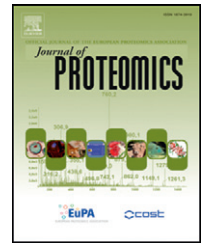


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RNASET2 — An autoantigen in anaplastic large cell lymphoma identified by protein array analysis

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ABSTRACT

Characterising tumour-associated antigens (TAAs) not only represents an important approach to the identification of new diagnostic/prognostic markers, but can also provide information on disease processes and additional potential therapeutic targets. Preliminary screening of a protein macroarray, containing more than 12,000 different proteins, with sera from anaplastic lymphoma kinase (ALK)-negative and ALK-positive anaplastic large cell lymphoma (ALCL) patients identified ribonuclease and tumour suppressor protein Ribonuclease T2 (RNASET2), phosphatase lipid phosphate phosphatase-related protein type 3 (LPPR3) and apoptotic adaptor molecule Fas-associating protein (FADD) as ALK-negative ALCL-associated TAAs. Further validation of these observations was confirmed using the ALCL sera in reverse ELISAs. The circulating anti-RNASET2 autoantibodies present in ALCL patients' sera also recognised eukaryotically expressed RNASET2 protein. RNASET2 expression was then investigated in normal tissues and in lymphomas to explore its clinical potential. RNASET2 protein and mRNA levels showed highest expression in the spleen, leucocytes and pancreas. RNASET2 protein expression was not restricted to ALK-negative ALCL (81%), being expressed in ALK-positive ALCL (65%) as well as in a number of other lymphomas. The immunological recognition of RNASET2, its expression in ALCL and other

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lymphomas together with its known tumourigenic properties suggest that further studies on this autoantigen are warranted.

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1. Introduction

The identification of tumour-associated antigens (TAAs) recognised by patients' immune responses represents an excellent approach to identify novel molecular mechanisms involved in cancer development, progression and drug responsiveness. Circulating autoantibodies have previously been used to identify TAAs that represent both diagnostic/prognostic markers [1–4] and also excellent biomarkers for the early detection of tumours [5,6]. TAAs, such as NY-ESO-1, additionally represent therapeutic targets for a number of tumours, [7,8]. Furthermore, TAAs may facilitate the monitoring of the efficacy of cancer treatment [9].

The recent development of protein array technology has provided the opportunity to investigate autoantibodies present in large numbers of patients against libraries containing thousands of different arrayed proteins [10–13]. This methodology has already played a significant role in analysing the relevance of circulating antibodies in a variety of autoimmune disorders [14], and identifying autoantibody profiles that can differentiate between symptomatic patients with and without colorectal cancer [4]. The ability to screen antibody responses to a large number of proteins in a high throughput technique has also led to the identification of autoantibody signatures that may represent disease subgroups, enabling early diagnosis in a range of pathological conditions, including cancer [2,6,15] and facilitated analyses of vaccine trials [9].

Anaplastic large cell lymphoma (ALCL) is a heterogeneous tumour of T-cell origin accounting for 10–15% of paediatric and 1–2% of adult non-Hodgkin's lymphomas [16,17]. Despite wide variations in morphology and presentation, 50–90% of cases are associated with abnormalities of the *anaplastic lymphoma kinase* (ALK) gene at 2p23 and constitute the tumour entity ALK-positive ALCL [18]. In previous studies we identified the ALK protein as an exceptionally antigenic TAA in ALCL patients [19–22]. Indeed, the presence of high titres of antibodies to ALK correlated with a decreased relapse risk in ALK-positive ALCL patients [22] permitting the use of ALK antibody titres for risk stratification in clinical trials for ALK-positive ALCL and provides a basis for the use and/or development of immunotherapeutic approaches [21,23,24]. In contrast, ALK-negative ALCLs remain a poorly characterised provisional entity [25]. With the exception of primary cutaneous ALCL, they have a poor prognostic outlook and risk factors are unknown [17,26]. There is, therefore, not only a need to be able to identify those patients who are at high-risk of relapse but also to identify new therapeutic targets for ALK-negative ALCL. One approach is through the identification of additional TAAs and/or through the development of a prognostic serum test.

This paper describes the identification of a number of novel ALCL-associated TAAs using protein array technology. In particular we provide the first description of RNASET2, a transferase-type 2 ribonuclease and tumour suppressor protein

[27] as a TAA, as well as the first report of RNASET2 protein expression in normal tissues and haematological malignancies.

2. Materials and methods

2.1. Samples

Serum samples: Serum samples were obtained from 39 lymphoma patients, namely, 6 follicular lymphoma (FL), 5 each of ALK-positive ALCL, ALK-negative ALCL, germinal centre derived diffuse large B-cell lymphoma (DLBCL), non-germinal centre-derived DLBCL, mantle cell lymphoma (MCL) and 4 each of peripheral T-cell lymphoma (PTCL) and transformed DLBCL (tDLBCL) patients attending the Department of Haematology, John Radcliffe Hospital, Oxford, Stoke Mandeville, Oxford. Ten control sera were used, 5 age- and sex-matched to the ALCL patients and 5 age- and sex-matched to the other lymphoma patients. Twenty-one sera from German patients entered into the NHL-BFM95 study and the European Inter-Group ALCL99 trial with a diagnosis of ALK-positive ALCL (n=19) or ALK-positive DLBCL (n=2) were also investigated. In addition, serum samples from 11 patients attending the Mater Misericordiae University Hospital, Dublin, Ireland) with stage 2 or greater invasive breast cancer (all female, average age 57) were investigated in the reverse ELISA validation step.

2.1.1. Normal tissues

Fresh tonsil was obtained from patients attending the Ear, Nose and Throat Department, West Wing, John Radcliffe Hospital, Oxford. Paraffin-embedded normal tissues were obtained from the Departments of Histopathology and Paediatric Pathology of the John Radcliffe Hospital, Oxford and the Centro Nacional de Investigaciones Oncológicas (CNIO), Madrid.

2.1.2. Haematological malignancies

Tissue microarrays (TMAs) of a variety of lymphomas were obtained from the CNIO whilst TMAs of ALK-positive and ALK-negative ALCL were obtained from the Department of Pathology, Würzburg.

All normal and neoplastic cells and tissues were obtained only after ethical approval and informed consent had been given.

2.2. Antibodies

2.2.1. Polyclonal antibodies

A polyclonal antibody to RNASET2 was generated to purified recombinant RNASET2 protein as described previously [28]. Anti-CD3 (DAKO-CD3 — diluted 1:100), anti-mouse Ig-conjugated to horseradish-peroxidase (HRP) diluted 1:100 and the Envision staining kit were obtained from

DakoCytomation (Glostrup, Denmark). Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (A1418—diluted 1:1000) was obtained from Sigma-Aldrich (St Louis, Missouri, USA). Goat anti-rabbit and -mouse isotype specific antibodies conjugated to fluorescein-isothiocyanate (FITC) (diluted 1:100) were obtained from Molecular Probes (Cambridge, UK).

2.2.2. Monoclonal antibodies

Mouse monoclonal antibodies to CD8 (C8/144), CD45 (2B11 and PD7/26) and CD68 (KP1) were produced in the authors' laboratories. Anti-p63 (VS38c) was a kind gift from Helen Turley (University of Oxford). Antibodies to CD4 (T4-10), CD20 (DAKO-L26) and CD138 (MI15) and β -actin were obtained from DakoCytomation. Mouse anti-human IgG antibody (GG-7) was obtained from Sigma-Aldrich whilst HRP-conjugated mouse anti- β -actin was obtained from Abcam (Cambridge, UK).

2.3. Cell lines

Clones from the murine B16 cell line stably transfected with cDNA encoding RNASET2 (Monti L., unpublished data) or with vector only as a control were cultured in RPMI 1640 containing 10% foetal calf serum and Gentamycin (50 μ g/ml, Sigma-Aldrich). The Thiel (myeloma), SUDHL-1, Karpas 299 (ALK-positive ALCL), FEPD (ALK-negative), Jurkat, PEER and CEM (T-acute lymphoblastic leukaemia), U937 and HL60 (acute myeloid leukaemia), germinal centre-SUDHL-6, SUDHL-10 and non-germinal centre RIVA and HBL-1 DLBCL-derived cell lines used were obtained and cultured as previously described [29]. The cultured cells were used to produce cytocentrifuge preparations, cell pellets or fixed in 10% formol-saline for paraffin embedding for the preparation of tissue sections of the cell lines.

2.4. Protein array methodology

The protein arrays (Source BioScience plc, Berlin, Germany) consist of His-tagged proteins expressed from the hEx1 library, which contains 37,200 clones of a cDNA library from human foetal brain cloned in the expression vector pQE30NST (GenBank Accession No. AF074376), transformed into *Escherichia coli* (E. coli) strain SCS1 (Stratagene, Stockport, UK) and grown on large PVDF membranes [10]. After induction of recombinant protein expression, the cells were lysed and the entire lysate, including the human protein, was fixed onto the membrane as previously described [13].

Screening the arrays with human serum was performed as previously described. Briefly, human serum samples were diluted to 1:100 in 2% (w/v) bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated with the arrays for 16 h at room temperature [4]. In the case of the pooled screen, the samples were first mixed in equal parts before diluting. After washing with TBST, the arrays were incubated with the following; mouse anti-human IgG antibody (GG-7, Sigma-Aldrich Company Ltd. Gillingham, UK), followed by 1:5000 dilution of alkaline phosphatase (AP)-conjugated goat antimouse IgG antibody (A1418, Sigma Aldrich). Human autoantibody complexes were then detected using the substrate Attophos (JBL Scientific, San Luis Obispo, California, USA). Images were captured with a Fuji LAS 3000 imager and positive clones identified using VisualGrid software (GPC

Biotech, Martinsried, Germany). In order to minimize false positive hits, each serum sample was screened twice and only hits that appeared in both screens were analysed. In this manner a unique antibody profile for each sample was generated.

2.5. Sequencing and analysis of cloned cDNA inserts

In order to confirm the identity of the protein expressed by clones of interest, plasmid DNAs were isolated for DNA sequencing using a QIAprep spin Miniprep kit (Qiagen, Netherlands) according to the manufacturer's instructions. Cloned cDNAs in the purified plasmid DNA were sequenced by Agowa Genomics Services (Berlin, Germany) using primers pQE-F (5'-CGGATAACAATTTCACACAG-3') and pQE-R (5'-GTTCTGAGGTCACTTACTGG-3'). Nucleotide and translated amino acid sequences were compared with known sequences using the NCBI BLAST algorithms.

2.6. Recombinant protein expression and FPLC purification

The recombinant *E. coli* clones expressing potential ALK-positive ALCL autoantigens (clones MPMGp800B07542, MPMGp800H15530, MPMGp800G08541, and MPMGp800J08578) were cultured and the human protein expressed and purified essentially as previously described [30]. The His-tagged recombinant human proteins were purified on a fast-performance liquid chromatography system (AKTA FPLC; GE Healthcare, UK) using Ni_2 -affinity chromatography (HisTrap; GE Healthcare), ion exchange (HiTrap Desalting Column; GE Healthcare), and gel filtration chromatography (Superdex 200 column; GE-Healthcare). The eluted fractions were analysed on Coomassie-stained 15% SDS-PAGE gels (Mini-PROTEAN II; Bio-Rad, Hemel Hempstead, UK) and the concentration of the soluble protein was determined (BCA Protein Assay Kit; Pierce, Rockford, IL).

2.7. ELISA

Seven hundred and fifty nanograms per well of recombinant protein were used to coat ELISA plates. Serum samples were then added for 1 h at room temperature. After washing an HRP-goat anti-human IgG was added for 30 min. Following six final washes (last two being in distilled water), the reaction was developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma-Aldrich).

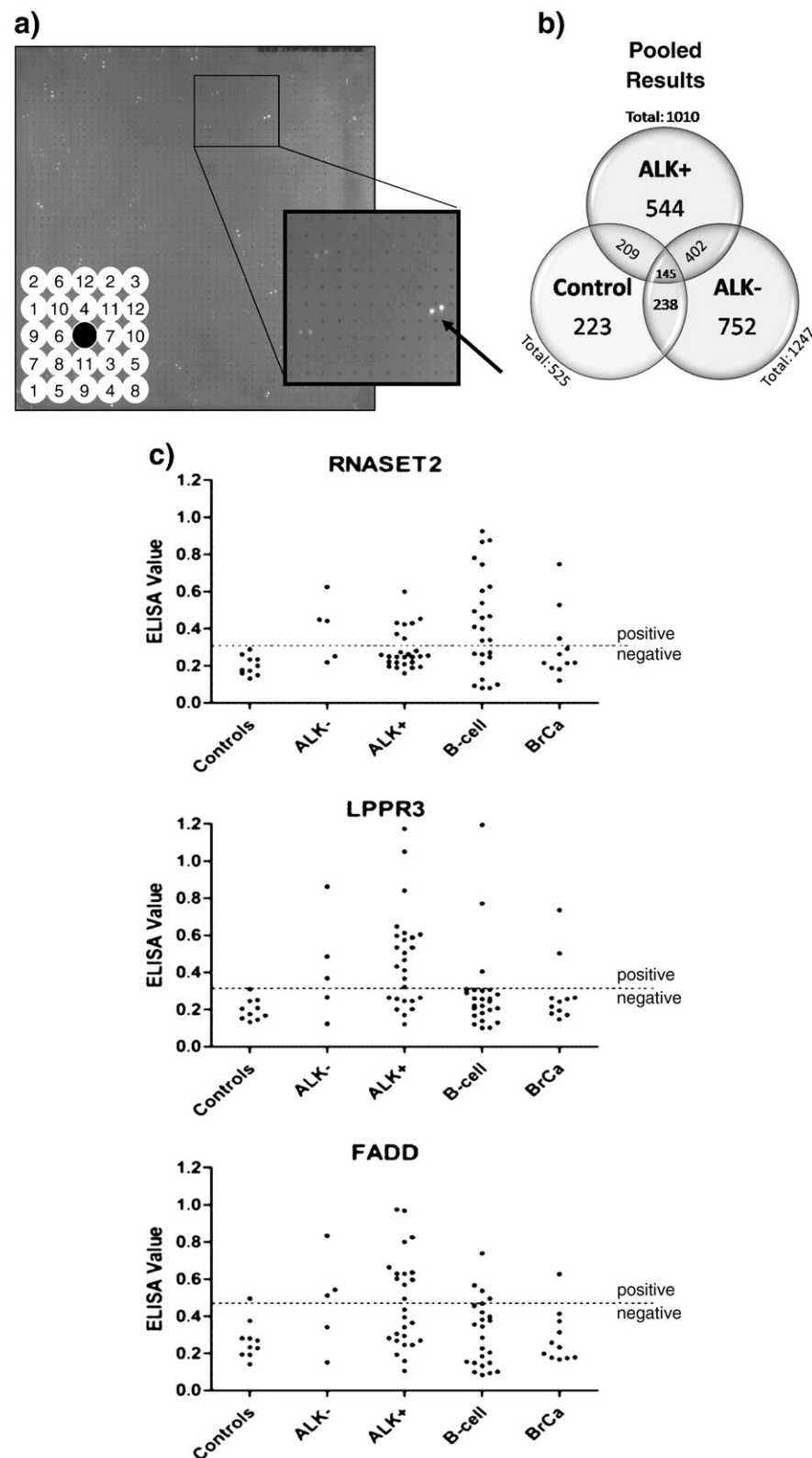
2.8. Reverse-transcription-polymerase chain reaction

RNASET2 transcripts were extracted from cell pellets containing 10^7 cells using the RNeasy Mini-kit from Qiagen, (Crawley, Sussex, UK) according to the manufacturer's instructions. Approximately 100 ng of mRNA was used as a template for cDNA synthesis using Superscript III (Invitrogen). Polymerase chain reactions (PCR) were set up using 2 μ l of cDNA template, 1 $\times$ buffer for KOD Hot Start Polymerase, 0.2 mM dNTPs, 1 mM MgSO_4 , 0.3 μ M of each primer, and 1 U KOD Hot Start Polymerase. Cycling parameters were 94°C for 2 min initial denaturation followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 10 min.

2.9. Real-time quantitative PCR (qRT-PCR)

qRT-PCR was performed using the MJ Research PTC200 Thermo Cycler. Primer pairs were validated in 20µl PCR reactions containing 2µl of testis cDNA (2µg) 10µl of Kapa qPCR Master Mix Sybr green (Kapa biosystem), and 0.3µmol/µl

of each primer. Cycling parameters were 95°C for 3 min initial denaturation followed by 40 cycles of 95°C for 10s and 60°C for 30s. The amplification step was followed by melting curve analysis. Each primer pair amplified a single product with a sharp melting peak at a temperature consistent with the calculated T_m of the predicted PCR product. Agarose gel



electrophoresis confirmed that the amplified products had the expected sizes. The oligonucleotide primers were all purchased from Invitrogen, UK. Primer pairs for each gene were:

RNASET2, 5'-AACTCCATCATGAAGTGTGACG-3' (forward) and
5'-GATCCACATCTGCTGGAAGG-3' (reverse);
for β -actin, 5'-GGGTTCTACGCAAAAGTCA-3' (forward) and
5'-GTTTCTTGGGTCGATGAT3' (reverse);
for GAPDH, 5'-GACAACAGCCTCAAGATCATCA-3' (forward) and
5'-GTCCTTCCACGATACCAAAGTT-3' (reverse);

Relative levels of mRNA expression in normal human tissues were determined using multiple tissue Clontech cDNA panels (MTC, Catalogue No 636742 and 636743) from Takara Bio Europe (St-Germain-en-Laye, France) in qRT-PCR reactions as described above. Termed cycle threshold (Ct) was determined for each sample using the software Real-time PCR Miner. Melting curve analysis confirmed amplification of only the expected product. To determine the quantity of RNASET2 gene-specific transcripts present in each tissue cDNA relative to testis, their respective Ct values were normalised after subtracting the Ct value from the GAPDH and β -actin controls. The concentration of gene-specific mRNA in a given tissue relative to testis was then calculated by subtracting the normalized Ct values obtained with each tissue from that obtained with testis (e.g. $-\Delta\Delta Ct = \Delta Ct$ of brain $-\Delta Ct$ of testis), and the relative concentration was determined (relative concentration = $2^{-\Delta\Delta Ct}$).

2.10. Immunolabelling of cells and tissue sections

2.10.1. Immunoperoxidase labelling

Cyocentrifuge preparations and cryostat sections of tonsil were stained using a two-stage immunoperoxidase technique as previously described [29]. Paraffin embedded tissue sections and microarrays were de-waxed and antigen retrieval carried out in 50mM Tris:2mM EDTA pH 9.0 by microwave pressure cooking for 2min [29]. The antibody to RNASET2 was applied at a dilution of 1:250 in phosphate buffered saline (PBS) for 30min at room temperature. Antibody binding was detected using the ChemMate™ DAKO EnVision™ Detection Kit, Peroxidase/DAB using the manufacturer's instructions. Cells were counterstained using haematoxylin and mounted in Aquamount (VWR International, Lutterworth, UK).

2.10.2. Double immunoperoxidase labelling

RNASET2 staining was performed on paraffin embedded tonsil sections as described above but omitting the counter-staining step. The tonsil sections were then incubated with one of the following monoclonal antibodies: anti-CD3, anti-CD20, anti-CD68, anti-p63 or anti-CD138 for 30min at room temperature. After washing the slides were incubated with the Vector S HRP substrate kit (Vector Laboratories, Peterborough, UK). After washing the slides were air-dried overnight and mounted in VectorMount (Vector Laboratories).

2.10.3. Single immunofluorescent labelling

Dilutions of patient's sera (1:50 to 1:250) were incubated for 60min at room temperature on cyocentrifuge preparations of either B16 RNASET2 transfectants or B16 vector only transfectant controls. After washing, the cells were incubated with a Texas Red conjugated rabbit anti-human IgG for 30min in the dark. Slides were mounted using an anti-fade mountant (DakoCytomation).

2.10.4. Double immunofluorescent labelling

Routinely fixed tonsil sections were incubated for 60 minutes with the antibody to RNASET2 and one of the following murine monoclonal antibodies anti-CD20 (IgG2a isotype), anti-CD3 or anti-CD68 (both IgG1 isotype). After washing, the slides were incubated for a further 30min with either a goat anti-mouse isotype specific or goat-anti-rabbit antibody conjugated to fluoroscein isothiocyanate (FITC) or Texas Red. The slides were washed, mounted and analysed as described above. All results were evaluated on a conventional fluorescent microscope equipped with a cooled CCD camera (Hamamatsu, Hamamatsu City, Japan) and the appropriate computer software (Improvision, Warwick, UK).

2.11. Western blotting

Protein lysates from mammalian cell lines were prepared by adding equal amounts of NuSep Tris Glycine SDS Sample Buffer (Generon Ltd, Maidenhead, UK) to each sample followed by sonication and boiling. SDS-PAGE was performed on 4%–20% gradient pre-cast gels (NuSep) and transferred to PVDF membranes at 100V for 1h in a transfer buffer containing 20mm Tris, 150mm glycine, and 20% methanol. The PVDF membranes were blocked in TBST containing 5% BSA for 1h and then incubated with primary antibodies for RNASET2 (1:500 dilution) in TBST and 5% BSA overnight at 4°C. The membranes were washed, incubated with a polyclonal

Fig. 1 – Analysis of results obtained from screening patient sera on the protein array containing 37,000 expression clones. a) Binding of autoantibodies from sera from an ALK-ve ALCL patient to recombinant proteins, each of which is arrayed in duplicate in a 5×5 pattern around ink guidedots. An example of autoantibodies binding a protein/antigen on the array detected by an alkaline phosphatase-labelled anti-human IgG antibody are shown (arrowed). **b)** Analysis of the results from pooled sera from each cohort of patients is displayed in a Venn diagram showing that multiple antigens were recognised by the sera from ALCL patients and control subjects. An example is the recognition of more than 752 antigens by autoantibodies present in sera only from ALK-negative ALCL. **c)** ELISA of reactivity of recombinant potential ALK-negative ALCL TAAs against sera from patients with ALK-negative (ALK-) and ALK-positive (ALK+) ALCL, as well as B-cell lymphoma and breast cancer (BrCa). A positive/negative cutoff was determined using the mean plus two standard deviations of the control values.

HRP-conjugated anti-rabbit (Dakocytomation) for 1 h at room temperature, and washed for 60 min with Tris-buffered saline-0.1% Tween. A HRP-conjugated mouse anti- β -actin (diluted 1:1000) was used to assess loading of the gels. Antibody complexes were visualised using the ECL chemiluminescence detection kit (Invitrogen).

3. Results

3.1. Protein array identification of ALCL-associated TAAs

Fig. 1a shows an example of screening a protein array with an individual serum sample from an ALK-negative ALCL patient. In order to test whether potential ALCL-associated autoantigens could be identified in this manner, we initially screened the arrays with the 5 pooled serum samples from each of the following: ALK+ve ALCL, ALK-ve ALCL (whose clinical details are summarised in Supplementary Table 1) and age- and sex-matched controls. This screening revealed a large number of potential disease-associated TAAs (Fig. 1b). For example, over 700 clones on the protein arrays expressed a protein which is an antigen bound by autoantibody molecules found only in the ALK-negative ALCL serum samples, when compared to normal controls or ALK-positive ALCL. Protein arrays were then screened individually with serum samples from the ALCL patients and also from 29 lymphoma patients, namely, GC-DLBCL (n=5),

non-GC-DLBCL (n=5), tDLBCL (n=4), MCL (n=5), FL (n=6), PTCL (n=4).

Screening the arrays with serum samples from individual ALCL patients enabled the antigens to be ranked based on the frequency of their immunological reactivity and the highest ranking are listed in Table 1. In order to identify the potentially most interesting autoantigens, the results from each cohort of patient sera were compared with each other and scored (data not shown). Of particular interest in the current study were the proteins recognized predominantly by autoantibodies in the serum of ALK-negative ALCL patients because of their poor prognosis. In order to identify the most relevant autoantigens, we focused on autoantigens that reacted with at least 3 patient serum samples and, at most, 1 control sample.

3.2. Protein expression and ELISA analyses of ALK-negative ALCL-associated TAAs

Our analysis of the protein array screen revealed six recombinant human clones expressing potential ALK-negative ALCL-associated autoantigens that were frequently recognised by patients' but not normal sera. Sequencing of these clones revealed that they represented four different potential autoantigens (Table 1): lipid phosphate phosphatase-related protein type 3 (LPPR3); Ribonuclease T2 (RNASET2); Transcription elongation factor A protein-like 2 (TCEAL2) and Fas-associating protein (FADD). Each of these autoantigens

Table 1 – Autoantigens detected in sera from patients with ALK-positive (n=5) and ALK-negative ALCL (n=5) and normal controls (n=5).

ALK-negative ALCL TAAs	Acc. no	Clone ID(s)	ALK+ve	ALK-ve	Normal	Location
Ribonuclease T2 (RNASET2)	O00584	MPMGp800H15530	0	3	0	Secreted
Lipid phosphate phosphatase-related protein type 3 (LPPR3)	Q6T4P5	MPMGp800L03577 MPMGp800B07542 MPMGp800F03529	0	3	0	Membrane
Transcription elongation factor A protein-like 2 (TCEAL2)	Q9H3H9	MPMGp800G08541	1	3	0	Nucleus
Fas-associating protein (FADD)	Q13158	MPMGp800J08578	1	3	1	Cytosol
ALK-positive ALCL TAAs						
Macrophage myristoylated alanine-rich C kinase substrate (MARCKS-like 1)	P49006	MPMGp800A08586	4	0	0	Plasma membrane
DnaJB1 (DnaJ homolog subfamily B member 1)	P25685	MPMGp800L22578	3	0	0	Cytoplasm, nucleus
Protein dispatched homolog 2 (DispB or Disp2)	A7MBM2	MPMGp800J16596	3	0	0	Membrane
Stromal interaction molecule 1 (STIM-1)	Q13586	MPMGp800E14534	3	0	0	Endoplasmic and plasma membrane
CpG-binding protein (CXXC1)	Q9P0U4	MPMGp800O05596	3	0	0	Nucleus
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P04406	MPMGp800K24586	3	0	0	Cytosol
ALCL TAAs						
Histone deacetylase 5 (HDAC5)(NY-CO-9)	Q9UQL6	MPMGp800C21550	4	2	0	Cytoplasm, nucleus
60S ribosomal protein L4 (RPL4)	P36578	MPMGp800N24592	3	2	0	ER, nucleolus
Inhibitor of growth protein 4 (ING4)	Q9UNL4	MPMGp800I19578	3	2	0	Nucleus
DNA helicase B (HELB)	Q8NG08	MPMGp800C09597	3	2	0	Nucleus
Pre-mRNA-splicing factor 38B (PRPF38B)	Q5VTL8	MPMGp800H16580	3	2	0	Nucleus

was recognised by a different combination of sera from three of five ALK-negative ALCL patients.

All six clones were expressed and purified as human His-tagged recombinant protein for use in biochemical studies. SDS-PAGE revealed a single purified protein for five of the six clones (data not shown). Expression of the clone MPMGp800G08541 (TCEAL2) revealed multiple His-tagged bands (presumably from rapid degradation) and was thus unsuitable for further validation.

Clone MPMGp800H15530 expressed a 15kDa protein consistent with the molecular weight of the C-terminal half of the 29.8kDa RNASET2 protein (aa 115–256). Clone MPMGp800J08578 expressed a 21kDa partial FADD protein, lacking the first 46 amino acids of the full length protein. Of the three clones expressing LPPR3, the clone expressing the largest protein was MPMGp800B07542, which expressed a 26kDa protein corresponding to the C-terminal region of the protein (aa 462–718), and this was chosen for further validation. Both of the other two LPPR3 clones completely overlap with this clone.

A number of these clones were also recognised by sera from patients with PTCL, which has a poor prognosis. MPMGp800H15530 (RNASET2) and MPMGp800G08541 (TCEAL2) were recognised by antibodies in two of the PTCL sera tested, whilst the clone for FADD protein (MPMGp800J08578) was recognized as a TAA in another PTCL serum sample.

ELISA studies using the original ALCL and normal control sera and the recombinant proteins RNASET2, LPPR3 and FADD confirmed the observations made with the protein arrays. The same serum samples found to be positive on the arrays were also positive in the ELISA assay. In order to estimate the specificity of these autoantigens, additional sera from 19 ALK-positive ALCL, 2 ALK-positive DLBCL, 25 B-cell derived lymphomas and 11 breast cancer patients and 5 additional age and sex-matched controls were also included. The study of serum samples from additional ALK-positive ALCL patients showed the presence of autoantibodies to RNASET2 in 5/19 (26%) of these patients, indicating that its immunological recognition was not specific to ALK-negative ALCL (Fig. 1c). It is of interest that one of these 5 patients possessed the ATIC-ALK gene rearrangement [31] whilst the 2 ALK-positive DLBCL patients with autoantibodies to RNASET2 possessed the (2;17) translocation encoding the (clathrin heavy chain) CLTC-ALK fusion protein [32] rather than the (t2;5) translocation encoding the NPM-ALK protein detected in 80–90% ALK-positive ALCL [18]. In contrast, the ELISAs using LPPR3 and FADD showed a higher frequency of autoantibodies against these proteins in ALK-positive ALCL sera, with over half of the patients' sera proving to be positive (Fig. 1c). RNASET2 was therefore chosen as the TAA for further study.

Interestingly, autoantibodies against RNASET2 were also observed in 15/25 serum samples tested from B-cell lymphoma patients (4 patients each with GC-DLBCL and non-GC-DLBCL, 2 patients each with tDLBCL and FL, and 3 patients with MCL), whereas only a relatively small number of patient samples had autoantibodies recognising LPPR3 or FADD (3 and 4 out of 25 patients, respectively). We also tested for the presence of autoantibodies in serum samples from 11 patients with breast cancer. Three out of 11 breast cancer patients were positive for the presence of RNASET2 autoantibodies, 2 for LPPR3 and only 1 for FADD. By comparing our results to previous studies using the same array technology carried out by our group,

we can confirm that no autoantibodies to RNASET2 or LPPR3 were observed in the sera studied from patients with glaucoma [30] or colorectal cancer [4]. Autoantibodies against the FADD-expressing clone MPMGp800J08578 were observed in approximately 10% of the much older population of glaucoma patients and in age- and sex-matched controls.

3.3. Normal tissue distribution of RNASET2

Results from the qRT-PCR studies are shown in Fig. 2a. Low levels of RNASET2 transcripts were detected in a wide range of normal tissues with the highest levels expressed in leucocytes, spleen and pancreas. Use of the anti-RNASET2 antibody (details of the validation of this antibody against recombinant RNASET2 are shown in Supplementary Fig. 1) confirmed the widespread distribution of RNASET2 protein in normal cells with the highest levels of RNASET2 protein also being observed in the spleen and pancreas (Fig. 2b). Strong staining was also noted in the spermatocytes and spermatids in the testis (data not shown). The majority of the cytoplasmic tonsillar staining was noted in the macrophages (arrowed) whilst punctate labelling was also detected in the cytoplasm of lymphocytes (arrow-head). This labelling was present in both T- and B-lymphocytes (insets). RNASET2 was not detected in terminally differentiated plasma cells (data not shown). Of interest was the variability in the subcellular distribution of RNASET2 in the normal tissues studied. Whilst predominantly nuclear labelling was observed in the epithelial cells, localised cytoplasmic labelling was also observed in some of these tissues. Weak nuclear labelling was also observed in some tonsil lymphocytes.

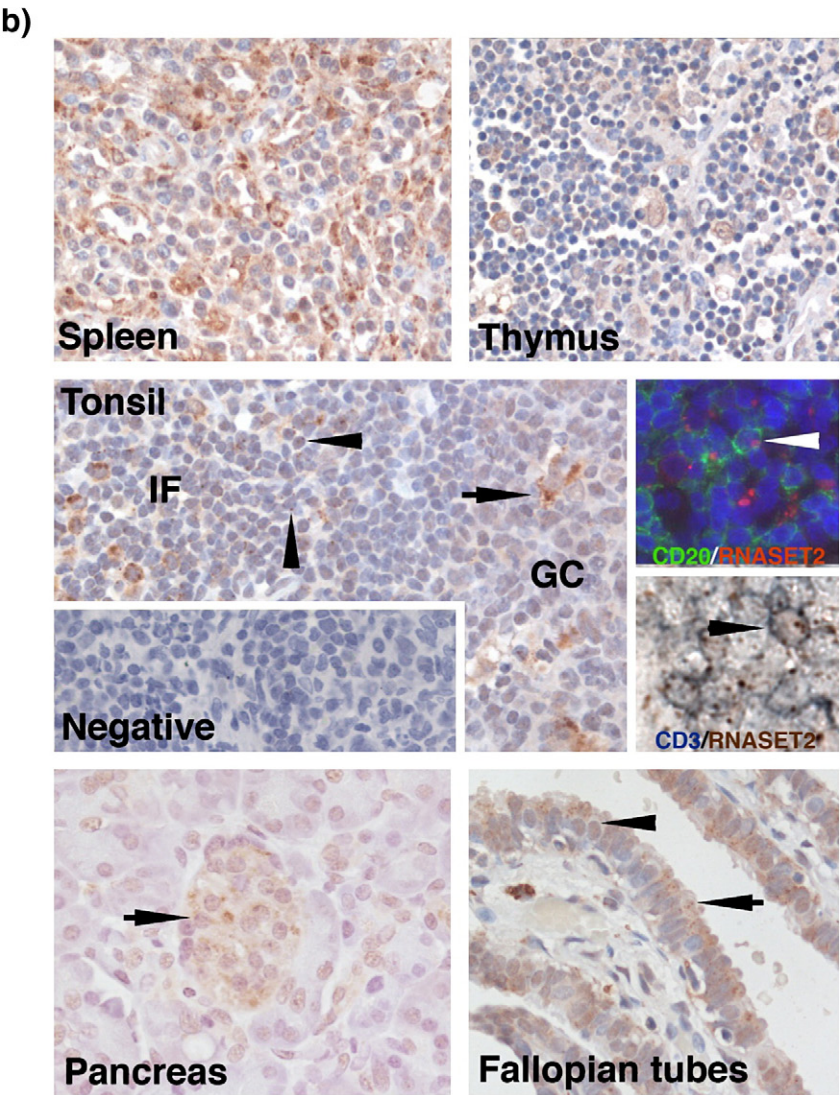
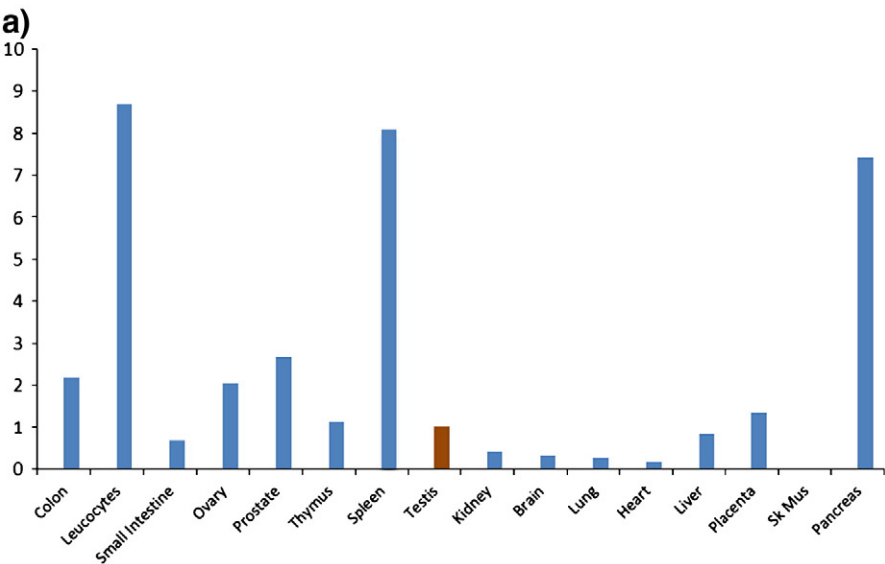
3.4. Expression of RNASET2 protein in haematological malignancies

3.4.1. Cell lines

RNASET2 expression was detected at both the transcript (Fig. 3a) and protein levels (Supplementary Table 2). Nuclear RNASET2 protein distribution was present in the ALK-negative ALCL-derived FE-PD cell line whilst both nuclear and cytoplasmic labelling was observed in the ALK-positive SUDHL-1 and Karpas 299 ALCL-derived cells. High levels of RNASET2 protein were present in the Jurkat, PEER and CEM T-ALL-derived cell lines. Western blotting studies confirmed the expression of the 31kD and 27kD RNASET2 proteins in the lymphoma-derived lines tested (example shown in Fig. 3b). The germinal centre-derived DLBCL lines SUDHL-6 and SUDHL-10 cells showed higher intensities of staining than the RIVA and HLY-1 non-germinal centre-derived DLBCL cells, a finding which is in agreement with the levels detected by qRT-PCR. RNASET2 was also detected in the U937 and the HL60 myeloid cell lines.

3.4.2. Lymphomas

RNASET2 protein expression in lymphomas is summarised in Table 2 and examples of labelling patterns are illustrated in Fig. 3c. RNASET2 was detected in 34/42 (81%) of ALK-negative ALCL and in 20/31 (65%) of ALK-positive ALCL. Of interest was the variation in staining patterns observed. Nuclear and cytoplasmic labelling were the most frequent patterns of RNASET2 expression in ALK-negative ALCL (Fig. 3a). In contrast,



strong granular cytoplasmic staining was observed in 7 of the ALK-positive ALCL cases but only in a single ALK-negative case. Labelling of other types of lymphomas was noted. Both nuclear and cytoplasmic staining was present in other tumours such as cases of DLBCL, HL, MCL, Burkitt's lymphoma (Fig. 3c) and PTCL.

3.5. Correlation of RNASET2 expression and autoantibodies in initial ALCL patients

Immunolabelling studies showed the expression of RNASET2 protein in the tumour cells of the one ALK-negative ALCL patient for whom a biopsy was also available (Fig. 4a) whilst the tumour cells of the ALK-positive ALCL patients were RNASET2-negative. Sera from patients were tested for reactivity against B16 RNASET2 transfectants using immunofluorescent labelling techniques. Sera from all 3 ALK-negative ALCL patients identified as possessing autoantibodies to RNASET2 from the protein microarray and ELISA studies labelled the RNASET2 transfectants in a punctate staining pattern (Fig. 4a). No comparable labelling was observed by sera from the 5 ALK-positive ALCL patients who were screened initially and who lacked an autoantibody response to RNASET2 on the protein microarray (Fig. 4b).

4. Discussion

A variety of different techniques using recombinant proteins, for example, ELISA, Western blotting [1,7] and SERological Proteome Analysis (SERPA) [33], have been developed to identify the targets of circulating autoantibodies. However, the generation of complex cDNA libraries producing large numbers of recombinant proteins has permitting the production and autoantibody screening of large numbers of recombinant proteins has resulted in the success of the SERological Analysis of Recombinant cDNA EXpression libraries (SEREX) technique [34–36] and, more recently, protein array technology (reviewed in [12]). The foetal brain cDNA library hEx1 used in the production of the microarray used in this study has previously identified disease-associated antigens in autoimmune conditions such as dilated cardiomyopathy and alopecia [14,37] and neurodegenerative disease [30] as well as characterising commercially available antibodies and identifying the target antigen of a monoclonal antibody [38].

Fifty–70% of patients with ALK-negative ALCL relapse with current chemotherapy regimens [17,26] and there are no prognostic factors to identify those higher risk ALK-negative

ALCL patients for whom alternative therapies might be appropriate. This is in contrast to the situation with ALK-positive ALCL where immunity to ALK is of clinical relevance [39]. In order to identify new TAAs as potential markers or therapeutic targets for high-risk ALK-negative ALCL patients, we selected an initial ALCL patient cohort of 5 ALK-negative and 5 ALK-positive ALCL for screening protein microarrays. RNASET2, LPPR3, TCEAL2 and FADD were identified as lymphoma-associated autoantigens being primarily being detected in those patients with ALK-negative ALCL. RNASET2, LPPR3 and FADD were subsequently confirmed as TAAs by ELISA where they each exhibited a higher frequency of reactivity with additional ALK-positive sera. Subsequent ELISA studies using ALCL sera showed that whereas autoantibodies to LPPR3 and anti-FADD autoantibodies were detected in over half the ALK-positive samples tested, anti-RNASET2 autoantibodies were observed in only 27% of ALK-positive sera.

RNASET2, the first mammalian member of the transferase-type 2 ribonucleases to be described [40–43] was chosen for further investigation. The members of this family exhibit a variety of subcellular distributions ranging from a cytoplasmic, lysosomal and/or nuclear localisation to being secreted proteins [44,45]. Such diversity of expression patterns suggests that these proteins may have different subcellular roles in addition to a ribonuclease function [46]. In keeping with this, RNASET2 has been shown to possess a role as a senescence inducing and class II tumour suppressor protein *in vivo*, although this role has yet to be shown *in vitro* [27,42,47–49].

Using qRT-PCR we demonstrated widespread expression of RNASET2 mRNA in normal tissues and confirmed its highest expression in leucocytes, pancreas and spleen. This is in agreement with previous reports using Northern blotting studies in which variable levels of 1.5 kB RNASET2 transcripts were reported in a wide range of normal non-haematopoietic tissues [27,41,48]. RNASET2 protein was also detected in a wide range of normal tissues. The high levels of RNASET2 protein in the tissues, such as the spleen, correlated with the distribution of RNASET2 mRNA. Although RNASET2 was present in both B and T cells, the highest protein levels were detected in macrophages, particularly within the tingible body macrophages in the tonsillar follicular germinal centres. This finding underscores a possible functional role of RNASET2 in cells from the monocyte/macrophage lineage, as recently suggested in a human ovarian cancer xenograft model [49].

Since the 36 kD form of RNASET2 is secreted, the RNASET2 protein observed in localised areas of the cytoplasm in the normal tissues probably represents the smaller 31 and 27 kD

Fig. 2 – Analysis of RNASET2 expression at the mRNA and protein levels in normal tissues. a) qRT-PCR studies of RNASET2 transcript expression in a normal human tissue cDNA panel. The data were normalized to that present in testis (red). b) Immunoperoxidase labelling with the polyclonal antibody to RNASET2 shows strong staining in red pulp macrophages and splenic sinusoids in the spleen and macrophages in the thymus. In the tonsil, high levels of RNASET2 were present in the germinal centre (GC) tingible body macrophages (arrowed) whilst punctate staining was observed of the lymphocytes particularly those in the interfollicular areas (IF) (arrowheads). Double immunofluorescent labelling shows that RNASET2 was present in the CD20-positive B cells (arrowed) whilst double immunoperoxidase staining demonstrates the presence of RNASET2 in the CD3-positive T cells (arrowhead). Punctate cytoplasmic labelling was also observed in both the Islets of Langerhans and the secretory acini of the pancreas, and epithelial cells of the Fallopian tubes. Nuclear labelling was also observed in some cells in these tissues (arrowhead).

proteolytic fragments reported to be present in the lysosome [42,43]. However, nuclear labelling was also observed in both haematopoietic and non-haematopoietic cells as well as the

RNASET2 B16 transfectants. Liu et al. previously described both nuclear and cytoplasmic patterns of RNASET2 labelling in RNASET2 transfectants [50] whilst more recently, there

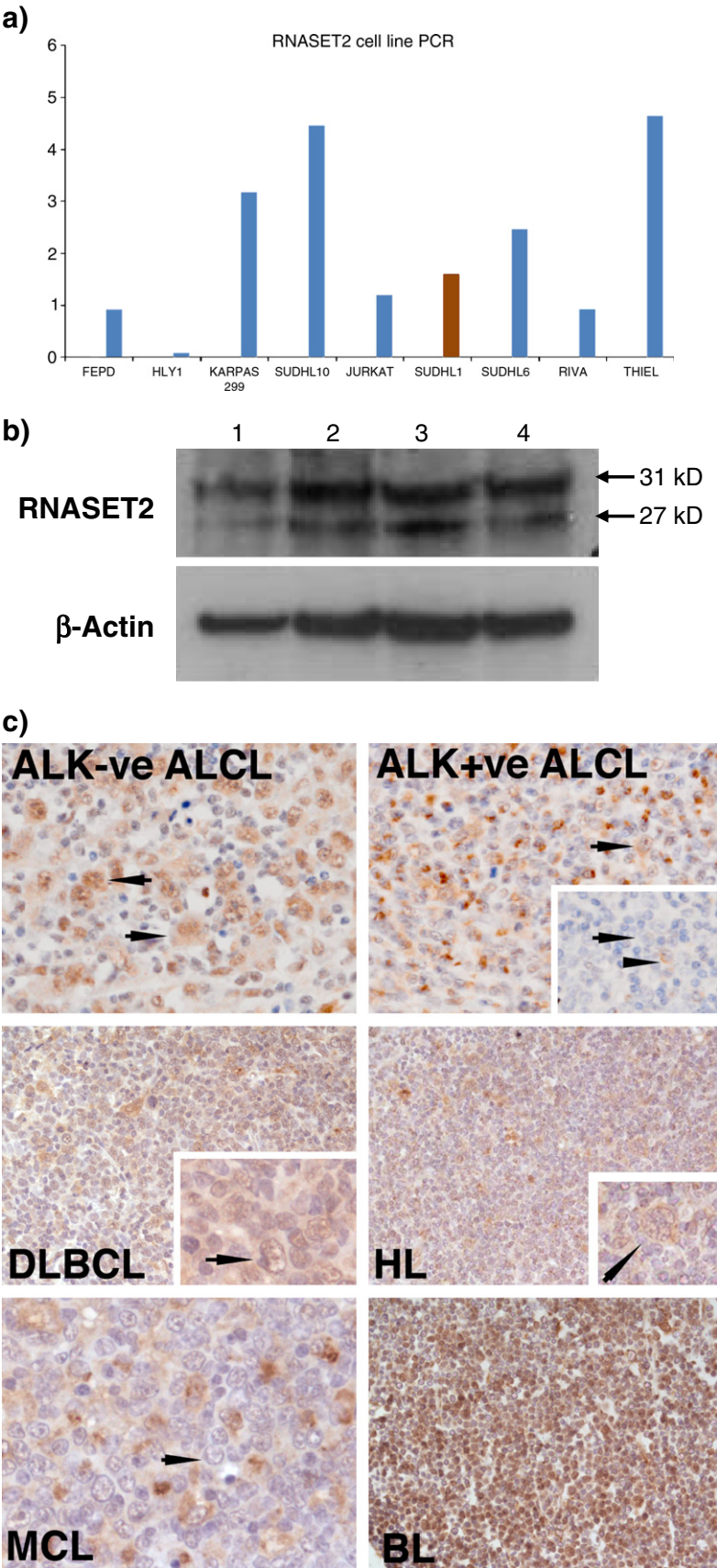


Table 2 – RNASET2 expression in haematological malignancies.

Lymphoma	RNASET2 expression		
	+	+/-	-
T cell			
ALK-negative anaplastic large cell lymphoma (n=42)	5 (C) 19 (N/C)	2 (C) 8 (N/C)	8
ALK-positive anaplastic large cell lymphoma (n=31)	10 (C) 7* (N/C)	1 (C) 2 (N/C)	11
T lymphoblastic lymphoma (n=1)	0	1 (N/C)	
Peripheral T-cell lymphoma (n=4)	2 (N/C)	2 (N/C)	0
B cell			
Diffuse large B-cell lymphoma (n=9)	2 (N/C)	5 (N/C)	2
Mantle cell lymphoma (n=7)	1 (N/C)	1(C) 3 (N/C)	2
Follicular lymphoma (n=7)	1 (N/C)	4 (N/C)	2
Burkitt lymphoma (n=7)	1 (N/C)	2 (C) 3 (N/C)	1
Hodgkin's lymphoma Lymphocyte predominant (n=3)	2 (N/C) 2 (N/C)	1 (N/C) 1 (N/C)	0 0
Mixed cellularity (n=3)	1 (N/C)	0	0
Nodular sclerosis (n=1)			

N = nuclear labelling, C = cytoplasmic labelling. *The tumour cells showed strong granular labelling for RNASET2.

appears to be a small amount of nuclear labelling in the HEK transfectants in the zebrafish study on RNASET2 [51]. Interestingly, analysis of the RNASET2 protein sequence shows the presence of a C-terminal nuclear localisation signal (TRG_NLS_MonoExtN_4: 249PPPKTKH256) which overlaps with a classical ER retention and retrieving signal (TRG_ER_dilys_1: 252KKTKH256) (<http://elm.eu.org/>). A very similar C-terminal arrangement of functional NLS and ER localisation/anchoring signals regulates the subcellular localisation of GRP58 [52], and could therefore play a similar role in RNASET2.

Downregulation of RNASET2 has been previously reported in primary ovarian tumours and cell lines [41,42,47,53] and in glioblastoma cell lines [54]. In the present study, however, RNASET2 protein was detected not only in cell lines derived from a wide range of haematological malignancies but also in a variety of primary lymphomas. Circulating autoantibodies to RNASET2 were also detected in lymphoma patients. This finding, combined with the low number of autoantibodies detected in breast cancer patients and the absence in patients with colorectal cancer, provides further evidence supporting a

difference for RNASET2 between haematological and solid tumours.

Of interest are the reports that chromosomal abnormalities at 6q27, the site of the RNASET2 gene are amongst the most frequent chromosomal changes in non-Hodgkin's lymphomas [55–58]. Although there is no evidence for RNASET2 mutations in lymphoma, downregulation of the RNASET2 gene has been reported [59]. The presence of a 5.5kB transcript in haematological tissues coupled with the report of RNASET2 mRNA encoding a splice variant lacking 27 codons in exon-1 in a range of cell lines [48,53] support the possibility of additional RNASET2 proteins with different subcellular localisations and aberrant tumour suppression functions. Thus, whilst there is a lack of evidence of mutations in RNASET2 or promoter methylation controlling RNASET2 expression in ovarian cell lines [27] the situation may differ in haematological malignancies.

Both nuclear and cytoplasmic labelling were observed in subsets of B- and other T-cell derived lymphomas. The heterogeneity of RNASET2 expression within haematological malignancies is of particular interest given reports of RNASET2

Fig. 3 – Analysis of RNASET2 expression in haematological malignancies. a) qRT-PCR analysis of RNASET2 transcript expression in a range of cell lines derived from haematological malignancies. The data were normalized to that present in the SUDHL-1 cell line (red). b) Western blotting of cell lines derived from ALCL and T-ALL. RNASET2 proteins of 31 and 27 kD were detected in the FEPD (Lane 1, ALK-negative), SUDHL-1 (Lane 2) and Karpas 299 (Lane 3, ALK-positive) ALCL-derived cell lines and the Jurkat (lane 4) T-ALL cell line. Actin was as used as a sample loading control. c) RNASET2 protein was detected in variety of lymphomas by immunoperoxidase labelling. The predominant pattern in ALK-negative ALCL was both nuclear and cytoplasmic. The case of ALK-positive ALCL shows the presence of localised areas of cytoplasmic staining in the tumour cells (arrowed). The inset shows a case of ALK-positive but RNASET2-negative ALCL (arrowed). RNASET2-positive macrophages can be observed (arrowhead). Nuclear and weak cytoplasmic labelling is shown in the case of DLBCL and the Reed-Sternberg cells in the HL (arrowed). In MCL, the RNASET2-negative tumour cells (arrowed) can be seen amongst the RNASET2-positive macrophages whilst the majority of the neoplastic cells in BL possess both a nuclear and cytoplasmic pattern of staining.

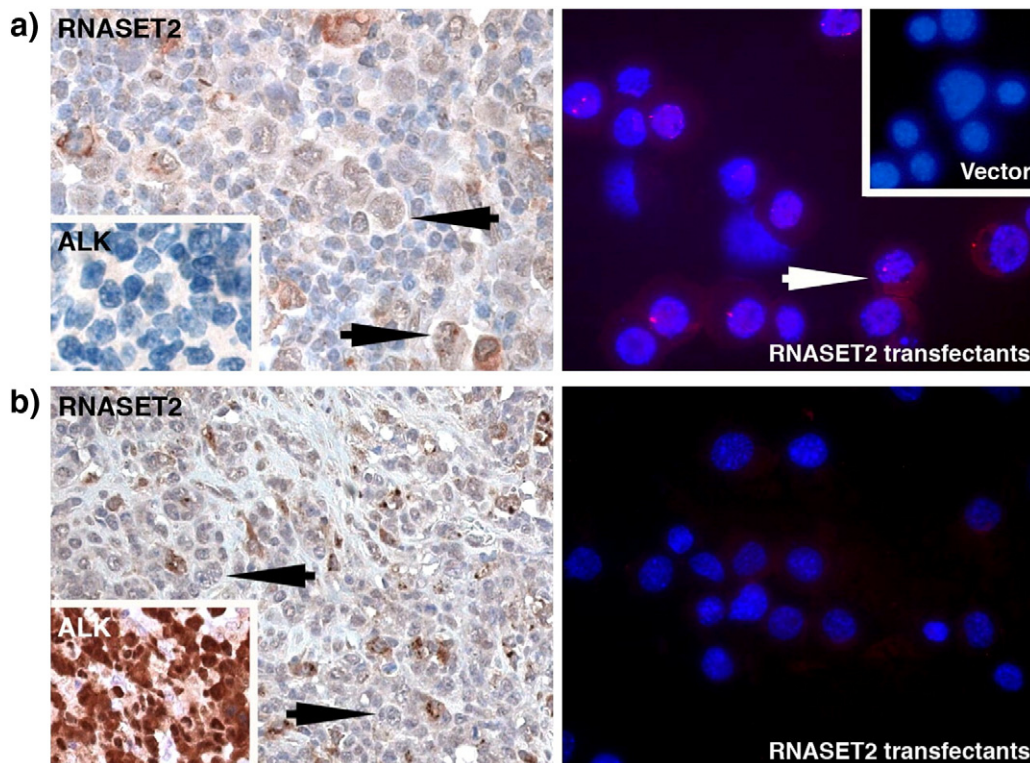


Fig. 4 – Examples of immunoperoxidase and immunofluorescent labelling studies on biopsies and RNASET2 transfectants obtained using ALCL serum samples used to screen the protein microarrays. **a)** Left-hand: Immunoperoxidase labelling shows RNASET2 expression in the tumour cells from a patient with ALK-negative ALCL (arrowed). Inset shows the ALK-negative tumour cells. Right-hand: Serum from this patient contained antibodies labelling the nuclei and cytoplasm of the RNASET2 transfectants (arrowed). In contrast, no labelling was detected in the vector only control (inset). **b)** Left-hand: The tumour cells in this ALK-positive ALCL patient were RNASET2 negative (arrowed) but expressed high levels of ALK (inset). Note the staining of RNASET2-positive macrophages (arrowhead). Right-hand: Strong labelling of the RNASET2 transfectants by the serum from this patient was not detected.

functioning as a tumour antagonising/malignancy suppressor gene [47,49]. Such proteins also appear to play a vital role(s) in the regulation of tumour growth by the tumour microenvironment *in vivo* [27,42,49,60–62]. This raises the possibility of RNASET2 as a possible therapeutic TAA given the importance of the role of the tumour microenvironment in lymphoma biology and disease progression [63,64].

RNASET2 is a 'self antigen' and immune responses to other 'self' antigens such as p53 [65], *Her2/neu* [66] and protein kinase A have been reported in disease situations [67]. The possibility of aberrant RNASET2 expression due, for example, to its subcellular distribution and the possible presence of splice variants with or without the effect of the tumour microenvironment, may therefore explain the presence of RNASET2 autoantibodies. Of interest are the recent publications describing the potential involvement of RNASET2 in vitiligo [68] and Graves disease [69], both of which are autoimmune disorders.

This paper describing the use of a protein macroarray and sera from lymphoma patients to identify lymphoma-associated TAAs is also the first report of RNASET2 as an ALCL-associated TAA. Additional studies on the biological and prognostic roles of the ribonuclease/tumour suppressor protein RNASET2 in larger cohorts of ALCL and other haematological malignancies are warranted. Such investigations may not only be of prognostic

significance but provide invaluable information on mechanisms underlying oncogenesis. RNASET2 represents an example of a potential autoantigen for use in the development of an ELISA-based and/or protein 'biochip' which might enable rapid non-invasive diagnostic procedures or methods of monitoring treatment efficacy.

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Contributions to the paper: SP, HC, LM, JS, DY, EG, EH, AL, KP, RT and FA – performed the research and analysed the results; GR, LM, FA, CSRH, RT and WW contributed essential reagents

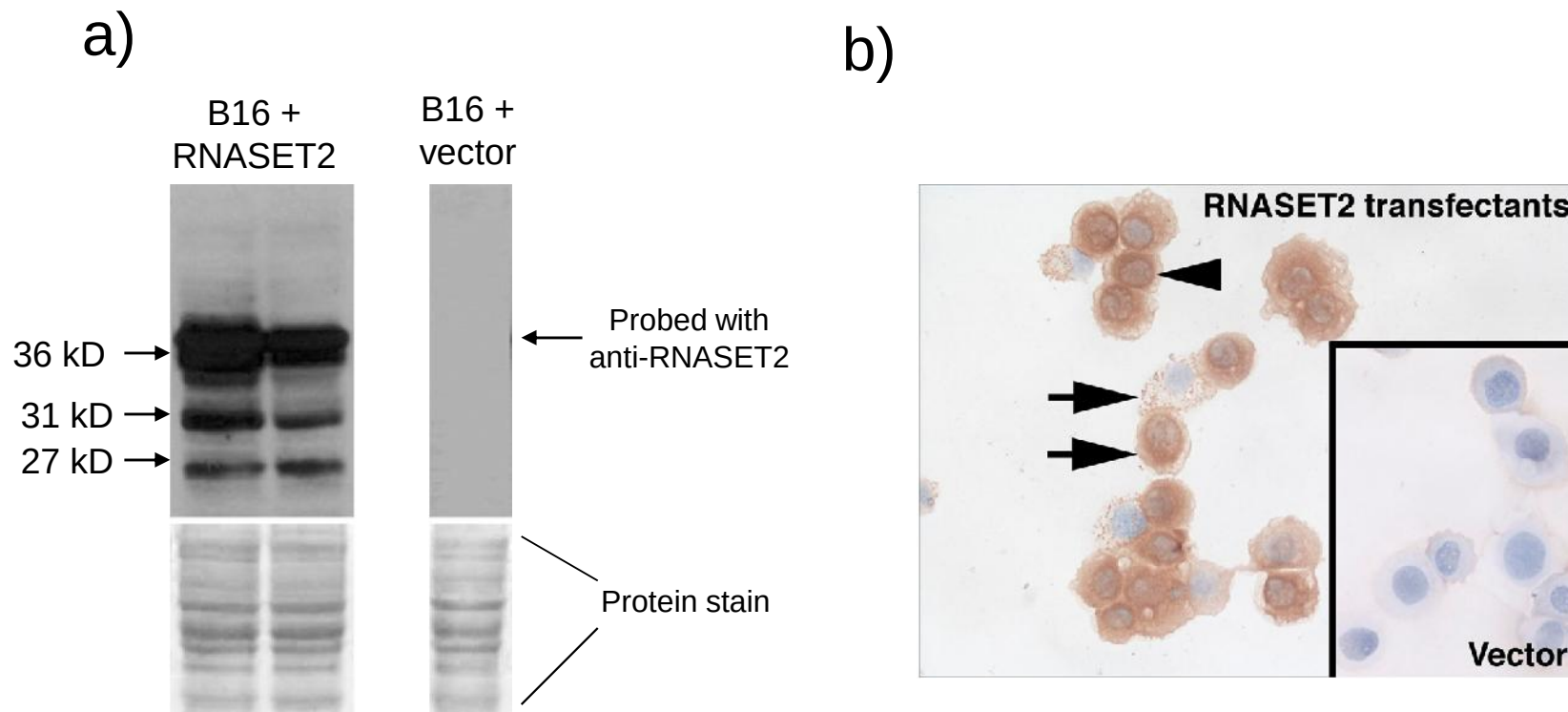
and/or tools; KP, AHB, AL and DM designed the research and analysed the data. All authors made an intellectual input and contributed to writing the paper.

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Supplementary Figure 1



Validation of the polyclonal anti-RNASET2 antibody using immunolabelling and Western blotting techniques.

a) The polyclonal antibody labelled RNASET2 transfectants but did not stain the B16 cells transfected with vector only using immunoperoxidase immunolabelling. Note the presence of nuclear (arrowhead) and cytoplasmic (arrowed) labelling of the transfected cells. b) Western blotting studies confirm the recognition of RNASET2 proteins of 36, 27 and 21 kD in the RNASET2 transfectants (arrowed). The presence of multiple bands suggests recognition by the antibody of a common epitope present on the RNASET2 isoforms.

Supplementary Table 1. Clinical details of ALCL patients whose sera were used in the initial screening studies on the protein microarray.

Patient	Sex	Age	Stage	Treatment	Response
ALK-positive					
001	M	41	II	CHOP	CR
002	M	16	I	ALCL'98	CR
003	F	34	II	CHOP, IDARAM/ESHAP, /TBI	CR
004	M	44	IIB	CHOP X 6	CR
005	F	58	III	CHOP X 8	DIED
ALK-negative					
006	F	65	N/A	N/A	DIED
007	F	53	N/A	BEAM/AUTO	DIED
008	M	28	IV	CHOP X 6 +RT	CR
009	F	49	IIB	CHOPX6/BEAM STEM CELL RESCUE	PD
010	M	52	Ie	CHOP X 6	DIED

CHOP –Cyclophosphamide, doxorubicin, vincristine, prednisolone; ALCL-98 - a modification of the NHL-BFM-B 90 treatment (Siedemann, et al 2001); IDARAM – idarubicin, dexamethosone, cytosine arabinoside, methotrexate; ESHAP – etoposide, methyprednisolone, cytarabine and cisplatin; TBI – total body irradiation; RT – Radiotherapy; BEAM - carmustine, etoposide, cytarabine, melphalan; AUTO – autologous transplant.

CR – Complete response; PR – Partial response:

Seidemann, K., et al. (2001) Short-pulse B-non-Hodgkin lymphoma-type chemotherapy is efficacious treatment for pediatric anaplastic large cell lymphoma: a report of the Berlin-Frankfurt-Munster Group Trial NHL-BFM 90. Blood, 97, 3699–706.

Supplementary Table 2. RNASET2 protein expression in cell lines derived from haematological malignancies.

Cell line	RNASET2 expression	
	Intensity of labelling	RNASET2 distribution
T-cell		
FEPD	+	+ Nuclei of 50% cells
SUDHL-1	+	Nuclei/cytoplasm of 20% cells
Karpas 299	++	Nuclei/ cytoplasm of 50% cells
Jurkat	+	Nuclei/cytoplasm of 50% cells
PEER	+++	Nuclei/cytoplasm of >95% cells
CEM	++	Nuclei/cytoplasm of 50% cells
B-cell		
SUDHL-4	+	Cytoplasm (50%)/nuclei (20%) of cells
SUDHL-6	+/-	Cytoplasm (30%)/nuclei (5%) of cells
SUDHL-10	+	Cytoplasm (100%)
RIVA	+/-	Nuclei/cytoplasm of 5% cells
HLY-1	+	10% Nuclei
Thiel	+	5% nuclei
Myeloid		
K562	+	Cytoplasm (50%)/Nuclei (10%) cells
HL60	++	Cytoplasm (<95%)/Nuclei (10%) of cells
U937	+/-	Cytoplasm (10%) cells

Intensity of labelling is as follows: ++ = strong, + = moderate and +/- = weak.