Elevated TRIB2 with NOTCH1 activation in paediatric/adult T-ALL

Elevated TRIB2 with NOTCH1 activation in pediatric/adult T-ALL

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Abstract

TRIB2 is a potent oncogene, elevated in a subset of human acute myeloid leukaemias (AML) with a mixed myeloid/lymphoid phenotype and NOTCH1 mutations. Although rare in AML, activating NOTCH1 mutations occur in 50% of all T-acute lymphoblastic leukaemias (T-ALL). **TRIB2** is a NOTCH1 target gene that functions in the degradation of key proteins and modulation of MAPK signaling pathways, implicated in haematopoietic cell survival and proliferation. We show that **TRIB2** expression level is highest in the lymphoid compartment of normal haematopoietic cells, specifically in T cells. Analysis of **TRIB2** expression across 16 different subtypes of human leukaemia demonstrated that **TRIB2** expression was higher in ALL phenotypes versus all other phenotypes including AML, CLL, MDS and CML. A T cell profile was distinguished by high **TRIB2** expression in normal and malignant haematopoiesis. High **TRIB2** expression was seen in T-ALL with normal karyotype and correlated with NOTCH signalling pathways. In a pediatric patient T-ALL cohort high **TRIB2** expression correlated with NOTCH1/FBXW7 mutations, strongly linking NOTCH1 activation and high **TRIB2** expression in paediatric T-ALL. The relationship between **TRIB2** and T cell signaling pathways uniquely identifies leukaemia subtypes and will be useful in the advancement of our understanding of T cell and ALL biology.

Introduction

Acute leukaemias of the lymphoid and myeloid lineage (ALL and AML) are the most common forms of childhood and adult leukaemia respectively. ALL is classified into genetic subtypes of the B and T lineage (B-ALL and T-ALL) according to cytogenetically defined chromosomal aberrations and genetic mutations. These important hallmarks of ALL disease are valuable for guiding treatment strategies of patients. The prognosis of T-ALL in children and adults has improved in recent years due to intensified therapies. However long-term remission figures suggest that there is still much to be gained with advancing our understanding of T-ALL biology (Kraszewska et al, 2012).

T-ALL accounts for 15% of paediatric and 25% of adult ALLs and these can be subdivided into certain classes based on the expression profiles of certain oncogenes (Ferrando et al, 2002), amongst which are TLX1 (HOX11), TAL1, LYL1, and LM01/2. NOTCH1 is the most frequently mutated gene in T-ALL with activating NOTCH1 mutations occuring in 50% (34-71%) of all adult and childhood T-ALL patients and in rare AML cases (Breit et al, 2006; Kraszewska et al, 2012; Weng et al, 2004; Wouters et al, 2007). The NOTCH1 gene encodes a transmembrane receptor that is activated through ligand binding, resulting in a series of proteolytic events releasing the intracellular portion of NOTCH1 from the plasma membrane into the nucleus (Aster et al, 2011). Most NOTCH1 mutations occur in the heterodimerisation domain (HD) and proline-glutamic acid-serine-threonine (PEST) domains. These mutations lead to the ligand-independent activation of NOTCH1 and decrease the degradation of intracellular NOTCH1 respectively, leading to upregulated NOTCH1 activation. FBXW7 is a component of ubiquitin ligase complex that induces the degradation of NOTCH1. Loss-of-function mutations in FBXW7 are frequently found in T-ALL with a prevalence of 8-16% (Kraszewska et al, 2012) which lead to the inability of FBXW7 to target NOTCH1 for degradation resulting in the prolonged half-life of active NOTCH1 (O'Neil et al, 2007; Thompson et al, 2007).

Despite significant progress made in recent years in our understanding of NOTCH1 mutations in T-ALL and T-ALL biology, the prognostic significance of NOTCH1 and/or FBXW7 mutations remains inconclusive in T-ALL. A good clinical outcome for paediatric patients harbouring NOTCH1 and/or FBXW7 mutations was reported in the ALL-BFM 2000 study (Breit et al, 2006) and in the JACLS ALL-97 and NHL-98 study (Park et al, 2009). Contrary to these it was reported that NOTCH1 mutations and/or FBXW7 mutations were not

predictive of outcome in paediatric (Erbilgin Y, 2010; van Grotel et al, 2007) or adult (Baldus et al, 2009) T-ALL. NOTCH1 mutations have been shown to correlate with poor prognosis in adults but not in paediatric patients in an independent study (Zhu et al, 2006).

TRIB2 was originally identified as a direct NOTCH1-regulated transcript in T-ALL (Wouters et al, 2007) **and recently as a target of PITX1, a leukemic gene recurrently activated in T-ALL (Nagel et al, 2011). TRIB2** proteins are emerging as important modulators of signalling and differentiation pathways through the modulation of MAPK signalling and C/EBP family members (Yokoyama & Nakamura, 2011). **TRIB2** can bind and target proteins for degradation through the proteasome and these functions are involved in myeloid differentation and cell survival pathways. Overexpression of **Trib2** in haematopoietic progenitors drives robust murine AML with a relatively short latency of 6 months and can cooperate with HOXA9 to accelerate AML in a murine bone marrow transplant model (Keeshan et al, 2006; Keeshan et al, 2008). Subsequently a survey of **TRIB2** mRNA expression in human AML patient samples identified elevated **TRIB2** expression in a specific subset of tumours with dysregulated C/EBPalpha and NOTCH1 mutations (Keeshan et al, 2006; Wouters et al, 2007). This subset of AML patients had a myeloid/lymphoid phenotype as determined by markers of the T cell lineage (Wouters et al, 2007).

In this study, we evaluated **TRIB2** expression in haematopoiesis and in myeloid and lymphoid leukaemias. Our results show that high **TRIB2** expression correlates with T-ALL, and specifically with normal karyotype T-ALL and ALL with t(1:19). Profiling of ALL subsets with high **TRIB2** expression reveal a correlation with T cell receptor (TCR) signalling pathways, and specifically T-ALL correlates with NOTCH signalling pathways. Furthermore **TRIB2** is normally expressed at the highest level in the lymphoid compartment, specifically in T cells. The analysis of the expression of **TRIB2** in a cohort of paediatric T-ALL revealed a significant correlation between high **TRIB2** expression and NOTCH1 activation. Our findings demonstrate that high **TRIB2** expression is an important marker of a subset of adult and paediatric T-ALLs, linking strongly with activated T cell pathways. Our data provide evidence for a potential role of **TRIB2** in the development of the T cell phenotype of ALL and specifically with activated NOTCH1 phenotypes.

Methods

Patient Samples

Bone marrow (BM) and peripheral blood (PB) samples were obtained from the Turkish BFM Study Group from 60 pediatric T-ALL patients. The study was approved by the ethical committee of Istanbul Medical Faculty, Istanbul University and informed consents were obtained from all participating patients as previously described (Erbilgin Y, 2010). The median age of diagnosis was 9 years (range 22 days- 16 years). The male to female ratio was 2.7:1.

RNA isolation and cDNA synthesis

BM and PB samples were stored at -80°C after homogenisation in RLT buffer. Total RNA was isolated using Qiagen Rneasy Plus Mini Kit (Qiagen, GmbH, Germany) and treated with DNAse (MBI Fermentas, Germany) to remove DNA contamination. cDNA was synthesised using 1ug of total RNA, random hexamers and MMLV reverse transcriptase in accordance with manufacturers protocol (MBI Fermentas Life Sciences, Lithuania).

Detection of NOTCH1 and FBXW7 mutations

Exons 7, 8, 9, 10, 11 and WD-40 of FBXW7 and HD-N (exon26), HD-C (exon 27) and PEST domain (exon 34) of NOTCH1 were amplified and denaturing high-performance liquid chromatography technique was performed in addition to direct sequencing as previously described (Erbilgin Y, 2010).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using SYBR Green **master-mix** on light cycler 480 instrument (Roche Applied Sciences, Manheim, Germany). 18s and ABL genes were selected for the house keeping normalization genes. Further primer details provided in supplementary data.

Statistical analysis

TRIB2 expression levels were normalised to 18s and ABL genes and RPMI 8402 T-ALL cell line was used as positive internal control. Each sample was normalised to the calibrator sample in accordance with **2-ΔΔCT** method **(Livak and Schmittgen, 2001). TRIB2** expression level in wildtype and mutant NOTCH1/FBXW7 T-ALLs were compared by 2-tailed student t-test. The 95% confidence value interval for differences in expression between

the groups was obtained. **For the box plots one-way ANOVA with Dunnett's post test was used to test the significance between TRIB2 levels in each leukemia phenotype and normal bone marrow.** All statistics were performed using GraphPad Prism5.

Bioinformatic Analyses

Expression profiles of the leukaemia subtypes were generated from the MILE Study (GSE13204) (Haferlach et al, 2010). **Two TRIB2 probe sets (202478_at and 202479_s_at) were present on the arrays. For each TRIB2 probe we analysed the expression levels of TRIB2 and values for each leukaemia subtype and normal bone marrow were plotted on a box plot with max and min whiskers using GraphPad and scaled to reflect the expression values in the Affymetrix chip. To determine the nearest-neighbours in the MILE dataset for ALL,** each patient sample was separated based on whether **TRIB2** expression was below the median expression of both probe sets (labelled 0) or above the median expression in both probe sets (labelled 2). A one-way ANOVA analysis was then preformed to determine genes with significantly different expression between the 0 and 2 group **(above versus below the median for both probe sets).** Unsupervised hierarchical clustering of these the top 1000 differentially expressed genes ($p<5x10^{-12}$) was then performed using Partek Genomics Suite (Version 6.6). GSEA with 1000 permutations was carried out using the GSEA program suite (version 2.07) available from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp) (Subramanian et al, 2005). **For GSEA analysis the dataset was first collapsed to max probe expression,** genes were then ranked using the Pearson correlation matrix with continuous phenotype labels for **TRIB2**, and run against the canonical pathway subset available for GSEA analyses in the Molecular Signalling Database (MSigDB) (v3.0), which include gene sets compiled from a number of sources (BioCarta, KEGG, Reactome, signalling gateway and more). GSEA was also run against the 80 modules of strongly coexpressed genes identified by Novershtern at al. in the haematopoietic cell lineages (Novershtern et al, 2011). GSEA with continuous phenotype labels was carried out for **TRIB2** specifically in T-ALL of the MILE study and run against a set of NOTCH signalling pathways derived from the MSigDB.

Results

Overexpression of Trib2 in a murine model leads to AML with a myeloid phenotype exclusively. However in the human disease, elevated **TRIB2** is associated with AML with a mixed myeloid and lymphoid phenotype (Wouters et al, 2007). Since we first identified **TRIB2** as a NOTCH1 target gene in a T-ALL cell line **(Keeshan et al, 2006),** we asked whether **TRIB2** plays a role in other types of human leukaemia. The collaborative Microarray Innovations in Leukaemia (MILE) study program incorporates gene expression profiles of 16 acute and chronic leukaemia subclasses, myelodysplastic syndromes (MDSs), and control groups that includes nonmalignant disorders and normal bone marrow from 2096 patients (Haferlach et al, 2010). We analysed the expression levels of **TRIB2** across the range of different leukaemias in this dataset. **TRIB2** was expressed at significantly higher levels in T-ALL (with normal karyotype, $p<0.001$) and in (pre-B) ALL with t(1;19) ($p<0.001$), relative to control normal bone marrow. The T-ALL subset contained ALL immature (pre), ALL immature (pro), ALL cortical, and ALL mature subclassess. Interestingly, significantly low levels of **TRIB2** expression were associated with (pre-B) ALL with t(12;21) ($p<0.001$) (Figure 1A).

As significant differences were observed in **TRIB2** expression in the ALL subtype of leukaemias **we sought to profile associated gene signatures with TRIB2 expression in these subsets further. TRIB2 expression was separated based on above the median versus below the median TRIB2 expression levels (see methods) and a one-way ANOVA was performed.** We then performed unsupervised hierarchical clustering on the top 1000 differentially expressed genes identified by the ANOVA analysis. Nearest–neighbour analysis revealed high **TRIB2** expression associated specifically with the T-ALL subtype (Figure 1B). Genes associated with high **TRIB2** ALL gene profile involved in T cell signalling and NOTCH1 signalling include CD7, ZAP70, LCK, CD3, CD28, LAT, SOCS2, PTPN(2, 4, 6, 7), NFAT, IK2F2, Deltex3 and T cell receptor genes TRD@ and TRBC1 (supplementary data). Gene set enrichment analysis (GSEA) showed that the gene expression pattern of high **TRIB2** ALL was significantly enriched for T cell receptor signalling pathway genes as described in the KEGG database (Figure 1C). An additional number of T cell pathways in the MsigDB showed significant enrichment in the high **TRIB2** ALL gene list, and represenative genes from these pathways are shown in Figure 1D. Interestingly, functional annotation of the top 100 high **TRIB2** gene neighbours showed enrichment of the

KEGG T cell receptor pathway and Haematopoietic cell lineage pathway in the ALL subsets (p value $7x10^{-11}$ and $7x10^{-5}$ respectively), in the AML subsets (p value $1.3x10^{-10}$ and $3x10^{-5}$ respectively), and in normal bone marrow (p value $7x10^{-3}$ and $4x10^{-2}$ respectively). No pathways were identified in the top 100 low **TRIB2** expressing gene lists (data not shown). These results are consistent with high **TRIB2** expression being associated, and possibly contributing to the T cell phenotype of human ALL, AML and normal haematopoiesis.

We next assessed the expression pattern of **TRIB2** in human haematopoiesis during normal development. Using the recently published dataset profiling human haematopoietic compartments into 80 network modules (Novershtern et al, 2011) we analysed the expression levels of **TRIB2** across the dataset (Figure 2A). The highest expression levels of **TRIB2** in all haematopoietic subsets were in the T cell subsets. The T cell subsets contain naïve $CD4^+$ and $CD8^+$ cells, and effective and central memory $CD4^+$ and $CD8^+$ cells. There was an increase in **TRIB2** expression as cells differentiated from the pre-B cell stage (containing early and pro-B cells) to the mature B cell stage (containing naïve and mature B cells). Interestingly, amongst the myeloid compartments **TRIB2** expression levels were higher in granulocytes compared to monocytes, and compared to the dendritic cell (DC) subset. The haematopoietic stem cell (HSC) compartment expresses low levels of **TRIB2** and as the cells commit to lineage development, **TRIB2** expression appears to increase in the common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP) and megakaryocyte erythroid progentior (MEP) populations. Overall, the levels of **TRIB2** expression in human haematopoietic subsets is variable and highest in the lymphoid lineage, specifically the T cell compartment.

To further dissect the expression profile of **TRIB2** in haematopoiesis we identified the module networks in the normal haematopoietic system that were enriched for the high **TRIB2** ALL gene profile. We performed GSEA comparing the high **TRIB2** ALL gene list identified in the MILE study with all the modules in the haematopoietic dataset. Modules 667, 757 and 829 were identified as being significantly enriched in the high **TRIB2** ALL gene set, with additional modules with T cell and T/B cell induction patterns identified (Figure 2B and C). These modules had GATA3, LMO2, BCL11B, MBP2, FOXJ3, IRF3 and HBP1 genes as the top regulators and determined by GSEA to contain genes involved in T-cell receptor signalling (Figure 2C)(Novershtern et al, 2011). Further GSEA analyses comparing the high **TRIB2** gene neighbours in the haematopoietic dataset (i.e. high **TRIB2**

expression in normal haematopoiesis) with the modules in the dataset reveal that 13 modules are significantly enriched that have an induction pattern in T/B cells (data not shown). Of interest, **TRIB2** itself is a member of module 769 defined in the Novershtern et al study which has an induction pattern in T/B cells and defined as the T/B cell module expressed in $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells and $CD56^+$ NK cells. These analyses reveal that normal **TRIB2** expression is highest in the lymphoid compartment, and the profile of high **TRIB2** expression in ALL closely resembles the normal T cell profile. These data suggest that **TRIB2** itself may function in lineage determination and/or the phenotype of the T cell compartment.

As NOTCH1 plays a major role in T cell development and leukaemogensis, and **TRIB2** is a NOTCH1 target gene, we analysed the **TRIB2** expression pattern specifically in T-ALL with normal karyoptype. GSEA showed that the gene expression pattern of high **TRIB2** T-ALL (gene list in supplementary data) identified in the MILE study was significantly enriched for NOTCH1 signalling pathways (Figure 3A), including NOTCH1 pathway genes Deltex2, 3, MAML1, 2, DLL1, JAGGED and ADAM10 (Figure 3B). We next addressed whether a relationship between **TRIB2** and NOTCH1 mutations exist in T-ALL. A total of 60 paediatric T-ALL patients were analysed for elevated **TRIB2** expression that contained NOTCH1 and FBXW7 mutation rates of 17% and 10%, respectively. **TRIB2** expression was measured by qRT-PCR and found to be significantly elevated in patients harbouring NOTCH1 and/or FBXW7 mutations versus patients wildtype for NOTCH1/FBXW7 (Figure 3C). **TRIB2** expression values were normalised to the geometric mean of two control genes (18s and ABL). The highest **TRIB2** expression ratios were identified in patients T84 and T110 that harboured a NOTCH1 PEST domain and HD-C mutation respectively. All mutations were previously described (Erbilgin Y, 2010). Therefore, activation of the NOTCH1 pathway via NOTCH1 or FBXW7 mutation correlates with higher **TRIB2** expression levels as compared to T-ALL samples without NOTCH1 activation. Together these data show that high **TRIB2** expression correlates with NOTCH1 signalling pathway and specifically in paediatric T-ALL with NOTCH1 and/or FBXW7 mutation and further supports a defining role of **TRIB2** in the lymphoid lineage and lymphoid malignancies.

Discussion

We demonstrate that high **TRIB2** expression can enrich for gene sets of the T cell lineage, in normal haematopoietic development and across all subtypes of leukaemia. Specifically, high **TRIB2** expression enriches for gene sets that define TCR signalling pathways in ALL and normal haematopoiesis. Moreover, high **TRIB2** expression enriches for NOTCH1 signalling pathways specifically in T-ALL (not in all ALL subsets, data not shown) and correlates with activation of NOTCH1 in paediatric T-ALL. We previously identified a correlation between NOTCH1 mutations and high **TRIB2** expression in AML (Wouters et al, 2007). The subtype of immature myeloid/T-lymphoid leukaemia reported originally as cluster 4 (Valk et al, 2004) has subsequently been analysed to reveal that this subset is a biological entity distinct from both AML and T-ALL, and has unique **epigenetic** features (Figueroa et al, 2009). **TRIB2** expression was originally identified to be elevated and a feature of the genetic signature of this cluster 4 (Keeshan et al, 2006; Wouters et al, 2007) along with NOTCH1 mutations and T-lymphoid markers. Here we show that high **TRIB2** expression alone can identify a T cell profile amongst all subtypes of leukaemia, and strongly correlates with activated NOTCH1 in paediatric T-ALL. While we do not know the mutation status of NOTCH1 or FBXW7 in the MILE study dataset which contains both paediatric and adult ALL samples, NOTCH1 mutations are reported to occur in ~50% of adult and paediatric T-ALL. Despite the relatively low prevalence of NOTCH1 mutation in the cohort of paediatric T-ALL analyzed here (17% versus 34-71%), the prevalence of FBXW7 mutations are similar to other reports (10% versus 8-16%) (Kraszewska et al, 2012). Therefore our data support the association of high **TRIB2** expression with an activated NOTCH1 phenotype, NOTCH1 signalling and T cell profile in both adult and paediatric T-ALL and suggest that **TRIB2** may represent a defining marker for malignancies with T cell features.

Understanding the precise function of **TRIB2** in the T cell lineage and specifically the NOTCH1 T-ALL phenotype will provide an important insight into our current understanding of **TRIB2**. High **TRIB2** levels were identified in T-ALL with normal karyotype, and our results suggest that **TRIB2** may function in the T cell phenotype of haematological malignancies. **TRIB2** is an important modulator of MAPK signaling pathways and functions in the degradation of C/EBP family members, key regulators of the myeloid lineage. **TRIB2** is therefore an important regulator of pathways involved in the survival, proliferation and differentiation of haematopoietic cells. **TRIB2** is also a direct target of NOTCH1, and

NOTCH1 activation leads to T-ALL in murine models and the human disease. Our data suggest that **TRIB2** may indeed be involved in these processes.

In our analysis of **TRIB2** expression during normal haematopoiesis, we observed an increase in **TRIB2** expression as cells differentiated from the pre-B cell stage (containing early and pro-B cells) to the mature B cell stage (containing naïve and mature B cells). We also see high levels of **TRIB2** expression associated with ALL with t(1;19), which results in the E2A-PBX1 fusion protein, one of the frequent recurring translocations in childhood ALL, present in about 5% of ALL cases, including 20–25% of pre-B ALL. Therefore, high **TRIB2** expression associated with a B and T-ALL in our study and **TRIB2** itself is a defining member of the T/B cell module, present in $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells, identified by GSEA in the Novasthern et al study. These data suggest that **TRIB2** functions in the lineage determination of T and B cells.

No significant difference in **TRIB2** expression was found in the mature B-ALL with t(8;14) subsets, whereas low **TRIB2** expression levels were statistically different in the pre-B ALL with t(9;22) and for AML with t(11q23)/MLL compared to normal bone marrow. **TRIB2** expression in AML subtypes in this study therefore trend towards low **TRIB2** expression levels with a relatively small subset of high **TRIB2** expressing samples. Similarly, our original findings reported a specific subset of high **TRIB2** expressing AMLs with a dysregulated C/EBPalpha and myeloid/lymphoid profile (Wouters et al, 2007). Functional annotation of the top 100 low **TRIB2** expressing subsets however did not reveal any enrichment of specific pathways. Our findings here support the ability of high **TRIB2** expression to reveal a T cell profile in both T-ALL and AML. That **TRIB2** expression levels can **distinguish** subtypes of leukaemia, for instance the T-ALL versus ALL with t(1;19) suggest that the molecular function of **TRIB2** in different cell types with different aberrations will be of future interest.

Remarkably **TRIB2** gene expression profiling distinguishes the T cell lineage from other lineages in both normal and leukaemia datasets. Overexpression of **TRIB2** in the murine model induced a block in myeloid differentiation and led to a myeloid leukaemia, yet normal granulocytes express **TRIB2** at significantly higher levels compared to the HSC compartment. Perhaps the cell of origin overexpressing **TRIB2** would determine the leukaemic phenotype that develops, and it would be interesting to test this further in *in vivo* models. In normal development **TRIB2** is expressed highest in T cells, and these data suggest

that **TRIB2** may in fact play a functional role in T cell lineage development and specifically associate with activated NOTCH1 signalling. **TRIB2** knockout or knockdown approaches will lend further insight into the potential functional role of **TRIB2** in lymphomagenesis.

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Conflict of interest

The authors report no potential conflict of interest

Legends

Figure 1: TRIB2 expression is highest in T-ALL and this subset is enriched for T cell signalling pathways (A) TRIB2 expression is highest in T-ALL with normal karyotype, in ALL with t(1;19) inversion, and lowest in ALL with t(12;21). **TRIB2** (202478_at) expression was calculated from the MILE dataset (GSE13204) for each leukemic subtype. Values for each leukaemia subtype and normal bone marrow were plotted on a **box plot with max and min whiskers.** Intersecting line indicates position of mean. ******* means p value is < 0.001 . **StDev and SEM values are available to download as supplementary data. (B)** T-ALL (red bar along top) is associated with high **TRIB2** expression (green bar along top) in ALL. The ALL samples from the MILE study were separated based on above the median versus below the median **TRIB2** expression levels of both **TRIB2** probes (202478_at and 202479_s_at), (2 = **TRIB2** has above median levels of **TRIB2** expression in both probe sets (green box). 1 = **TRIB2** has above median levels of **TRIB2** expression in one probe set (yellow), 0 = **TRIB2** expression has below the median expression of **TRIB2** in both probe sets (red)). Using Partek software 1-way ANOVA testing determined 1000 genes which were significantly differentially expressed between the high and low **TRIB2** subsets (red and blue box along bottom bar, see supplementary data for gene list) with a p value < $5x10^{-12}$. These genes were then hierarchically clustered using Partek. **(C)** T-cell receptor signalling pathways are enriched in the high **TRIB2** expressing subset of ALL. GSEA comparing the high **TRIB2** ALL subset from the MILE study and the KEGG T cell receptor signalling pathway dataset. The normalized enrichment scores (NES), p-value and q-value (FDR) are indicated on each plot. A p-value below 5% and a q-value below 25% indicate that the result is significant. **(D)** Heatmap of genes present in the high **TRIB2** subset of ALL enriched in a number of statistically significant T-cell signalling and activation pathways identified from MSigDB (high expression in red and low in blue). A white box indicates that these genes are not present in that particular pathway.

Figure 2: TRIB2 expression is highest in the T-cell compartment of the haematopoietic system. (A) TRIB2 expression analysis across normal human haematopoietic compartments. **TRIB2** expression was assessed in the array dataset including human hematopoietic stem and progenitor cells, terminally differentiated cells, and intermediate state cells (GSE24759, noveshtern et al). Values for each compartment were plotted on a **boxplot with max and min whiskers.** Intersecting line indicates position of mean. **StDev and SEM values are available**

to download as supplementary data. (**B and C**) GSEA comparing the high **TRIB2** ALL gene list from the MILE study and Module 667 **(B)** and 757 **(C)** haematopoietic compartments (Novershtern et al, 2011)(modules comprised of highly expressed genes in normal human T-cells and NK cells). The normalized enrichment scores (NES), p-value and q-value (FDR) are indicated on the plot. A p-value below 5% and a q-value below 25% indicate that the result is significant. **(C)** Table describing the induction pattern module 667, 757 and 829. Classification of this module was determined by GSEA (Novershtern et al, 2011) which identified that the genes present in the module are involved in T-cell receptor activity.

Figure 3: High TRIB2 expression associates with NOTCH1 signalling in T-ALL. (A) NOTCH signalling pathways are enriched in the high **TRIB2** expression subset of T-ALL. GSEAs comparing the high **TRIB2** T-ALL subset of the MILE study and genes involved in NOTCH signalling, derived from the Reactome pathway database (left plot), based on the gene ontology (GO) database (middle plot) or derived from the KEGG pathway database (right plot). The normalized enrichment scores (NES), p-value and q-value (FDR) are indicated on each plot. A p-value below 5% and a q-value below 25% indicate that the result is significant. **(B)** Heatmap of genes present in the high **TRIB2** subset of T-ALL enriched for the NOTCH signalling pathway identified from MSigDB **(C)** Elevated **TRIB2** expression in paediatric patients harbouring mutated NOTCH1 and/or FBXW7 mutations. Relative expression ratios to the geometric mean of two housekeeping genes for **TRIB2** gene in NOTCH1/FBXW7 mutant and wild type T-ALL patients. The relative expression levels for each sample was used to calculate the means (Avg), standard deviation (SD) and standard error of the mean (SEM) for each genotype. According to Student t test, p= 0.027 P value of 0.05 or less (two sided) was considered to indicate a statistically significant.

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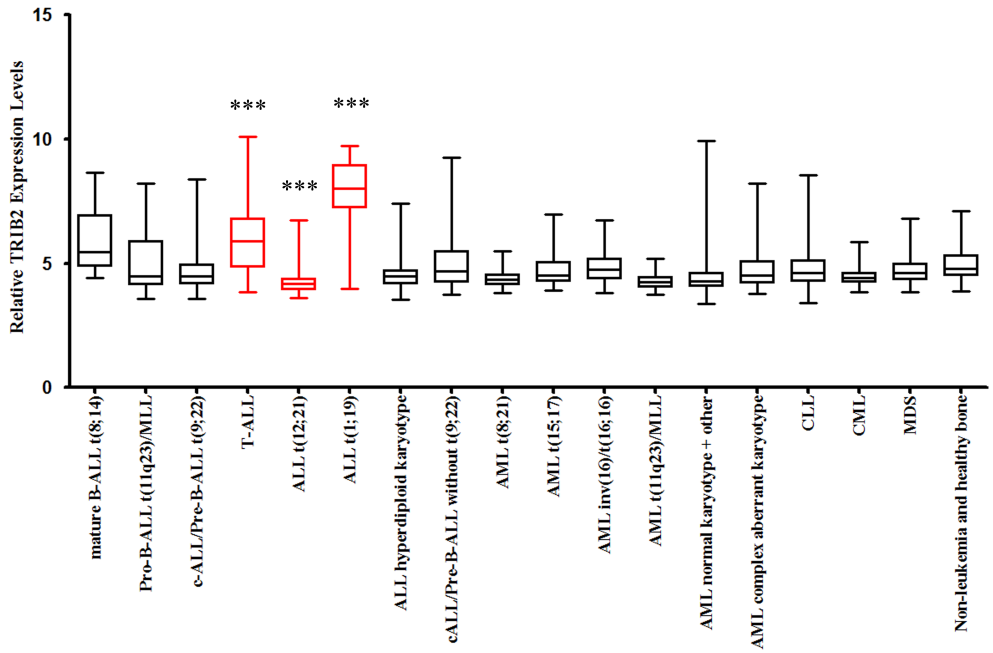
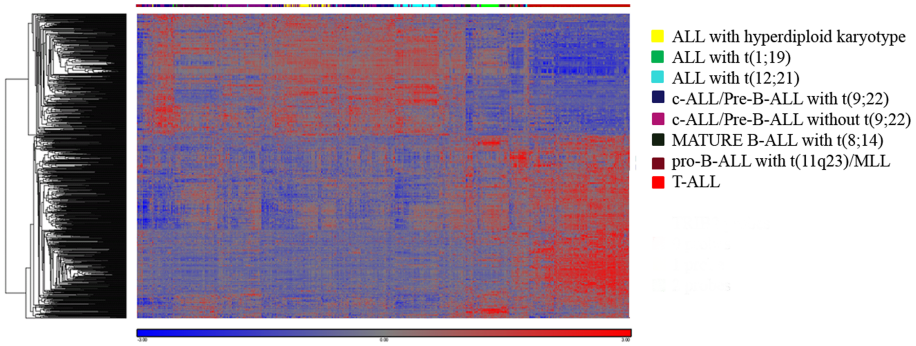
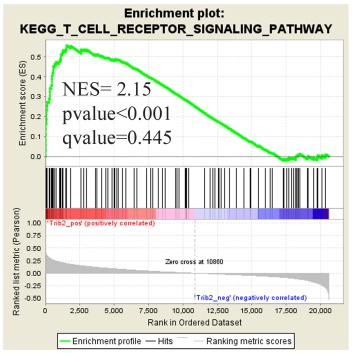
(A)


Relative TRIB2 Expression Levels
15
10
5
0
***
***
***
mature B-ALL t(8;14)
Pro-B-ALL t(11q23)/MLL
c-ALL/Pre-B-ALL t(9;22)
T-ALL
ALL t(12;21)
ALL t(1;19)
ALL hyperdiploid karyotype
cALL/Pre-B-ALL without t(9;22)
AML t(8;21)
AML t(15;17)
AML inv(16)/t(16;16)
AML t(11q23)/MLL
AML normal karyotype + other
AML complex aberrant karyotype
CLL
CML
MDS
Non-leukemia and healthy bone

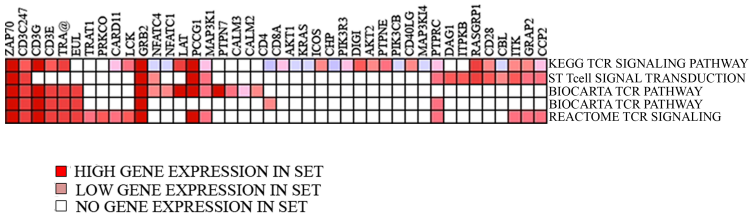
(B)


ALL with hyperdiploid karyotype
ALL with t(1;19)
ALL with t(12;21)
c-ALL/Pre-B-ALL with t(9;22)
c-ALL/Pre-B-ALL without t(9;22)
MATURE B-ALL with t(8;14)
pro-B-ALL with t(11q23)/MLL
T-ALL

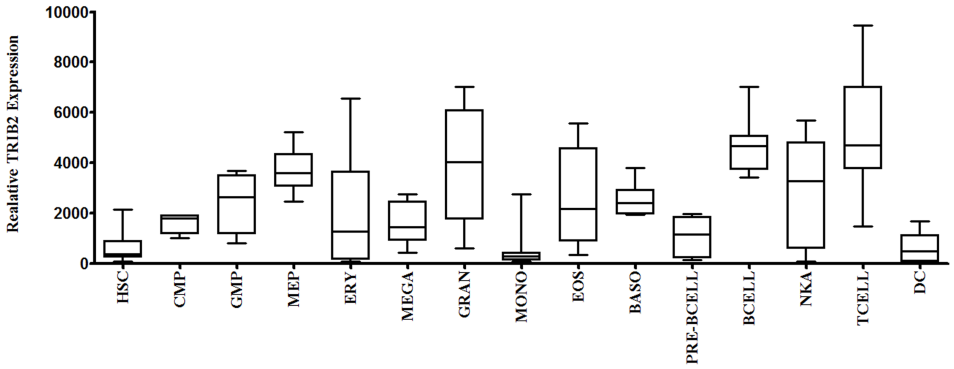
(C)


Enrichment plot:
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY
NES= 2.15
pvalue<0.001
qvalue=0.445
Enrichment score (ES)
Ranked list metric (Pearson)
Trib2_pos' (positively correlated)
Zero cross at 10860
Trib2_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

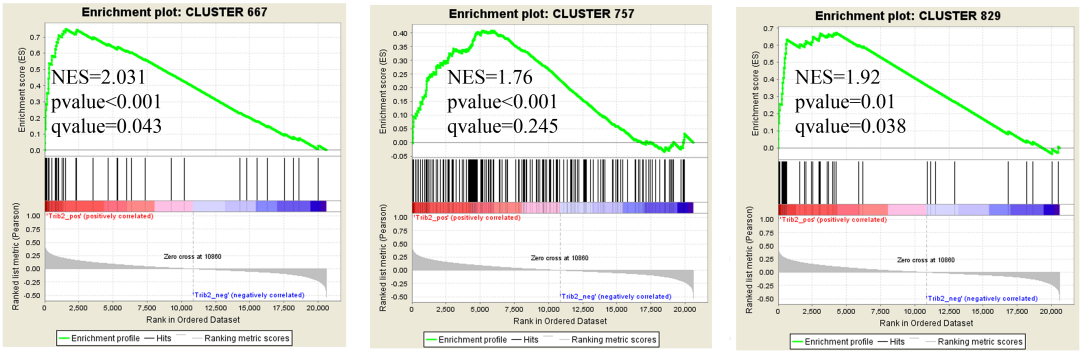
(D)


ZAP70
CD3G
CD3E
TRAC
EIL
TRAT1
PRKCQ
CARD11
LCK
GRB2
NFATC4
NFATC1
LAT
PCCG1
MAP3K1
PTPN7
CALM3
CALM2
CD4
CD8A
AKT1
KRAS
FOS
JUP
PIK3R3
DIGI
AKT2
PTPNE
PIK3CB
CD40LG
MAP3K14
PTPRC
DAG1
ITPKB
RASGRP1
CD28
CBL
ITK
GRAP2
CCP2
KEGG TCR SIGNALING PATHWAY
ST Tcell SIGNAL TRANSDUCTION
BIOCARTA TCR PATHWAY
BIOCARTA TCR PATHWAY
REACTOME TCR SIGNALING
HIGH GENE EXPRESSION IN SET
LOW GENE EXPRESSION IN SET
NO GENE EXPRESSION IN SET

(A)



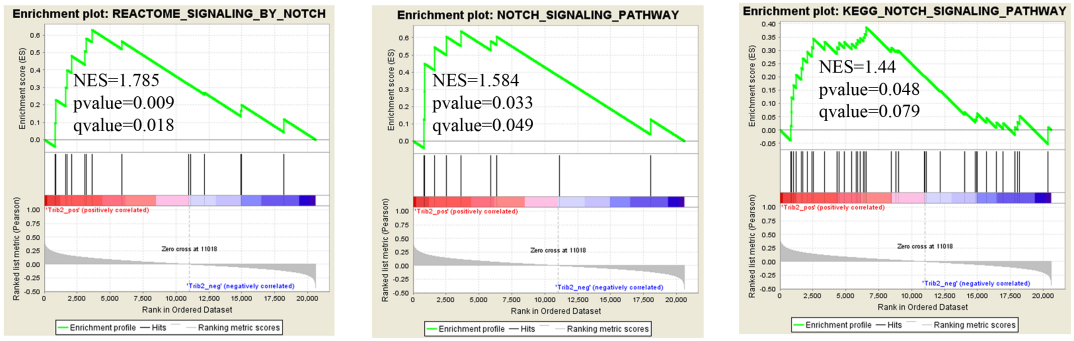
(B)



(C)

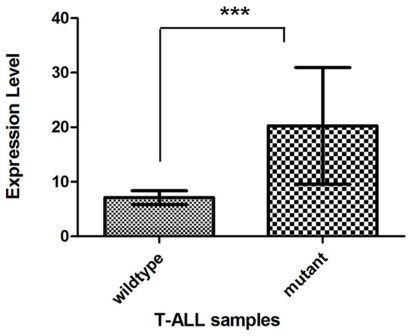
Name	Induction Pattern	Classification
Cluster 667	Tcell+NK	TCR
Cluster 757	Tcell+NK	
Cluster 829	Tcell+NK	TCR
Cluster 955	Tcell+NK	
Cluster 769	T/B cell	

(A)

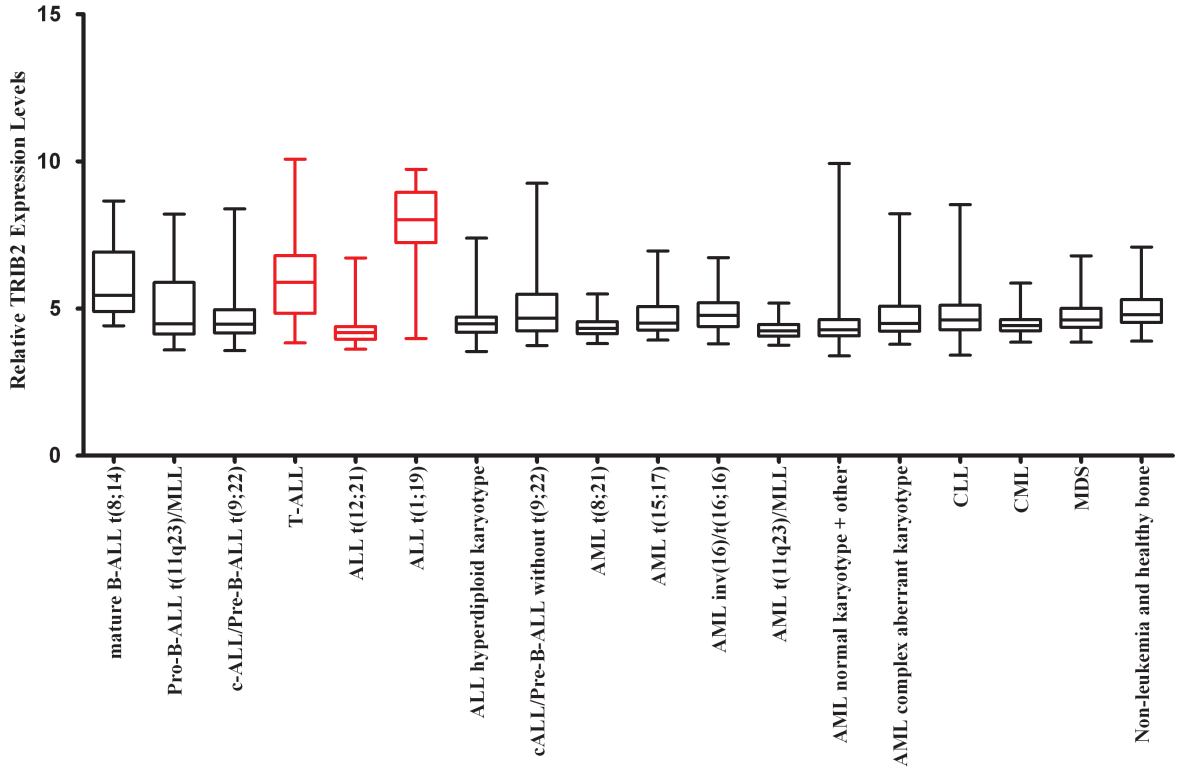


(B)

(C)



Box plot other TRIB2 probe


Relative TRIB2 Expression Levels
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Pro-B-ALL t(11q23)/MLL
c-ALL/Pre-B-ALL t(9;22)
T-ALL
ALL t(12;21)
ALL t(1;19)
ALL hyperdiploid karyotype
cALL/Pre-B-ALL without t(9;22)
AML t(8;21)
AML t(15;17)
AML inv(16)/t(16;16)
AML t(11q23)/MLL
AML normal karyotype + other
AML complex aberrant karyotype
CLL
CML
MDS
Non-leukemia and healthy bone