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Three childhood malignancies with striking morphologic and phenotypic similarities

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Chapter 2

High expression of calcium binding proteins, S100A10, S100A11 and CALM2 in anaplastic large cell lymphoma

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Summary

Anaplastic large cell lymphomas (ALCL) are characterized by the presence of CD30-positive large cells, which usually are of T-cell type. Based on the presence or absence of translocations involving the anaplastic lymphoma kinase (ALK) locus, ALCL cases can be divided in two groups. To gain more insight in the biology of ALCL, we applied serial analysis of gene expression (SAGE) on the Karpas299 cell line and identified 25 up- and 19 downregulated genes. Comparison of the differentially expressed genes with DNA copy number changes in Karpas299 revealed that two overexpressed genes, S100A10 and S100A11, were located in an amplicon suggesting that the increased mRNA levels were caused by DNA amplification. Quantitative reverse transcription polymerase chain reaction on 5 ALCL cell lines and 12 ALCL tissues confirmed the SAGE data for 13 out of 14 up- and one out of four downregulated genes. Immunohistochemical staining confirmed the presence of S100A10, a calcium binding protein, in three out of five ALK+ and all seven ALK− ALCL cases. S100A11 staining was confirmed in all ALK+ and six of seven ALK− ALCL cases. Three of the upregulated genes represented calcium-binding proteins, which suggest that altered intracellular signaling might be associated with the oncogenesis of ALCL.

Introduction

Anaplastic large cell lymphoma (ALCL) was first described as an entity in 1985.[1] In general, ALCL are defined by the presence of a cohesive proliferation of CD30-positive large cells with abundant cytoplasm and pleomorphic, often horseshoe-shaped nuclei (reviewed in Stein *et al*.[2]). Most ALCL cases are of T-cell type, but also null cell type ALCL occurs.[3,4] It has become apparent that two forms of ALCL can be distinguished, based on the presence or absence of a translocation involving the anaplastic lymphoma kinase (ALK) locus on chromosome 2 that leads to over expression of the ALK protein.[5,6] ALK-positive cases occur more frequently in children and young adults and have a relatively good prognosis with appropriate chemotherapy. ALK-negative ALCL occurs in older individuals and has a poor prognosis.[7,8]

Little is currently known about the biology of ALCL, except for the role of ALK. Identification of genes specifically expressed in tumors has been shown to be a useful method to understand the molecular mechanisms underlying pathogenesis. In ALCL, cDNA expression arrays have revealed a high expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) in 5 ALCL cell lines as compared to normal stimulated T-cells.[9] In pathological states TIMP-1 may inhibit tumor invasion and metastasis, and control tumor growth through the regulation of the extracellular matrix (ECM) homeostasis.[10,11] In another study using cDNA arrays, overexpression of CLU (Clusterin) was observed specifically in ALCL.[12] Clusterin has been implicated in several biological processes, including complement regulation, cell aggregation, lipid transport, apoptosis and response to cell stress or injury, but a definitive mechanism of action and role has not yet emerged.[13] Subtractive suppression hybridization and serial analysis of gene expression (SAGE) revealed high expression of MCL1, an anti-apoptotic member of the BCL2 family, in ALK+ ALCL.[14,15] These studies all demonstrate the power of gene expression profiling to identify biologically relevant genes in ALCL.

We applied SAGE[16] on the CD4+ ALCL derived cell line Karpas299 and used flow sorted resting CD4+ T-cells, as well as polarized activated T-helper 1 (Th1) and T-helper 2 (Th2) cells as controls. The SAGE technique enables the construction of a comprehensive expression profile and results in the quantification of expression levels of the corresponding genes.[16] Array comparative genomic hybridization (CGH)[17] was applied to detect DNA copy number changes and relate genomic aberrations to gene expression differences. The differentially expressed genes were

verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry.

Materials and methods

Cells and cell lines

CD4^{+} T-cells were isolated from the buffy-coats of healthy donors using the Fluorescence-activated cell sorter (FACS; MoFlo cytomation, Fort Collins, Colorado USA). After sorting, the CD4+ T-cells were incubated in Roswell Park Memorial Institute medium containing 10% fetal calf serum (RPMI-10% FCS) for 24 hr at 37°C. Five ALK+ anaplastic large-cell lymphoma derived cell lines were used to study the gene expression in ALCL; Karpas299 (American Type Culture Collection, Rockville, MD, USA) was used for SAGE and qRT-PCR; SU-DHL-1, SR786, SUP-M2 and DEL (Deutsche Sammung von Mikro-organismen und Zellkulturen (DSMZ), Braunschweig, Germany) were used for qRT-PCR. Array CGH was applied for Karpas299 to relate gene expression with genomic copy number changes. Frozen and paraffin-embedded ALCL tissue specimens were obtained from the Tissue Bank of the Department of Pathology from the University Medical Center Groningen. We randomly selected 5 ALK+ and 7 ALK− ALCL cases for qRT-PCR and immunohistochemical analysis. All protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research.

Serial analysis of gene expression

A detailed protocol for the SAGE procedure and a computer program (SAGE2000 version 4.12) for the analysis of gene-specific tags were kindly provided by Dr. K.W. Kinzler (John Hopkins Oncology Center, Baltimore, MD, USA).[16] We analyzed the Karpas299 ALCL cell line and CD4^{+} cells as their normal counterpart. SAGE libraries of polarized Th1 and Th2 cells constructed previously were used for comparison to identify activation-related genes.[18] Briefly the method comprises the isolation of mRNA followed by the synthesis of double stranded cDNA and digestion with NlaIII. The most 3-primed fragments were isolated and ligated to a linker sequence followed by a second restriction digest with BsmFI, resulting in 50 bp fragments containing a linker sequence and 10 to 12 gene-specific nucleotides downstream the most 3-primed NlaIII site (tag). After filling in of the sticky ends, the blunt-ended

DNA fragments were ligated, resulting in DNA fragments consisting of 2 tags flanked by the linkers. These fragments were amplified and digested with NlaIII, resulting in the so-called ditags. Ditags were ligated overnight to obtain concatemers and cloned in the pUC19 vector. The resulting clones were sequenced and analyzed with the SAGE2000 software.[16] The expression profiles were compared using the SAGE2000 software and Microsoft-access, SAGE tags with fivefold increased or decreased counts in Karpas299 when compared with CD4^{+} T-cells were selected for qRT-PCR. Activation related genes were excluded for qRT-PCR based on expression at similar levels in Karpas299 compared with average expression in Th1 and Th2 cells.[18] The 14 bp gene-specific sequences were linked to the Unigene library (**http://www.sagenet.org/resources/genemaps.htm**; Human 03/01) to identify the corresponding genes.

Array CGH

The genome-wide microarray used in this study consists of the 1-Mb BAC collection obtained from Dr Nigel Carter (Wellcome Trust Sanger Institute, UK) supplemented with a subset of the clones from the Human BAC Resource Consortium_1 Set (Dr Pieter de Jong, Children's Hospital Oakland Research Institute,CA, USA), and with a small selection of BACs to fill a few remaining gaps. For the positioning of the BACs relative to the human sequence we have used the May 2004 human reference sequence (University of California, Santa Cruz (UCSC) version hg16) that is based on National Center for Biotechnology Information (NCBI) Build 35.

BAC DNA isolation, amplification and spotting were carried out as reported previously[19] using epoxy-coated slides (Schott Nexterion, Mainz, Germany) and a MicroGrid II arrayer (BioRobotics, Cambridge, UK). Cot1 DNA and *Drosophila* BACs DNA were spotted as control.

Genomic DNA was labeled with either Cy3- or Cy5-dUTP (Perkin Elmer, Boston, MA, USA) using the BioPrime DNA Labeling System (InVitrogen, Breda, the Netherlands). Reference DNAs (male or female) were pools of 20 different individuals. Hybridization was performed overnight at 65°C for 40 hours as described previously.[19] Post hybridization washes were carried out as recommended by the manufacturer of the slides. Slides were dried by spinning for 3 min at 800 r.p.m. at room temperature.

After appropriate washing steps, arrays were scanned using the Affymetrix 428 scanner (Affymetrix, High Wycombe, UK) and the images were analyzed with

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Imagene software package 5.0 (BioDiscovery inc., El Segundo, CA, USA). Data were further processed with specifically designed data-analyses software.[19]

qRT-PCR analysis

Total RNA from the cell lines was isolated with Trizol (Life Technologies Inc., Gaithersburg, MD) and from the ALCL tissue samples using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA). The first-strand cDNA synthesis, primed with random primers, was performed using the protocol provided by the manufacturer (Life Technologies Inc.). Quantitative PCR was performed for differentially expressed genes, using primers and probes listed in table 1. RNA polymerase II (RPII) was used as a positive control and for normalization. Real-time PCR reactions were performed in triplicate in a 20 µl reaction volume containing $1 \times$ SYBRgreen mix (Applied Biosystems, Foster City, CA, USA), 600 nmol/l primers, and 1 ng cDNA. Reactions were performed on an ABI7900HT Sequence Detection System device (Applied Biosystems) using the standard program (10 min at 95°C followed by 40 cycles of 15 sec at 95°C, and 60 sec at 55°C). All PCR reactions were performed in triplicate, positive and negative controls were included in each run. Fluorescence was quantified with the sequence detection system software (SDS, version 2.0, Applied Biosystems). Mean cycle threshold values (Ct) and standard deviations (SD) were calculated for all genes. The amount of target gene was normalized relative to the amount of RPII ($\Delta Ct = \Delta Ct_{(gene)} - \Delta Ct_{(RPII)}$) and the SD of the ΔCt (SD(ΔCt)) was calculated (SD(ΔCt)=$\sqrt{((SD_{gene})^2 + (SD_{RPII})^2)}$). The factor difference is calculated ($2^{-\Delta Ct}$).

Immunohistochemistry

Immunohistochemistry was applied using standard laboratory procedures. S100A10 staining was performed using an antibody against Annexin light chain (clone 148, BD Transduction, Erebodegem, Belgium). As a detection system Envision (DAKO, Glostrup, Denmark) was used in combination with 3-amino-9-ethylcarbazol (AEC, Sigma-Aldrich, Zwijndrecht, The Netherlands). The guinea pig anti-S100A11 polyclonal antibody, which was developed and characterized as described elsewhere in Ref.[20], was used to demonstrate the presence of S100A11 in ALCL. Rabbit ant-Guinea pig Ig peroxidase labeled, followed by Goat anti-Rabbit Ig peroxidase labeled antibodies (DAKO) were used with AEC as substrate for detection. Galectin-1 staining was performed using anti-Galectin-1 (clone25C1, Novocastra, Newcastle

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upon Tyne, UK). On frozen sections Rabbit anti-Mouse Ig Peroxidase labeled, followed by Goat anti-Rabbit Ig Peroxidase labeled antibodies (DAKO) were used with AEC as substrate for detection. On paraffin sections antigen retrieval by microwave was performed using EDTA pH 9 as buffer, similar secondary antibodies as on frozen sections with diaminobenzidin as substrate. CD7 was stained using clone M-T701 (BD Biosciences, Erebodegem, Belgium) with Rabbit anti-Mouse Ig and Goat anti-Rabbit Ig (both peroxidase labeled) as secondary antibodies and AEC as substrate. We routinely included positive control sections and negative staining controls (first incubation step without primary antibody) in each run.

Table 1. Primers selected for qRT-PCR

Gene	Forward primer	Reverse primer	Amplicon length	Exon
S100A10	aggagttccctggatttttgg	tacactggtccaggtccttcatta	78	2-3
CLU	tcctaccagtggaagatgctcaa	cttcgccttgcgtgaggtt	100	7
SUI1	cgtatcgtatgtccgctatcca	caggtcatcacccttacttgca	74	1-2
MRCL3	ggtagcagggtggtgtgatagc	ttgttcttttgctcgacatggt	104	1/2-2
CALM2	tggtcgcgtctcggaaac	tctttgaattctgcaatctgctctt	78	1-2
SERPINA1	ctgggcatcactaaggtcttcag	agtccctttctcgtcgatggt	120	3-4
LGALS1	catcctcctggactcaatcatg	tcgcactcgaaggcactct	82	1-2
IFITM3	cgtctggtccctgttcaacac	caaccatcttcctgtccctagact	98	1/2-2
IARS	cacgtgactggacaatttcca	ttccgccactgacccaat	104	14-15
ZNF9	tgcctgctataactgcggtaga	gtcgcagtcacgagccagat	121	3/4-4
HPCAL1	gccgcagcgagatgct	tccggcatcttcatcacaga	70	3-4
ADFP	cctgctcttcgcctttcg	tgcaacggatgccattttt	68	1-2
ZNF169	gggaggtgatgctggagaactac	tgttcgatgagttttggtttgg	76	2-3
E2IG5	tcatatggtcaggtcgctttcaa	tgttctttgcagcatcaagca	77	2-3/4
S100A11	tacagaactagctgccttcacaaag	ctgttggtgtccagtttcttcatc	81	2-3
UBA52	gagcccagtgacaccattga	gctgctggtcaggtgggata	73	1-2
LAPTM5	catgaactcggtggaggagaa	caaagacagggcttcctcgta	81	7-8
FTL	gcaggcctcctacacctacct	cgccttccagagccacat	68	1-2
NRAS-related gene	tgatccaacacagactgagtacca	caaatggatagacctcacctttca	80	17-18
RPII	cgtacgcaccacgtccaat	caagagagccaagtgtcggtaa	139	16-17

Results

SAGE

The SAGE technique was applied to study gene expression differences in the ALK+ ALCL derived cell line Karpas299 in comparison to resting $CD4^+$ T-cells isolated from total blood. The SAGE data were also compared to the previously constructed SAGE libraries of polarized activated Th1- and Th2-cells.[18] For Karpas299 10 678 tags

were sequenced (representing 5090 different genes), for CD4+ T-cells 8425 tags (representing 4467 different genes), for Th1-cells 8821 tags (representing 4868 different genes) and for Th2-cells 7934 tags (representing 4417 different genes). The SAGE tags were linked to the Unigene database, which revealed identification of the corresponding genes for the vast majority of SAGE tags. Application of the selection criteria (5 fold difference) revealed 25 upregulated and 19 downregulated genes in Karpas299 as compared to CD4+ T-cells (Tables 2 and 3). Of the 25 upregulated genes 11 demonstrated a similar expression level in the Th1 and Th2 SAGE libraries. For 15 of the 19 downregulated genes a low tag frequency was also observed in Th1 and Th2 cells. The Karpas299 specific upregulated and downregulated genes were selected for qRT-PCR.

Array-CGH

Array CGH was used to characterize genomic copy number changes in Karpas299. Gain or loss of chromosomal material was observed for several regions (Fig 1A). High level amplifications were detected at three genomic loci, 1q12~q21, 10p12 and 19q13. Intergration of the chromosomal positions for the 4-fold upregulated (25 + 34) and downregulated (19 + 43) genes into the array CGH data revealed co-localization of S100A10 with the amplicon at 1q12~q21 (Fig 1B). A second gene mapping to this amplicon, S100A11, was slightly upregulated in Karpas299 (Table II). Loss of one allele was observed for the telomeric region of chromosome arm 19q (Fig 1C) and three of the down regulated genes, EMP3, FTL and CD37 all mapped within this region. For the remaining differentially expressed genes no clear relation was observed between copy number changes and expression levels.

QRT-PCR on cell lines

ALK+ ALCL cell lines Karpas299, SUP-M2, SR786, SU-DHL-1 and DEL were analyzed by qRT-PCR for 14 up-regulated genes plus S100A11, and for four down-regulated genes. Fourteen of 15 up-regulated genes indeed demonstrated higher expression levels in KARPAS299 confirming the SAGE results. For ZNF9, a similar mRNA level was observed in resting CD4+ cells as in KARPAS299. For 2 upregulated genes ADFP and MRCL3, a high expression level was observed only in Karpas299 and not in the other ALCL cell lines (Fig 2A). For 12 upregulated genes CALM2, CLU, E2IG5, HPCAL1, IARS, IFITM3, LGALS1, SERPINA1, SUI1, S100A10, S100A11 and ZNF169, a high expression was observed in Karpas299 and in at least one of the other ALCL
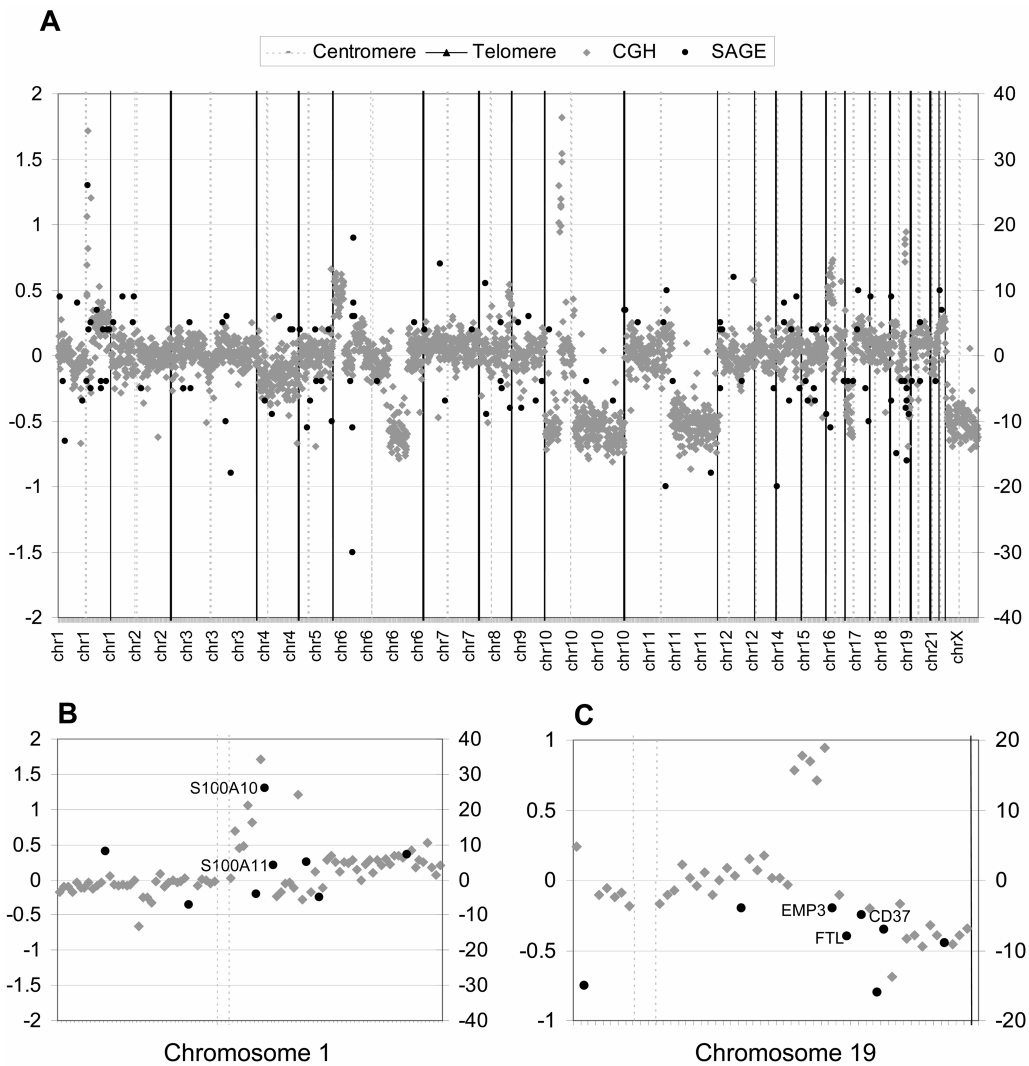
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cell lines compared to CD4+ T-cells.

Figure 1. Comparison of array CGH results and differentially expressed genes obtained with SAGE on ALCL derived cell line Karpas299. A; Schematic representation of SAGE (including all 4 fold up- and downregulated genes) and array CGH data for all chromosomes. B; Schematic representation of the chromosome 1q12~q21 amplicon. The two upregulated genes S100A10 and S100A11 and MCL1 previously reported to be upregulated map within the amplified region. C; Schematic representation of the telomeric region of chromosome 19 with loss of one allele. Three down regulated genes, EMP3, FTL and CD37, map within the deleted region. The black spots indicate the genes obtained with SAGE and the gray diamonds indicate the ratio's observed for the BACs and their position on the genome. The left scale indicates the CGH ratio and the right scale indicates the relative expression factor of the 4-fold up- and down-regulated genes in Karpas299 compared to CD4+ T-cells.

Table 2. Genes at least 5 times up-regulated in Karpas299 compared to CD4+ T-cells

Tag sequence	K299 TH‡	CD4	Gene symbol	Gene	Biological Process
GTTCACATTA	46	7	0	CD74	CD74 antigen	Immune response
GGGGAAATCG	26	21	5	TMSB10	thymosin, beta 10	Cell growth and/or maintenance
TTGGGGTTTC	22	6	4	FTH1	ferritin, heavy polypeptide 1	Transport
CCTAGCTGGA	14	27	0	PPIA	peptidylprolyl isomerase A	Protein metabolism
GTGCTGAATG	12	7	1	MYL6	myosin, light polypeptide 6	Cell growth and/or maintenance
AACGCGGCCA	10	12	1	MIF	macrophage migration inhibitory factor	Signal transduction/ Cell communication
GAAGCAGGAC	10	6	0	CFL1	cofilin 1 (non-muscle)	Cell growth and/or maintenance
GTAGCAGGTG	9	3	0	M6PRBP1	mannose 6 phosphate receptor binding protein 1	Transport
TACAGTATGT	7	6	0	GLUL	glutamate-ammonia ligase	Metabolism/ Energy pathways
ACCATTGGAT	7	4	0	IFITM1	interferon induced transmembrane protein 1 (9-27)	
TAGTTGAAGT	5	2	0	UQCRB	ubiquinol-cytochrome c reductase binding protein	Energy and metabolism
AGCAGATCAG	53	0	2	S100A10	S100 calcium binding protein A10	Signal transduction; Cell communication
CAACTAATTC	11	0	0	CLU	clusterin	Immune response
TGAAGTAACA	10	0	1	SUI1	putative translation initiation factor	Translation regulation/ Protein biosynthesis
CCCTTAGCTT	9	2	0	MRCL3	myosin regulatory light chain MRCL3	Cell growth and/or maintenance
TTGTTGTTGA	9	0	0	CALM2	calmodulin 2	Signal transduction; Cell communication
GGAAAAGTGG	9	0	0	SERPINA1	serine (or cysteine) proteinase inhibitor, clade A	Protein metabolism
GCCCCCAATA	7	2	0	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)	Immune response
ACCTGTATCC	7	0	0	IFITM3	interferon induced transmembrane protein 3 (1-8U)	Immune response
TTGGGAGCAG	6	1	0	IARS	isoleucine-tRNA synthetase	Protein metabolism
CGTGTTAATG	6	0	0	ZNF9	zinc finger protein 9	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
TGTGCCCTGA*	5	0	0	HPCAL1	hippocalcin-like 1	Signal transduction; Cell communication
TGTGCCCTGA*	5	0	0	ZNF169	zinc finger protein 169	Regulation of transcription, DNA dependent
ATGCTTGCTT	5	0	0	ADFP	adipose differentiation-related protein	Transport
GTTATTGAGG	5	0	0	E2IG5	growth and transformation-dependent protein	
CAGGCCCCAC†	4	1	0	S100A11	S100 calcium binding protein A11	Signal transduction; Cell communication

*This SAGE tag matches reliable with two genes.
†S100A11 was included in qRT-PCR analysis based on its location in the 1q amplicon.
‡Tag frequencies in the TH cell library represent the average frequency obtained in TH1 and TH2 cells.[18]
The grey rows indicate the genes that are selected for qRT-PCR.

Table 3. Genes at least 5 times downregulated in Karpas299 compared with CD4+ T-cells

Tag sequence	K299	TH	CD4	Gene Symbol	Gene	Biological process
CAGATCTTTG	1	10	15	UBA52	ubiquitin A-52	Protein metabolism
GCGGTTGTGG	0	9	13	LAPTM5	Lysosomal-associated multispanning membr prot 5	Signal transduction; Cell comunication
CCCTGGGTTC	0	4	8	FTL	ferritin, light polypeptide	Transport
CAAATGAGGA	0	3	7	D1S155E	NRAS-related gene	Regulation of transcription, DNA dependent
GTGGCAGGCG	1	3	11	GP2	glycoprotein 2	Regulation of cell adhesion/migration
GCCCAGCTGG	2	3	17	EEF1D	eukaryotic translation elongation factor 1 delta	Signal transduction; Cell comunication
CACCTGTAGT	0	1	10	DHX8	DEAH (Asp-Glu-Ala-His) box polypeptide 8	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
GCCAAGGAGG	0	0	10	CD7	CD7 antigen	Immune response
TGGAAGCACT	0	0	9	IL8	interleukin 8	Immune response
CCCAACGCGC	0	0	9	HBA2	hemoglobin, alpha 2	Transport
GGATGTGGAG	0	0	7	TLE3	transducin-like enhancer of split 3	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
AGACAAGCTG	0	1	7	SFRS5	splicing factor, arginine/serine-rich 5	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
TAGAAAGGCA	0	2	7	GEMIN7	gem (nuclear organelle) associated protein 7	mRNA processing
AGGCTCCGTG	0	0	7	HA-1	minor histocompatibility antigen HA-1	Immune response
GCCTTCCAAT	0	2	5	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
GTGGTACACA	0	1	5	CHDH	choline dehydrogenase	Metabolism; Energy pathways
TACTAGTCCT	0	2	5	HSPCA	heat shock 90kDa protein 1, alpha	Protein metabolism
CAATTTGTGT	0	0	5	IL1B	interleukin 1, beta	Immune response
ACGCTCTCGA	0	0	5	CD37	CD37 antigen	Immune response

The grey rows indicate the genes that are selected for qRT-PCR.

For the downregulated genes, SAGE data could only be confirmed for LAPTM5, which showed a consistent low expression level for all 5 cell lines (Fig 2B). The other three genes could not be confirmed by qRT-PCR possibly due to different donors used for SAGE and qRT-PCR or incorrect SAGE to gene linking.

QRT-PCR for ALCL tissue samples

Genes consistently up- or downregulated in two or more ALCL cell lines compared to CD4+ T-cells were selected for qRT-PCR on 5 ALK+ and 7 ALK– ALCL cases (Fig 3A). A mean expression level higher than the expression level observed for the housekeeping gene RPII was observed for CALM2, CLU, IFITM3, LGALS1, SERPINA1, SUI1, S100A10 and S100A11 in ALCL tissues. An expression level similar to the RPII gene was observed for 3 genes, E2IG5, HPCAL1 and IARS. The average expression level of ZNF169 was lower than the expression level observed for RPII. Differences between ALK+ and ALK– groups were tested by a non-parametric Mann-Whitney *U*-test and revealed a significant difference only for ZNF169 (p=0·018). Expression levels were higher in the ALK– ALCL cases. In comparison with normal reactive lymph node tissue, increased expression levels were observed only for LGALS1, S100A11 and SERPINA1 (Fig 3B). LAPTM5 demonstrated reduced expression levels in 9 out of 12 ALCL cases, with no relation to presence or absence of ALK protein expression (Fig 3C).

Immunohistochemistry

Immunohistochemical staining for S100A10 revealed positive cytoplasmic and membrane staining in all ALCL cell lines and based on the morphology also in the tumor cells of three out of five ALK+ cases and of all seven ALK– cases (Fig 4A and 4B). The pattern of staining was similar in cell lines and tumor cells with an evenly distributed cytoplasmic stain and a stronger staining immediate under or at the cell membrane. Staining for S100A11 showed positive cytoplasmic staining in all ALCL cell lines and in all or part of the tumor cells in five ALK+ cases and in six out of seven ALK– cases (Fig 4C and 4D). Positive staining for galectin-1 was not observed in ALCL cell lines and the tumor cells of ALCL tissue samples. However, a strong staining was observed in part of the stromal cells of all ALK+ and ALK– cases (Fig 4E). As it is known that galectin-1 can induce apoptosis by binding to CD7 in T cells, we also stained the ALCL cases for CD7 (Fig 4F); this revealed no CD7 expression in the tumor cells of the ALCL cases.
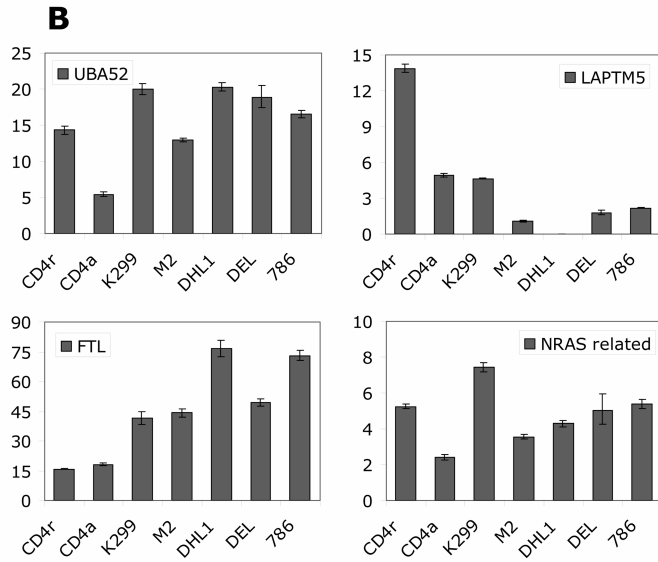
A

B
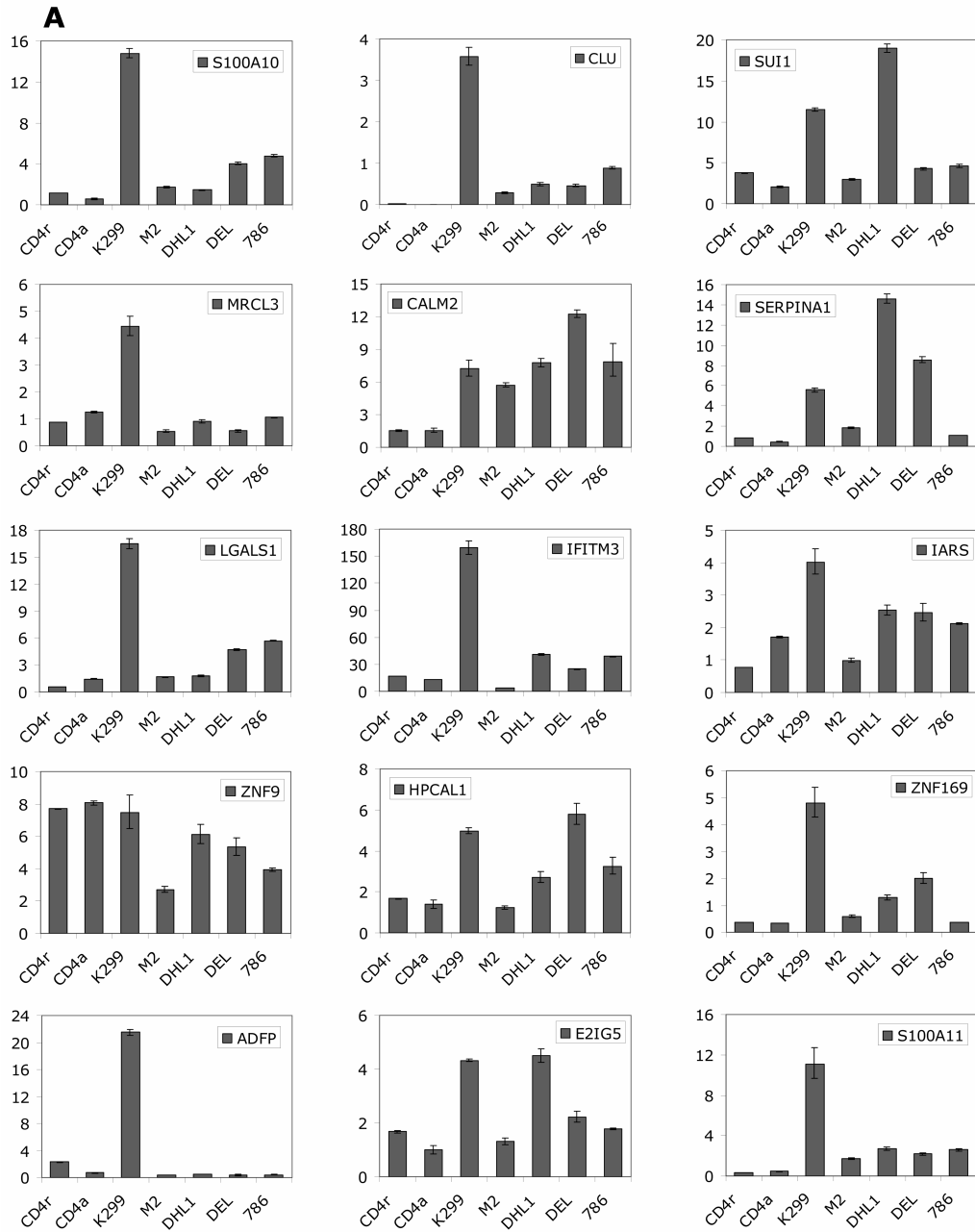
Figure 2. qRT-PCR results for the upregulated and downregulated genes on 5 ALCL derived cell lines and as comparison resting CD4+ T-cells (CD4r) and activated CD4+ T-cells (CD4a). **A**; Relative S100A10, CLU, SUI1, MRCL3, CALM2, SERPINA1, LGALS1, IFITM3, IARS, ZNF9, HPCAL1, ADFP, ZNF169, E2IG5 and S100A11 expression in ALK+ ALCL derived cell lines Karpas299, SUP-M2, SU-DHL-1, DEL and SR786. **B**; Relative UBA52, LAPTM5, FTL and NRAS related gene expression in ALK+ ALCL derived cell lines Karpas299, SUP-M2, SU-DHL-1, DEL and SR786. The bars indicate the relative expression levels.
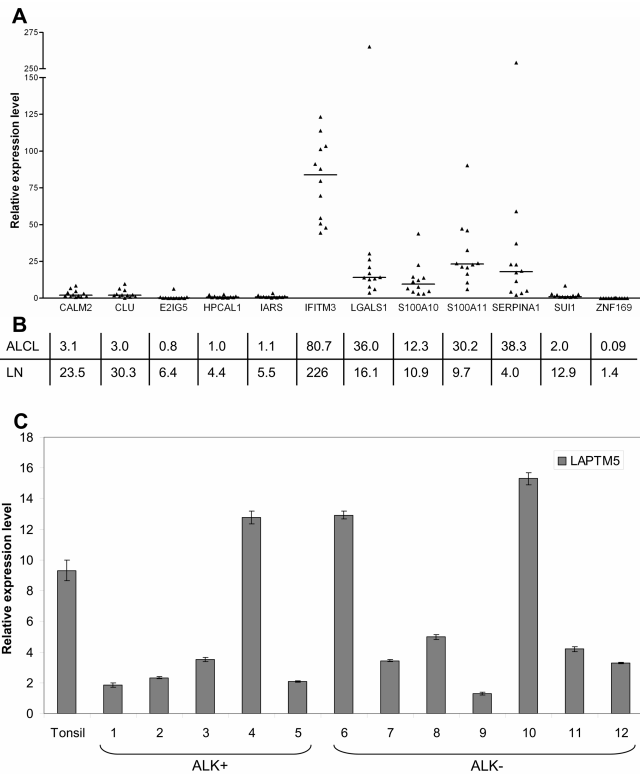

	CALM2	CLU	E2IG5	HPCAL1	IARS	IFITM3	LGALS1	S100A10	S100A11	SERPINA1	SUI1	ZNF169
ALCL	3.1	3.0	0.8	1.0	1.1	80.7	36.0	12.3	30.2	38.3	2.0	0.09
LN	23.5	30.3	6.4	4.4	5.5	226	16.1	10.9	9.7	4.0	12.9	1.4

Figure 3. qRT-PCR results for the up-regulated and down-regulated genes on ALK+ and ALK- ALCL cases. **A**; Median relative CALM2, CLU, E2IG5, HPCAL1, IARS, IFITM3, LGALS1, S100A10, S100A11, SERPINA1 and ZNF169 expression in ALCL cases. **B**; Mean relative expression level of the ALCL cases (12) and lymph nodes (LN)(3) for each gene in A. **C**; qRT-PCR results for LAPTM5 in the individual ALCL cases in comparison to normal control tissue.

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Discussion

In this study, comprehensive gene expression profiles from the ALCL derived cell line Karpas299 and normal CD4+ T-cells were analyzed and compared to identify differentially expressed genes in ALCL. We identified 25 upregulated and 19 down-regulated genes in Karpas299. For most of the selected genes differential expression was confirmed by qRT-PCR in Karpas299. In comparison to the other ALCL cell lines the expression levels were in general more pronounced in Karpas299, this is not surprising as we used the SAGE library of Karpas299 for the selection of the most differentially expressed genes. According to the ontology database (http://www.hprd.org), the differentially expressed genes appear to be involved in a variety of biological processes (Tables 2 and 3). The most striking difference between the up- and downregulated genes with respect to their biological function is the presence of four genes (TMSB10, MYL6, CFL1 and MRCL3) related to cell growth and/or maintenance which are identified only in the group of upregulated genes.

Most of the differentially expressed genes mapped at genomic loci with a normal copy number. This suggests that overall the deregulated expression is not caused by the copy number changes, but by transcriptional, post-transcription and other (epigenetic) factors like (de-)methylation of DNA, deacetylation of histone proteins, over or under expression of transcriptions factors.[21-23] Three regions had a higher copy number gain. A 4·5 Mb amplicon at 1q12~21 covered the genomic loci of S100A10 and S100A11, both of which demonstrated increased expression in Karpas299. Moreover, MCL1, previously reported to be highly expressed in Karpas299 and ALCL cases[14,15] also mapped within this chromosomal region. This suggests that gain of chromosome 1 material is associated with the increased expression levels of these three genes in Karpas299. Overexpression of S100A10 and S100A11 was also observed in the other ALCL cell lines and in most ALCL cases in our study. It is not clear if the association with 1q amplifications is a general finding in ALCL. Gains of 1q have been reported for the ALCL cell lines Karpas299 (1q~12-22) and DEL (1q21~q44) using conventional CGH.[24] Although gain of 1q has not been described as a common finding in primary ALCL[25,26] small amplicons like the one we observed in Karpas299 may have been missed due to lower resolution of cytogenetic and conventional CGH techniques.

Increased expression levels in comparison with CD4+ T-cells were observed for 12 genes in at least two of the ALCL cell lines. Comparison of ALCL tissues to normal lymphoid tissue revealed a consistent upregulation for three genes, e.g. S100A11,

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SERPINA1 and LGALS1. Despite the consistent increased expression levels in ALCL cell lines, the expression levels in ALCL tissues was similar to or reduced in comparison to normal lymphoid tissue for nine of the genes. It can be argued that reactive lymphoid tissue may not be the best control to establish increased expression levels because of admixed B-cells and stromal cells in the tissues. This is also nicely illustrated for CLU, which has been reported previously to be upregulated consistently in ALCL,[12] but in fact shows reduced mRNA expression levels when compared to normal lymphoid tissue (see Fig 3A). In comparison with the expression level of the house keeping gene RPII, which is fairly abundant in most cells and tissues, eight genes demonstrated a markedly increased expression level and three genes demonstrated an expression level similar to the housekeeping gene, indicating the presence of high transcript levels in ALCL tissues for these 11 genes. A reduced expression level as compared to the housekeeping gene was only observed for ZNF169.

The high expression of CLU and SERPINA1 (α1-antitrypsin) that was observed in ALCL was consistent with previous studies.[12,27-29] Analysis of 31 hematopoietic cell lines of B- and T-cell origin by cDNA expression arrays revealed expression of CLU specifically in the ALCL cell lines.[12] In ALCL tissue samples, a distinct Golgi staining pattern was demonstrated in all cases and is not or only rarely observed in other lymphoma subtypes.[12,30] Clusterin has been implicated in a variety of functions, including complement regulation, cell aggregation, lipid transport, apoptosis and response to cell stress or injury.[13] Although contradictory functions have been reported for Clusterin with respect to cell death dependent on cellular context, apoptotic stimulus or site of action,[31] it is generally accepted that it does play a role in oncogenesis.

SERPINA1 (α1-antitrypsin) is an inhibitor of serine proteases which is mainly produced by hepatocytes in the liver and neutralizes the effect of proteases in several organ systems. Its primary target is elastase, which is secreted by activated neutrophils and can be inactivated by binding to α1-antitrypsin. In oncogenesis, the imbalance of elastase and α1-antitrypsin might result in activation of matrix metalloproteinases leading to tumor invasion and metastasis and reduced response to tumor necrosis factor-α signaling leading to reduced apoptosis sensitivity (reviewed in Ref. Sun & Yang[32]). We demonstrated high expression of SERPINA1 mRNA in four out of five ALCL cell lines indicating that ALCL tumor cells are capable of producing α1-antitrypsin. At the protein level, several previous studies have

reported expression of α1-antitrypsin in tumor cells of ALCL cases and in normal T-cells upon activation.[27,28]
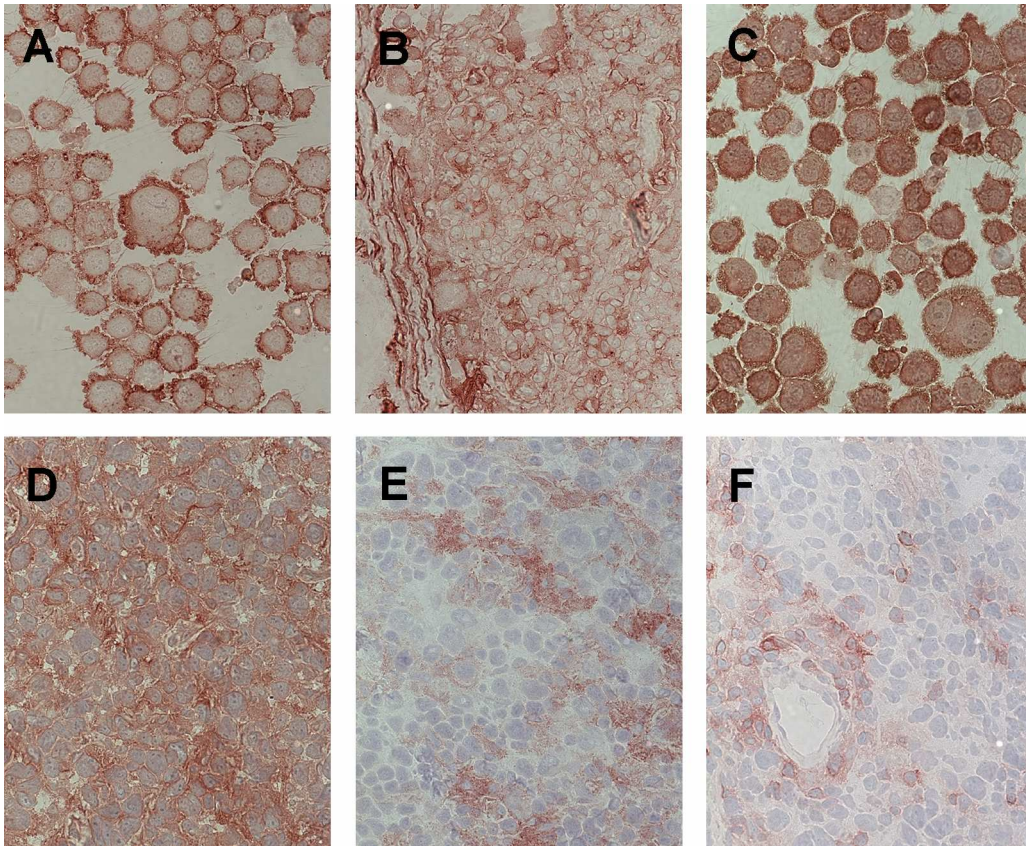

Figure 4. Immunohistochemical staining of S100A10, CD7 and Galectin1 in Karpas299 and in a representative case of ALCL. **A**; S100A10 positive cytoplasmic and membrane staining in Karpas299. **B**; S100A10 positive staining in an ALCL case. **C**; S100A11 positive cytoplasmic staining in Karpas299. **D**; positive cytoplasmic staining in an ALCL case. **E**; Galectin-1 staining in part of the stromal cells of an ALK+ ALCL case. **F**; Lack of CD7 staining in tumor cells of a representative ALCL case.

LGALS1 (galectin-1) is an endogenous lectin that binds beta-galactoside residues present on various T cell membrane markers. Via binding to the glycoprotein receptor CD7, galectin-1 triggers apoptosis in T-cells.[33,34] Our SAGE data revealed an increased expression level of LGALS1 and a reduced expression level of CD7 in Karpas299 (Tables 2 and 3). It is tempting to speculate that downregulation of CD7 acts as a protective mechanism against galectin-1 induced apoptosis in ALCL. At the mRNA level we observed high expression levels of LGALS1 in 3/5 ALCL cell lines and

in all ALCL cases. Immunohistochemical staining revealed positive staining for galectin-1 only in the surrounding cells and not in the tumor cells in ALCL tissues and no staining of CD7 in the tumor cells of both ALCL cases and cell lines. To our knowledge this is the first study describing galectin-1 expression in ALCL tissues. Lack of CD7 was observed previously in 13 of 19 ALCL cases confirming our data.[35] Based on our data we can speculate that in the presence of galectin-1 protein, produced by either the tumor cells or the normal surrounding cells, lack of CD7 protects the tumor cells from galectin-1 induced apoptosis.

High expression of IFITM3 (1-8u) was observed in 3/5 ALCL cell lines and in all ALCL cases. IFITM3 belongs to the 1-8 gene family and is involved in the transduction of antiproliferative signals.[36] In a previous study overexpression of IFITM3 was observed in colon cancer, with no relation to the extent and duration of the disease.[37] At present the function of IFITM3 is still unknown.

The only consistently downregulated gene in ALCL in the present study was LAPTM5 (Clast6/E3). LAPTM5 is a lysosomal-associated muIti-spanning membrane protein that is preferentially expressed in hematopoietic cells[38] and is involved in cellular differentiation and in apoptotic pathways. Inactivation of LAPTM5 was also observed in the majority of multiple myeloma cases,[39] whereas increased expression was observed in B cell lymphoma.[40]

Perhaps the most striking finding in the present study was the increased expression level of three EF-hand calcium binding proteins, CALM2, S100A10 and S100A11. These proteins have multiple target genes and play an important role in intracellular calcium signaling, which regulates a variety of cellular processes such as cell proliferation and gene transcription.[41] The expression level of CALM2 (Calmodulin) was higher than RPII in all ALCL cell lines and cases, albeit only moderately increased in some cases. There are no previous data on expression of CALM2 or other members of the Calmodulin family in ALCL or other lymphomas and its potential role in cancer development has not yet been elucidated. Treatment of breast cancer cell lines with a Calmodulin antagonist inhibits the *in vitro* growth, supporting a potential role for Calmodulin in proliferation of cancer cells.[42]

Several members of the S100 gene family map to the q-arm of chromosome 1 and altered expression levels, which are partly caused by rearrangements or deletions, have been observed in several forms of cancer.[43] We observed S100A10 and S100A11 overexpression in the ALCL cell lines and tissues, which was most noticeably for S100A11. S100 proteins regulate intracellular processes such as cell

growth and motility, cell cycle regulation, transcription and differentiation.[44] Involvement of S100A10 and S100A11 in oncogenesis is supported by the elevated expression in various cancers.[45-47] The functional S100A10 complex consists of two S100A10 and two annexin-A2 subunits. Besides its role in regulation of plasma membrane ion channels, S100A10 can also be expressed on the extracellular surface where it functions as a plasminogen receptor resulting in plasminogen activation.[48] This can promote invasiveness of the tumor.[49] S100A11 has been reported to cause increased expression levels of p21$^{CIP1/WAF1}$, which is a negative regulator of cell growth.[50]

In summary, we report a gene expression profile of the ALCL cell line, Karpas299. Only one consistently downregulated gene, LAPTM5, was observed in the ALCL cell lines and in 9/12 tissues. A consistently increased expression level was observed for most differentially expressed genes in the cell lines and tissues, including three calcium-binding proteins. This suggests that altered calcium-dependent intracellular signaling might be associated with the oncogenesis of ALCL. Increased expression levels observed for S100A10 and S100A11 genes in Karpas299 might be related to the amplicon present at 1q12~q21.

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