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Posted Date: 17 February 2025

doi: 10.20944/preprints202502.1306.v1

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Article

Adult B-Cell Acute Lymphoblastic Leukaemia Antigens and Enriched Pathways Identify New Targets for Therapy

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Simple Summary: Adult acute lymphoblastic leukaemia is a cancer of the immature white blood cells that reside in the bone marrow. It mostly affects children, with a peak incidence at 0-4 years but 4 in 10 people affected by this type of leukaemia are adults. For adults with acute lymphoblastic leukaemia, the 5-year survival rates for those over 40 years of age is only 25% and this disease desperately needs some new treatments to help improve the chances of survival. To this end we have used a number of different methods to try to identify which proteins are altered in adult acute lymphoblastic leukaemia and which of the normal cell behaviours are affected by the disease process. Our study identified a number of proteins and cell behaviours that are affected in acute lymphoblastic leukaemia and these are worthy of further investigation as targets for new treatment strategies.

Abstract: Background: Adult B-cell acute lymphoblastic leukaemia (aB-ALL) is characterised by abnormal differentiation and proliferation of lymphoid progenitors. Despite a significant improvement in relapse-free and overall survival for children with B-ALL, aB-ALL has a particularly poor prognosis with a 5-year survival rate of 20%. First remission is achieved for most patients but relapse is common with a high associated mortality. New treatments such as immunotherapy offer an opportunity to extend remission and prevent relapse. **Methods:** aB-ALL antigens were identified using different sources - immunoscreening, protoarrays, two microarrays and one cancer-testis antigen (CTA) database, and a review of the genomic analyses of aB-ALL. 385 aB-ALL-associated gene products were examined for their association with patient survival. **Results:** We identified 87 transcripts with differential expression between aB-ALL and healthy donors, and 42 that were associated with survival. Enrichment analysis showed that the Transforming Growth Factor- β (TGF β), Wnt and hippo pathways were highly represented ($p < 0.02$). We found that SOX4 was upregulated in all types of B-ALL, while ROCK1 was upregulated in all types of B-ALL ($p < 0.001$ except t(8;14)) and along with SMAD3 and TEAD4 were associated with survival ($p = 0.0008$, 0.05 and 0.001 respectively). Expression of each aB-ALL antigen was verified by qPCR but only TEAD4 showed significant transcript upregulation in aB-ALL compared to healthy donor CD19⁺ cells ($p = 0.01$). **Conclusions:** We have identified a number of antigens and their pathways that play key roles in aB-ALL and may act as useful targets for future immunotherapy strategies.

Keywords: adult B-cell acute lymphocytic leukaemia; antigen discovery; cancer vaccines; SMAD3; TEAD4; immunotherapy

1. Introduction

Adult B-cell acute lymphoblastic leukaemia (aB-ALL) occurs due to an impairment in B-cell differentiation leading to an accumulation of blast cells in the bone marrow. ALL has a bimodal distribution in terms of age, with peak incidences in children aged 2-5 years, and in adults (those aged 18 and over) when they over 40 years of age. Adults over 60 account for 20% of ALL cases and 50% of ALL-related deaths [1]. Although early induction treatment can result in first remission for 80-90% of B-ALL patients, long-term survival is hampered by resistance, relapse and extramedullary infiltration [2]. For this reason, the monitoring of minimal residual disease has been used to determine prognosis, best practise treatment options and risk of relapse for patients [3].

First line treatment for aB-ALL involves pre-phase and induction therapy followed by consolidation and maintenance. Although 80-90% aB-ALL patients achieve first remission more than half relapse. In recent years a number of immunotherapy strategies including antibody and chimeric antigen receptor (CAR) T-cell therapies have been used to break immunological tolerance and changed treatment outcomes for patients with relapsed/refractory (R/R) ALL following chemotherapy and haematopoietic stem cell transplant. Blinatumomab, a bispecific T cell engager that directs CTL to CD19 expressing cells, inotuzumab ozogamicin, an anti-CD22 antibody conjugated to calicheamicin, and tisagenecel, a CAR T cell therapy have all achieved improved outcomes for patients with R/R ALL when compared to standard therapies [4–6]. Despite its efficacy, CAR-T cell therapy is associated with adverse effects including B-cell aplasia, increased infection susceptibility due to the impairment of antibody production, and more serious events such as cytokine release syndrome leading to neurotoxicity and multiple organ dysfunction [7]. In addition, the time-consuming process of CAR-T cell engineering, antigenic variability due to ALL being a heterogeneous disease and trying to develop off-the-shelf therapies represent a real challenge [8].

Recently the paradigm has shifted to immune-checkpoint inhibitors, although these can lead to excessive immune stimulation [9]. To address this issue, "armoured" chimeric antigen receptors (CARs) that secrete PD-1-blocking cytokines or co-express dominant-negative transforming growth factor-B receptor type II to create a proinflammatory environment [10] were utilised. However, long-term use of immunotherapy can lead to the selection of leukaemic clones that are resistant to treatment. This evolutionary process hampers the effectiveness of immunotherapy by selecting subclones that are unresponsive to current therapy, resulting in escape variants and relapse. This phenomenon has been observed in patients with the *KMT2A* rearrangement who were treated with CAR-T cells and CD19 targeting-immunotherapy for B-ALL, which eventually became ineffective due to the presence of an expanding population of CD19 negative clones [11].

In addition to the immunotherapy targets supra vide, the identification of antigens associated with survival in leukaemia, especially for subtypes associated with poor prognosis and phenotypic plasticity, remains an attractive approach for B-ALL treatment. Sero-profiling of B-ALL compared to age- and sex-matched healthy donor samples revealed three differentially recognised tumour antigens, bone marrow tyrosine kinase (BMX), DCTPP1, and VGLL4 [12]. BMX is found on chromosome Xp22.2, and expressed in bone marrow cells. It belongs to the Tec kinase family which is a non-receptor tyrosine kinase involved in many signal transduction processes such as PI3K and TNF pathways. In addition to its expression in bone marrow cells, BMX is upregulated in prostate cancer and non-malignant conditions such as wound healing where it causes the proliferation of skin keratinocytes [13]. Targeting of BMX has been achieved using the epidermal growth factor receptor inhibitor, BMX-IN-1, and CTN06, which are small molecule inhibitors of both BMX and BTK, suppressing tumour growth and migration via the induction of autophagy and apoptosis. Ibrutinib and zanubrutinib are BTK inhibitors, the latter with improved specificity for BTK, and both of which have been used in clinical trials as treatments for mature B-malignancies such as R/R chronic lymphoblastic leukaemia and small lymphocytic lymphoma [14]. Ibrutinib was found to cause a higher than expected level of adverse events and premature discontinuation in clinical trials [14] while zanubrutinib has been found to have a more acceptable safety profile with improved response rates and progression-free survival [15,16].

Boullosa et al. [17] found that baculoviral IAP repeat containing 5 (*BIRC5*)/Survivin was expressed in aB-ALL but not healthy donors ($p= 0.015$). This anti-apoptotic protein is found at low levels in terminally differentiated healthy tissues but is upregulated in many cancers such as lung and breast cancer due to the high expression of oncogenes (JAK/STAT, Akt/PI3K and TCF- β -catenin pathways) and loss of tumour suppressor genes (P53 pathway) promoting tumour proliferation and survival [18]. *BIRC5* overexpression is also associated with chemotherapy resistance and tumour aggression while *BIRC5* knockdown results in the induction of apoptosis of leukaemia cells and increased chemosensitivity in-vitro [19]. *BIRC5* and BMX are newly identified TAAs in B-ALL [12,17] and offer an opportunity for existing therapies to be repurposed to aB-ALL.

Due to the existence of immune escape variants, the heterogeneity between and within aB-ALL tumour cells, and the limitations with existing immunotherapy treatments (generating a lasting anti-tumour immune response), it is essential to broaden the number, specificity and sensitivity of TA targets available for future clinical use [20]. The search for leukaemia associated and ideally cancer-restricted antigens, that are effective targets to stimulate the immune destruction of aB-ALL cells, may help in the development of new treatments that boost anti-leukaemia immune responses, while retaining the specificity needed to minimise off-target effects. To maximise the identification of new targets for treatment, and enable the repurposing of already approved drugs for B-ALL patients, aB-ALL TAAs were identified from six sources - immunoscreening, protoarrays, two microarrays and one cancer testis-antigen (CTA) database, and a review of the genomic analyses of aB-ALL, and their relationship with patient survival was determined.

2. Materials and Methods

2.1. Patient Samples

This study received local ethical approval (REC Reference: 07/H0606/88; LREC 228/02/T) and samples were collected (Tables 1 and 2) following informed consent. On the day of sample receipt, serum was collected from clotted PB samples following centrifugation at 1200xg for 10 min. Bone marrow (BM) and peripheral blood (PB) samples collected in K₂-EDTA tubes, were incubated with 5 volumes of red cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 30 min at room temperature. Leukocytes were isolated by centrifugation for 10 min at 800xg. Healthy donors were self-selecting individuals who responded to a local request for samples. They had no signs of illness and were not undergoing any investigations by a health care professional. CD19⁺ B-cells were used as a control as pre-normal B cells could not be sourced. CD19⁺ is expressed on most B-ALL cells, normal pre-B and B-cells up until maturation to plasma cells and were purchased from Cambridge Bioscience (Cambridge, UK) after isolation via negative immunomagnetic separation from leukophoresate. The donor was a female aged 18-66 and cells were shipped on dry ice. Isolated leukocytes were resuspended at 5 x 10⁶/ml in PBS and 5ul spotted onto two sites on each microscope slide. Once dried for 4-16h, slides were saran wrapped and stored at -20°C for future immunocytochemistry (ICC) analysis. Remaining leukocytes, were pelleted and along with sera samples were immediately stored at -80°C for later use. K562 (ATCC) and A549 (Sigma-Aldrich Co. Ltd) were cultured in RPMI or DMEM, respectively, 10% foetal calf serum, 1% L-Glutamine and 1% penicillin streptomycin (all Sigma-Aldrich Co Ltd) in a 5% CO₂ in a humidified chamber. K562 cells were used as a positive control for the expression of all antigens of interest in this study except YAP1, while A549 was used as a positive control for YAP1 expression.

2.2. Antigen Identification

Genes and antigens were identified from six sources as follows:-

(i) Serological analysis of recombinant cDNA expression libraries (SEREX)

The testis cDNA expression library was made using the ZAP Express® cDNA synthesis and ZAP Express® cDNA Gigapack® III Gold cloning kit (Stratagene Europe) as described previously [21]. SEREX was performed following an optimised protocol [22] based on the original method [23]. The reactivity of serum was confirmed through agglutination tests and >10⁶ plaques in the testes cDNA library were immunoscreened using pre-cleared serum from five representative aALL patients (ALL004;005;010;017;018; Table 1). cDNA in plaques that were reactive in two independent rounds of immunoscreening were amplified using T3 and T7 primers and Ready Mix Taq PCR Reaction Mix with MgCl₂, (Sigma Aldrich, UK). PCR products were gel purified (QIAQuick gel extraction kit, Qiagen Ltd., Lancashire, UK), Sanger sequenced (Department of Biochemistry, University of Cambridge) and analysed using Applied Biosystems' Sequencing Analysis Software. Sequences were compared to expressed sequence tag (EST) and protein databases, including the National Centre for Biotechnology Information (NCBI) BLAST and these aALL antigens were added to the pool for survival and pathways analysis.

(ii) & (iii) Differentially expressed genes (DEGs) identified through the analysis of two microarray databases

DEGs in aB-ALL were identified using (i) the GSE38403 [25] and (ii) the Microarray Innovations in leukaemia (MILE; GSE13204) microarray datasets via BloodSpot.eu. 649 aB-ALL samples and 74 HVs were utilised to compare gene transcription levels in ALL patient subgroups. Patient samples were collected from eighteen diagnostic gold standard categories. The read count was downloaded from GEO and gene expression was calculated by the Fragments per Kilobase per Million Mapped Fragments (FPKM) method. Data was normalised using the limma method and DEGs were identified (fold change ≥ 1.5 for upregulated genes and ≤ 1.5 for downregulated one) by the DESeq2 method which is part of the 'Bioconductor' package. The adjusted p-value (qp) and p-value ≤ 0.05 were calculated using the Fisher's test [24].

The MILE study includes a limited number of samples from childhood B-ALL (cB-ALL) for C3 (c-ALL/pre-B-ALL with t(9;22)) and C8 (c-ALL/pre-B-ALL without t(9;22)) which is known as Philadelphia (Ph) and Philadelphia-like ALL (Ph-like ALL) cytogenetics subclasses [27] as well as a pre-ponderance of aB-ALL.

Table 1. Patient information.

ID	Disease stage	Cytogenetics	WCC (10 ⁹ /L)	BM blast %	Relapse	Survival post-sample (mo) [‡]	Age*	Sex	Sample type
ALL001	Diagnosis Ph+ ALL	Ph+ ALL: t(9;22)	4.9	40	NK	NK	39	M	PB
ALL002	Diagnosis T-ALL	46,XY,t(1;7)(p36;p15)	232	88	No	Alive (post allo)	19	M	PB
ALL003	Diagnosis B-ALL	t(1;19)	28.6	91	No	Alive	26	F	PB
ALL004*	Diagnosis T-ALL	Complex karyotype	591	NK	No	Died 19mo (post allo)	19	M	PB
ALL005*	‡Post-allo T-ALL	No result	NK	NK	No	Died 3.5mo (post allo)	46	M	PB
ALL007	Diagnosis Pre B-ALL	Loss of one copy of ETV6 (12p13) and gain of one copy of ABL1 (9q34) by FISH	8.2	92	No	Alive	24	M	PB
ALL008	Diagnosis Pre-B-ALL	46XY, 5,del(5)(q15q33),dic(9;16)(p11;q11),del(13)(q12q14)	10.4	72	No	Alive	19	M	PB
ALL009	Diagnosis Pre-B-ALL	46,XY,t(1;7)(q25;q3?5),add(3)(p1?3)	48.1	89.6	Yes	Died 94mo (post allo, CART)	19	M	BM
ALL010*	Diagnosis Pre-B-ALL	Complex including t(4;11)	229.1	85	Yes	Died 3mo	64	M	PB
ALL011	Diagnosis Pre-B-ALL	No result – external referral	NK	NK	NK	NK	19	F	PB
ALL012	Diagnosis Pre-B-ALL	t(11;14)(q24;q32)	4.3	83	No	Alive (post allo)	33	M	BM
ALL014	Diagnosis Pre-B-ALL	47,XY,+2,add(2)(p1)[3]/46,XY[47].nucish (CRLF2)x2[100]	4.9	53	Yes	Alive (post allo & CART)	56	M	BM
ALL015	Diagnosis Pre-B-ALL	Gain of one copy of CRLF2 (Xp22.3/Yp11.3) and loss of one copy of CSFR1 (5q32) and EBF1 (5q33.3) detected by FISH	68.0	94	No	Alive	20	F	PB
ALL016	Diagnosis Pre-B-ALL	Hyperdiploid; 56-57 XX +X,+4,+6,+9,+10,+14,+17,+18,+21,+marker	1.9	96	No	Alive	27	F	BM
ALL017 [‡]	Diagnosis, Pre-B-ALL	No cytogenetics available, no FISH	3.0	96	No	Alive	19	F	PB
ALL018 [‡]	Diagnosis, T-ALL	Normal cytogenetics, SET/CAN fusion detected by FISH	58.8	74	No	Alive	22	M	PB
ALL020	Diagnosis Pre-B-ALL	46,XY, t(1;7)(q25;q3?5), add(3)(p1?3) TCF3 ex16-PBX1 ex3 fusion transcript detected	31.9	92	Yes	Died 34.5mo (Post allo, relapse, salvage chemo then CART & relapse)	56	F	PB/BM

* - age at time of sampling; ‡ - status on 30 Oct 2024; Allo: allogeneic stem cell transplant; NK: not known; * - samples used in immunoscreening, qPCR and ICC; †: serum samples used for immunoscreening only.

Table 2. Healthy volunteer demographics.

HV control	Age [#]	Sex	Sample type
HV008	40	F	PB
HV010	22	M	PB
HV012	46	F	PB
HV021	34	M	PB
HV043	NK	M	PB
CD19 ⁺ cells	18-66	F	PB

BM: bone marrow; HV: healthy volunteer; NK; not known; PB: peripheral blood.

(iv) A review of genomic studies

We also analysed all of the genes identified by Iacobucci and Mullighan's [28] review of genomic studies. Genes with a corrected Spearman's correlation false detection rate (FDR) corrected to $p > 0.05$ were filtered out and the remaining genes were arranged in accordance with decreasing Spearman's value, thus creating a ranked correlation file. These aB-ALL antigens were added to the pool of genes for survival and pathways analysis.

(v) Proto-array analysis

18 antigens had previously been identified using protoarrays, by virtue of their preferential recognition by antibodies in patients but not healthy donor sera [12] and these were added to the pool of antigens for further analysis.

(vi) the CTA database

We were particularly interested in CTAs because of their cancer-restricted expression (recently reviewed in [29]). We identified 78 antigens identified in the CTA database [30,31] and these were added to the pool of aB-ALL antigens for survival and pathways analysis.

2.3. Pathway Analysis and Survival

The molecular pathways utilised by the antigens were determined using Enrichr [33,34] and the core genes in these selected pathways were examined for their protein-protein interaction networks using the Search Tool for the Retrieval of Interacting Genes (STRING) analytical program [35]. We determined which antigens, when transcribed at above and below median levels were associated with aB-ALL patient survival using the MILE dataset accessed via bloodspot.eu [32]. This identified gene expression data from 649 B-ALL patients. We then priority-ranked the list of cancer antigens based on predefined and preweighted objective criteria following the analytical hierarchy process developed by a panel of 80 experts and described by Cheever et al [36].

2.4. qPCR

RNA extraction was performed on 11 PB and 5 BM samples from 15 aALL patients and 6 PB samples from healthy donors, including one sample of CD19⁺ purified cells, using the RNeasy Mini Kit® (Qiagen) and following the manufacturer's guidelines. The genomic DNA elimination mix was prepared in a RNase and DNase free PCR tube according to the manufacturer's instructions (MBI Fermentas). Quantitative PCR (qPCR) was performed using the RT² SYBR® Green Master-mix, RT² qPCR Primer Assay and cDNA synthesis reaction (all Qiagen) on a 96-well qPCR plate (Applied Biosystems, USA). TATA-box binding protein (*TBP1*) and protein kinase cGMP-dependent 1 (*PRKG1*) were used as reference genes following MIQE guidelines [37]. Primers from Qiagen were used to detect each transcript: SRY-Box Transcription Factor 4 (*SOX4*; PPH01950A), Rho associated

coiled-coil containing protein kinase 1 (*ROCK1*; PPH01966C), Yes-associated protein (*YAP1*; PPH13459A), TEA Domain Transcription Factor 4 (*TEAD4*; PPH10558A-200), SMAD family member 3 (*SMAD3*; PPH01921C), and T cell receptor 4 (*TCF4*; PPH02770A) and *BIRC5* (PH00271E) and at least one human cancer cell line that was known to express the gene of interest, based on previously published studies [38,39] was used as a positive control. Every sample was analysed for expression of each gene in triplicate. 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 H₂O. Otherwise the reaction volumes were 10 μ L 2X QuantiNova SYBR green PCR Mix, 2 μ L QN ROX reference dye, 2 μ L QuantiTect primer assay and 5 μ L RNase-free H₂O, making a total volume of 19 μ L added to each well in the 96-well plate. In 1 μ L final volume, \leq 100 ng of template cDNA was added to achieve a final volume of 20 μ L. 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 collected using StepOne software v2.0 (Applied Biosystems) and the results normalised using the *TBP1* and *PRKG1* housekeeping genes (Δ CT = CT genes of interest – CT housekeeping genes). The $2^{-\Delta\Delta$ CT method [40] log transforms the $\Delta\Delta$ CT (where $\Delta\Delta$ CT = Δ CT patient sample – Δ CT healthy donor) to determine the relative expression of the seven genes of interest (*BIRC5*, *ROCK1*, *SMAD3*, *SOX4*, *TCF4*, *TEAD4* and *YAP1*).

2.5. Immunocytochemistry

Immunocytochemistry was performed as described previously [41] using monoclonal rabbit anti-human antibodies against *TEAD4* (1D10) and *SMAD3* (2C12) (ThermoFisher Scientific, Leicestershire, UK)(Table S1). Mouse anti-human monoclonal Actin antibody (*ACTN05* (C4), Fisher Scientific) was used as a positive control for immunolabelling, *BIRC5* as a comparator [17], while isotype controls (mouse or rabbit anti-human IgG antibodies) were used to determine whether there was any non-specific antibody binding. Secondary antibodies were provided as part of the EnVision+ System/HRP, Rabbit(DAB+) kit (DAKO, USA). Lillies-Mayer Haematoxylin was used as a counterstain. Staining intensities were scored according to a five-tiered scale described originally by [42] 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 scored over \geq 5 microscopic views by the value for staining intensity within the same [43].

3. Results

3.1. Immunoscreening Identified 72 aB-ALL Associated Antigens

Primary immunoscreening of the testes cDNA library with pre-cleared aB-ALL patient sera identified 310 sero-positive plaques. In total 134 phage colonies were confirmed as expressing immunogenic polypeptides through a second independent round of immunoscreening. PCR amplification of cDNA inserts identified 69 independent known genes (Table S2) and three novel sequences (UOH-ALL-104, UOH-ALL-105, UOH-ALL-106). The function and distribution of the identified genes in cancer and healthy tissues were determined (Table S3) through a comprehensive search of the literature. The expression of SEREX identified genes were examined using data from the MILE study and compared to bone marrow from HVs from the same (Table S3).

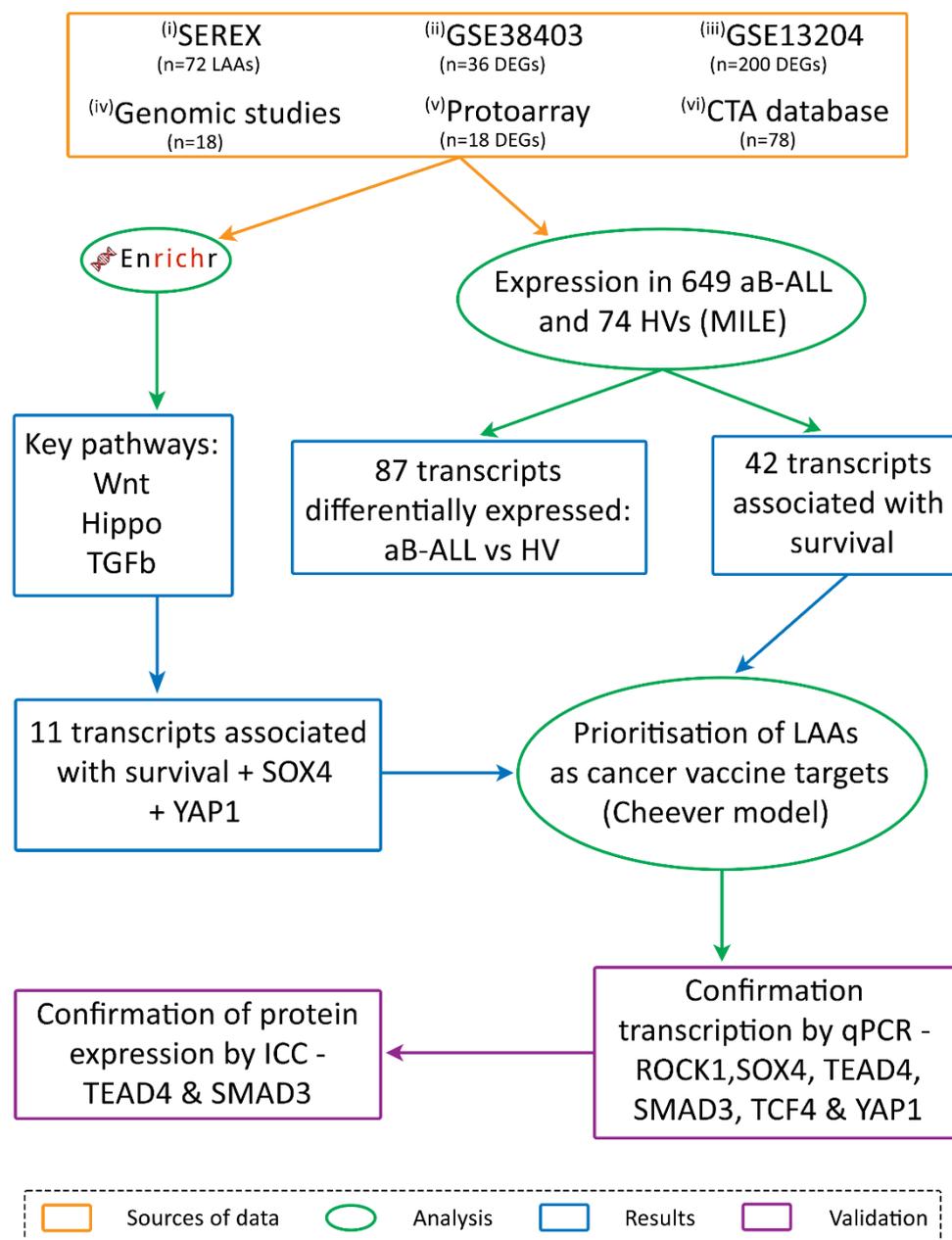


Figure 1. Workflow for the identification and analysis of aB-ALL antigens from six sources. Antigens were analysed for their differential expression, expression in gene pathways and association with survival. Antigens were prioritised using the model devised by Cheever et al [55]. From the final antigens analysed for RNA transcription and translation, we found SMAD3 and TEAD4 to be elevated in aB-ALL samples compared to healthy donor samples.

3.2. Association Between Leukaemia Associated Antigen (LAA) Expression in aB-ALL Cells and Patient Survival

Of the 385 genes identified from the six sources ((i) SEREX; (ii) GSE38403; (iii) GSE13204; (iv) Iacobucci and Mullighan's review of genomic studies [28]; (v) proto-array analysis and (vi) CTA database; Figure 1), 87 transcripts were found to have differential expression (Figure 2) and 42 of these had expression levels (above and below media) that were associated with survival in aB-ALL patients, determined using the MILE study data (Table S4).

3.3. TGF β , Wnt and Hippo Pathways Were Highly Represented by the DEGs in aB-ALL Samples

Transforming Growth Factor- β (TGF β), Wnt and hippo pathways were highly represented by the genes ($p \leq 0.001$, ≤ 0.01 and ≤ 0.01 respectively), when analysed by Enrichr (Figure 3A). The core genes from these pathways (TGF β , Wnt and hippo pathways) were then examined using the MILE dataset for their association with patient survival ($n=11$) (Table S4) and literature searches were performed to identify genes known to be involved in B-ALL. SOX4 and YAP1 were added to the antigen ranking as SOX4 was considered to be non-conical activator for three pathways [44] whereas YAP1 has restricted expression in healthy tissues (excluding stem/progenitor populations) [45].

STRING showed that PMP2, PMP4, CDKN2B, RBX, CUL1 were involved in TGF β enrichment (Figure S1) while Wnt2B, CDKN2B, RBX, PMP2, PTGS2 were involved in cancer pathways. SEREX identified genes that were involved in ubiquitination including UBC, UBE2C, UBE2D2 and CUL1 and genes involved in regulating the intrinsic apoptosis process (BCLAF2, PARL, SERINC3, TRAP1, TPT1, MCL1 and PTPN1).

Enrichr showed that the SEREX-identified antigens were mostly involved in molecular functions involving binding and catalytic activity, and the Wiki pathway (Table 3). In addition, envelope proteins (TCF7L2, Wnt2B) essential to maintaining the structure and function of the nucleus, mainly correlated with Emery-Dreifuss muscular dystrophy (EDMD). From MSigDB Hallmark 2020, the mitotic spindle pathway had a high p -value of 0.001 and this pathway plays a crucial role in cell division and chromosomal separation. Using PANTHER.db, the most enriched pathway was the Cadherin signalling pathway (14.3%) and PTPN1, TCF7L2, FER and Wnt2B were also identified by SEREX.

3.4. Most of the aB-ALL Antigens Were Differentially Expressed in Solid Tumours

Of the antigens that were ranked by the Cheever system (Figure 2A), ubiquitin C (HMG20/UBC) and MOB kinase activator 1B (MOB1B) were rarely detected in the healthy tissues (Figure S2A), while SOX4 was found at low to moderate levels in most solid tumours. ROCK1 and YAP1 had very similar patterns of expression in solid tumours except lymphomas where ROCK1 was upregulated and YAP1 was not detected. SMAD3 was upregulated in renal cancers. TGF β 1 was not found in most solid tumours or found at low levels, while TCF4, HMG20 and septin 9 (SEPT 9) weren't found in any of the solid tumours investigated (Figure S2B).

3.5. SOX4 and TGF β 1 Were the Top-Ranking aB-ALL Vaccine Targets

aB-ALL antigens that impacted survival were ranked using the criteria described by Cheever et al [36] to prioritise cancer antigens on their capacity to act as vaccine targets in clinical studies (Figure 3B). SOX4 (0.89) and TGF β 1 (0.81) had the top scores while ROCK1, YAP1, TEAD4, SMAD3 and TCF4 had the next highest cumulative scores of 0.41, 0.36, 0.34, 0.33, and 0.32 respectively. TGF β 1 was downregulated in aB-ALL compared to healthy donor samples ($p < 0.0001$) reflecting its activity as a tumour suppressor [46] and was excluded from further analysis.

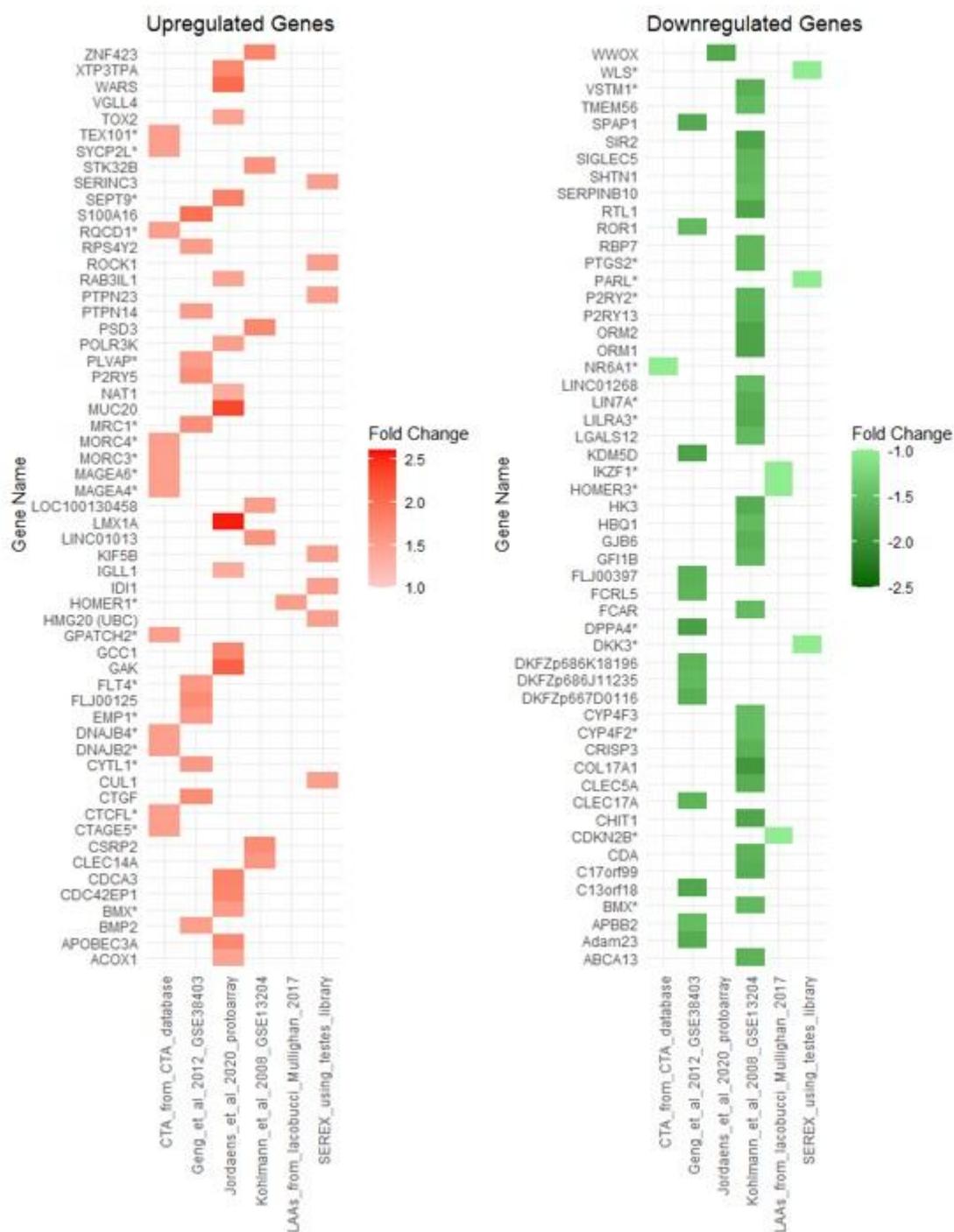


Figure 2. Relative expression of aB-ALL associated genes from each source. Differentially expressed genes which were upregulated (red) or downregulated (green) are shown as a fold change (FC) and by a key, ranging in FC from +1 to +2.5 and -1 to -2, respectively. The * indicates genes that were associated with survival ($p < 0.05$). Only BMX was common between two studies (protoarray and MILE study).

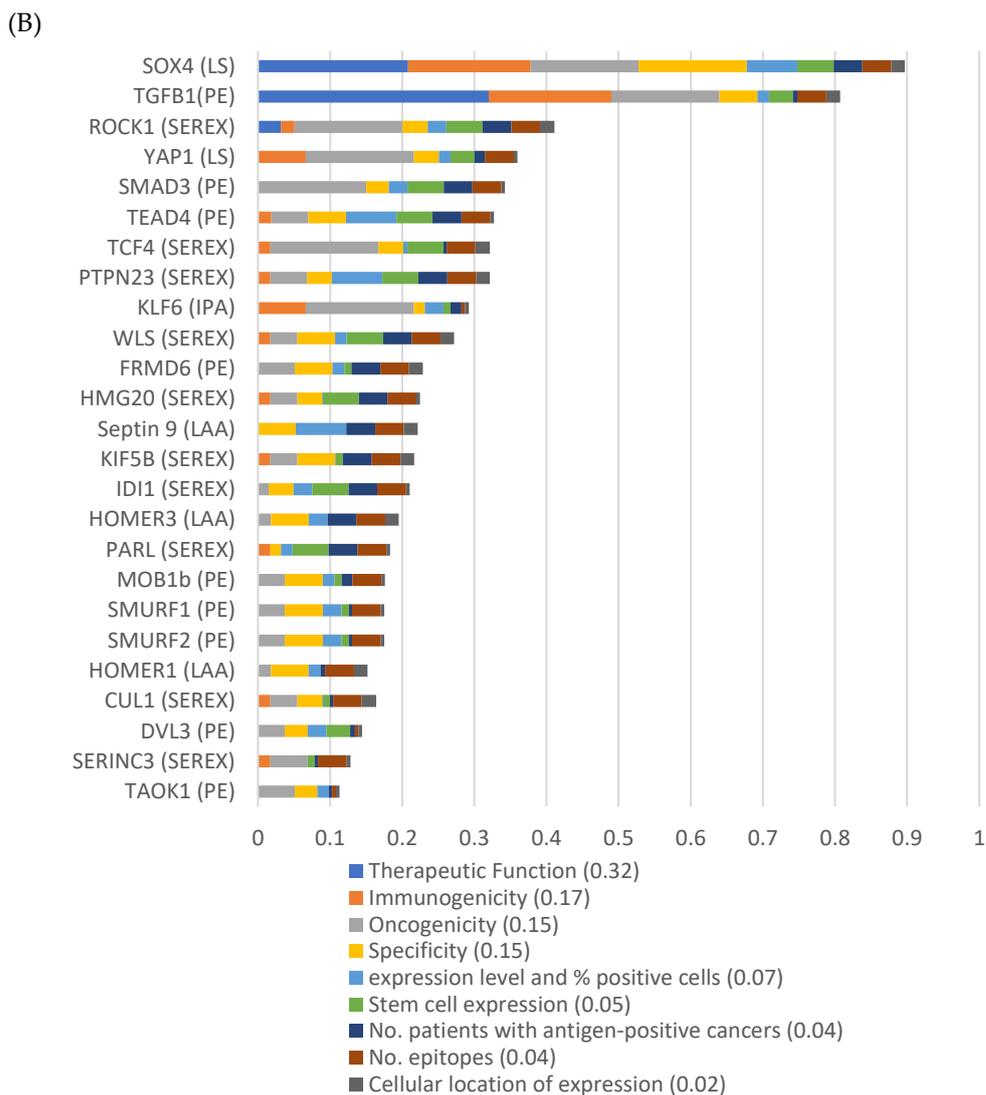
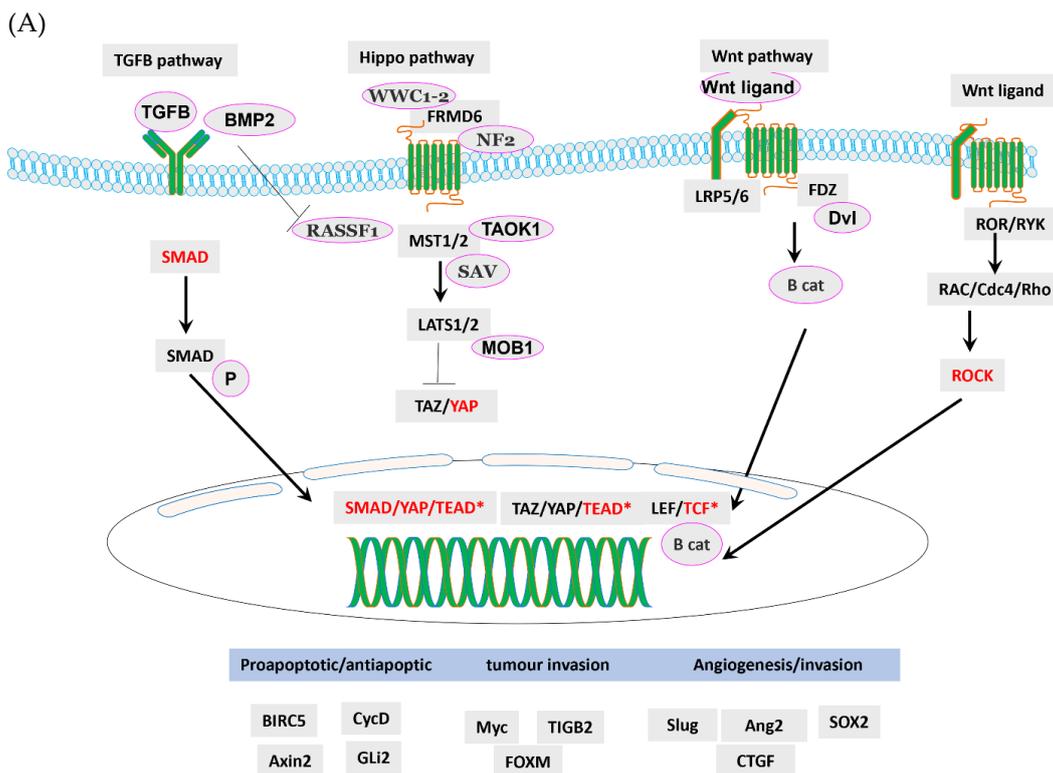


Figure 3. Role of gene products in key pathways and prioritising of gene products to act as vaccine targets. (A) Three key pathways were found to be enriched within the list of genes identified. These were Hippo, TGF β and Wnt pathways each involved in promoting leukaemogenesis via the upregulation of anti-apoptotic genes and pro-proliferative genes that causes blasts to continue growing and resist to apoptosis; (B) Antigens that were identified using SEREX, pathway enrichment (PE), literature search (LS), CTA database and microarrays (LAA). Key shows evaluation criteria and the maximum attainable score in parenthesis; Genes indicated in red font were ranked highly within the Cheever system and were selected for further analysis.

Table 3. Pathways identified as involving members of the SEREX identified genes.

Pathway	p-value	q-value	SEREX-identified genes involved in this pathway	Database used
Envelope proteins and their potential roles in EDMD physiopathology	0.0007	0.0892	TCF7L2, WNT2B	Wiki Pathway
Hematopoietic stem cell gene regulation	0.001	0.064	DNMT1, MCL1	
TGF β signalling pathway	0.015	0.229	TRAP1, ROCK1, CUL1	
Mitotic spindle	0.001	0.038	ROCK1, KIF5B, FLNB, KIF1B, MYH10	MSigDB
Notch signalling	0.007	0.117	TCF7L2, CUL1	
Apoptosis	0.026	0.286	ROCK1, TIMP1, MCL1	Hallmark 2020

3.6. TCF4, SOX4 and SMAD3 Transcription Was Increased in Almost All aB-ALL Subtypes

We analysed the expression levels of the six most highly ranked aB-ALL antigens (SOX4, ROCK1, TCF4, TEAD4, SMAD3 and YAP1), identified using the Cheever system, and used BIRC5 as a comparator, in 16 aALL PB and BM samples compared to four HV PB and one CD19⁺ sample by qPCR. The K562 cell line was used as a positive control for BIRC5, TCF4, SMAD3 and ROCK1 expression while A549 cells were used as a positive control for YAP1. Transcription of all aALL-antigens were elevated, while BIRC5, SMAD3 and TEAD4 had the highest mean fold change in aALL patient samples compared to HV samples (Figure 4A).

In the MILE study, ROCK1 was found to be upregulated in all types of aB-ALL ($p < 0.05$), except t(8;14)/t(1;19)/Pre-B-ALL t(9;22) which were not significant (NS). TEAD4 was upregulated in aB-ALL with t(8;14), t(1;19), c-/Pre-B-ALL t(9;22) - $p < 0.001$. There was no consistent difference in YAP1 expression between aB-ALL and HV samples, with variation occurring depending on the probe set used. TCF4 was upregulated in all subtypes of aB-ALL- $p < 0.001$, SOX4 was upregulated in all subtypes of B-ALL - $p < 0.001$ except t(8;14) and SMAD3 was upregulated in all subtypes of aB-ALL- $p < 0.05$.

3.7. TEAD4 and SMAD3 Protein Expression Was Elevated in aB-ALL Samples

ICC was performed on six B-ALL samples compared to HV CD19⁺ B-cells to examine SMAD3 and TEAD4 expression. TEAD4 and SMAD3 levels were significantly higher in aB-ALL samples compared to HV CD19⁺ B-cells ($p < 0.05$; Figure 4B). TEAD4 protein was mostly found in the nucleus whereas SMAD3 was found in both the nucleus and cytoplasm. TEAD4 protein levels were significantly different between PB and BM. We found no statistical significance in immunoreactivity scores between TEAD4, SMAD3 and historical BIRC5 data, when we compared the levels of TEAD4 and SMAD3 in this study and BIRC5 levels in the same three patient samples analysed in our previous study [17].

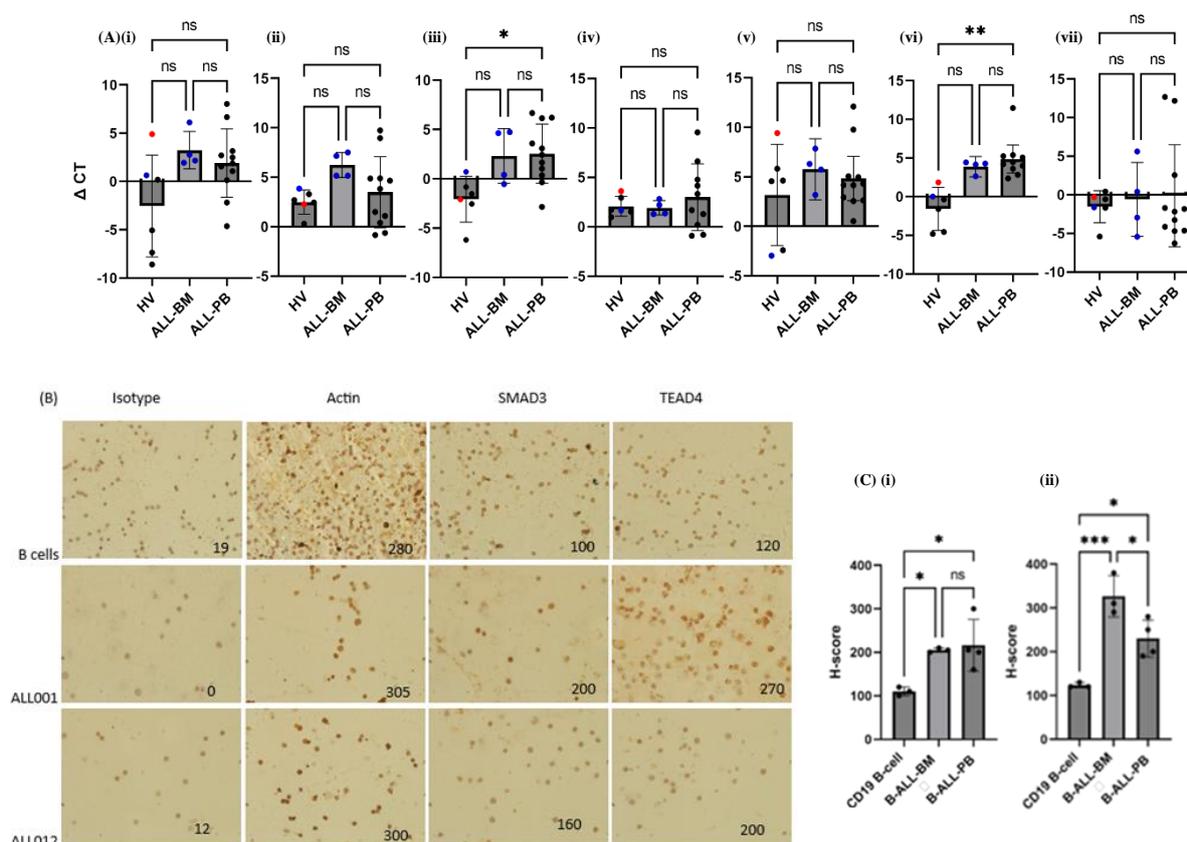


Figure 4. Expression of the key genes in aALL patient samples. (A) $2^{-\Delta\Delta CT}$ was used to determine relative gene expression of (i) *BIRC5*; (ii) *ROCK1*; (iii) *SMAD3*; (iv) *SOX4*; (v) *TCF4*; (vi) *TEAD4* and (vii) *YAP1* when compared to the average of two reference genes – *TBP* and *PRKG1*. Expression was analysed in 11 PB and five BM samples from ALL patients and five PB samples from healthy donors. (B) Immunolabelling of SMAD3 and TEAD4 in HV CD19⁺ B-cells and patient samples at X 400 magnification were represented by an immunoreactivity score (0-400; shown in black text on the bottom right corner of each image). Isotype caused little immunolabelling while actin provided a positive control for immunolabelling (stained dark brown). (C)(i) SMAD3 and (ii) TEAD4 immunolabelling were detected in all patient samples with immunoreactivity scores (y-axis) of 200-350, indicating their varied but elevated levels in aB-ALL samples when compared to CD19⁺ cells from the PB of a HV.

4. Discussion

A number of immunotherapy strategies have been used to improve treatment outcomes for patients with relapsed/refractory (R/R) ALL following chemotherapy and haematopoietic stem cell transplant. However, long-term use of immunotherapy can lead to the selection of leukaemic clones that are resistant to treatment. This evolutionary process hampers the effectiveness of immunotherapy by selecting subclones that are unresponsive to current therapy, resulting in escape variants and relapse. To overcome their existence, the heterogeneity between and within aB-ALL tumour cells, and the limitations with existing immunotherapy treatments, it is essential to broaden the number, specificity and sensitivity of tumour antigen targets available for future clinical use. To this end we immunoscreened a testes cDNA library with sera from five representative aALL sera. We identified 134 sero-positive plaques that encoded 72 independent sequences and three (UOH-ALL-104, UOH-ALL-105 and UOH-ALL-106) were novel providing a source of previously uncharacterised gene products for future analysis.

The majority of SEREX-identified antigens (67/72) had already been shown to be involved in tumour pathogenesis, four were CTAs (TUBA3C/UOH-ALL-3, C10orf82/UOH-ALL-20,

CT152/UOH-ALL-83 and PRM1/UOH-ALL-101), three were highly enriched in the testis and involved in spermatogenesis (TEX43/UOH-ALL-43, CCDC89/UOH-ALL-49 and LOC338963/UOH-ALL-85) and three were non-coding RNAs that had testis-restricted expression (LINC00661/UOH-ALL-58, LOC338963/UOH-ALL-85 and LINC00251/ UOH-ALL-98).

In addition we collated CTAs and LAAs from our previous studies [12] and that of others [25,26,28,30,31], determined the pathways most often utilised by the antigens and the core genes in these selected pathways, and then examined the association between levels of expression as determined by the MILE study in aB-ALL samples with survival. We priority ranked the antigens using the system described by Cheever et al [36] that assesses antigens for their utility as vaccine targets, however, this mathematical model has not been updated since 2009, has the disadvantage of giving a high weighting to therapeutic function and may not be precise in reflecting the fairness and reliability of the data [36,47]. However, in the absence of a better criteria, we used the model to identify the five aB-ALL antigens that scored highest for vaccine target potential and the correlation of their expression with patient survival. Levels of *SOX4* and *YAP1* expression levels were not associated with survival, while *TGFβ1*, *ROCK1* and *TEAD4* were. We found that *SOX4* and *TEAD4* were upregulated in aB-ALL patients samples while lower *SOX4* expression showed a trend towards better outcomes and survival ($p=0.07$). *SOX4* belongs to the sex-determining region Y-related HMG box family. Its abnormal expression is associated with cancer, reflecting its role in regulating cell stemness, proliferation and differentiation in healthy cells. *SOX4* is known to regulate several cancer-promoting signalling pathways including Wnt, TGFβ and PI3K [48] and the TGFβ, Wnt and hippo pathways were all highly represented by the aB-ALL antigens. SEREX also identified *ROCK1/UOH-ALL-65* which has been shown previously to regulate normal haematopoiesis through the negative regulation of erythropoietic stress and inflammation [49]. *ROCK1* is an established oncogene in AML, where it's overexpression is associated with poor survival ($p < 0.01$) [50]. Of note, using data from the MILE study we identified *TEAD4*, *SMAD3*, *SOX4* and *TCF4* as being upregulated in cB-ALL compared to HVs, while *ROCK1* and *YAP1* were not. *ROCK1* oncogenes may be an attractive target for future treatments as a small molecule inhibitor, GSK269962A, has already been investigated in an AML mouse model [51]. GSK269962A was shown to inhibit tumour growth, apoptosis and clonogenicity, significantly prolonging survival with some mice remaining disease-free for more than 140 days. Knocking out *ROCK1* was shown to at least double the survival time in a KIT(D814V)-induced myeloid leukaemia model when compared to controls [52]. The anti-proliferative activity of GSK269962A occurs via the inhibition of cell cycle kinase (CDK6) which induces cell cycle arrest. It also causes apoptosis via increased expression and phosphorylation of p53 as well as decreased expression of antiapoptotic genes including survivin, Bcl-xL and it has been shown to cause the cleavage of PARP in AML cells [51].

TGFβ signalling in haemopoietic and lymphoid cells is complex and context dependent [53]. The TGFβ1 pathway is involved in a variety of cellular functions, including inhibiting cell proliferation, and is often inactivated in lymphoma and acute myeloid leukaemia [54]. Although the MILE study showed that *TGFβ1* was downregulated in aB-ALL, in contrast to paediatric B-ALL [55], TGFβ1 acts as a potent immune suppressant that inhibits NK effector function and TGFβ blocking monoclonal antibodies have been shown to reverse ALL-mediated NK cell dysfunction [55]. In contrast, adding exogenous TGFβ1 to healthy donor NK cells induced an inhibitory phenotype similar to ALL-NK cells that has been shown to act through the SMAD signalling pathway [55].

The Wnt/β-catenin pathway regulates cell proliferation, survival and differentiation in hematopoietic stem cells (HSCs), while deregulation of this pathway leads to haematological malignancies including B-ALL [56]. SEREX identified four Wnt pathway components (WLS, Wnt2b, TCF7L2, and DKK3) that were recognised by antibodies in aB-ALL patient sera. Targeting Wnt ligands and receptors can reduce leukaemic stemness [57] while knock-down of *FZD6/WNT10B* reduced the proliferation of leukaemic stem cells [58]. However, TGFβ and NFAT pathways sustain Wnt activation increasing the leukaemic burden [58]. KIBRA, is a promising tumour suppressor in drosophila and mammalian cells [59] and an upstream regulator of the hippo

pathway. It was found to be frequently silenced due to hypermethylation in cB-ALL [60], suggesting one mode by which the Hippo pathway could be targeted.

We also identified eukaryotic initiation factor 2 α (eIF2 α) through the immunoscreening of a testis cDNA library using ALL sera. The activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) leads to the phosphorylation of eIF2 α . This phosphorylation event promotes the translation of activating transcription factor 4 (ATF4) while reducing overall protein synthesis [61]. ATF4, as a transcription factor, has the ability to increase the expression of CCAAT/enhancer-binding protein homologous protein (CHOP). ATF4 and CHOP together can also upregulate the production of growth arrest and DNA damage-inducible protein-34 (GADD34), which acts to dephosphorylate phospho-eIF2 α [61]. Therefore, prolonged endoplasmic reticulum (ER) stress, without the restoration of protein synthesis homeostasis, can ultimately result in apoptotic cell death which may be worthy of further investigation in the context of treating aB-ALL.

Our investigation of genes that can act as possible targets for immunotherapy has identified antigens that are involved in patient survival (ROCK1, TEAD4, SMAD3, and TCF4), in maintaining tumour stem cell features (SOX4, YAP1 and TCF4) as well as the tumour microenvironment (ROCK1, YAP1 and TEAD4). Several of the antigens we identified have been found to play a role in childhood B-ALL (SMAD3 [62]; TCF4 [63]) and demonstrate the broad potential of treatments targeting these proteins/pathways.

The identification of immunogenic epitopes within antigens and their capacity to stimulate the immune response are the usual next step in the development of immunotherapy strategies for patient treatment [64,65]. However, there is a need to break immune tolerance which is exacerbated by the immunosuppressive microenvironment created by tumour cells, through the secretion of IL-10 and TGF β [66]. The direct targeting of proteins such as TEAD4 and SMAD3 may be especially challenging because they are involved in the maintenance of homeostatic functions and may cause significant off-target effects. Additional investigations using small inhibitors/protein-protein interaction targeting are necessary to determine the clinical utility of direct targeting and the specificity of such treatment for diseased cells [67]. Although BIRC5 is a novel target, phase I trial using antisense oligonucleotide targeting BIRC5 has been examined in children who had relapsed with B-ALL (cB-ALL). However, the trial was discontinued due to high toxicity and off-target effects [19]. One reason for this limited success may be that many immunodominant TAAs are self-proteins. As such, they are subject to both central and peripheral tolerances that dampen immune responses to avoid autoimmunity. This situation demonstrates the need to break self-tolerance in a controlled manner, replicating stem cell transplant strategies that have been used and evolved to effectively treat at least a proportion of patients with haematological malignancies since the 1930s.

5. Conclusions

Current treatments for aB-ALL have involved integrating chemotherapy and targeted therapy. B-ALL is a complex disease with various factors influencing the overall prognosis, including cytogenetic abnormalities, MDR, and response to therapy. Simply targeting the tumour antigens on and in malignant cells is not enough to eradicate tumour growth, as the tumour microenvironment within the BM plays a crucial role in treatment outcomes. Sensitivity to immunotherapy in B-ALL is not solely determined by intrinsic biological factors but also by the diverse interactions between leukaemia cells and the bone marrow microenvironment [68]. Leukaemia cells exploit this microenvironment to sustain their proliferation and survival, taking advantage of the tightly controlled signalling pathways and transcriptional factors that regulate normal lymphopoiesis. Breaking immune tolerance towards leukaemic cells will require a multifaceted approach that minimizes the opportunities for escape mutants to survive the treatment process.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Table S1 Antibodies used for ICC; Table S2 Characterisation of cDNA sequences confirmed by two rounds of immunoscreening; Table S3 Expression of SEREX-identified genes and

their function in health and cancer tissues; Table S4 Gene expression and association with above and below median expression using MILE data; Figure S1 Protein-protein interaction of LAAs using STRING. Most of the LAAs were involved in binding followed by transcription regulator activity. PMP2, PMP4, CDKN2B, RBX, CUL1 involved in TGF β enrichment. Wnt2B, CDKN2B, RBX, PMP2, PTGS2 were involved in cancer pathways. The lack of interactions among other genes in this signature is likely explained by a variety of other biological pathways utilised within B-ALL. SEREX identified genes that were involved in ubiquitination included UBC, UBE2C, UBE2D2, CUL1, RBX1 and SKP2. Genes involved in regulating the intrinsic apoptosis process were BCLAF2, PARL, SERINC3, TRAP1, TPT1, MCL1 and PTPN1; Figure S2. In silico analysis of the antigens revealed the levels of expression of each shortlisted antigen in healthy and cancerous tissues. Heatmap showing the expression levels of each protein in (A) healthy cells and (B) different tumour types, with the y-axes represent the proportion of patients expressing each tumour antigen. The purple, red and orange colours indicate very high to low levels of expression, pale yellow represents no expression detected, and white represents no data available in the corresponding histology in Human Protein Atlas.

Author Contributions: Conceptualization, E.M. and B.G.; methodology, E.M. and B.G.; validation, E.M., S.G. and L.C.; formal analysis, E.M., S.G. and L.C.; investigation, E.M. S.G. and L.C.; resources, V.B.B. and K.H.O.; data curation, E.M., D.M.F., K.I.M. and K.H.O.; writing—original draft preparation, E.M., S.G., L.C. and B.G.; writing—review and editing, E.M., and B.G.; visualization, E.M. and B.G.; supervision, B.G.; project administration, B.G.; funding acquisition, B.G. All authors have read and agreed to the published version of the manuscript.

Funding: E.M. and L.C. were funded by University of Hull cluster PhD studentships and V.B. by Breakthrough Cancer Research (previously known as Cork Cancer Research Centre). We would like to thank the patients for their kind donation of samples.

Institutional Review Board Statement: Informed consent and the collection of aB-ALL samples was performed according to the guidelines of the Declaration of Helsinki, and approved by a Local Ethics Committee in the UK as well as the Southampton University Hospitals Trust and University of Hull ethics committees.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We would like to thank the patients for their kind donation of samples.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

aB-ALL	Adult B-cell acute lymphoblastic leukaemia
BIRC5	baculoviral IAP repeat containing 5
BMX	bone marrow tyrosine kinase
CAR	chimeric antigen receptor
cB-ALL	children with B-ALL
CTA	cancer testis antigen
DEGs	Differentially expressed genes
HV	Healthy volunteer
LAA	leukaemia associated antigens
NS	Not significant
PB	Peripheral blood

PRKG1	protein kinase cGMP-dependent 1
R/R	relapsed/refractory
ROCK1	Rho associated coiled-coil containing protein kinase 1
SMAD3	SMAD family member 3
SOX4	SRY-Box Transcription Factor 4
TAAAs	Tumour associated antigens
TBP1	TATA-box binding protein
TCF4	T cell Factor 4
TEAD4	TEA Domain Transcription Factor 4
TGFβ	Transforming Growth Factor-β
YAP1	Yes-associated protein1

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