vs Healthy BM)	Linear Contrast (P-value)	Fold Difference (vs Combined B-lineage ALL)	Linear Contrast (P-value)
Non-leukemic healthy BM	74	4.29	0.02				
Hyperdiploid	68	4.54	0.08	1.2	0.1		
MLL-R <sup>+</sup>	95	7.40	0.18	8.7	<0.0001	6.1	<0.0001
t(1;19)	61	5.12	0.10	1.8	<0.0001		
t(12;21)	58	4.92	0.10	1.6	0.0005		
t(8;14)	13	4.30	0.05	1.0	0.9		
t(9;22)	123	5.10	0.10	1.8	<0.0001		

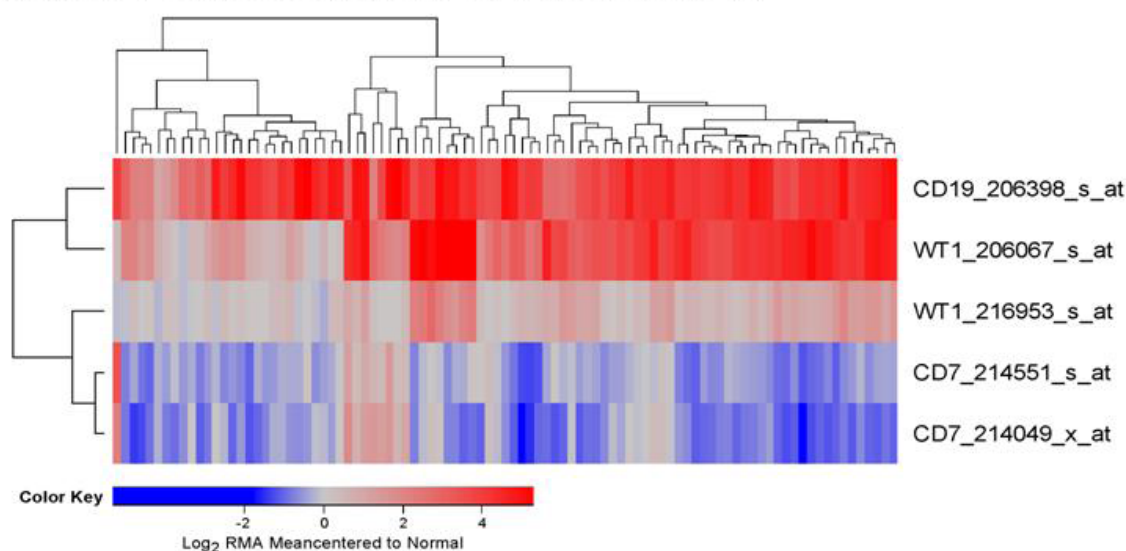
**Figure 3.** Augmented expression of *WT1* in cytogenetically distinct B-lineage acute lymphoblastic leukemia (ALL) subsets. Increased levels of *WT1* expression in ALL subsets compared to normal samples from the in-house database. Gene expression profiling data utilizing the Affymetrix Human Genome U133 Plus 2.0 Array from the robust multi-array analysis (RMA) normalized in-house database were interrogated for *WT1* expression in ALL samples relative to normal non-leukemic healthy bone marrow samples. A: Log<sub>2</sub> transformed RMA values for the *WT1* probeset, 206067\_s\_at in ALL subsets are depicted using mean and standard error values presented in the bar chart. ALL with *MLL-R* exhibited the highest expression levels for *WT1*. Sample sizes for t(9;22), t(8;14), t(12;21), t(1;19), *MLL-R*<sup>+</sup>, Hyperdiploid, Non-leukemic healthy BM were 123, 13, 58, 61, 95, 68 and 74 respectively; B: one way ANOVA was performed across 7 subgroups of ALL cases in the in-house database and statistical significance was assessed utilizing linear contrasts of each subgroup vs. nonleukemic control samples, and for *MLL-R*<sup>+</sup> subgroup vs. the other B-lineage ALL subgroups combined. Depicted are the mean values fold differences in expression and the corresponding *P*-values

lineage ALL subtypes (Figure 3; ~6.1-fold increase vs. combined B-lineage ALL,  $P < 0.0001$ ).

In leukemic B-cell precursors from *MLL-R*<sup>+</sup> B-lineage ALL patients, the gene for the pan-B lineage marker antigen CD19 that served as a positive control was expressed at > 16-fold higher expression levels, whereas the negative control gene *CD7*, a pan-T lineage marker, was expressed at significantly lower levels than in normal hematopoietic cells [Figure 4]. Notably, both of the *WT1* probesets exhibited increased expression



### A. MLL-R<sup>+</sup> Pediatric B-lineage ALL (N=95) versus Normal (N=74)



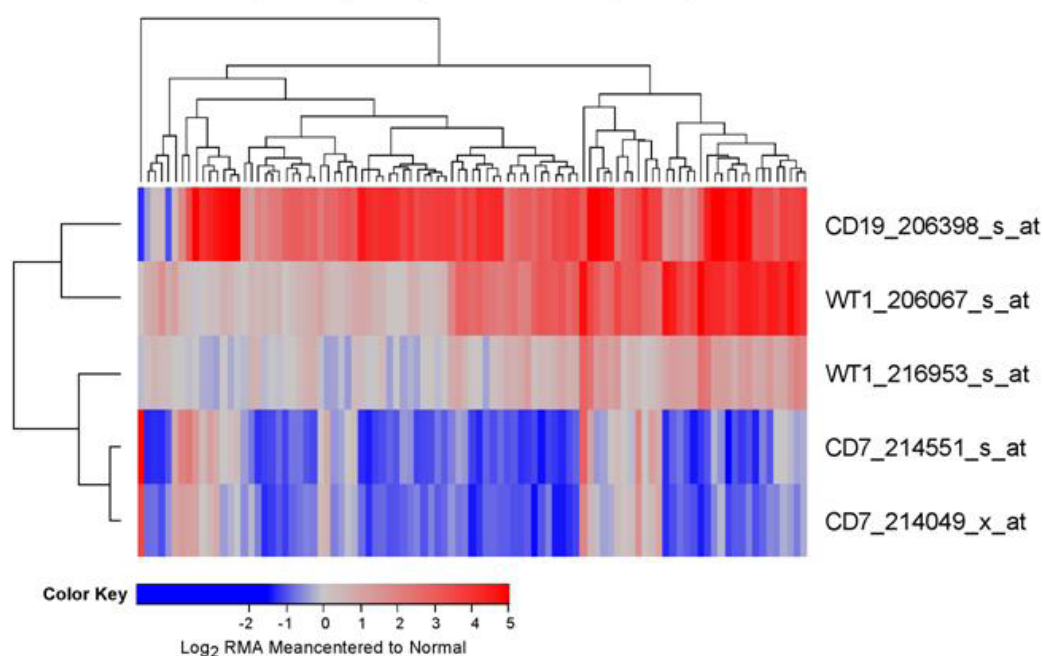
### B. Representation of the WT1 Interactome in MLL-R<sup>+</sup> ALL Transcriptome

Gene_AffyID	Fold Difference (vs Healthy BM)	Linear Contrast (P-value)
CD19_206398_s_at	16.6	<1.0x10 <sup>-16</sup>
WT1_206067_s_at	8.7	<1.0x10 <sup>-16</sup>
EWSR1_229966_at	2.1	<1.0x10 <sup>-16</sup>
TP53_201746_at	2.0	<1.0x10 <sup>-16</sup>
EWSR1_210012_s_at	1.8	<1.0x10 <sup>-16</sup>
WT1_216953_s_at	1.7	5.3x10 <sup>-15</sup>
TP53_211300_s_at	1.5	6.0x10 <sup>-11</sup>
EWSR1_209214_s_at	1.3	1.8x10 <sup>-5</sup>
EWSR1_210011_s_at	1.3	2.5x10 <sup>-4</sup>
U2AF2_218381_s_at	1.3	3.1x10 <sup>-4</sup>
CD7_214551_s_at	0.8	5.0x10 <sup>-4</sup>
CD7_214049_x_at	0.7	1.0x10 <sup>-7</sup>

**Figure 4.** Augmented expression of *WT1* in MLL-R<sup>+</sup> pediatric B-lineage acute lymphoblastic leukemia (ALL). Expression of log<sub>2</sub>-transformed, robust multi-array analysis (RMA) normalized values for leukemia samples from 95 MLL-R<sup>+</sup> patients were mean centered to the mean expression of 74 normal control samples and clustered using a two-way algorithm (average distance metric) to determine co-regulation of *WT1* and the control genes. A: The heat map ranging from blue to red represents expression lower than normal to greater than normal in ALL samples respectively for the transformed expression values. *CD19* and *WT1* were upregulated and *CD7* was downregulated in these B-lineage ALL samples; B: expression levels were also examined for the members of the WT1 interactome identified from the HPRD database. A 3 factor Mixed Model Analysis of Variance analysis was utilized to compare the expression of the WT1 interactome plus the control genes in leukemia samples from ALL patients vs. normal samples extracted from the in-house RMA normalized database (36 probesets analyzed to determine least square error of the expression measurements used to calculate *P*-values). Fold change and linear contrast *P*-values are reported for the *WT1*, *CD19* and *CD7* probesets

in MLL-R<sup>+</sup> leukemic cells (8.7-fold for 20607\_s\_at ( $P < 1 \times 10^{-16}$ ) and 1.7-fold for 216953\_s\_at ( $P = 5.3 \times 10^{-15}$ ) with highly significant linear contrast *P*-values. We also used a 3-factor mixed model ANOVA to compare the expression of the WT1-directed interactome from the Oncomine HPRD interaction set in leukemic BCP vs. normal hematopoietic cells and found that the WT1-regulated *EWSR1*, *TP53*, *U2AF2*, and *WTAP* genes were also upregulated in MLL-R<sup>+</sup> leukemic cells [Figure 4B].

We extended our analysis to 97 MLL-R<sup>+</sup> infant B-lineage ALL cases by using the dataset GSE68720. *WT1*

A. *MLL-R<sup>+</sup>* Infant B-lineage ALL (N=97) versus Normal (N=74)

## B.

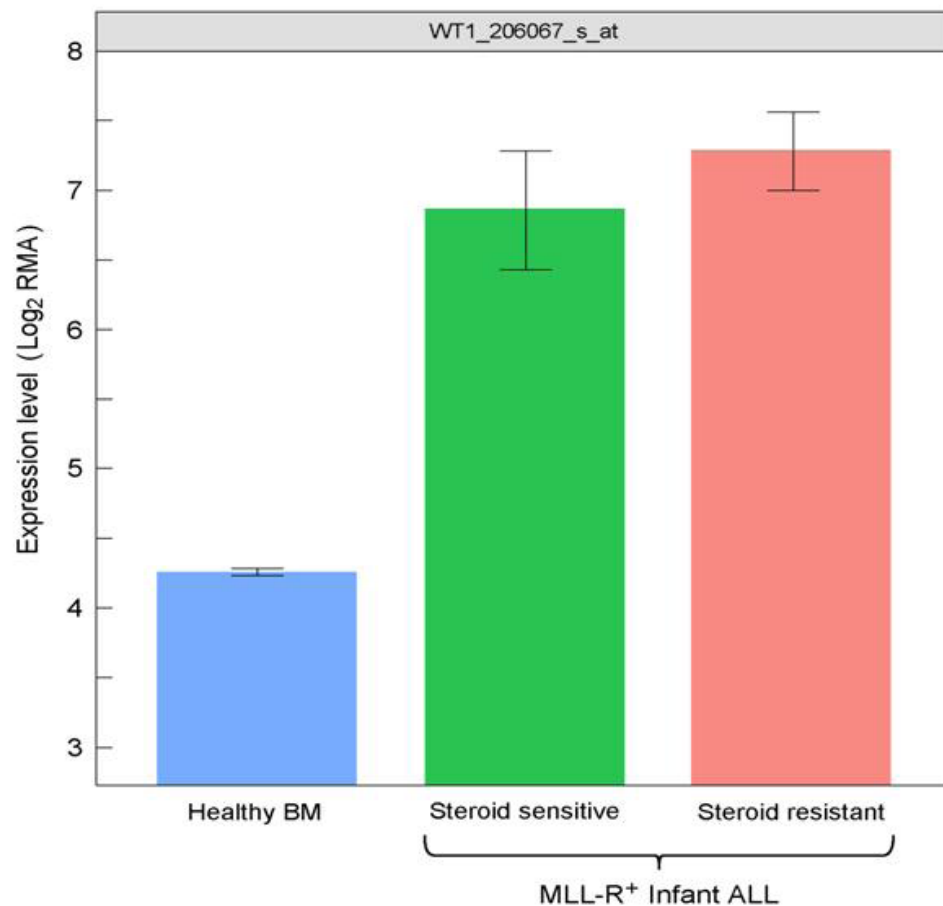
Probeset	Gene	Fold Difference (vs Normal)	Linear Contrast (P-value)
206398_s_at	CD19	9.6	$<1.0 \times 10^{-16}$
206067_s_at	WT1	4.2	$<1.0 \times 10^{-16}$
216953_s_at	WT1	1.5	$1.9 \times 10^{-4}$
214551_s_at	CD7	0.8	$6.8 \times 10^{-3}$
214049_x_at	CD7	0.7	$2.4 \times 10^{-3}$

**Figure 5.** Augmented expression of *WT1* in *MLL-R<sup>+</sup>* infant B-lineage acute lymphoblastic leukemia (ALL). A: Expression of log<sub>2</sub> transformed, robust multi-array analysis (RMA) normalized values for 97 *MLL-R<sup>+</sup>* infants with B-lineage ALL (GSE68720) were mean centered to the mean expression of 74 normal control samples and clustered using a two-way algorithm (average distance metric) to determine co-regulation of *WT1* and the control genes; B: a 3 factor Mixed Model Analysis of Variance analysis was utilized to compare the expression of the *WT1* plus the control genes in infant ALL patients with normal samples extracted from the in-house RMA normalized database of 1,556 patients of which nonleukemic control samples and leukemic samples from *MLL-R<sup>+</sup>* infant ALL cases were interrogated (5 probesets analyzed to determine least square error of the expression measurements used to calculate *P*-values). Fold change and linear contrast *P*-values are reported for the *WT1*, *CD19* and *CD7* probesets

was expressed at 1.5-4.2-fold higher levels (Figure 5;  $P < 0.001$ ) in *MLL-R<sup>+</sup>* infant leukemia cells than in normal hematopoietic cells and the *WT1* expression level was independent of the steroid resistance of leukemia cells (Figure 6; Fold difference  $> 6$  compared to non-leukemic healthy bone marrow cells).

### Amplified expression of *WT1* in pediatric *Ph<sup>+</sup>* and *Ph*-like B-lineage ALL

Examination of *WT1* expression in patients diagnosed with Philadelphia translocation in chromosome 22 of BPL leukemia cancer cells (*Ph<sup>+</sup>*) revealed increased expression of *CD19* (Table 2; Fold difference vs. control samples = 20.1,  $P < 1 \times 10^{-16}$ ) and *WT1* 206067\_s\_at probeset (Fold difference = 1.8,  $P < 1 \times 10^{-16}$ ; *WT1*\_216953\_s\_at displayed borderline significance with 1.1 fold increase,  $P = 0.08$ ). *WT1*-directed interactome from the Oncomine HPRD interaction set also exhibited increased expression of *EWSR1* (2 probesets),

**A. GSE32962 Infant MLL-R<sup>+</sup> B-lineage ALL****B.**

Subset	Sample Size (N)	Fold Difference (vs Healthy BM)	Linear Contrast (P-value)
Steroid resistant	24	8.1	<0.0001
Steroid sensitive	19	6.1	<0.0001

**Figure 6.** Increased levels of *WT1\_206067\_s\_at* probeset expression in MLL-R<sup>+</sup> infant B-lineage acute lymphoblastic leukemia (ALL) vs. normal samples for both steroid sensitive and resistant subsets of Infant B-lineage ALL patients. A: Expression of *WT1* probeset, 206067\_s\_at that were log<sub>2</sub> transformed and RMA-normalized were compared for 24 MLL-R<sup>+</sup> infants with B-lineage ALL that were steroid-resistant, 19, 24 MLL-R<sup>+</sup> infants with B-lineage ALL that were steroid-sensitive (GSE32962) vs. 74 normal control samples (mean and standard errors depicted in the bar chart); B: a 3 factor Mixed Model Analysis of Variance analysis was utilized to compare the expression of the *WT1\_206067\_s\_at*

*PAWR* (4 probesets), *WTAP* and *TP53* (2 probesets).

In addition to identifying and increase in *WT1* expression in *BCR-ABL/Ph<sup>+</sup>* patients, we also evaluated *WT1* expression in *Ph<sup>+</sup>*-like cases that were previously identified using a 110 probeset expression signature developed with the Affymetrix U133 plus 2.0 microarray platform. In our study, expression values for each of the 110 probesets were averaged across 123 *BCR-ABL<sup>+</sup>* cases from the normalized database of 1,416 patients

**Table 2. Representation of the WT1 interactome in Ph<sup>+</sup> B-lineage acute lymphoblastic leukemia transcriptome**

Gene_AffyID	Fold difference (vs. healthy BM)	Linear contrast <i>P</i> -value
CD19_206398_s_at	20.1	$< 1 \times 10^{-16}$
EWSR1_229966_at	2.2	$< 1 \times 10^{-16}$
WT1_206067_s_at	1.8	$< 1 \times 10^{-16}$
PAWR_204005_s_at	1.5	$1.2 \times 10^{-10}$
WTAP_1560274_at	1.5	$2.2 \times 10^{-10}$
EWSR1_210012_s_at	1.4	$2.1 \times 10^{-8}$
PAWR_204004_at	1.4	$2.3 \times 10^{-8}$
PAWR_226223_at	1.4	$3.4 \times 10^{-8}$
TP53_201746_at	1.3	$7.5 \times 10^{-5}$
PAWR_229515_at	1.3	$5.9 \times 10^{-4}$
TP53_211300_s_at	1.2	$6.2 \times 10^{-3}$
WT1_216953_s_at	1.1	$8.6 \times 10^{-2}$
CD7_214551_s_at	0.8	$1.5 \times 10^{-5}$
CD7_214049_x_at	0.7	$7.2 \times 10^{-8}$

A 3 factor Mixed Model Analysis of Variance analysis was utilized to compare the expression of the WT1 interactome plus the control genes in Ph<sup>+</sup> B-lineage acute lymphoblastic leukemia patients ( $n = 123$ ) with normal control samples ( $n = 74$ ) extracted from the in-house robust multi-array analysis normalized database (36 probesets analyzed to determine least square error of the expression measurements used to calculate *P*-values) and the most significantly affected expression levels are depicted in the table. Fold change and linear contrast *P*-values are reported in the table for the *WT1*, WT1 interactome, *CD19* and *CD7* probesets

**Table 3. Representation of the WT1 interactome in Ph<sup>+</sup>-like B-lineage acute lymphoblastic leukemia transcriptome**

Gene_AffyID	Fold difference (vs. healthy BM)	Linear contrast <i>P</i> -value
CD19_206398_s_at	15.6	$< 1.0 \times 10^{-16}$
WTAP_227621_at	2.4	$< 1.0 \times 10^{-16}$
WT1_206067_s_at	2.2	$< 1.0 \times 10^{-16}$
WTAP_1560274_at	1.8	$< 1.0 \times 10^{-16}$
EWSR1_210012_s_at	1.7	$< 1.0 \times 10^{-16}$
EWSR1_229966_at	1.7	$2.2 \times 10^{-16}$
TP53_211300_s_at	1.3	$6.6 \times 10^{-6}$
TP53_201746_at	1.2	$1.4 \times 10^{-3}$
U2AF2_218381_s_at	1.2	$7.2 \times 10^{-3}$
WT1_216953_s_at	1.2	$1.1 \times 10^{-2}$
CD7_214551_s_at	0.8	$7.3 \times 10^{-5}$
CD7_214049_x_at	0.7	$6.1 \times 10^{-12}$

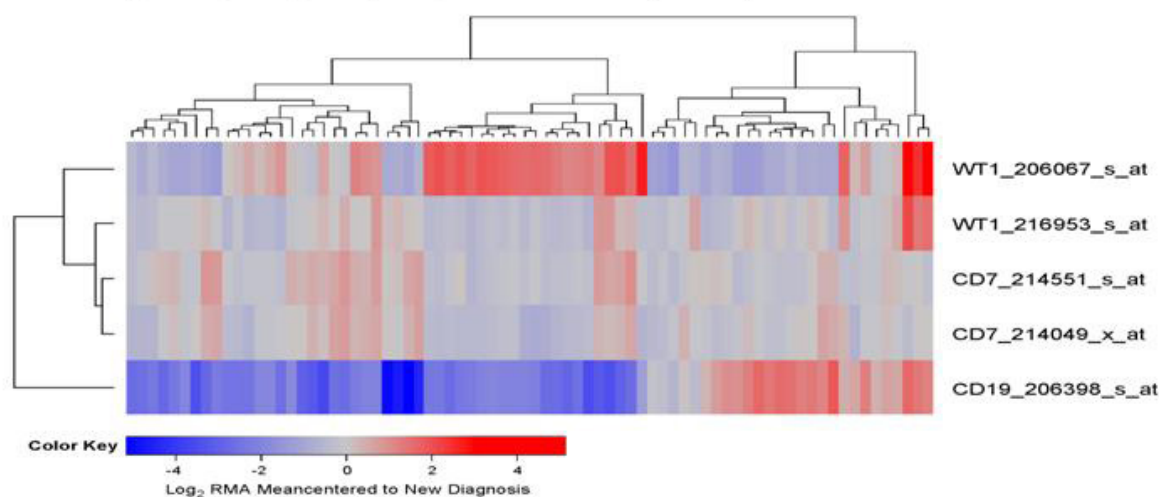
Ph<sup>+</sup>-like acute lymphoblastic leukemia (ALL) cases were previously identified using a set of 110-gene probeset gene expression signature developed utilizing the Affymetrix U133 plus 2.0 microarray platform<sup>[27,28]</sup>. The expression values for each of the 110 probesets were averaged across 123 *BCR-ABL* positive cases from the normalized database of 1,416 patients to provide an expression template for subsequent correlation analysis to identify the Ph<sup>+</sup>-like subset. Expression of log<sub>2</sub> transformed, robust multi-array analysis normalized values for 370 Ph<sup>+</sup>-like ALL patients were compared to the normal control samples for *WT1*, WT1 interactome, *CD19* and *CD7* probesets using A 3 factor Mixed Model Analysis of Variance model

to provide an expression template for subsequent correlation analysis to identify Ph<sup>+</sup>-like patients ( $n = 370$ ). This patient subset also exhibited increased expression of *CD19* (Fold difference = 15.6,  $P < 1 \times 10^{-16}$ ) and *WT1* (206067\_s\_at probeset, Fold difference = 2.2,  $P < 1 \times 10^{-16}$ ) (*WT1*\_216953\_s\_at probeset showed a 1.2 fold increase,  $P = 0.011$ ) as well as the WT1-directed interactome [Table 3; *WTAP* (2 probesets), *EWSR1* (2 probesets), *TP53* (2 probesets) and *U2AF2*].

### Amplified expression of *WT1* in pediatric B-lineage ALL in relapse

Chemotherapy-resistant primarily leukemic cells from relapsed B-lineage ALL patients ( $n = 76$ ) exhibited > 1.25-fold higher expression levels of *WT1* (detected by probe 206067\_s\_at) than primarily leukemia cells from newly diagnosed B-lineage ALL patients ( $n = 474$ ) ( $P = 0.001$ ) [Figure 7].

**A. B-lineage ALL, Relapsed (N=76) versus New Dx (N=474)**



**B.**

Probeset	Gene	Fold Difference (vs NewDiag)	Linear Contrast (P-value)
206067_s_at	WT1	1.3	$1.2 \times 10^{-3}$
214551_s_at	CD7	1.0	0.6
216953_s_at	WT1	1.0	0.9
214049_x_at	CD7	0.9	0.4
206398_s_at	CD19	0.4	$< 1 \times 10^{-16}$

**Figure 7.** Augmented expression of *WT1* in relapsed pediatric B-lineage acute lymphoblastic leukemia (ALL). Gene expression profiling data on primary leukemia cells from relapsed ( $n = 76$ ; combined datasets from GSE18497 and GSE28460) vs. newly diagnosed ( $n = 474$ ) B-lineage ALL patients were compared. Mixed ANOVA model was utilized to determine significant differences in expression between new diagnosis and relapse cases. A: A two-way agglomerative hierarchical clustering technique was used to organize expression patterns using the average distance linkage method such that probesets having similar matched pair differences across patients and samples having similar matched pair differences levels were grouped together. A heat map was constructed to depict change in expression level for each patient ranging from blue to red representing expression lower than at new diagnosis to greater than at new diagnosis for relapsed samples respectively for Log<sub>2</sub> transformed robust multi-array analysis (RMA) values; B: probeset level and sample level Mixed Model Analysis of Variance analysis was utilized to compare the expression of the WT1 interactome plus the control genes in B-lineage ALL patients extracted from the in-house RMA normalized database. Fold change and linear contrast *P*-values are reported for the *WT1*, *CD19* and *CD7* probesets

## DISCUSSION

Our meta-analysis of normal vs. 801 pediatric ALL samples deposited in the Oncomine database combined with an in-depth gene expression analysis using our in-house database of 1,416 pediatric ALL cases as presented herein have provided unprecedented evidence that primary leukemia cells from *MLL-R*<sup>+</sup> patients express highest levels of *WT1* within the high-risk subsets of pediatric B-lineage ALL. The in-house database was constructed by combining raw Affymetrix gene chip files harboring expression profiling data from 6 datasets. We utilized robust and reproducible normalization of expression values across 6 independent datasets permitting direct comparison of *WT1* expression in primary leukemia cells of high-risk subsets of ALL patients vs. normal hematopoietic cells. This procedure ensured that the median and interquartile range of expression values, and the median and interquartile standard error range of expression values, were consistent for each of the genes across all gene chips and datasets such that significance of expression changes were not due to batch artifacts arising from the processing of the raw data from different studies. Notably, *MLL-R*<sup>+</sup> patients exhibited > 6-fold higher expression of *WT1*, 206067\_s\_at probeset compared to



the other B-lineage ALL subtypes combined ( $P < 0.0001$ ). Our findings in 97 *MLL-R*<sup>+</sup> infant B-lineage ALL cases uniquely demonstrated that *WT1* is expressed at 1.5-4.2-fold higher levels in *MLL-R*<sup>+</sup> infant leukemia cells than in normal hematopoietic cells and revealed that *WT1* expression level was > 6-fold higher in steroid-resistant infant leukemia cells when compared to non-leukemic healthy bone marrow cells. Furthermore, our study demonstrates for the first time that the *WT1*-regulated *EWSR1*, *TP53*, *U2AF2*, and *WTAP* genes (i.e., *WT1* interactome) were differentially upregulated in *MLL-R*<sup>+</sup> leukemic cells illustrating that the *MLL*-regulatory pathway is aberrantly upregulated in *MLL-R* pediatric B-lineage ALL. These novel insights provide compelling rationale for targeting *WT1* in second line treatment of *MLL-R*<sup>+</sup> pediatric B-lineage ALL, including *MLL-R*<sup>+</sup> infant ALL. To our knowledge, this is the largest study of *WT1* expression in pediatric cancer patients that also characterized the expression of the *WT1* interactome in cancer cells from meta-analysis of independent gene expression studies. Furthermore, the normalization procedure at the gene chip level across independent datasets enabled comparisons of expression of *WT1* that could not be performed between high-risk cancer subtypes and normal cells in the original independent datasets. Our study significantly extends the observations of Boublikova *et al.*<sup>[13]</sup> who evaluated *WT1* expression in 125 patients with pediatric ALL.

Within the B-lineage ALL subset, we found that leukemia cells from 370 Ph-like patients had enhanced *WT1* expression when compared to normal hematopoietic cells. To our knowledge, this is the first such report for the Ph-like ALL subset. Likewise, we found amplified *WT1* expression in primary leukemia cells from 123 pediatric *BCR-ABL/Ph*<sup>+</sup> patients thereby confirming and extending an earlier report of high *WT1* expression in adult *BCR-ABL/Ph*<sup>+</sup> ALL<sup>[12]</sup>. Finally, our findings demonstrate for the first time that chemotherapy-resistant primarily leukemic cells from relapsed B-lineage ALL patients exhibit higher expression levels of *WT1* than primarily leukemia cells from newly diagnosed B-lineage ALL patients ( $P = 0.001$ ). Thus, *WT1* gene product may serve as a target for immunotherapy in these high risk/poor prognosis subsets of newly diagnosed as well as relapsed pediatric B-lineage ALL.

*WT1* has been implicated in DNA damage response of T-lineage leukemia cells<sup>[37]</sup>. Neumann *et al.*<sup>[38]</sup> reported high level *WT1* expression in patients with early T-cell precursor ALL (ETP-ALL), a high-risk subgroup of acute T-lineage ALL with a high rate of *FLT3*-mutations and low *GATA3* expression and a low rate of *TCR* gene rearrangements consistent with a pluripotent prothymocyte stage in T-cell ontogeny. In our study, we examined the expression of *WT1* in primary leukemic cells from 299 T-lineage ALL patients in the Oncomine database and 189 T-lineage ALL patients in the archived datasets GSE13159, GSE13351, and GSE13159. Our results presented herein significantly expanded the current knowledge of *WT1* expression in T-lineage ALL and provided new evidence that *WT1* gene and its interactome are expressed in T-lineage ALL cells at significantly higher levels than in normal hematopoietic cells. This previously unknown differential expression profile uniquely indicates that the *WT1* gene product would be an attractive molecular target for treatment of T-lineage ALL.

*WT1* antigen may contribute to a graft-versus-leukemia (GVL) effect after allogeneic HSCT. The immunogenicity of *WT1* after HSCT for ALL highlights the potential for *WT1* vaccines to boost GVL after SCT for ALL<sup>[39]</sup>. Both central memory (CD27<sup>+</sup> CD57<sup>-</sup>) and effector memory (CD27<sup>-</sup> CD57<sup>+</sup>) CD8<sup>+</sup> T-cell responses against *WT1* have been reported for patients with ALL after allogeneic HSCT. *WT1*-specific CD8<sup>+</sup> T cells were only found in patients who had *WT1* gene expression in their peripheral blood before transplantation<sup>[39]</sup>. The emergence of *WT1*-specific CD8<sup>+</sup> T cells was associated with a reduction in leukemia load as assessed by *WT1* expression, supporting the immunogenicity of *WT1* as a potential target for immunotherapeutic approaches in ALL. The prompt CD8<sup>+</sup> T-cell response to *WT1* antigen suggests that the *WT1* gene product could be a useful target for post-HSCT immunotherapy of ALL. Furthermore, the vigorous homeostatic proliferation of donor T cells after allogeneic HSCT, especially in a T-depleted setting, may represent a hitherto unconsidered window of opportunity for post-HSCT vaccination.

## DECLARATIONS

### Authors' contributions

Participated in the review of relevant literature, drafting of the manuscript, review and revisions of the final draft: Uckun FM, Qazi S

Performed the statistical and bioinformatics analyses: Qazi S

### Availability of data and materials

The archived gene expression datasets GSE11877, GSE13159, GSE13351, GSE18497, GSE28460, GSE7440, GSE32962 and GSE68720 used in this meta-analysis are publicly available (Gene Expression Omnibus; <https://www.ncbi.nlm.nih.gov/geo/>) as is the online data mining tool, Oncomine<sup>TM</sup>research edition (<https://www.oncomine.org/resource/main.html>).

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### Conflicts of interest

Both authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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