Serum profiling identifies ibrutinib as a treatment option for adults with B-cell acute lymphoblastic leukaemia

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ABSTRACT

Acute lymphoblastic leukaemia (ALL) is a haematological malignancy that is characterized by the uncontrolled proliferation of immature lymphocytes. 80 % of cases occur in children where ALL is well understood and treated. However it has a devastating affects on adults, where multi-agent chemotherapy is the standard of care with allogeneic stem cell transplantation for those who are eligible. New treatments are required to extend remission and prevent relapse to improve patient survival rates.

We used serum profiling to compare samples from presentation adult B-ALL patients with age- and sex-matched healthy volunteer (HV) sera and identified 69 differentially recognised antigens ($p \leq 0.02$). BMX, DCTPP1 and VGLL4 showed no differences in transcription between patients and healthy donors but were each found to be present at higher levels in B-ALL patient samples than HVs by ICC. BMX plays a crucial role in the Bruton's Tyrosine Kinase (BTK) pathway which is bound by the BTK inhibitor, ibrutinib, suggesting adult B-ALL would also be a worthy target patient group for future clinical trials.

We have shown the utility of proto-array analysis of adult B-ALL patient sera to help characterise the B-ALL immunome and identified a new target patient population for existing small molecule therapy.

Keywords: acute lymphoblastic leukaemia, antigen identification, serum profiling, immunotherapy, survivin, bone marrow tyrosine kinase on chromosome X (BMX).

INTRODUCTION

B-cell acute lymphoblastic leukaemia (B-ALL) is a clonal malignant disease that is characterised by the accumulation of genetically damaged precursors of B lineage lymphocytes. This lineage of ALL affects immature B-cells, suppressing normal haematopoiesis and affecting the ability of healthy immature B-cells to differentiate and mature into functional B and plasma cells. Like all leukaemias, ALL rapidly spreads throughout the body making localised treatments of little benefit. In addition, most ALL patients will achieve first remission but experience high relapse rates with long term disease free survival ranging from 15 – 45 % (Sant et al., 2014) depending on patient age.

Immunotherapy is currently used to treat ALL patients through allogeneic haematopoietic stem cell transplant and can improve overall survival for patients up to 27-65 % (Fielding et al., 2009, Goldstone et al., 2008, Moorman et al., 2007). To boost the graft versus host effect and maximise the chances of successful transplantation of the donor stem cells, patients are given donor leukocyte infusions to support the graft. However, over one third of patients will still relapse and the mortality rates associated with stem cell transplantations remain high. Additional immunotherapy strategies for B-ALL include those targeting CD20, a surface marker of B-lineage lymphocytes present in 25 % of patients with pre-B ALL and nearly all mature ALL cells (Wei et al., 2017). Rituximab, a humanized anti-CD20 antibody (Pui and Jeha, 2007) was replaced by second-generation anti-CD20 mAbs, Ofatumumab and Obinutuzumab, to overcome its limitations (Wei et al., 2017). Athough the ubiquitous B-cell marker, CD19 is unsuitable as a target for naked mAbs (Wei et al., 2017), antibody-drug conjugates including SAR3419, which fuses a humanized anti-CD19 antibody and maytansin, and subsequently SGN-CD19A (Denintuzumab) have each shown promise (Wei et al., 2017). CD22 is expressed on leukemic blasts in 90 % of patients with pre-B ALL and mature ALL. Inotuzumab ozogamicin is an antibody-drug conjugate developed to target CD22 and couples an engineered humanized monoclonal immunoglobulin G4 antibody against CD22 conjugated to calicheamicin (Wei et al., 2017, Schwarzbich and Witzens-Harig, 2017).

Bispecific T cell engagers (BiTEs) conjugate the antigen binding domains of two monoclonal antibodies recognizing leukaemic cells and cytotoxic T cells to direct cytotoxic T cells against malignant B cells. Blinatumomab was the first clinically approved bispecific T cell engagers for use in ALL [recently reviewed by (Hathaway et al., 2018)]. Most recently chimeric antigen receptor (CAR) T cells, T cells that have been genetically modified to

express a T-cell receptor with a specific antigen binding site have shown positive results in clinical trials (Wei et al., 2017, Bassan et al., 2018, Gokbuget, 2018, Horvat et al., 2018) albeit that on-tumour off-target effects caused by CD19 expression on both normal and malignant B-lymphocytes mean that CAR T cells do not distinguish between tumour and healthy cells (Wei et al., 2017).

Despite the number of existing therapies, new treatments targets need to be identified to further personalise treatments and enhance survival rates for patients with adult B-ALL. Antigen identification has the potential to extend the use of existing therapeutic strategies and identify biomakers that enable more personalised treatment. To this end we used proto-arrays that contain over 9,000 individally purified human proteins to determine the preferential recognition of antigens by antibodies in adult B-ALL patient sera.

MATERIALS AND METHODS

Patient samples. Adult B-ALL samples were collected from patients, attending the Departments of Haematology at Southampton University Hospitals Trust, Portsmouth Hospitals NHS Trust and the Royal Devon and Exeter Foundation Trust, following informed consent and local ethical approval (REC 07/H0606/88). Leukaemic blasts, mononuclear cells and sera were isolated from bone marrow (BM) or peripheral blood (PB) and were collected in EDTA or from clotted blood. Sera and white blood cells were also isolated from age and sex-matched healthy volunteer (HV) samples following informed consent and local ethical approval (LREC 228/02/T), or provided by the North London Blood Transfusion Centre (NLBTC) and all samples were stored at -80^{o}C for later use. Red blood cells were lysed using red cell lysis buffer, the cells were washed in phosphate buffered saline and either 2.5 x 10^4 spotted onto two sites on glass slides and allowed to dry for 4-16 h prior to being double wrapped in cling film and stored at -20^{o}C for ICC or snap-frozen as pellets at 10^5-10^6 cells per tube at -80^{o}C.

Cell culture. Cell lines were obtained from and cultured as per ATCC instructions. Cell lines were cultured in RPMI 1640 (K562) or DMEM (Hek293T, MDA-MB-231 and MDA-MB-468), 10 % foetal calf serum, 1 % penicillin/streptomycin (all Sigma-Aldrich Co. Ltd, Dorset, U.K.)

Serum profiling using protein microarrays. We performed serum profiling on 13 samples from nine B-ALL patients, eight of which were prior to treatment, as well as nine age and

sex-matched HV samples (**Table 1**). Each ProtoArray - Human Protein Microarray v5.0 (Life Technologies) slide was immunoscreened with a single serum sample while one ProtoArrayR Control Microarray (Life Technologies) was used with control protein and one slide was assayed with secondary antibody alone. Signals from all peptides were analysed on the ProScanArray (PerkinElmer) using ProtoArray® Prospector v5.2 software (Life Technologies).

Prioritisation of B-ALL antigens. To determine which genes warranted further investigation, we examined the antigens that were more frequently recognised by antibodies in sera from adult B-ALL than age- and sex-matched HV samples (at a p value of ≤0.02) using a systematic peer review process [described in (Cooksey, 2018)]. Briefly, we used Scopus and NCBI, and the search terms used were, where X represents each protein individually, 'X', 'X AND cancer', 'X AND leukaemia', 'X AND 'acute lymphoblastic leukaemia'', 'X AND function'; where available appropriate aliases were also searched in the same manner. In addition we performed STRING analysis (https://string-db.org/) to identify the processes these antigens engage in that are involved in cancer.

cDNA preparation from human samples. To evaluate the expression of the most promising antigens in HV and B-ALL cells, we isolated RNA from BM and PB samples using QIAGEN RNeasy® Plus mini kit (QIAGEN Ltd.) according to the manufacturer's protocol. cDNA was prepared using the MBI Fermentas RevertAid First Strand cDNA synthesis kit (MBI Fermentas Ltd, Helena BioSciences Ltd, Sunderland, U.K.) which was DNase I treated (Roche Products Ltd, Herts, U.K.), cleaned using a RNeasy kit (Qiagen), checked on a 1 % agarose-TBE gel and quantified using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific, UK).

Quantitative–Polymerase Chain Reaction (qPCR) analysis. QPCR was performed using Quantinova™ SYBR® Green kit and QuantiTect Primers (all Qiagen). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* and TATA-box binding protein (*TBP)* were used as reference genes, chosen based on previous publications (Lossos et al., 2002) and in keeping with MIQE guidelines (Bustin et al., 2009, Bustin and Wittwer, 2017). Each primer was tested on at least one human cancer cell line that was known to express that antigen of interest (**Table 2**).

To control for gDNA contamination within the qPCR reagents, a no cDNA control was included on every qPCR plate whereby cDNA was replaced by RNase-free H_2O. In addition,

each sample was analysed in triplicate on the 96-well qPCR plate (Applied Biosystems, USA). The reaction volumes were 10 µL 2X QuantiNova SYBR green PCR Mix, 2 µL QN ROX reference dye, 2 µL QuantiTect primer assay and 5 µL RNase-free H_2O, making a total volume of 19 µL added to each well in the 96-well plate. In 1 µL final volume, ≤100 ng of template cDNA was added to achieve a final volume of 20 µl. The plate was then loaded into the thermocycler (StepOne Plus Real-Time PCR system, Applied Biosystems).

The cycle was 2 minutes at 95°C, then 40 cycles of 5 s at 95°C followed by 10 s at 60°C. This was immediately followed by a melt curve stage of 15 s at 95°C, 1 min at 60°C and 15 s at 95°C, to verify the specificity of the amplification. Data was compared using StepOne software v2.0 (Applied Biosystems) and the comparative C_T method.(Livak and Schmittgen, 2001) We used the relative quantification (also comparative C_T) method to analyse the qPCR data.

Immunocytochemistry. Immunocytochemistry was performed as described previously(Khan et al., 2015) using the antibodies detailed (**Supplementary Table A1**). Actin was used as a positive control for the successful performance of ICC while isotype, no primary and no secondary antibody immunolabelling acted as negative controls, used to detect non-specific staining. Lillies-Mayer Haematoxylin was used as a counterstain.

Staining intensities were scored according to a five-tiered scale [described originally by (Biesterfeld et al., 1996)] as follows:- 0: no staining; 1: background; 2: weak staining; 3: moderate staining; 4: strong staining. The percentage of positively stained cells was based on the cell count of stained cells per microscopic view, and represented on a five-tiered scale (0: 0 %; 1: 1-10 %; 2: 11-50 %; 3: 51-80 %; 4: > 80 %). The final immunoreactivity score was obtained by multiplication of the percentage of positive stained cells per microscopic view by the value for staining intensity (0 = negative, 1-29 = weak, 30-143 = moderate, 144-228 = high and > 228 = very high staining)(Deng et al., 2014).

Microarray analysis of gene expression and clinical parameters. Finally we analysed a publically available microarray expression data (GSE38403)(Geng et al., 2012) which encompassed 215 adult B-cell ALL and 12 pre-B samples (CD19+ and VpreB+) isolated from the bone marrows of HVs. For microarray analysis, the files were imported from GEO in the Partek Genomic Suite, normalised and subjected to ANOVA analysis.

RESULTS

Serum profiling using proto-arrays. The intensity values were used to undertake a per chip / per gene normalisation process and then ANOVA was performed on the 3,998 proteins, that had showed differences in recognition by HV and patient sera, across the 22 samples analysed (excluding the control). ANOVA analysis identified 69 significant genes that had a $p<0.05$ in terms of their recognition by ALL versus HV sera (**Supplementary Table A2**). The mean value and standard deviation of each signal was calculated to produce a z-score and showed that 19 proteins were more frequently recognised (**Supplementary Table A2i)** and 50 proteins were less frequently recognised by antibodies in B-ALL (**Supplementary Table A2ii)** compared with HV sera. Two proteins that had fold changes of less than 1.5 (POLK3K and RAB2IL1) were included as they were significantly different in their recognition by ALL versus HV sera. However, we focussed on antigens that were more frequently recognised by antibodies in B-ALL than HV sera because of our interest in identifying antigenic targets for immunotherapy.

Hierarchical cluster analysis was performed using Wards method (**Fig 1A**) and showed that B-ALL patients could be differentiated from HVs on the basis of protein recognition by sera. Principal component analysis (PCA)(**Fig 1B**) showed that apart from one HV sample, there was a good cluster distinction between the proteins recognised significantly more or less frequently by ALL compared with HV sera. Interestingly, the ALL cluster in the PCA plot is "tighter" than the HV cluster probably reflecting the homogeneity of the proteins uniquely recognised by B-ALL sera and the relative heterogeneity of the proteins recognised by antibodies in the HV samples.

Prioritisation of proto-array identified antigens. Through a systematic review of the literature (Cooksey, 2018) we identified bone marrow tyrosine kinase on chromosome X (BMX) also known as epithelial and endothelial tyrosine kinase (Ekt), dCTP pyrophosphatase 1 (DCTPP1) also known as XTP3-transactivated protein A (XTP3TPA) and vestigial like 4 (VGLL4) for further expression analysis. Each antigen was investigated by STRING analysis and shown to have pathways associated with subverted processes in cancer (**Fig 2A**). These three antigens were then analysed for their expression in samples from adult B-ALL and HVs by qPCR and ICC.

qPCR analysis of antigen expression in B-ALL and HV samples. We determined the relative expression of transcripts encoding the antigens of interest in the following cell lines that we

used as controls (K562, HEK293T, MDA-MB-231 and MD-MB-468)(**Table 2**). We included survivin in this study based on the promising results we had observed with this antigen, in terms of elevated expression, in B-ALL compared with HV samples previously (Boullosa et al., 2018). All cell lines expressed all housekeeping genes as well as the four antigens under investigation, except BMX which was not expressed in any of the cells lines.

All samples from B-ALL patients and HVs, except for HV025, showed convincing GAPDH expression (**Table 3**) and all antigens tested (BMX, DCTPP1, Survivin and VGLL4) were expressed at lower levels than GAPDH, as expected. Survivin was expressed at higher levels than TBP in all cell lines, and DCTPP1 and VGLL4 were expressed at higher levels than TBP in K562 and Hek293T. TBP was recommended as a housekeeping gene for qPCR by other studies however we found TBP expression was not consistent between the samples. However the differences in expression between the HV samples were much smaller, after excluding HV025 from our analysis.

Patients ALL005 and ALL019 were not found to express detectable levels of any of the antigens studied (BMX, DCTPP1, survivin and VGLL4)(**Fig 2B**) while ALL020 (PB and/or BM) expressed all four antigens. Of the other B-ALL patient samples analysed, two had detectable BMX expression (ALL008 and ALL020 PB), three patients expressed DCTPP1 (ALL007, ALL008 and ALL020 BM) and three patients expressed VGLL4 and survivin (ALL007, ALL008 and ALL020 PB and BM). ALL007 and ALL008 had also been examined by our group previously (Boullosa et al., 2018) and while ALL007 was found to have detectable survivin transcripts in both studies, ALL008 was negative for detectable survivin transcripts in our previous study. None of the HVs had consistently detectable levels of BMX, while all expressed DCTPP1 and VGLL4. In contrast to our previous findings, where four of five HVs did not express detectable levels of survivin in this study transcripts were detected in three of four HVs (whom we had not analysed previously).

Immunolabelling of antigen expression in B-ALL using immunocytochemistry. We used actin as a positive control for immunolabelling and found it, as expected, to be present in all four control cell lines at high levels (**Table 2**), while the negative controls for this experiment (cells only, no primary antibody and isotype control) were all negative. HEK293T cell line was used to demonstrate we could immunolabel BMX (van Oosterwijk et al., 2018) and VGLL4 (Jiao et al., 2017), but only showed a moderate cytoplasmic expression of BMX and were negative for VGLL4; weak immunolabelling of survivin was also detected. The K562

cell line was used to demonstrate the immunolabelling of DCTPP1 (Song et al., 2015) and survivin (Schmidt et al., 2003), and showed moderate cytoplasmic levels of both. MDA-MB-231 cell line was used to demonstrate we could immunolabel DCTPP1 (Song et al., 2015), while MDA-MB-468 was chosen as an immunolabelling control for VGLL4 (Zhang et al., 2017), but both were negative for the detection of these antigens. Of note VGLL4 was not found in any cell line, MDA-MB-231 and MDA-MB-468 did not express DCTPP1 or survivin, while each had transcripts from these antigens that were detectable by qPCR. In contrast BMX was detectable in Hek293T by ICC in agreement with previously published Western blotting data (van Oosterwijk et al., 2018).

Each antigen was found at moderate to high levels in patient samples (BMX - 4 of 6; DCTPP1 – 2 of 2; survivin - 1 of 2 and VGLL4 - 1 of 2) while each antigen was only found at low levels in HV samples (n=7)(**Table 4; Fig 3**). Of note ALL005 had also been examined for survivin expression in our previous study and was found to be positive by ICC in this study also.

Gene expression microarray analysis. The antigens that were significantly more frequently recognised by B-ALL than HV sera ($p \leq 0.02$) and were present in the microarray database (GSE38403)(Geng et al., 2012) was examined for their association with above and below median levels of expression at disease diagnosis, and overall patient survival in the following 5 years post-diagnosis. Only cyclin G associated kinase (GAK) and high mobility group box family member 2 (TOX2) showed a significant association between above median expression at disease diagnosis and 5-year overall survival ($p=0.024$ and $p=0.0056$ LogRank and $p=0.056$ and $p=0.028$ by Wilcoxon-Gehan, repectively; **Fig 4A**). When we analysed the levels of expression, segregated into quartiles, we found that only expression of TOX2 in the third quartile (Q3 - above median-low) was associated with 5 year survival while expression in the top quartile (Q4 - above median-high) was not (**Fig 4B**).

In addition a number of clinical features (stage and cytogenetic abnormalities) were found to be significantly associated with above or below median antigen expression at disease diagnosis (**Supplementary Table A3**). Of particular note was the high level of statistical significance ($p<0.001$) for the following antigens and gender (BMX), stage (APOBEC3A, DCTPP1, MUC20, SEPT9, TOX2 and WARS), early pre-B versus mature B-ALL (WARS), early pre-B versus early Pro-B (DCTPP1, MUC20, SEPT9, TOX2), early pre-B versus transitional Pre-B (VGLL4), mature B-ALL versus Pro-B (APOBEC3A), pre-B-ALL versus

pro-B (MUC20), MLL mutation (DCTPP1, MUC20 and TOX2), BCR-ABL (DCTPP1, IGLL1, LMX1a, TOX2 and VGLL-4), other cytogenetic abnormalities (LMX1A), no cytogenetic abnormalities (IGLL1 and TOX2) and CD25 positivity (CDCA3, IGLL1, and TOX2).

DISCUSSION

Despite the recent development of a number of strategies for the treatment of adult B-ALL, survival rates remain low. This is mostly due to the toxicity of treatment to older adult B-ALL patients, the absence of B-ALL cell specific therapies, co-morbidities associated with the level of fitness of patients, treatment related toxicities and death due to disease recurrence. The circumvention of relapse due to improved treatments including matched unrelated stem cell transfusions, donor lymphocyte infusions and more recent immunotherapy strategies have had significant impacts on patient survival but they still have associated toxicity and antibody based therapies, especially, can be expensive (Hernandez et al., 2018). As part of the effort to identify new targets for immunotherapy, that can help delay if not prevent relapse after first remission, and to help us better understand the biological basis of adult B-ALL, we sero-profiled adult B-ALL patients and age and sex-matched HV sera. We identified three antigens that had been shown to play a significant role in cancer(s) and examined their expression in patient and HV samples. We have previously shown that survivin had significantly higher expression in B-ALL samples compared with healthy controls by qPCR analysis (p=0.015) and gene expression analysis ($p = 0.013$)(Boullosa et al., 2018). However there were no statistically significant differences between the expression of the four antigens examined in this study (BMX, DCTPP1, survivin or VGLL4) in patient samples compared with HVs by qPCR. Gene expression array analysis confirmed that BMX, DCTPP1, survivin and VGLL4 transcript levels were not different between B-ALL patients compared with healthy donors (data not shown), while ICC analysis showed elevated protein levels in patients compared with healthy donors.

The discordance between detection of transcripts by qPCR and immunolabelling of antigens by ICC has been observed by scientists in other fields of research. It appears that there is a linear relationship between gene transcription and protein levels in *Drosophila,* (Lemos et al., 2005) that uncouples with evolutionary complexity, maximising in chimpanzees and humans

(Fu et al., 2007) and is partially explained by post-transcriptional regulation and differences in mRNA and protein turnover rates. Although qPCR is often used to semi-quantitate gene expression, we found that protein levels, the ultimate target of immunotherapy strategies, are a more convincing indicator of the presence of an antigen in a cell.

BMX was more frequently recognised by B-ALL patient compared to HV sera (p=0.035) and is a particularly appealing target for immunotherapy, in accordance with the National Cancer Institute (NCI) priority list (Cheever et al., 2009), by virtue of it's location on the X chromosome. In addition, BMX has been shown to play a central role in the immune response, inflammation and cytokine signalling by regulating TNF-dependent signalling at a central node, that affects the efferent signalling branches of JNK, p38 MAPK and NK-kappa-B (Gottar-Guillier et al., 2011, Holopainen et al., 2012, Li et al., 2017). BMX has been reported to regulate multiple proteins (Saharinen et al., 1997, Chen et al., 2013, Holopainen et al., 2012, Wang et al., 2017, Li et al., 2017) and these associations were confirmed by our STRING analysis. As a tyrosine kinase BMX is a target for inhibitors including CI-1033, an epidermal growth factor receptor (EGFR) inhibitor, BMX-IN-1 (Liu et al., 2013) and CTN06 (Guo et al., 2014), a small molecule dual inhibitor of BMX and Bruton's Tyrosine Kinase (BTK), the latter shown to be effective in autophagy and apoptosis induction, as well as the inhibition of the growth and migration of prostate cancer cells. In addition, BMX is a target of ibrutinib, a small molecule therapy that targets the BTK pathway. Ibrutinib is being used in several clinical trials for patients with mature B-cell malignancies including relapsed/refractory chronic lymphocytic leukaemia (Coutre et al., 2019, Nuttall et al., 2019, Shanafelt et al., 2019), small lymphocytic lymphoma (Coutre et al., 2019), and mantle cell lymphoma (Wang et al., 2013) and BMX expression in 4/6 adult B-ALL patients in this study, the antigenicity of BMX as shown by proto-array analsys and recent study showing the ability of ibrutinib to target pre-BCR signalling in B-ALL (Kim et al., 2017) all suggest the value of using ibrutinib to treat adults with B-ALL.

The other two antigens investigated were DCTPP1 an intracellular regulator of 5-methyl-dCTP metabolism that has been associated with DNA hypermethylation and gene silencing (Song et al., 2015). DCTPP1 is downregulated when the PI3K/AKT/mTOR pathway is activated and autophagy activity is suppressed (Lu et al., 2018) with its' expression being associated with poor prognosis and lower overall survival of solid cancer patients (Zhang et al., 2013, Morisaki et al., 2014, Song et al., 2015, Lu et al., 2018). DCTPP1 has also been

shown to increase chemotherapy resistance in gastric cancer suggesting a role as a indicative biomarker of chemoresistance in patients (Xia et al., 2016). VGLL4 is a tumour suppressor gene located on 3p25.3-3p25.2 and was deemed worthy of further investigation because of it's roles in proliferation, migration and cell death in a number of cancer types (Deng and Fang, 2018). VGLL4 is a co-factor for TEA domain-containing transcription factors (TEADs)(Zhang et al., 2017) and this association was re-itterated by our STRING analysis.

It was also notable that WWOX appeared as one of the antigens that was less frequently recognised by patient than HV sera and this correlated with previous findings that WWOX was partially expressed or absent in over half of haematopoietic malignancies tested (Ishii et al., 2003, Cui et al., 2013). Some debate remains as to whether WWOX is affected in leukaemias by virtue of it's location on 16q23.3-24.1 in the common fragile site FRA16D, however recent studies have shown reduced WWOX expression in leukaemia cells, with WWOX inhibition repressing proliferation and increased rates of apoptosis (Lin et al., 2013).

Due to the relative rarity of adult B-ALL (Inaba et al., 2013) we used exisiting gene expression data (GSE38403)(Geng et al., 2012) to examine associations between gene transcript levels, disease stage and cytogenetic abnormalities. Only two antigens, GAK and TOX2, had above median expression at disease diagnosis that significantly correlated with improved overall survival. We have seen similar results previously in acute myeloid leukaemia (AML) at disease presentation with SSX2IP, RHAMM and Survivin (Guinn et al., 2009) and reasoned that the elevated antigen expression could induce enhanced anti-tumour responses in patients following conventional treatment. We propose that as dead and dying leukaemia cells are mopped up by immune cells and their peptide contents are presented on MHC in the context of 'danger' (inflammation) leading to more effective anti-tumour responses. However this isn't always the case with antigens such as PRAME showing a Goldilocks effect in terms of both very high and very low expression being associated with poor survival in myelodysplastic syndome patients (Liberante et al., 2013), perhaps reflecting the impact of high PRAME expression on tumourigenicity and low PRAME levels enabling immune evasion following conventional treatment.

In summary, we have used serum profiling for the first time to characterise the recognition of antigens by antibodies in adult B-ALL sera, in direct comparison to age and sex-matched HV sera. We have identified new targets for treatment (BMX), provided new insights into the the

biology underlying this rare and difficult to treat disease (loss of WWOX antigen recognition in adult B-ALL) as well as identifying new biomarkers for survival (GAK, TOX2).

ABBREVIATIONS

AML: acute myeloid leukaemia; B-ALL: B-cell acute lymphoblastic leukaemia; BMX: bone marrow tyrosine kinase on chromosome X; BTK: Bruton's Tyrosine Kinase; CAR: chimeric antigen receptor; DCTPP1: dCTP pyrophosphatase 1; GAK: cyclin G associated kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HV: healthy volunteer; PB: peripheral blood; PCA: Principal component analysis; qPCR: quantitative polymerase chain reaction; TBP: TATA-box binding protein; TEAD: TEA domain-containing transcription factors; TOX2: TOX high mobility group box family member 2 (TOX2) variant 3; VGLL4: vestigial like 4.

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CONTRIBUTION: S.J., and L.C.: designed and performed experiments, analysed results, made figures and contributed to the writing of the paper; S.B. and L.O., designed and performed experiments; K.I.M. analysed results, made the figures, contributed to writing of paper; K.H.O., designed experiments, contributed to the writing of the paper; BG: designed and performed experiments, analysed results, made the figures and wrote the paper. Thanks to Dr Mat Arno and the Genomics Centre, Kings College London; Dr Cindy Lee and Dr Hannah Wickenden for help and support.

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(A) *(B)*

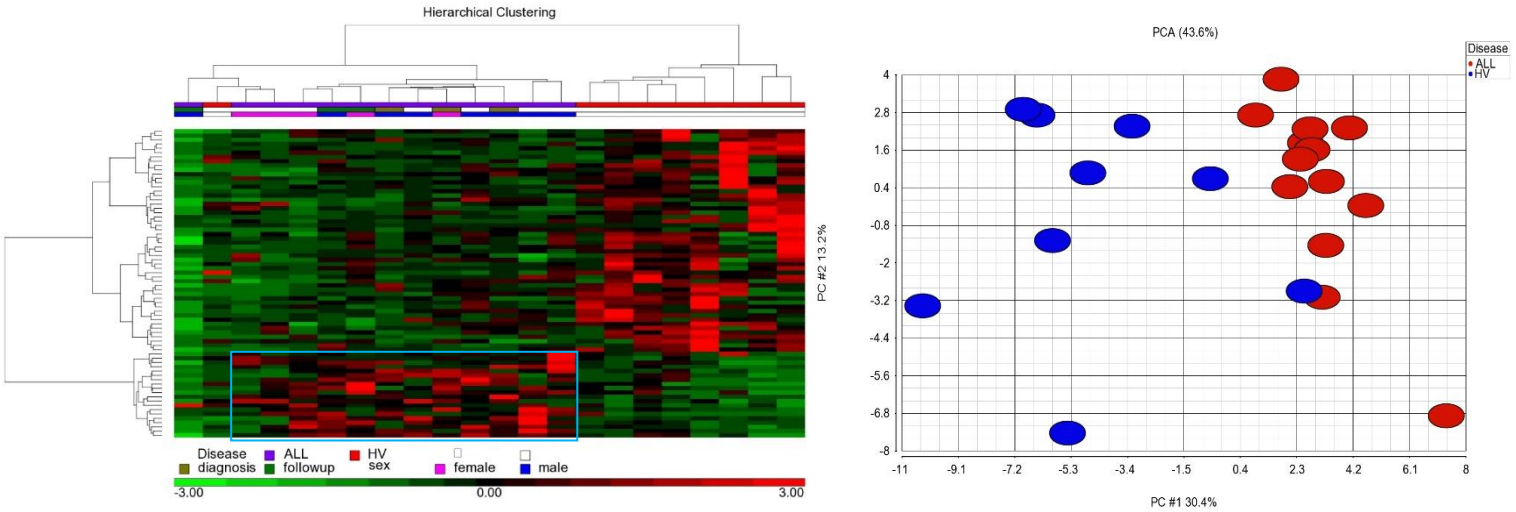
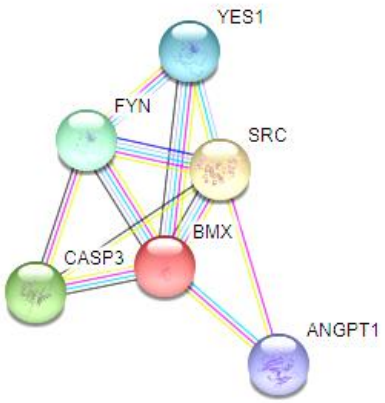
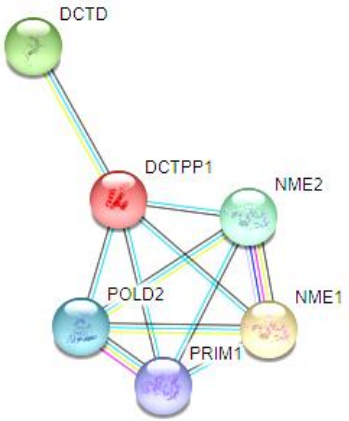


Fig 1 Immunoprofiling using sera from adult B-ALL patients and HVs. (A) Hierarchal Clustering of proteins shows that adult B-ALL and HV samples may be differentiated based on antibody recognition of antigens and that antigen recognition to adult B-ALL sera (blue box) may be less heterogeneous than previously thought; **(B)** PCA evidences the distinctness of features of samples from HVs (blue circles) and patient samples (red circles) bar one HV sample.

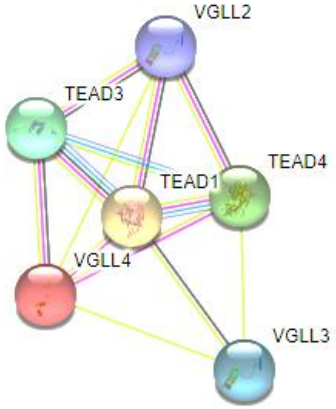
(A)(i)


YES1
FYN
SRC
BMX
CASP3
ANGPT1

(ii)


DCTD
DCTPP1
NME2
POLD2
NME1
PRIM1

(iii)


VGLL2
TEAD3
TEAD4
TEAD1
VGLL4
VGLL3

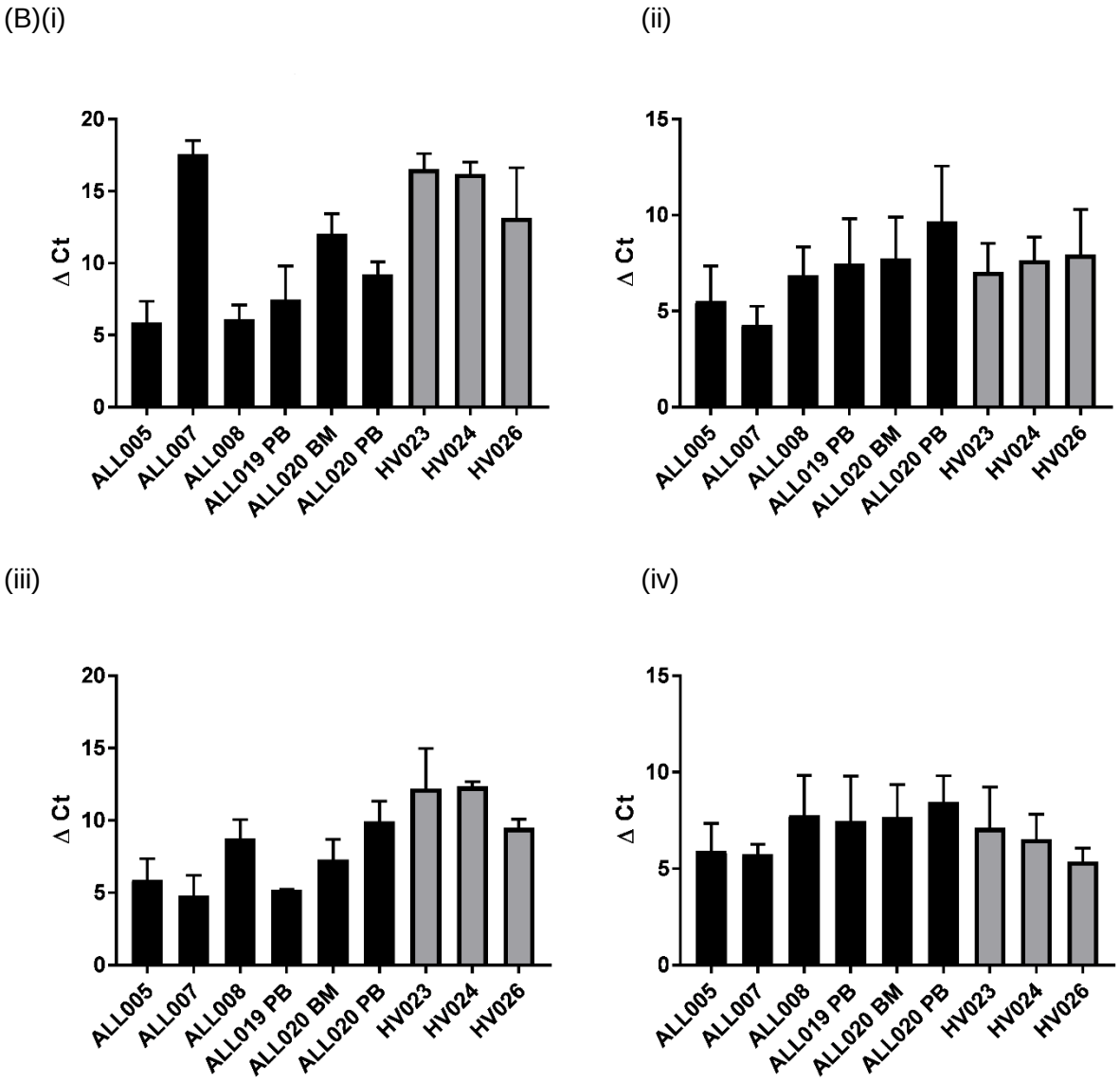
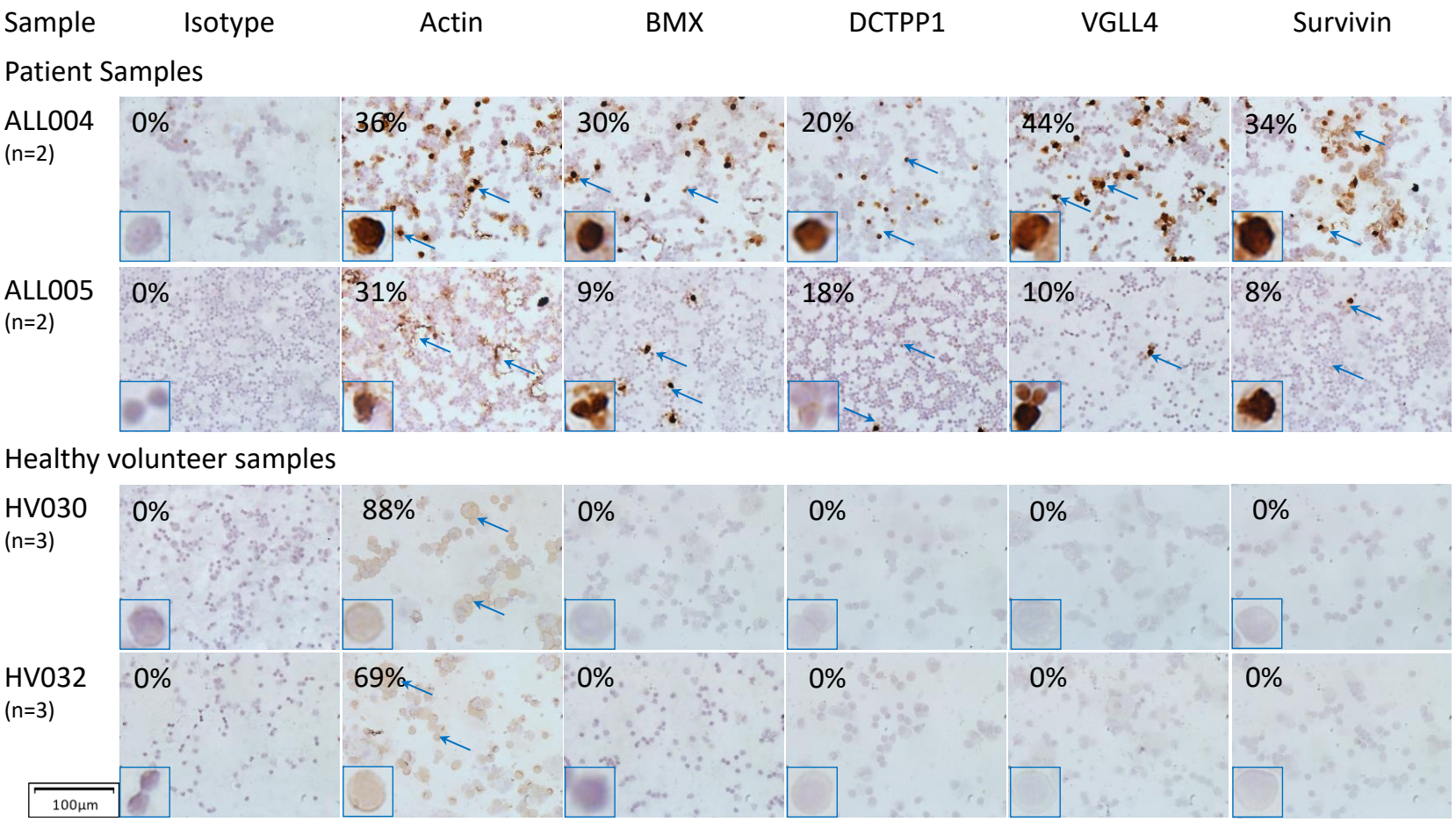


Fig 2. Analysis of the protein interactions and gene expression of prioritised antigens. (A) STRING analysis identified the experimentally determined protein interactions by each antigen of interest (represented as a red ball). Panel **(i)** BMX showed interactions with multiple components involved in cell-adhesion, cell growth, cell survival, signaling pathways and apoptosis, including ES1, FYN, SRC and CASP3; Panel **(ii)** DCTPP1 (red ball) interacts with NME1 and 2. NME1 is involved in cell proliferation, differentiation and development while NME2 acts as a transcriptional activator for the MYC gene, a family of regulator genes and proto-oncogenes; Panel **(iii)** VGLL4 interacts with TEA domain family members (TEAD1, 2 and 4), transcriptional factors that play key roles in the Hippo signaling pathway. The Hippo pathway is involved in controlling tumor suppression by restricting proliferation and promoting apoptosis; **(B)** Expression of each antigen is shown as follows **(i)** BMX; **(ii)** DCTPP1; **(iii)** Survivin and **(iv)** VGLL4 relative to GAPDH as determined by qPCR.


Sample
Isotype
Actin
BMX
DCTPP1
VGLL4
Survivin
Patient Samples
ALL004
(n=2)
0%
36%
30%
20%
44%
34%
ALL005
(n=2)
0%
31%
9%
18%
10%
8%
Healthy volunteer samples
HV030
(n=3)
0%
88%
0%
0%
0%
0%
HV032
(n=3)
0%
69%
0%
0%
0%
0%
100µm

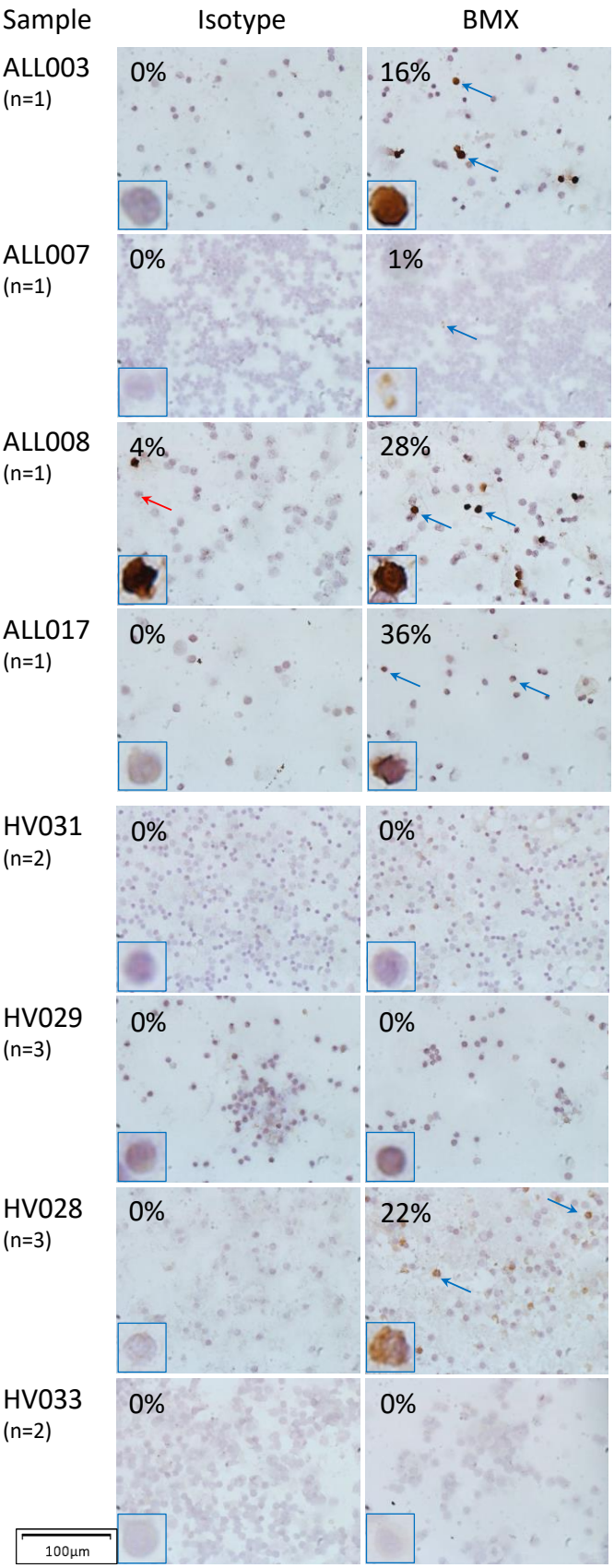

Sample
Isotype
BMX
ALL003
(n=1)
0%
16%
ALL007
(n=1)
0%
1%
ALL008
(n=1)
4%
28%
ALL017
(n=1)
0%
36%
HV031
(n=2)
0%
0%
HV029
(n=3)
0%
0%
HV028
(n=3)
0%
22%
HV033
(n=2)
0%
0%
100µm

Figure 3. Immunolabelling to detect expression of the prioritised antigens in primary adult B-ALL patient samples. Blue arrows indicate the B-ALL patient cells that show antigen expression (also indicated by the formation of a brown precipitate). Actin acted as the positive control for immunolabelling. The two isotype matched antibodies were used as negative controls. Panel (**A**) shows patient and HV samples immunolabelled for expression of BMX, DCTPP1, survivin and VGLL4 while panel (**B**) shows samples immunolabelled for BMX expression and with isotype control antibody only. Percentages shown are the averages of positively stained cells of the 'n' independent experiments. The blue arrows point at positive stained cells and the blue squares contain one-cell-zoom pictures from the original picture (scale bar is 100μm). All pictures were taken at 400X magnification.

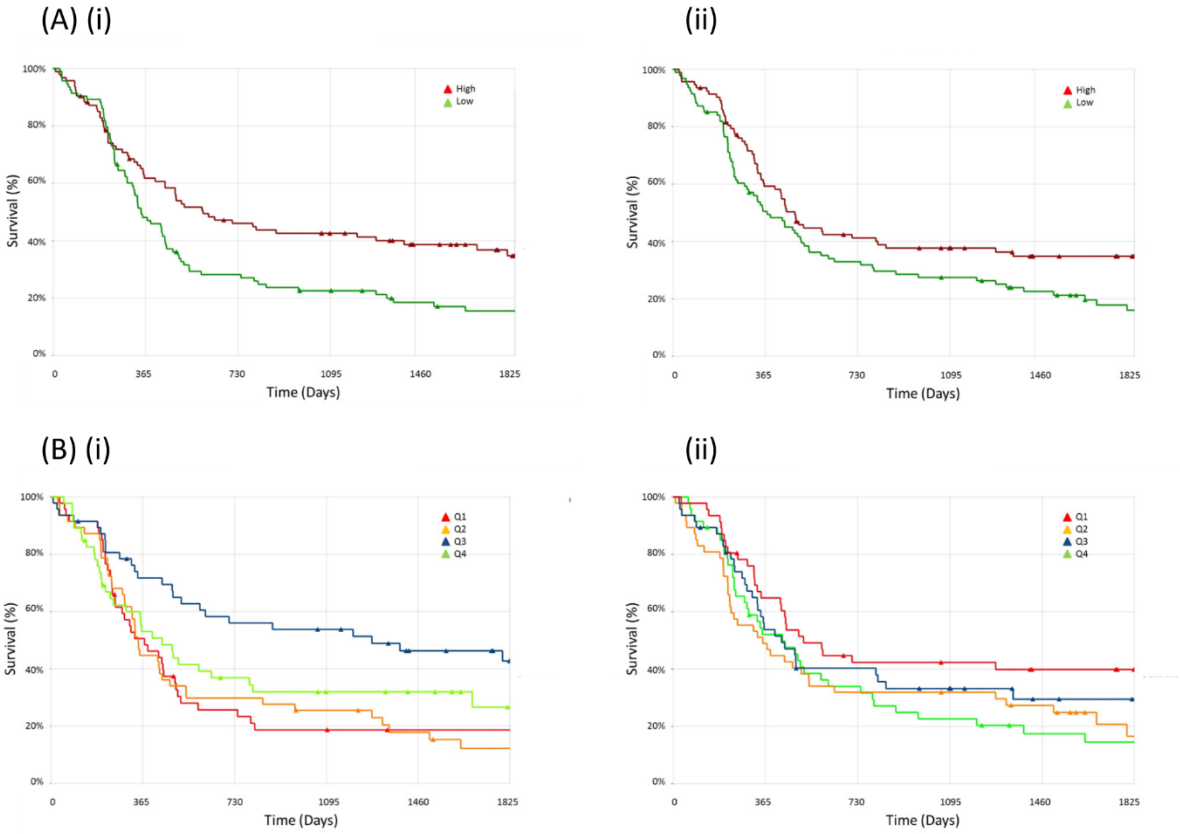


Fig 4. Gene expression microarray analysis of antigen expression and overall survival (over 5 years) in adult B-ALL patients. Overall survival was found to be significantly associated with above median (high) levels of (**A**)(i) TOX2 (Log-Rank p-value = 0.075; Wilcoxon-Gehan p-value = 0.034) and (ii) GAK (Log-Rank p-value 0.024; Wilcoxon-Gehan p-value = 0.034); When expression levels were divided into quartiles (**B**) Q1 represents low-low level expression, Q2 median-low; Q3 median-high and Q4 high-high levels of expression, relative to the median. (i) TOX2 expression was significantly associated with improved survival for the patients expressing median-high (Q3) levels of expression (Log-Rank p-value 0.011; Wilcoxon-Gehan p-value = 0.032) but not for patients expressing the high-high levels (Q4). The same association was not seen with (ii) GAK expression although a trend towards improved survival was seen in patients with the high-high levels of expression (Log-Rank p-value = 0.11; Wilcoxon-Gehan p-value = 0.19).

Table 1. Patient and HV information

ID	Disease stage	Age (at sampling)	Sex	HV control†	Age	Sex
ALL001	Diagnosis – Ph+ ALL	39	M	**HV013**	40	M
ALL002	Follow-up	19	M	**HV007**	19	M
ALL003	Diagnosis	26	F	**HV006**	26	F
ALL003-FU	Follow-up - 4 months post-allo	26	F	**HV006**	26	F
ALL004	Diagnosis	22	M	**HV015**	22	M
ALL005	Diagnosis	64	M	**HV009**	64	M
ALL007	Diagnosis	19	M	**HV007**	19	M
ALL008	Pre-treatment	19	F	**HV011**	19	F
ALL009	Diagnosis	49	M	**HV014**	45	M
ALL009-FU	Follow-up – 4 months post-allo	49	M	**HV014**	45	M
ALL010	Diagnosis	19	M	**HV015**	22	M
ALL010-FU	Follow-up	19	M	**HV015**	22	M
ALL017	Post-treatment	46	M			
ALL018	Pre-treatment	37	F			
ALL019	Pre-treatment, hyperdiploid	18	F			
ALL020	Pre-treatment	33	M			

†: age and sex-matched control for immune profiling on proto-arrays

Table 2. Cell line controls used for qPCR and ICC analysis of the antigens of interest, existing results on expression from the literature and corresponding qPCR and ICC data from our study

Cell line	Antigens expressed	qPCR data						ICC data*				
		GAPDH	TBP	BMX	DCTPP1	Survivin	VGLL4	Actin	BMX	DCTPP1	Survivin	VGLL4
Hek293T	BMX VGLL4	+	+	-	+	+	+	+ Mod	+ Mod	-	+ Weak	-
K562	DCTPP1 Survivin	+	+	-	+	+	+	+ V.high	-	+ Mod	+ Mod	-
MDA-MD-231	DCTPP1	+	+	-	+	+	+	+ Weak	-	-	-	-
MDA-MD-468	VGLL4	+	+	-	+	+	+	+ Mod	-	-	-	-
	Total	**4/4**	**4/4**	**0/4**	**4/4**	**4/4**	**4/4**	**3/4**	**1/4**	**1/4**	**1/4**	**0/4**

*Only scores of moderate and v.high are included in the total; Mod.: Moderate; V.high: very high.

Table 3. Antigen expression in B-ALL patient and HV samples as detected by qPCR.

Patients	GAPDH	TBP	BMX	DCTPP1	VGLL4	Survivin (#)	HVs	GAPDH	TBP	BMX	DCTPP1	VGLL4	Survivin
ALL005	+	-	-	±	-	-	**HV023**	+	+	-	+	+	+
ALL007	+	±	-	+	+	+ (+)	**HV024**	+	+	-	+	+	+
ALL008	+	±	+	+	+	+ (-)	**HV025**	±	±	±	+	+	±
ALL019 PB	+	-	-	-	-	±	**HV026**	+	+	±	+	+	+
ALL020 PB	+	+	+	±	+	+	**Total**	**3/4**	**3/4**	**0/2**	**4/4**	**4/4**	**3/4**
ALL020 BM	+	+	±	+	+	+							
Total*	**5/5**	**1/5**	**1/5**	**3/5**	**3/5**	**3/5**							

*Data is representative of two independent experiments. GAPDH and TBP were used as reference genes. +, antigen expression; +/-, one experiment showed no expression while the second showed expression; -, no antigen expression. *The total number of patients that expressed the antigen is shown at the bottom of the table, in this result the ± samples are excluded. (#): results obtained by qPCR on the same samples in our previous work.(Boullosa et al., 2018)*

Table 4. Immunoreactivity scores (and immunolabelling intensity in parenthesis) of the antigens of interest in (A) cell lines and (B) primary samples as determined by ICC.

Patient samples	BMX	DCTPP1	VGLL4	Survivin
ALL003	49 (Moderate)	ND	ND	ND
ALL004	91 (Moderate)	59 (Moderate)	154 (High)	68 (Moderate)
ALL005	23 (Weak)	35 (Moderate)	20 (Weak)	15 (Weak)
ALL007	1 (Weak)	ND	ND	ND
ALL008	84 (Moderate)	ND	ND	ND
ALL017	73 (Moderate)	ND	ND	ND
Total*	**4/6**	**2/2**	**1/2**	**1/2**

HVs	BMX	DCTPP1	VGLL4	Survivin
HV027	0 (Negative)	15 (Weak)	0 (Negative)	22 (Weak)
HV028	22 (Weak)	3 (Weak)	0 (Negative)	11 (Weak)
HV029	0 (Negative)	0 (Negative)	0 (Negative)	0 (Negative)
HV030	0 (Negative)	0 (Negative)	0 (Negative)	0 (Negative)
HV031	0 (Negative)	20 (Weak)	0 (Negative)	10 (Weak)
HV032	0 (Negative)	0 (Negative)	0 (Negative)	0 (Negative)
HV033	0 (Negative)	0 (Negative)	0 (Negative)	0 (Negative)
Total*	**0/7**	**0/7**	**0/7**	**0/7**

*ND, not done;. *: the total number of individuals that express the antigen at moderate and high levels are shown.*

Supplementary data

Table A1. Antibodies used for ICC

Antibody specificity	Clone name	Species	Isotype	Catalogue number	Manufacturer	Working dilution
Primary antibodies						
Actin	AC-15	Mouse anti-human	Monoclonal, IgG_1	sc-69879	Santa Cruz Biotechnology; Santa Cruz, CA (USA)	1/100
BMX	Y396	Rabbit anti-human	Monoclonal, IgG	ab32153	Abcam	1/100
DCTPP1	A10058	Rabbit anti-human	Polyclonal, IgG	HPA002832	Sigma-Aldrich	1/100
Survivin	EP2880Y	Rabbit anti-human	Monoclonal, IgG	ab76424	Abcam	1/100
VGLL-4	N/A	Rabbit anti-human	Polyclonal, IgG	ab243518	Abcam	1/100
Isotype control for actin	MOPC-21	Mouse anti-human	Monoclonal, IgG_1	ab18443	Abcam	1/250
Isotype control for BMX, DCTPP1, Survivin, VGLL4	SP137	Rabbit anti-human	Monoclonal, IgG_1	ab125938	Abcam	1/250
Secondary antibodies*						
Anti-mouse		Anti-mouse	Polyclonal, Ig	K4007	Dako	N/A
Anti-rabbit		Anti-rabbit	Polyclonal, Ig	K4011	Dako	N/A

***Supplied as part of the EnVision kit

Table A2 Analysis of the antigens that were differentially recognised by patient compared with HV sera identified antigens that were (i) more frequently recognised and (ii) less frequently recognised by sera from B-ALL patients compared with age and sex-matched HVs.

(i)

Gene Symbol	Protein Name	HV vs. ALL		
		p-value	Mean Ratio	Fold Change
GCC1	GRIP and coiled-coil domain-containing protein 1	0.016	0.57	-1.76
VGLL4	vestigial like 4 (Drosophila)	0.020	0.36	-2.76
ACOX1	acyl-Coenzyme A oxidase 1, palmitoyl (ACOX1)	0.022	0.69	-1.45
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	0.022	0.58	-1.73
TOX2	TOX high mobility group box family member 2, transcript variant 3	0.024	0.69	-1.45
MUC20	Mucin-20	0.025	0.43	-2.32
WARS	Tryptophanyl-tRNA synthetase, cytoplasmic	0.025	0.49	-2.03
DCTPP1	XTP3-transactivated protein A	0.027	0.58	-1.73
GAK	cyclin G associated kinase	0.030	0.47	-2.11
SEPT9	septin 9	0.031	0.55	-1.81
CDC42EP1	Cdc42 effector protein 1	0.033	0.57	-1.77
BMX	BMX non-receptor tyrosine kinase, transcript variant 2	0.035	0.65	-1.55
IGLL1	immunoglobulin lambda-like polypeptide 1, transcript variant 1	0.039	0.73	-1.36
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	0.044	0.74	-1.35
LMX1A	LIM homeobox transcription factor 1, alpha	0.046	0.39	-2.55
CDCA3	cell division cycle associated 3	0.046	0.55	-1.80
POLR3K	polymerase (RNA) III (DNA directed) polypeptide K, 12.3 kDa	0.047	0.67	-1.49
RAB3IL1	RAB3A interacting protein (rabin3)-like 1	0.050	0.69	-1.46

(ii)

Gene Symbol	Protein Name	(HV vs. ALL)		
		p-value	Mean Ratio	Fold Change
KIAA0020	Pumilio domain-containing protein KIAA0020	0.0003	8.65	8.65
CTTN	cortactin, transcript variant 2	0.003	2.31	2.31
MTA1	Metastasis-associated protein	0.003	1.76	1.76
RTN4IP1	Reticulon-4-interacting protein 1	0.005	1.66	1.66
BRSK2	BR serine/threonine kinase 2	0.005	3.01	3.01
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	0.007	1.82	1.82
FCHSD1	FCH and double SH3 domains 1	0.008	1.46	1.46
DUSP22	dual specificity phosphatase 22	0.009	2.16	2.16
NR4A1	nuclear receptor subfamily 4, group A, member 1, transcript variant 1	0.011	1.64	1.64
RASL11B	RAS-like, family 11, member B	0.013	1.82	1.82
CENTD2	centaurin, delta 2	0.014	1.58	1.58
DBR1	Lariat debranching enzyme	0.015	2.64	2.64
CCDC69	coiled-coil domain containing 69	0.015	2.16	2.16
SELK	Selenoprotein K	0.015	1.58	1.58
UTP18	U3 small nucleolar RNA-associated protein 18 homolog	0.016	1.57	1.57
C20orf18	RanBP-type and C3HC4-type zinc finger-containing protein 1	0.016	2.10	2.10
SCG3	secretogranin III	0.016	2.10	2.10
MGC50811	Uncharacterized protein C2orf62	0.017	2.04	2.04
CDK7	cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)	0.018	1.42	1.42
SCYE1	small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	0.020	1.42	1.42
FLJ46266	FLJ46266 protein, mRNA.	0.020	1.68	1.68
H2AFY2	H2A histone family, member Y2	0.021	1.54	1.54
RGS10	regulator of G-protein signaling 10, transcript variant 1	0.022	2.30	2.30
CPEB4	Cytoplasmic polyadenylation element-binding protein 4	0.022	1.52	1.52
CHCHD2	coiled-coil-helix-coiled-coil-helix domain containing 2	0.025	2.02	2.02
SF1	Splicing factor 1	0.026	1.31	1.31

C17orf44	chromosome 17 open reading frame 44	0.026	1.43	1.43
ODF3L2	Outer dense fiber protein 3-like protein 2	0.027	1.44	1.44
WWOX	WW domain containing oxidoreductase, transcript variant 3	0.028	1.72	1.72
HTF9C	HpaII tiny fragments locus 9c protein	0.033	1.71	1.71
VSTM2	V-set and transmembrane domain containing 2A	0.034	2.26	2.26
MGC31957	hypothetical protein MGC31957	0.038	1.96	1.96
TMEM136	transmembrane protein 136	0.038	1.37	1.37
PLK2	Serine/threonine-protein kinase	0.038	1.46	1.46
MIER1	mesoderm induction early response 1 homolog (Xenopus laevis)	0.039	1.79	1.79
SUV420H1	suppressor of variegation 4-20 homolog 1 (Drosophila)	0.040	1.28	1.28
CENTG2	centaurin, gamma 2	0.040	3.46	3.46
BTN1A1	Butyrophilin subfamily 1 member A1	0.041	1.84	1.84
IMP3	IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast)	0.041	1.43	1.43
CCDC82	coiled-coil domain containing 82	0.042	1.56	1.57
RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4	0.044	1.55	1.55
ZMYND11	zinc finger, MYND domain containing 11	0.044	1.30	1.30
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3	0.044	1.56	1.56
C8orf53	chromosome 8 open reading frame 53	0.045	1.40	1.40
ARID3B	AT rich interactive domain 3B (BRIGHT-like)	0.046	2.25	2.25
SULF2	sulfatase 2	0.046	1.33	1.34
LRRC1	leucine rich repeat containing 1	0.049	2.09	2.09
SMYD2	SET and MYND domain-containing protein 2	0.049	3.43	3.43
CDC7	Cell division cycle 7-related protein kinase	0.049	1.39	1.39

Table A3 ANOVA analysis of proteins identified on proto-array using published gene expression microarray dataset. Each protein was subjected to further analysis of its expression in B-ALL compared with healthy pre-B bone marrow cells using microarray data from GSE38403.(Geng et al., 2012) The tables show associations between antigen expression and (A) disease stage or (B) cytogenetic abnormalities. Cells highlighted are those that have a p value of <0.05. Values in parenthesis indicate whether the relationship between gene expression and the feature is significant (p value of <0.05) and positive (1) highlighted orange or negative (-1) and highlighted green.

(A)

Gene Symbol	Stage	CD56+-B-ALL vs. Early-Pre-B	CD56+-B-ALL vs. Mature-B-ALL	CD56+-B-ALL vs. Pre-B-ALL	CD56+-B-ALL vs. Pro-B	CD56+-B-ALL vs. Transitional-Pre-B	Early-Pre-B vs. Mature-B-ALL	Early-Pre-B vs. Pre-B-ALL	Early-Pre-B vs. Pro-B	Early-Pre-B vs. Transitional-Pre-B	Mature-B-ALL vs. Pre-B-ALL	Mature-B-ALL vs. Pro-B	Mature-B-ALL vs. Transitional-Pre-B	Pre-B-ALL vs. Pro-B	Pre-B-ALL vs. Transitional-Pre-B	Pro-B vs. Transitional-Pre-B
ACOX1	0.06	0.03 (-1)	0.42 (-1)	0.10 (-1)	0.14 (-1)	0.96 (-1)	0.29 (1)	0.43 (1)	0.19 (1)	0.03 (1)	0.53 (-1)	0.67 (-1)	0.44 (1)	0.73 (1)	0.11 (1)	0.15 (1)
APOBEC3A	2.00E-03	0.13 (1)	0.36 (-1)	0.64 (1)	0.05 (1)	0.70 (-1)	0.01 (-1)	0.04 (-1)	0.21 (1)	0.04 (-1)	0.10 (1)	1.86E-03 (1)	0.60 (1)	0.01 (1)	0.34 (-1)	0.01 (-1)
BMX	0.64	0.91 (1)	0 .42 (1)	0.41 (1)	0.88 (1)	0.68 (1)	0.32 (1)	0.12 (1)	0.92 (1)	0.65 (1)	0.83 (-1)	0.37 (-1)	0.69 (-1)	0.24 (-1)	0.77 (-1)	0.71 (1)
CDC42EP1	0.11	0.02 (1)	0.19 (1)	0.03 (1)	0.14 (1)	0.48 (1)	0.64 (-1)	0.78 (1)	0.13 (-1)	0.19 (-1)	0.58 (1)	0.82 (-1)	0.53 (-1)	0.17 (-1)	0.18 (-1)	0.56 (-1)
CDCA3	0.41	0.18 (1)	0.99 (1)	0.45 (1)	0.49 (1)	0.53 (1)	0.19 (-1)	0.31 (-1)	0.20 (-1)	0.64 (-1)	0.45 (1)	0.49 (1)	0.54 (1)	0.89 (-1)	0.97 (1)	0.91 (1)
DCTPP1	3.84E-03	0.39 (-1)	0.04 (-1)	0.16 (-1)	0.02 (-1)	0.50 (-1)	0.05 (-1)	0.20 (-1)	3.66E-04 (-1)	0.94 (-1)	0.22 (1)	0.76 (1)	0.17 (1)	0.10 (-1)	0.60 (1)	0.14 (1)
GAK	0.55	0.26 (-1)	0.38 (-1)	0.72 (-1)	0.43 (-1)	0.91 (-1)	0.92 (-1)	0.14 (1)	0.54 (1)	0.33 (1)	0.44 (1)	0.72 (1)	0.44 (1)	0.47 (-1)	0.82 (1)	0.52 (1)
GCC1	0.16	0.22 (-1)	0.34 (-1)	0.06 (-1)	0.52 (-1)	0.93 (-1)	0.91 (-1)	0.09 (-1)	0.24 (1)	0.27 (1)	0.49 (-1)	0.54 (1)	0.38 (1)	0.03 (1)	0.07 (1)	0.60 (1)
IGLL1	0.20	0.46 (-1)	0.78 (-1)	0.16 (-1)	0.97 (1)	0.35 (-1)	0.72 (1)	0.13 (-1)	0.09 (1)	0.59 (-1)	0.30 (-1)	0.70 (1)	0.51 (-1)	0.01 (1)	0.84 (1)	0.22 (-1)
LMX1A	0.17	0.65 (1)	0.21 (1)	0.30 (1)	0.19 (1)	0.23 (1)	0.20 (1)	0.18 (1)	0.04 (1)	0.23 (1)	0.57 (-1)	0.76 (-1)	0.96 (-1)	0.64 (1)	0.62 (1)	0.81 (1)
LMX1A†	0.90	0.33 (1)	0.48 (1)	0.47 (1)	0.49 (1)	0.86 (1)	0.99 (1)	0.71 (-1)	0.61 (-1)	0.47 (-1)	0.86 (-1)	0.82 (-1)	0.59 (-1)	0.93 (-1)	0.62 (-1)	0.65 (-1)
MUC20	1.47E-04	0.62 (1)	0.05 (1)	0.41 (1)	0.01 (1)	0.34 (1)	0.02 (1)	0.44 (1)	5.89E-06 (1)	0.42 (1)	0.08 (-1)	0.90 (-1)	0.29 (-1)	4.91E-03 (1)	0.69 (1)	0.22 (-1)
NAT1	0.01	0.13 (-1)	0.02 (-1)	0.03 (-1)	0.56 (-1)	0.94 (-1)	0.09 (-1)	0.10 (-1)	0.05 (1)	0.16 (1)	0.42 (1)	0.02 (1)	0.03 (1)	0.01 (1)	0.04 (1)	0.63 (1)
SEPT9	1.69E-03	0.30 (-1)	0.87 (-1)	0.64 (-1)	0.41 (1)	0.57 (1)	0.42 (1)	0.28 (1)	4.80E-05 (1)	0.07 (1)	0.80 (-1)	0.30 (1)	0.46 (1)	0.03 (1)	0.23 (1)	0.94 (-1)
RAB3IL1	0.67	0.49 (1)	0.55 (1)	0.23 (1)	0.44 (1)	0.87 (-1)	0.88 (1)	0.22 (1)	0.78 (1)	0.36 (-1)	0.67 (1)	0.98 (-1)	0.44 (-1)	0.43 (-1)	0.16 (-1)	0.33 (-1)
TOX2	1.29E-03	0.42 (1)	0.26 (-1)	0.70 (-1)	0.53 (-1)	0.38 (-1)	0.02 (-1)	0.01 (-1)	1.41E-03 (-1)	0.04 (-1)	0.29 (1)	0.41 (1)	0.81 (1)	0.68 (-1)	0.46 (-1)	0.61 (-1)
VGLL4	0.01	0.58 (-1)	0.18 (1)	0.80 (-1)	0.72 (-1)	0.07 (1)	0.02 (-1)	0.57 (1)	0.70 (1)	2.73E-03 (1)	0.05 (-1)	0.04 (-1)	0.65 (1)	0.87 (-1)	0.01 (1)	0.01 (1)
WARS	3.02E-03	0.65 (-1)	0.02 (-1)	0.14 (-1)	0.19 (-1)	0.09 (-1)	3.69E-03 (-1)	0.02 (-1)	0.04 (-1)	0.05 (-1)	0.10 (1)	0.06 (1)	0.47 (1)	0.74 (1)	0.47 (-1)	0.36 (-1)

(B)

Gene Symbol	Gender (M vs. F)	MLL_X (vs. 1)	BCR-ABL (vs. 1)	TEL-AML1 (vs. 1)	Other cytogenetic abnormalities (vs. 1)	Hyperdiploidy (neg vs. pos)	No abnormalities (vs. 1)	CD25 pos/neg (neg vs. pos)
ACOX1	0.56 (-1)	0.16 (1)	0.07 (-1)	0.56 (1)	0.007 (1)	0.54 (-1)	0.75 (-1)	0.4 (-1)
APOBEC3A	0.31 (1)	0.01 (1)	0.012 (-1)	0.24 (-1)	0.37 (-1)	0.57 (1)	0.2 (1)	0.5 (-1)
BMX	0.001 (-1)	0.83 (-1)	0.31 (-1)	0.45 (1)	0.11 (-1)	0.36 (-1)	0.27 (1)	0.14 (1)
CDC42EP1	0.51 (1)	0.04 (-1)	0.32 (1)	0.71 (1)	0.11 (-1)	0.65 (1)	0.41 (1)	0.13 (1)
CDCA3	0.61 (-1)	0.51 (-1)	0.14 (1)	0.53 (-1)	0.008 (-1)	0.41 (1)	0.95 (1)	3.73E-05 (1)
DCTPP1	0.09 (-1)	9.39E-07 (-1)	5.03E-05 (1)	0.39 (1)	0.89 (-1)	0.55 (1)	0.45 (-1)	0.47 (1)
GAK	0.87 (-1)	0.39 (-1)	0.023 (1)	0.32 (1)	0.09 (1)	0.23 (-1)	0.044 (-1)	0.18 (1)
GCC1	0.98 (-1)	0.38 (1)	0.097 (1)	0.12 (-1)	0.18 (-1)	0.17 (-1)	0.1 (-1)	0.0024 (1)
IGLL1	0.03 (1)	0.09 (1)	1.17E-05 (1)	0.59 (-1)	0.024 (-1)	0.049 (1)	7.76E-07 (-1)	1.93E-05 (-1)
LMX1A	0.07 (1)	0.01 (1)	2.33E-08 (-1)	0.26 (-1)	0.0005 (-1)	0.1 (1)	0.002 (1)	0.03 (1)
LMX1A†	0.49 (1)	0.69 (-1)	0.06 (1)	0.53 (1)	0.73 (1)	0.54 (1)	0.43 (-1)	0.1 (-1)
MUC20	0.56 (-1)	0.0001 (1)	0.09 (-1)	0.84 (-1)	0.04 (-1)	0.28 (1)	0.66 (-1)	0.65 (-1)
NAT1	0.91 (1)	0.11 (1)	0.1 (-1)	0.51 (-1)	0.7 (-1)	0.56 (-1)	0.62 (1)	0.25 (1)
RAB3IL1	0.34 (1)	0.1 (1)	0.9 (1)	0.24 (-1)	0.31 (-1)	0.86 (-1)	0.03 (-1)	0.95 (-1)
SEPT9	0.53 (1)	0.02 (1)	0.93 (-1)	0.84 (1)	0.43 (1)	0.03 (-1)	0.08 (-1)	0.4 (-1)
TOX2	0.9 (-1)	0.0006 (-1)	1.01E-13 (1)	0.29 (-1)	0.07 (-1)	0.7 (-1)	9.54E-05 (-1)	6.07E-14 (1)
VGLL4	0.43 (1)	0.49 (-1)	0.004294 (-1)	0.13 (-1)	0.02 (1)	0.34 (1)	0.01 (1)	0.018 (-1)
WARS	0.93 (1)	0.41 (-1)	0.09 (1)	0.049 (1)	0.5 (1)	0.97 (1)	0.08 (-1)	0.42 (1)

†Transcript variant 2