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High level of *cannabinoid receptor 1*, absence of *regulator of G protein signalling 13* and differential expression of *Cyclin D1* in mantle cell lymphoma

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Mantle cell lymphoma (MCL) is a moderately aggressive B-cell lymphoma that responds poorly to currently used therapeutic protocols. In order to identify tumour characteristics that improve the understanding of biology of MCL, analysis of oligonucleotide microarrays were used to define specific gene expression profiles. Biopsy samples of MCL cases were compared to reactive lymphoid tissue. Among genes differentially expressed in MCL were genes that are involved in the regulation of proliferation, cell signalling, adhesion and homing. Furthermore, some genes with previously unknown function, such as C11orf32, C2orf10, TBC1D9 and ABCA6 were found to be differentially expressed in MCL compared to reactive lymphoid tissue. Of special interest was the high expression of the cannabinoid receptor 1 (CB1) gene in all MCL cases analysed. These results were further confirmed at the cellular and protein level by immunocytochemical staining and immunoblotting of MCL cells. Furthermore, there was a reduced expression of a regulator of G protein signalling, RGS13 in all MCLs, with a complete absence in the majority of cases while present in control lymphoid tissue. These results were further confirmed by PCR. Sequencing of the RGS13 gene revealed changes suggesting polymorphisms, indicating that down-regulation of the expression of RGS13 is not related to mutations, but may serve as a new specific marker for MCL. Moreover, comparison between individual cases of MCL, revealed that the CCND1 gene appears to be differently expressed in MCL cases with high *vs* low proliferative activity.
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Introduction

Mantle cell lymphoma (MCL) is characterised by centrocytic morphology and expression of the B-cell markers CD19, CD20, CD79a together with the pan-T-cell marker CD5. These lymphomas lack the expression of CD10 and CD23. The majority of MCL has rearranged, but unmutated immunoglobulin genes, suggesting that the tumour originates from mature B cells that have not yet encountered antigen.[1] The current hypothesis is that the tumour cells are derived from the mantle zone of the B-cell follicles.

The incidence of MCL has been estimated to be 5% of all non-Hodgkin's lymphomas.[2] In many instances, advanced stage with bone marrow involvement is present already at the time of diagnosis and the median survival time is only 3–4 years.[2] However, the disease is heterogeneous and some cases have an indolent course.

The typical growth pattern of MCL is a nodular tumour proliferation with residual small germinal centres. However, many MCLs have an entirely diffuse growth pattern. The so-called blastoid MCL variant is characterised by larger, blastoid tumour cells and a high proliferative activity. The most characteristic feature of all MCL variants is the overexpression of the Cyclin D1/CCND1 (BCL1, PRAD1) gene[3] due to its translocation to the IgH locus, t(11;14)(q13;q32).[4] This results in an upregulation of Cyclin D1 nuclear protein that is detectable by immunohistochemistry. Cyclin D1 regulates the transition from the G1 to the S phase of the cell cycle by binding to CDK4, which results in the phosphorylation of the Rb1 protein and release of the E2F family of transcription factors. Although overexpression of CCND1 probably is an important event in MCL, it is not sufficient for malignant transformation.[5] It is therefore important to identify additional tumour abnormalities that would improve our understanding of the pathogenesis and factors involved in disease progression in MCL.

Transcription profiling using microarray analysis is a recently defined method that has been successfully used for subclassifying high-grade malignant lymphoma[6–9] and to define apoptotic pathways,[10] and alterations in genes regulating differentiation and trafficking[11] in MCL. In a recent study, MCL gene expression profiles were associated to patient survival.[12] To further define the changes in gene expression and the biology of the disease, we have performed oligonucleotide microarray analysis and compared the genes expressed in MCL to those in reactive lymphoid tissue. The results from the microarray analysis were validated by comparison to results obtained by flow cytometry and immunohistochemistry. The expression analysis revealed that a number of genes involved in G protein signalling were differentially expressed in MCL compared to reactive lymphoid tissue, and these results were confirmed by RT-PCR and immunocytochemistry. Further insights into the molecular pathogenesis of the disease could provide prognostic information or suggest targets for new treatment modalities.

Materials and methods

Clinical data and tumour characteristics

Patients

Lymph node biopsies from nine patients with MCL were investigated (Table 1). The biopsies were part of enlarged lymph nodes removed for diagnostic purposes and were snap-frozen upon arrival to the Department of Pathology and subsequently stored at −70°C. All morphologic diagnoses were confirmed by flow cytometry and by immunohistochemical staining for Cyclin D1. In most cases, t(11;14) translocation was confirmed by fluorescent *in situ* hybridisation (FISH) analysis.

The age of the patients ranged from 52–79 years at diagnosis (Table 1). In all cases except one (MCL A), the tumour material

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Table 1 Overview of the included MCL cases

	Clinical information			*Morphology of the included material*			
	Gender	*Age at diagnosis (years)*	*Percentage of B cells*	*Light chain*	*Cyclin D1*	*Blastoid*	*Percentage of MIB1+ cells*
MCL A	Male	64	93	Lambda	+	No	50
MCL B	Male	79	90	Lambda	+	No	20
MCL C	Female	63	94	Kappa	+	Yes	90
MCL D	Male	52	86	Kappa	+	No	30
MCL E	Male	78	85	Kappa	+	Yes	40
MCL G	Female	58	59	Lambda	+	No	40
MCL H	Female	79	85	Lambda	+	Yes	90
MCL I	Male	72	58	Lambda	+	No	40
MCL J	Male	66	87	Lambda	+	No	30

represented the original diagnostic biopsy, before the patient had received any treatment for the disease. The patient MCL A was initially treated at another centre and the material investigated here represented tumour material from the first relapse.

Controls

Control tissue were from nonmalignant hyperplastic tonsil tissue (control 1), tonsil cells (control 2) or lymph nodes (controls 3–5). The age of the control patients ranged from 9 to 64 years of age.

This study was approved by the Ethics Committee at Karolinska Institutet (Etikkommitté Syd).

Cell lines

The Ramos cell line, a mature B-cell line from a Burkitt lymphoma, and the Nalm 6 human B-cell precursor leukaemia cell line were cultured in RPMI 1640 with 10% FCS and 0.5 mg/ml of gentamicin.

Two MCL cell lines were used, the Granta 519[13] and the Rec-1.[14] The Granta 519 is an EBV- transformed MCL cell line. It was obtained from DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen and cultured in Dulbecco's MEM (4.5% glucose) with 10% NBCS and 2 mM L-glutamine. The Rec-1 cell line was kindly donated by Dr Christian Bastard, Rouen, France and cultured in RPMI 1640 with 10% NBCS and 2 mM L-glutamine. In both MCL cell lines, the translocation t(11;14) was confirmed by FISH analysis and upregulation of Cyclin D1 protein by immunocytochemistry.

The SK-MM-2 is a human plasma cell leukaemia cell line, which was obtained from DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen was cultured in RPMI 1640 with Glutamax I and 25 mM HEPES with 10% FBS and 0.1 mg/ml gentamicin. It carries a t(11;14) translocation and overexpresses Cyclin D1.

The AtT20 cell line transfected with cDNA for the CB1 receptor[15] was kindly provided by Dr Ken Mackie (Department of Anaesthesiology, University of Washington, Seattle, WA, USA) and cultured in Dulbecco's MEM with 10% NBCS. Selection was carried out with 400 μg G418. These cells are adherent and trypsination was used to bring the cells into suspension to allow cytospin preparation.

Immunohistochemistry

Immunohistochemical stainings were performed on formalin-fixed paraffin-embedded sections by routine procedures. All stainings were semiautomated and performed on a TechMate 500 plus (DAKO, Glostrup, Denmark) by using the DAKO ChemMate Detection Kit, Peroxidase/DAB, Rabbit/Mouse polylinker as recommended by the manufacturer.

Primary monoclonal antibodies were as follows: mouse monoclonal antibodies to BCL-6 (clone PG-B6p), to Ki-67 antigen (clone MIB-1), to p53 (clone DO-7), to BCL2 oncoprotein were all obtained from DAKO. For Cyclin D1 staining, we used clone D2D11F11 from Novocastra (Newcastle upon Tyne, UK). Monoclonal antibody to CD5 (clone 4C7) was from Novocastra. Polyclonal rabbit antibodies to CD3, kappa light chain and lambda light chain were all obtained from DAKO. Pretreatment protocols for antigen demasking were EDTA buffer pH 8.0 at 60°C overnight for Cyclin D1 and BCL-6 and for all other antigens, microwave treatment in citrate buffer pH 6.0 for 3+15 min was used.

Immunofluorescent staining for CB1

Cells on cytospin preparations or imprints from lymph nodes were fixed in 3.7% formaldehyde (Sigma, St Louis, MA, USA) during 4 min, rinsed × 3 in PBS and incubated for 30 min in PBS containing 0.1% Triton X-100 (Sigma) and 0.1% BSA (Sigma). Rabbit polyclonal antiserum to CB1 was kindly provided by Dr Ken Mackie and diluted 1:400 in PBS–Tween–BSA before being added to the cell preparation for 1 h at room temperature in a moist chamber. Thereafter, cells were rinsed × 3 in PBS and incubated with Cy3-conjugated donkey–anti–rabbit antiserum (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:250 in PBS–Tween–BSA. DAPI (4′,6-diamidino-2-phenylindole, Sigma) was used to stain cell nuclei.

Immunoblot analysis

Total cell lysates from Granta 519 and SK–MM–2 cells were prepared using ice–cold lysis buffer (20 mM HEPES, 5 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulphonylfluoride, 5 mM NaF, 0.2 mM Na_3VO_3, 1% Triton-X−100). Lysates were clarified by centrifugation at 14 000 rpm for 10 min at 4°C. A measure of 15 μl of total cell lysate was size fractionated by SDS–PAGE gel. Following electrophoresis, proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with polyclonal anti–Cannabinoid 1 antibody, CB1 (Chemicon, Hampshire, UK) 1:1000 dilution. The specific protein was detected with ECL.

Flow cytometry

Three- or four-colour flow cytometry was performed on freshly prepared cells according to standard procedures. Directly

fluorochrome-conjugated monoclonal antibodies to CD45 and to antigens on B cells (CD19, CD20, CD10, CD23, CD5) and on T cells (CD3, CD5) were obtained from Becton-Dickinson (BD) (Mountain View, CA, USA). Data acquisition was made on a FACScan or a FACScalibur (BD) using the Cell Quest software (BD), both for acquisition and analysis. For each sample, 10 000 cells were acquired. Samples were analysed by setting appropriate side and forward gates around the lymphoid population.

Fluorescent in situ *hybridisation*

Interphase FISH was performed on imprint material from lymph node biopsies by using the Vysis LSI IGH/CCND1 dual fusion probe (Vysis, Richmond, UK) essentially according to the recommendations from the manufacturer. In short, after fixation in methanol, pretreatment with 1% pepsin in 0.01 M HCl and postfixation in 1% formaldehyde, the samples were dehydrated by serial incubation with ethanol and air-dried. The hybridisation was performed by adding the probe diluted in hybridisation buffer (Vysis) and incubation at 80°C for 5 min followed by incubation at room temperature overnight. After stringency washes in 2 × SSC, and 0.4 X SSC, the samples were mounted in antifading mounting medium containing DAPI (Vector Laboratories, Peterborough, UK) and the slides were examined in a Nikon Microphot-FXA microscope equipped with an FITC/TRITC v2 filter with a scan range of 470–670 nm (Chroma Technology Corp., Brattelboro, VT, USA).

RNA isolation and oligonucleotide array hybridisation

Total RNA was prepared using TRIzol method as directed by the supplier. Test arrays were performed with all samples prior to using the U95Av.2 arrays containing more than 12 500 genes. The target synthesis and hybridisations were performed using Affymetrix GeneChip™ high-density oligonucleotide human arrays according to standard Affymetrix protocols at the core facility at Department of Biosciences, Karolinska Institutet, Novum, Huddinge, Sweden. Each MCL was compared to all the controls. The only exception was MCL A and control 2 that were only compared between themselves as 8 μg of RNA was used in them, while in the rest, 5 μg of total RNA was used as starting material. Comparative analyses were performed on the expression data using Affymetrix Datamining Tool (DMT) version 3.0. A cutoff of 1.5 signal log ratio was used.

Data analysis

The data were analysed using Affymetrix Microarray Suite version 5.0, MicroDB 3.0 and DMT 3.0 as well as a beta test version of Geneweaver, Affibody/Inforsense.

For hierarchical clustering, genes that showed three-fold or more differential expression between normal and MCL in at least five of the nine MCL samples were first selected. Then a hierarchical clustering program; Geneweaver, was used to cluster these differentially expressed genes.

cDNA synthesis and RT-PCR

To confirm the downregulation of RGS13 determined by microarray analyses, RT-PCR was performed. A total of 4 μg of total RNA from tonsil, lymph nodes and MCLs was used for cDNA synthesis using First-strand cDNA synthesis kit (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's protocol. The following primers were used, forward: 5′-CAGAGTGCCATCTAAGGTA-3′ and reverse: 5′-CAAGATCACGATGAGTTCAC-3′. 2 μl cDNA was amplified in a volume of 20 μl containing 0.5 μM of each primer, 100 μM of each dNTP, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U *Taq Gold* Polymerase (Applied Biosystems, Stockholm, Sweden). The initial denaturation was for 6 min at 94°C. Amplification was for 40 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min and 30 s and the resulting PCR product was 1465 bp. As an assessment of cDNA quantity, β-actin cDNA was amplified as described.[16]

Sequence analysis

To find out whether RGS13 was mutated in MCLs, we performed sequencing of genomic DNA from MCLs, A, B, D, G, H and I. DNA was extracted by using the QIAamp DNA Minikit (Qiagen, Crawley, West Sussex, UK) according to the instructions from the manufacturer. The template for sequencing was prepared by using primers for each exon in the RGS13 gene (Table 2). As exon 7 is 923 bp, that exon was divided into two parts. The PCR conditions for all exons were as follows. A total of 50 ng DNA was amplified in a volume of 20 μl containing 0.5 μM of each primer, 100 μM of each dNTP, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.5 U *Taq Gold* Polymerase (Applied Biosystems). Initial denaturing was for 6 min at 94°C. Amplification was for 35 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR products were purified by using Jetquick PCR Purification Spin kit (Genomed GmbH, bad Oeynhausen, Germany) according to the manufacturer's protocol. Direct sequencing was performed by using Amersham's Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Table 2 PCR primers used for sequencing of RGS13

Exon	*Forward primer*	*Reverse primer*	*PCR fragment (bp)*
1	5′-CAGAGTGCCATCTAAGGTA-3′	5′-CCTCTGTTAACACTCCCAG-3′	254
2	5′-CCAGGAAGCACACAATAATC-3′	5′-CCCAGTGAAACAGTGATCTA-3′	195
3	5′-GGTGCTATTAAACTGTCGAT-3′	5′-GGAGATTAACCAAGGGGAA-3′	283
4	5′-CCTTAGAAACTGACTGTAAC-3′	5′-GCTCTTCTGATGAAATGACT-3′	288
5	5′-CCAACCAAAGCCACACACAA-3′	5′-GCATACATTCATCATCTGGC-3′	282
6	5′-GGTGATTTTGACCCATGAAA-3′	5′-GGCACTTATGGATGCTTGA-3′	295
7:1	5′-CCACTGAAAAGGAGAGCAG-3′	5′-GCTTGTATCATGAAGAAAGAC-3′	551
7:2	5′-CAGCAAGCCTATGTAGTTC-3′	5′-CAAGATCACGATGAGTTCAC-3′	509

The forward primer used for exon 1 and the reverse primer used for exon 7:2 are the same for RT-PCR and sequencing.

Results

Characterisation of the material and validation of the expression data

In order to characterise the tumour population and to be able to estimate the proportion of clonal tumour B cells as well as nonmalignant B and T cells in the tumour samples and controls, flow cytometry was performed on cells suspended from fresh biopsies. The frequency of clonal B cells in the tumour biopsies is shown in Table 1. The frequency of B cells varied from 58 to 94% in the MCL samples and from 12 to 57% in the reactive lymph nodes. The remaining cells were mainly $CD3^+$ T cells (data not shown).

Flow cytometry demonstrated that six out of nine MCL were clonal for the lambda light chain (Table 1). Analysis of expression data from the oligonucleotide arrays gave similar results considering lambda light-chain expression in the tumours, demonstrating the validity of the expression data (data not shown).

Differentially expressed genes between MCL and reactive lymphoid tissue

To identify genes involved in mantle cell differentiation or tumour development in MCL, a total of 33 comparisons were made between control and tumour samples. All transcripts over- or underexpressed at least three-fold (1.5 average signal log ratio) compared to the controls in at least five of the nine MCLs were considered. The differentially expressed genes are listed in Table 3a and b and categorised based on described functions.

Several of the overexpressed genes in Table 3a are associated with cellular growth regulation. The midkine gene, a secreted growth factor binding to the anaplastic lymphoma kinase (ALK),[17] previously detected in solid tumours and in Reed–Sternberg cells of Hodgkins lymphoma,[18] was found to be highly expressed in MCL. The expression of RORalpha, a transcription factor that belongs to the family of orphan nuclear receptor tyrosine kinases, was increased in MCL. Response elements for this factor are present in cell cycle regulating genes such as N-myc,[19] suggesting that it is involved in the control of cell proliferation. Furthermore, RORalpha has been shown to negatively regulate inflammatory responses.[20,21] Furthermore, in MCL, genes involved in G protein-mediated signalling such as cannabinoid receptor 1 (CB1) and G protein gamma-11 subunit was highly expressed.

There was also an increased expression of genes for matrix proteins such as fibulin-2 and collagen alpha 3-type IX and adhesion molecules such as CD24 (the ligand for P-selectin). Among the underexpressed genes in Table 3b were the adhesion molecule L-selectin, IL-7 receptor, RGS13 and several genes encoding T-cell-specific receptor molecules such as CD7, CD3 epsilon and zeta chains. The reduced expression of the T-cell-related genes reflects the low level of tumour-infiltrating T cells in MCL. T cells can express mRNA for cytolytic proteins granzyme B and granzyme M and the expression of these genes was decreased compared to control lymphoid tissue.

Hierarchical clustering of differentially expressed genes and cases

To represent the gene expression differences between MCLs and reactive lymphoid tissue, a hierarchical clustering of the genes listed in Table 3 and the individual MCL cases and controls was performed. Two major clusters representing differentially expressed genes were identified (Figure 1). Among the different cases, all controls formed one cluster and control 1, representing tonsil tissue, and control 2, representing tonsil cells, clustered together. Among the MCL cases, the two highly proliferative tumours, MCL C and MCL H clustered next to each other.

High expression of CB1 in MCL

Both types of CB were expressed in MCL. In particular, the so-called central type of CB, CB1, was highly expressed in a majority of MCL (Table 3a). In order to confirm the microarray data, we therefore performed immunofluorescent stainings on the Rec-1 and Granta 519 MCL cell lines that were both found to express CB1 (Figure 2b). As positive control cell line, AtT20 stably expressing CB1[15] was used (Figure 2c and d). In all three cell lines, a strong granular intracellular staining was detected, demonstrating the presence of CB1 at the protein level. The CB1 protein expression was further confirmed by immunoblotting that detected a specific band in Granta 519, while a Cyclin D1-positive plasma cell leukaemia cell line, SK-MM-2, lacked expression of CB1 (Figure 2a). There were no imprints from the MCL A – J available. However, during the course of this study, two other MCL cases were diagnosed, and imprints from these cases were subjected to immunofluorescent staining for CB1. A similar type of granular intracellular stainings as in MCL cell lines were detected also in the cells from these newly diagnosed MCL cases (Figure 2e).

In order to confirm the data, we investigated the expression of CB1 and CB2 in six additional MCLs that are part of another study that is to be published separately. These six additional MCLs were analysed by microarray analysis on the HG U133a Affymetrix chip. The probe sets for CB1 and CB2 are not identical between HG U95 Av.2 and U133a chips, but map to very similar areas of the CB1 RNA. Also, all the six additional MCL tumours were strongly positive for CB1 mRNA (Figure 3). Moreover, the peripheral receptor, CB2, was also highly expressed (Figure 3). These results clearly demonstrate that high expression of CB1 is a characteristic feature of MCL.

Low expression of regulator of G protein signalling 13 (RGS13) in MCL

RGS13, one of the members of a family of regulators of G protein signalling, was almost absent in a majority of MCL while present in the controls (Table 3b). Analysis of the expression of RGS13 was further confirmed on the six additional MCL tumours and the result from the total set of 15 tumours is shown in Figure 4. In contrast, another RGS member, RGS 14, was expressed at similar levels in MCLs and controls (Figure 4).

The low expression of RGS13 in MCL was confirmed by RT-PCR. RGS13 was expressed in control sample from tonsil cells but not in MCL (Figure 5a). Furthermore, strong expression of RGS13 was seen in the mature B-cell line Ramos but not in the Nalm 6 pre-B cells (Figure 5b).

To further analyse the low RGS13 expression, we performed direct sequencing of the entire RGS13 gene in six different MCL tumours. We did not detect any mutation in the coding region or the corresponding splice sites. A couple of silent polymorphisms were found in exon 1, intron 1, exons 2 and 6 (Table 4).

Differential expression of CCND1 in MCL

As expected, CCND1 was overexpressed in all MCL (Table 3a). The Affymetrix U95Av.2 chip contains three different probe sets

Table 3 (a) Genes overexpressed and (b) underexpressed in MCL compared to control lymphoid tissue

GenBank access #	*Probe set*	*Map*	*Name*	*Description*
(a) Genes overexpressed in MCL				
Growth regulation				
M64349	2017_s_at	11q13	CCND1	CCND1, Cyclin D1
M73554	2020_at	11q13	CCND1	CCND1, BCL-1
X59798	38418_at	11q13	CCND1	CCND1, PRAD1 mRNA for cyclin
X55110	38124_at	11p11	HSNOPMR	NEGF2, neurite outgrowth-promoting protein, midkine
M97675	213_at	1p32-p31	HUMROR1A	Ror1, retinoic acid receptor-related orphan receptor (ROR) nuclear receptor tyrosine kinase
U73304	36906_at	6q14-q15	CNR1	CB1 cannabinoid receptor
L08096	34054_at	19p13	HUMLIGAND	CD27 ligand, CD70, TNF ligand superfamily costimulatory molecule
U31384	37908_at		GNG11	G protein gamma-11 subunit
Matrix proteins				
X82494	32783_at	3p25-p24	FBLN2	Fibulin-2, extracellular matrix protein
L41162	36455_at	20q13	COL9A3	Collagen alpha 3 type IX
Antigen presentation				
L38820	3856_at	1q21-q23	HUMMHCD1	CD1D, exon 6, antigen presentation
M16276	36108_at		HUMMHDRQ	MHC class II HLA-DR2-Dw12mRNA DQw1-beta
Adhesion molecules				
L33930	266_s_at	6q21	HUMCD24B	CD24, ligand for P-Selectin
AF002246	34193_at	3p?	AF002246	Neural cell adhesion molecule (CALL)
Miscellaneous				
AF039397	38285_at			Mu-crystalline gene, exon 8 and complete cds
AF073308	41872_at	7p15	DFNA5	Nonsyndromic hearing impairment protein, ICERE-1
Z38026	36710_at	3p21	HSFALL-39	FALL-39, peptide antibiotic
AB000115	36927_at		AB000115.1	mRNA expressed in osteoblast
U30521	39710_at		HSU30521	P311
J03600	307_at	10	HUMLOX5	Human 5-lipoxygenase
L48215	32052_at		HUMHBB5E28	Beta-globin gene with a to c allele
D17530	37981_at		HUMDRE	Debrin E
U59185	39260_at		HSU59185	Putative monocarboxylate transporter (MCT) mRNA
Recently described genes with genes with unknown functions	*Map*	*Name*	*Description*	
AB023159	35794_at		KIAA0942 protein, similar to EFA GR?	
AB018287	37483_at		KIAA0744 protein, similar to HDA C9, histone deacetylase?	
AF052145	33601_at	C2orf10[a]	Chromosome 2 open reading frame 10	
AB020689	38254_at	TBC1D9[a]	TBC1 domain family, member 9	
D79994	37225_at		KIAA0172, similar to KANK, kidney ankyring repeat containing protein?	
AI651024	35390_at		cDNA, 3 end clone, similar to ATP binding cassette transporting protein?	
U66680	35389_s_at	ABCA6	ATB binding cassette, subfamily A, member 6	
(b) Genes underexpressed in MCL				
Growth regulation	*number*			
Y09392	41189_at	1p36	TNFRSF12	Apo1,WSL-LR, WSL-S1 and WSL-S2 proteins, apoptosis-mediating receptor
L13720	37658_at		GAS6	Growth-arrest-specific protein (GAS)
AB002409	36503_at	9p13	CCL21	Secondary lymphoid tissue chemokine, SLC
Y13710	32128_at	17q11	CCL18	Pulmonary and activation induced chemokine

Table 3 (*continued*)

GenBank access #	*Probe set*	*Map*	*Name*	*Description*
Transcription factors				
AF055376	41504_s_at	16q22-q23	MAF	c-maf
Adhesion molecules/homing receptors				
M25280	245_at	1q23-q25	SELL	L-selectin
AL023657	38147_at	Xq25	SH2D1A	SH2D1A (DSHP) SAP. Duncan's disease (lymphoproliferative syndrome)
Receptors/signalling molecules				
M29696	1370_at	5p13	IL7R	Interleukin-7 receptor
AF043129	36227_at			Interleukin-7 receptor precursor (IL7R) gene, exons 7 and 8 and complete cds
L27071	1600_at	4p12	TXK	Tyrosine kinase (TXK)
L10717	1478_at		ITK	IL-2 inducible T-cell kinase
U33017	33513_at		SLAM	Signalling lymphocytic activation molecule (SLAM)
L01087	38949_at	10p15	PRKCQ	Protein kinase C-theta
AF030107	35459_at	1q31.2	RGS13	Regulator of G protein signalling (RGS13)
T/NK cell specific				
L23134	32264_at	19p13	GZMM	Granzyme M, Met-ase
X06026	39226_at	11q23		CD3 gamma
U11276	35449_at	12p13-p12	KLRB1	Killer-cell lectin-like receptor (NKR-P1A)
X02883	432_s_at	14q11		T-cell receptor alpha chain C
X00437	32793_at		TRB	T-cell receptor beta locus
X76220	38051_at	2cen-q13		T-lymphocyte maturation ass. protein (MAL)
J04132	37078_at	1q22-q23	CD3Z	T-cell receptor zeta chain
M17016	37137_at	4q11	GZMB	Granzyme B
D00749	771_s_at	17q25		CD7
M23323	36277_at	11q23		CD3-epsilon
M21624	1110_at	14q11	TRD	T-cell receptor delta chain
L23134	32264_at	19p13.3	GZMM	MET-1, metase, granzyme M
X59871	32649_at		TCF7	TCF-1 mRNA for T-cell factor 1 (splice form C)
B-cell specific				
U80114	34094_i_at			Immunoglobulin heavy-chain variable region (V4-31)
AF015128	35566_f_at			IgG heavy-chain variable region (Vh26)
D84143	31315_at		IGLJ3	Immunoglobulin (mAb59) light-chain V region
Miscellaneous				
Y08110	32140_at	11q23-q24	SORL1	Mosaic protein LR11, SORL1
X61118	32184_at	11p13	LMO2	Rhombotin 2 (RBTN2)/translocated in T-cell gene 2 (ttg-2), a member of the LIM family of proteins
U13616	36965_at		ANK3	Ankyrin G (ANK-3), neuronal protein
Recently described genes and genes with unknown function				
AL049409	36021_at	4q23-q25		CDNA DKFZp586H0919, similar to LEF1?
D13626	33462_at	3q21-q25	GPR105	G protein-coupled receptor 105
U90916	38411_at		C11orf32[a]	Chromosome 11 open reading frame 32
U51712	39698_at	4q11-q12	HOP	Human normal gingiva Homo sapiens cDNA
D50915	33528_at	14q32.33		KIAA0125 gene

[a]Suggested new name, approved by the Human Gene Nomenclature Database, <http://www.gene.ucl.ac.uk/nomenclature/>.

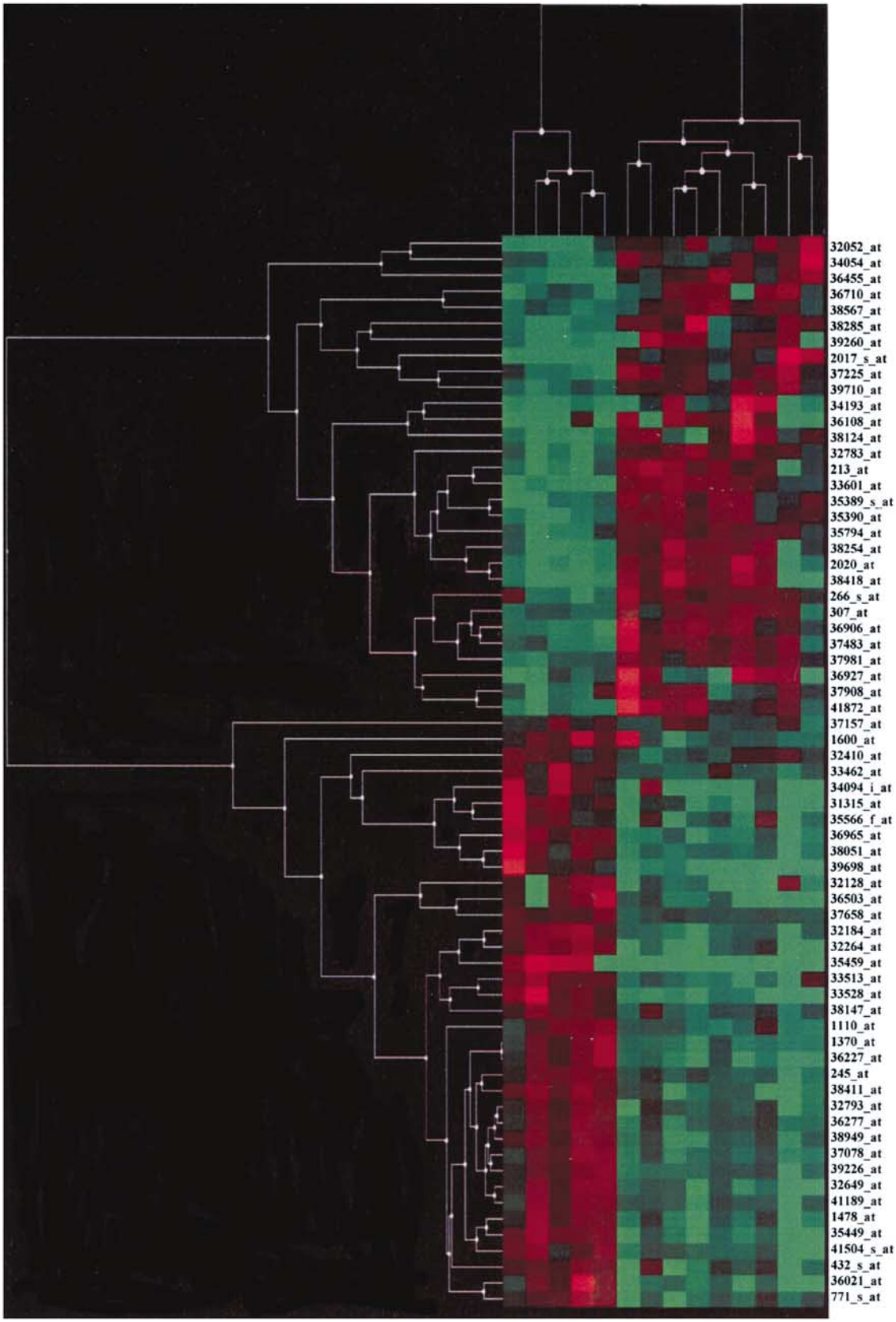

Figure 1 Hierarchical clustering of genes differentially expressed between MCLs and reactive lymphoid tissue. Genes differentially expressed $\geqslant 3$ times in at least 5/9 MCLs were hierarchically clustered using the Geneweaver program. Overexpressed genes are in red, underexpressed in green, no change is represented by black. The order of the samples are, from left to right; control 1, control 2, control 3, control 4, control 5, MCL A, MCl G, MCL B, MCL D, MCL J, MCL E, MCL I, MCL C and MCL H. Probe set numbers are indicated.

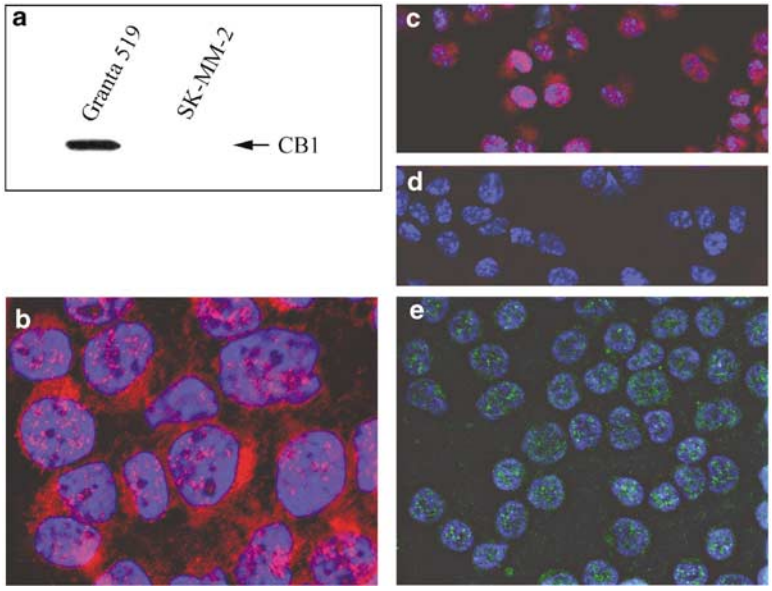

Figure 2 Immunoblot and immunofluorescence stainings for CB1. Immunoblot for CB1 (a) shows high protein expression in the Granta 519 MCL cell line compared to no expression in the SK-MM-2 plasma cell leukaemia cell line. Whole-cell lysates of Granta 519 and SK-MM-2 was prepared, resolved by SDS-PAGE and immunoblotted with anti-CB1 antibody as described in Materials and methods. The MCL cell line Granta 519 (b) and the CB1-transfected AtT20 cell line stably expressing CB1 receptors (c) were stained for CB1 as described in Materials and methods. A strong specific staining (red) is detected in both cell lines as compared to cells stained with an irrelevant control antibody (d). Nuclei are stained blue by DAPI. Imprints from a primary MCL was stained for CB1 (e) resulting in a specific granular staining (green).

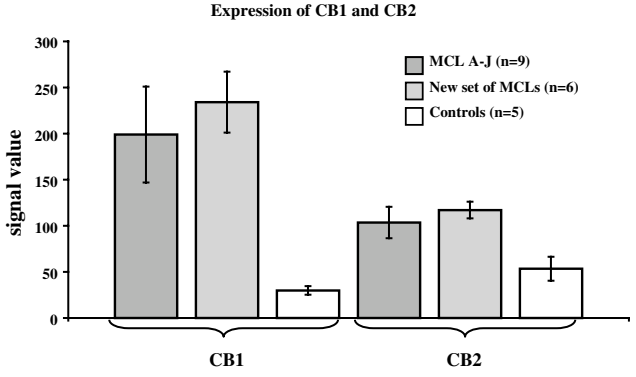

Figure 3 Average expression of cannabinoid receptors in MCLs and controls. All nine MCL tumours (MCL A–H) have high expression levels of CB1 and this finding was confirmed in a new set of six MCLs analysed with Affymetrix U133a chip. Also, CB2 expression is higher in MCLs compared to controls. The *Y*-axis represents absolute signal values.

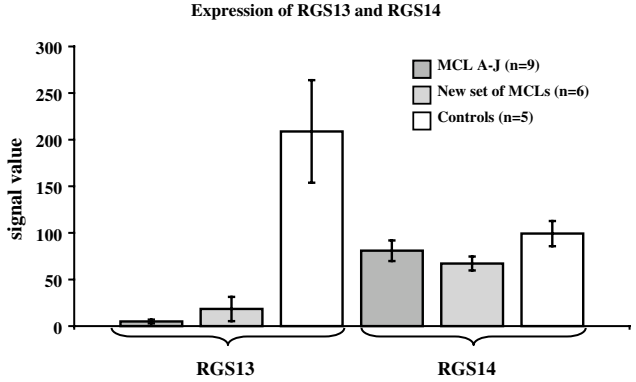

Figure 4 Average expression levels of RGS13 and RGS14 in MCL. In 7/9 MCL, RGS13 expression was absent while RGS14 had similar expression levels as found in reactive lymphoid tissue. The findings were confirmed in a new set of six MCLs analysed with Affymetrix U133a chip. The *Y*-axis represents absolute signal values.

for CCND1 (38418_at = 'PRAD1', 2020_at = 'BCL 1' and 2017_s_at = 'Cyclin D1').Two of the probe sets ('PRAD1' and 'BCL 1') were partially overlapping at the very 3′ end of the gene, while the third probe set ('Cyclin D1') recognised a more central part of the mRNA approximately 75 bases 3′of exon 5 (Figure 6a). Interestingly, among the nine MCLs investigated, there were two different patterns of hybridisation to these three probe sets (Figure 6b). Seven MCLs had high expression levels of the two overlapping probe sets that are specific for the very 3′ end ('PRAD1' and 'BCL 1'), while the two MCLs with a higher proliferative rate (90% of the cells positive for Mib 1), MCL C and MCL H, (Table 1) both had a much lower signal with these two probe sets but a higher expression level of the third probe set, 'Cyclin D1' (Figure 6b). Using immunohistochemistry, all MCL were positive for Cyclin D1 protein and exhibited a characteristic nuclear staining (data not shown).

Discussion

In this study, overexpression of Cyclin D1 protein and a characteristic morphology and phenotype were used as criteria for the inclusion of lymphomas of MCL type. Although Cyclin D1 overexpression may be a necessary event in MCL tumour development, other genetic changes are probably needed.

Here we report two novel characteristics associated with MCL; the absence of RGS13 expression and high CB1 expression. The expression of RGS13 has, to our knowledge,

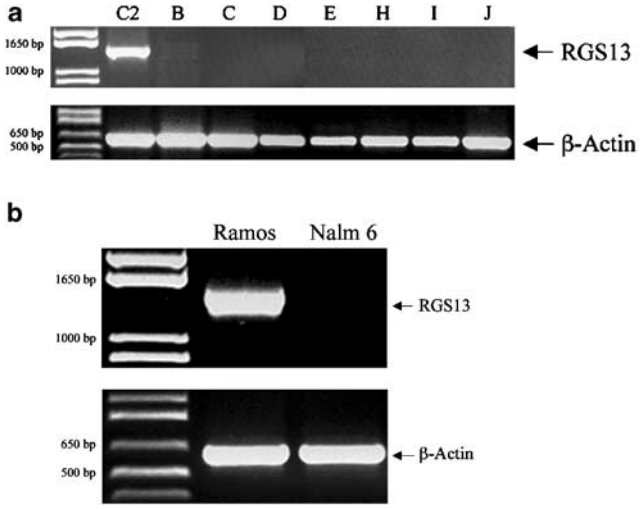

Figure 5 Confirmation of downregulation of RGS13 by using RT-PCR. RGS13 was amplified in the control sample 2 from tonsil cells and MCL samples (a). Furthermore, the expression of RGS13 was present in a B-cell line, Ramos, but absent in a pre-B-cell line, Nalm 6 (b).

Table 4 Polymorphisms found in the RGS13 gene[a]

Exon/intron	*Base substitution*	*Sample*
Exon 1	44 C>T	MCL A, D, I
Intron 1	166+60 C/G[b]	MCL B, G
Intron 1	166+60 C>G[b]	MCL H
Exon 2	219 A/G	MCL D
Exon 6	509 G/A[c]	MCL B

[a]According to *Homo sapiens* chromosome 1 reference genomic contig with accession number NT 004671 region 607562 to 631670 is the entire RGS13 gene sequence. Base 607652 in that sequence is equal to base 1 in our numbering. Start codon is located in exon 4. Numbering according to cDNA sequence.
[b]The polymorphism is located 60 bp from the 3′ end of exon 1 following cDNA nucleotide 166.
[c]Results in silent mutation R76R.

not been previously analysed in malignant lymphoma. During the course of our study, an article by Ek *et al*[11] also reported high CB1 expression levels in MCL using microarray analysis. However, their findings were not confirmed at the protein level. The high level of CB1 receptor expression in all MCL tumours led us to analyse the cellular expression of CB1, taking advantage of the availability of the two MCL-derived cell lines, Rec-1 and Granta 519. CB1 expression was evident both by immunofluorescence and Western blot. Furthermore, in two newly diagnosed cases of MCL, CB1 receptor expression was detected by immunofluorescence, indicating that this may be a characteristic finding in MCL. On average, B-CLL express comparatively lower levels of CB1 mRNA (M Merup, S Fält, A Wennborg, personal communication, April 2003), indicating that CB1 positivity is a characteristic finding in MCL.

Our results suggest that genes coupled to G protein signalling pathways may be dysregulated in MCL. In contrast to our findings in MCL, CB2 and not CB1 has been reported to be preferentially expressed in lymphoid tissue. CB1 was originally described as predominantly expressed in the brain and to a lower extent in human bone marrow, thymus and tonsils.[22] The expression level of CB2 mRNA has been reported to be 10–100-fold higher than that of CB1[22] in lymphoid tissue, but absent in

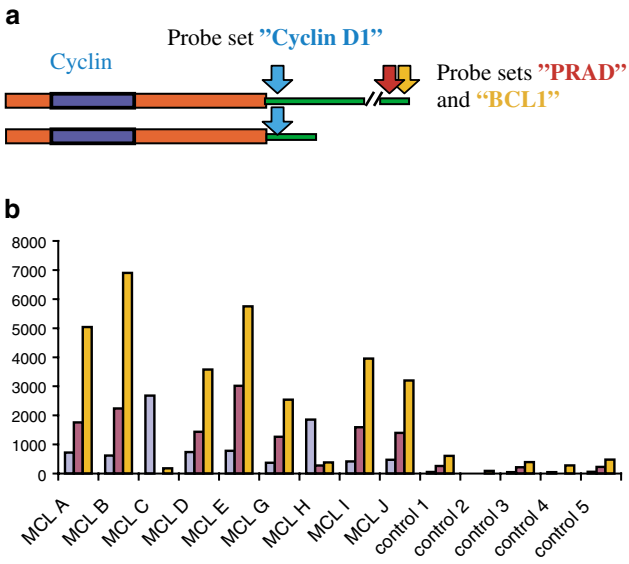

Figure 6 (a) Mapping of Affymetrix HG U95Av.2 three probe sets for CCND1 (2017_s_at = 'Cyclin D1', 2020_at = 'BCL 1' and 38418_at = 'PRAD1'). The coding region for the cyclin box is shown in blue. (b) The expression levels of the three probe sets for CCND1. All MCLs had high expression levels of CCND1 compared to reactive lymphoid tissue. However, in MCL C and MCL H, a different expression pattern was seen compared to the other seven MCLs, with high levels of the 'Cyclin D1' probe set and very low expression of the two probe sets that hybridise at the 3′ end of the gene, 'PRAD1' and 'BCL 1'. The *Y*-axis represents absolute signal values.

the brain.[22,23] Galiegue *et al*[22] also ranked the CB2 mRNA levels in human blood cells as B cells > NK cells > monocytes > polymorphonuclear cells > T8 cells > T4 cells.

Several endogenous ligands for CB1 and CB2 have been identified (reviewed in Howlett *et al*[24]). Many of those are metabolites of arachidonic acid such as arachidonyl ethanolamide, also known as anandamide, and 2-arachinodyl glycerol. Cannabinoids have been reported to stimulate B-cell growth. However, it is not clear if this stimulation is mediated only through cannabinoid receptors or if other receptors, such as vanilloid receptors, are involved as well (reviewed in Howlett *et al*[24]). Selective antagonists and agonists to CB1 and CB2 have recently been produced and used to elucidate CB1- and CB2-specific responses. CB2 receptors have been reported to be functionally involved during B-cell differentiation (reviewed in Howlett *et al*[24]). Carayon *et al*[23] reported that CB2 receptor expression was upregulated in virgin and germinal centre B cells in response to CD40-mediated activation. Noe *et al*[25] reported that CB1 mRNA was upregulated in mouse splenocytes in response to CD40 ligation. These reports suggest that CB receptors have biologically important roles during B-cell differentiation and activation and together with our findings of an upregulation of CB1 receptor in MCL indicates that modulation of CB1 might be a candidate for tumour therapy.

CB receptors are coupled to heterotrimeric G proteins, $G\alpha\beta\gamma$ subunits (reviewed in Howlett *et al*[24]). Signalling is activated by $G\alpha$ binding of GTP and the release of $G\beta\gamma$. The heterotrimeric G proteins are in turn regulated by regulator of G protein signalling (RGS) proteins, a family of approximately 25 proteins that share a conserved domain (RGS domain) that bind directly to activated $G\alpha$ subunits (reviewed in Hollinger *et al*[26]). Initially, the RGS proteins were found to attenuate G protein-mediated signalling by acting as GTPase-activating proteins (GAPs) for $G\alpha$

subunits. However, there is now emerging evidence that at least some of the RGS proteins link active Gα subunits to other signalling pathways thereby acting as modulators of G protein signalling rather than functional inhibitors only. There are today 17 known subunits of Gα that on the basis of amino-acid similarities are classified into four major subclasses namely Gs, Gi/o, Gq/11 and G12/13 (reviewed in Hur and Kim[27]). Interestingly, RGS13,[28] that affects signalling through Gi/o, was recently described as highly expressed in murine germinal centres,[29] but absent from the marginal zones. Furthermore, CD40 ligation was found to upregulate RGS13 expression in human tonsil B lymphocytes. Moreover, RGS13 was found to reduce signalling through the MAP kinase induced by chemokine receptors CXCR4 and CXCR5, suggesting a role in response to chemoattractants in the germinal centre.[29]

Our microarray findings of reduced RGS13 expression in 9/9 MCLs while RGS14 was unchanged, was confirmed by RT-PCR and by microarray analysis of six additional MCLs. Moreover, we found by RT-PCR that the mature B-cell line Ramos expressed RGS13 while the pre-B-cell line Nalm 6 lacked expression. These results are thus in accordance with those by Shi *et al*,[29] suggesting that RGS13 expression is regulated during B-cell differentiation, being absent in pre-B cells and virgin B cells. Furthermore, RGS13 has been reported to be absent also in T lymphocytes.[29] RGS13 expression is also relatively low in B-CLL (M Merup and co-workers, personal communication, April 2003). To further analyse the low RGS13 expression in MCL, the entire gene was sequenced in six MCLs. Apart from a few polymorphisms in exon 1, intron 1, exons 2 and 6, the sequence was identical to NT 004671 (Table 4). These results suggest that the low expression of RGS13 is a result of regulatory events reflecting the differentiation stage of the cells from which MCL originates. However, it is possible that the lack of RGS13 in cells of this developmental stage make them particularly susceptible to activation through CB receptors.

We found two different patterns of CCND1 expression using the three probe sets for this gene on the Affymetrix U95Av.2 chip, two of which targeted the very 3′ end of the CCND1 transcript, while the third probe set (Cyclin D1, 2017_s_at) lies at a much more 5′ region. There is also a possible second polyadenylation site just 3′ to the region where this probe set maps. This is interesting in as much as the two MCLs that had a high signal from this more 5′ probe set (MCL C and MCL H) had lower signals from the other two probe sets. A possible explanation for the findings could therefore be that there is an alternative splicing or truncation of the transcripts in MCL C and MCL H and that the binding pattern of these three CCND1 probe sets could reflect the presence of shorter Cyclin D1 mRNA lacking the 3′ AU-rich region that contains sequences involved in mRNA stability.[14] Moreover, these MCLs were the most highly proliferative and had blastoid morphology, suggesting that the presence of a shorter CCND1 transcript might influence the cell cycle control (Sander B. *et al*. Blood 2002; **100**: ASH abstract 4676). Following the submission of this manuscript, Rosenwald *et al*[12] reported very similar findings. In their large series of MCLs, a subset of lymphomas had a truncated Cyclin D1 transcript lacking the 3′ end. These tumours had a higher proliferative rate and also expressed 20 genes that constituted a 'proliferation signature' associated with significantly shorter survival,[12] further emphasising the clinical importance of perturbations of cell cycle regulation in MCL. Of the 20 genes in this 'proliferation signature', 13 are represented on the HG95Av.2 chip. When the expression level of these genes were investigated in our material, we found the highest 'proliferation signature average' in MCL C and MCL H, thus confirming the interesting results published by Rosenwald *et al*.

Our key finding in MCL of high CB1 expression and the absence of RGS13 expression is interesting, since these two molecules potentially could participate in a common signalling pathway. The absence of RGS13 expression may reflect the expected expression of 'normal' mantle cells that in the context of upregulated CB1 may provide necessary additional signals of importance to malignant phenotype. We can, however, not at present discriminate between the two possible explanations for these findings, namely that the expression pattern reflects the differentiation status in the cell type that give rise to MCL rather than tumour-specific events. Clearly, further experiments are needed in order to clarify the precise role of signalling through cannabinoid receptors in MCL and if these signalling pathways possibly can constitute new targets for antitumour therapy.

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References

1 Walsh S, Thorselius M, Johnson A, Söderberg O, Jerkeman M, Björk E *et al*. Mutated VH genes and preferential VH3-21 usage define new subsets of mantle cell lymphoma. *Blood* 2003; **101**: 4047–4054.
2 Leonard JP, Schattner EJ, Coleman M. Biology and management of mantle cell lymphoma. *Curr Opin Oncol* 2001; **13**: 342–347.
3 Rosenberg CL, Wong E, Petty EM, Bale AE, Tsujimoto Y, Harris NL *et al*. PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. *Proc Natl Acad Sci USA* 1991; **88**: 9638–9642.
4 Raffeld M, Jaffe ES. bcl-1, t(11;14), and mantle cell-derived lymphomas. *Blood* 1991; **78**: 259–263.
5 Lovec H, Grzeschiczek A, Kowalski MB, Moroy T. Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. *EMBO J* 1994; **13**: 3487–3495.
6 Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A *et al*. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; **403**: 503–511.
7 Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC *et al*. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. *Nat Med* 2002; **8**: 68–74.
8 Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E *et al*. Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA* 1999; **96**: 2907–2912.
9 Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X *et al*. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. *J Exp Med* 2001; **194**: 1639–1647.
10 Hofmann WK, de Vos S, Tsukasaki K, Wachsman W, Pinkus GS, Said JW *et al*. Altered apoptosis pathways in mantle cell lymphoma detected by oligonucleotide microarray. *Blood* 2001; **98**: 787–794.

11 Ek S, Hogerkorp CM, Dictor M, Ehinger M, Borrebaeck CA. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. *Cancer Res* 2002; **62**: 4398–4405.
12 Rosenwald A, Wright G, Wiestner A, Chan WC, Connors JM, Campo E *et al*. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell* 2003; **3**: 185–197.
13 Jadayel DM, Lukas J, Nacheva E, Bartkova J, Stranks G, De Schouwer PJ *et al*. Potential role for concurrent abnormalities of the cyclin D1, p16CDKN2 and p15CDKN2B genes in certain B *cell non-Hodgkin's* lymphomas. Functional studies in a cell line (Granta 519). *Leukemia* 1997; **11**: 64–72.
14 Rimokh R, Berger F, Bastard C, Klein B, French M, Archimbaud E *et al*. Rearrangement of CCND1 (BCL1/PRAD1) 3′ untranslated region in mantle-cell lymphomas and t(11q13)-associated leukemias. *Blood* 1994; **83**: 3689–3696.
15 Hsieh C, Brown S, Derleth C, Mackie K. Internalization and recycling of the CB1 cannabinoid receptor. *J Neurochem* 1999; **73**: 493–501.
16 Schmidt EE, Ichimura K, Messerle KR, Goike HM, Collins VP. Infrequent methylation of CDKN2A(MTS1/p16) and rare mutation of both CDKN2a and CDKN2B(MTS2/p15) in primary astrocytic tumours. *Br J Cancer* 1997; **75**: 2–8.
17 Stoica GE, Kuo A, Powers C, Bowden ET, Sale EB, Riegel AT *et al*. Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. *J Biol Chem* 2002; **277**: 35990–35998.
18 Kato H, Watanabe K, Murari M, Isogai C, Kinoshita T, Nagai H *et al*. Midkine expression in Reed-Sternberg cells of Hodgkin's disease. *Leukemia Lymphoma* 2000; **37**: 415–424.
19 Dussault I, Giguere V. Differential regulation of the N-myc proto-oncogene by ROR alpha and RVR, two orphan members of the superfamily of nuclear hormone receptors. *Mol Cell Biol* 1997; **17**: 1860–1867.
20 Delerive P, Monte D, Dubois G, Trottein F, Fruchart-Najib J, Mariani J *et al*. The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. *EMBO Rep* 2001; **2**: 42–48.
21 Missbach M, Jagher B, Sigg I, Nayeri S, Carlberg C, Wiesenberg I. Thiazolidine diones, specific ligands of the nuclear receptor retinoid Z receptor/retinoid acid receptor-related orphan receptor alpha with potent antiarthritic activity. *J Biol Chem* 1996; **271**: 13515–13522.
22 Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P *et al*. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 1995; **232**: 54–61.
23 Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A *et al*. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. *Blood* 1998; **92**: 3605–3615.
24 Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA *et al*. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacol Rev* 2002; **54**: 161–202.
25 Noe SN, Newton C, Widen R, Friedman H, Klein TW. Anti-CD40, anti-CD3, and IL-2 stimulation induce contrasting changes in CB1 mRNA expression in mouse splenocytes. *J Neuroimmunol* 2000; **110**: 161–167.
26 Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev* 2002; **54**: 527–559.
27 Hur EM, Kim KT. G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. *Cell Signal* 2002; **14**: 397–405.
28 Johnson EN, Druey KM. Functional characterization of the G protein regulator RGS13. *J Biol Chem* 2002; **277**: 16768–16774.
29 Shi GX, Harrison K, Wilson GL, Moratz C, Kehrl JH. RGS13 regulates germinal center B lymphocytes responsiveness to CXC chemokine ligand (CXCL)12 and CXCL13. *J Immunol* 2002; **169**: 2507–2515.