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Gene expression profiling of mantle cell lymphoma cells reveals aberrant expression of genes from the PI3K-AKT, WNT and TGFβ signalling pathways

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Summary

Microarray studies have revealed the differential expression of several genes in mantle cell lymphoma (MCL), but it is unknown which of these differences are dependent on the transformed MCL cell itself or on the tumour microenvironment. To investigate which genes and signalling pathways are aberrantly expressed in MCL cells we used oligonucleotide microarrays to perform gene expression profiling of both purified leukaemic MCL cells and their normal counterparts, the naive B cells. A total of 106 genes were differentially expressed at least threefold in MCL cells compared with naive B cells; 63 upregulated and 43 downregulated. To validate the microarray results in a larger set of samples, we selected 10 differentially expressed genes and quantified their expression by real-time polymerase chain reaction in peripheral blood of MCL patients ($n = 21$), purified MCL cells ($n = 6$) and naive B cells ($n = 4$), obtaining fully concordant results. A computer-assisted approach was used to procure specific molecular signalling pathways that were aberrantly expressed in MCL cells. Several genes related to apoptosis and to the PI3K/AKT, WNT and tumour growth factor β signalling pathways were altered in MCL cells when compared with naive B cells. These pathways may play a significant role in the pathogenesis of MCL and deserve further investigation as candidates for new therapeutic targets.

Keywords: non-Hodgkin lymphoma, gene expression, transforming growth factor-β.

Mantle cell lymphoma (MCL) is associated with the translocation t(11;14)(q13;32) and consequent cyclin D1 overexpression (Bosch *et al*, 1994). The cell of origin of MCL is the pre-germinal-centre naive B-cell, a subset of B-lymphocytes which populates primary follicles and the mantle zone of secondary follicles in lymphoid organs (Kuppers *et al*, 1999). MCL represents 6% of all non-Hodgkin lymphoma (The Non-Hodgkin Lymphoma Classification Project, 1997), and has a median survival of 3–4 years (Pittaluga *et al*, 1996). Disease is predominantly disseminated at diagnosis and a frank leukaemic phase is detected in more than one-third of the cases (Argatoff *et al*, 1997; Orchard *et al*, 2003).

Although cyclin D1 overexpression plays a pivotal role in the pathogenesis of MCL (Bosch *et al*, 1994), it is not sufficient by itself to cause lymphoma (Bodrug *et al*, 1994), and the elucidation of the additional genetic lesions may provide insights towards a specific therapy. Aiming to elucidate these additional lesions, DNA microarrays have been used as a genomic approach to MCL (Hofmann *et al*, 2001; Ek *et al*, 2002; Islam *et al*, 2003; Martinez *et al*, 2003; Rosenwald *et al*, 2003; Thieblemont *et al*, 2004). However, most MCL cases investigated by microarrays to date were obtained from lymph node or spleen biopsy specimens, which comprise a mix of malignant and non-transformed cells. Microarray studies have indicated the differential expression of several genes in MCL compared with normal lymphoid tissue (Hofmann *et al*, 2001; Ek *et al*, 2002; Islam *et al*, 2003; Martinez *et al*, 2003), but it is unknown which of these differences are dependent on the transformed MCL cell itself or on the tumour microenvironment. In addition, although the identification of genes whose expression is markedly different in tumour versus normal tissue experiments provides valuable information, more relevant biological meaning could be obtained by identification of groups of altered genes functionally connected through signalling pathways. To investigate which genes and signalling pathways are aberrantly expressed in MCL cells compared

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specifically with their normal counterparts, the naive B cells, we used oligonucleotide microarrays to evaluate gene expression profiling of MCL cells and naive B cells isolated from the peripheral blood (PB) of patients with leukaemic MCL and from normal tonsils respectively.

Materials and methods

Patients

The PB samples were collected at diagnosis from 21 patients with leukaemic MCL. Patients had a median (range) age of 66 years (34–81); 15 (71%) patients were male. The median lymphocyte count was $47{\cdot}4 \times 10^9$/l (range, $6{\cdot}7$–$256{\cdot}6 \times 10^9$/l) and all patients had evidence of cyclin D1 overexpression as detected by real-time polymerase chain reaction (PCR).

Tonsils were obtained from routine tonsillectomies in four children (2–6 years old) free of any medications, including topical corticoids. The study protocol was approved by the local institutional review board, and subjects or their guardians gave informed consent to participate.

Cell line and cell culture

Granta-519 cell line (Jadayel *et al*, 1997) cells were grown in 90% Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 2 mmol/l L-glutamine, penicillin and streptomycin. Cells were kept in cell culture flasks in a humidified incubator at 37°C and 5% CO_2.

Study design

As a screening strategy to determine which genes were differentially expressed in MCL compared with naive B cells, we performed gene expression profiling of MCL cells purified from the PB of three patients with leukaemic MCL and of naive B cells purified from tonsils of three normal individuals. Experiments were run in duplicates, in such a way that six hybridisations were obtained from each group. Genes were considered differentially expressed by MCL cells if up or downregulated by at least threefold compared with naive B cells. To validate microarray results and extend the analysis to a larger set of clinical samples, we selected 10 differentially expressed genes and quantified their expression by real-time PCR in the PB of MCL patients ($n = 21$), in purified MCL cells ($n = 6$) and in naive B cells ($n = 4$). After validation of microarray results, we used a computer-assisted approach to search for specific molecular signalling pathways altered in MCL cells when compared with naive B cells.

Magnetic cell sorting

Tonsil specimens were kept on ice immediately after surgical removal. After mincing in cooled Roswell Park Memorial Institute (RPMI) 1640 medium, mononuclear cells (MNC) were harvested by centrifugation on a Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA) density gradient. Naive B cells were isolated according to the currently accepted immunophenotypical characterisation of this subset (Pascual *et al*, 1994; Klein *et al*, 1998), using magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany), by depletion of CD10, $CD38^{high}$, CD27 and CD14-positive cells, followed by enrichment of IgD-positive cells, as previously described (Klein *et al*, 2003). Briefly, tonsillar MNC were stained with purified mouse IgG_1 anti-CD27 and anti-CD10 monoclonal antibodies (Becton Dickinson, San Jose, CA, USA). After washing, cells were incubated with rat anti-mouse IgG_1-microbeads and CD14-microbeads and passed over lymphocyte depletion columns (Miltenyi Biotec) for depletion of labelled cells. The flow-through was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-IgD (Becton Dickinson Pharmingen, San Diego, CA, USA), and in a second staining step with anti-FITC-microbeads (Miltenyi Biotec). The cell suspension was then passed over a lymphocyte separation (LS) column (Miltenyi Biotec) for positive selection of labelled cells.

MCL cells were purified from 6 of the 21 patients with leukaemic MCL using the CD19 MultSort Kit (Miltenyi Biotec), according to the manufacturer's protocol.

Homogeneity of naive B cells (Fig 1) and purified MCL samples was confirmed by flow cytometry, and purity of more than 95% was obtained in all samples.

RNA isolation

From all 21 MCL patients, total RNA was extracted either from total PB or from the PB MNC fraction. In 13 patients, RNA was isolated from fresh PB, whereas in eight patients it was isolated from samples of PB MNC cryopreserved at −80°C in RPMI 1640 with 10% FBS and 10% dimethylsulphoxide. Total RNA was also isolated from purified MCL and naive B cells, as well as from the Granta-519 MCL cell line. For RNA extraction of the Granta-519 cell line, cells were harvested by centrifugation at 4°C and washed twice with cold phosphate-buffered saline. From all samples, RNA was extracted using the Trizol LS reagent (Invitrogen, Carlsbad, CA, USA).

Microarray procedures

Gene expression profiling was performed with Amersham CodeLink UniSet Human I BioArrays (Amersham Biosciences, Piscataway, NJ, USA), containing 10 000 probes. After DNase treatment and purification with the RNeasy kit (Qiagen, Valencia, CA, USA), RNA quality was assessed by denaturing agarose gel electrophoresis and using a Bionalyzer RNA chip (Agilent, Palo Alto, CA, USA). RNA concentration was quantified by spectrophotometry and 2 μg were used from each studied subject. Target synthesis and hybridisations were performed with the CodeLink Expression Assay Reagent Kit (Amersham), following the manufacturer's protocol. Briefly,

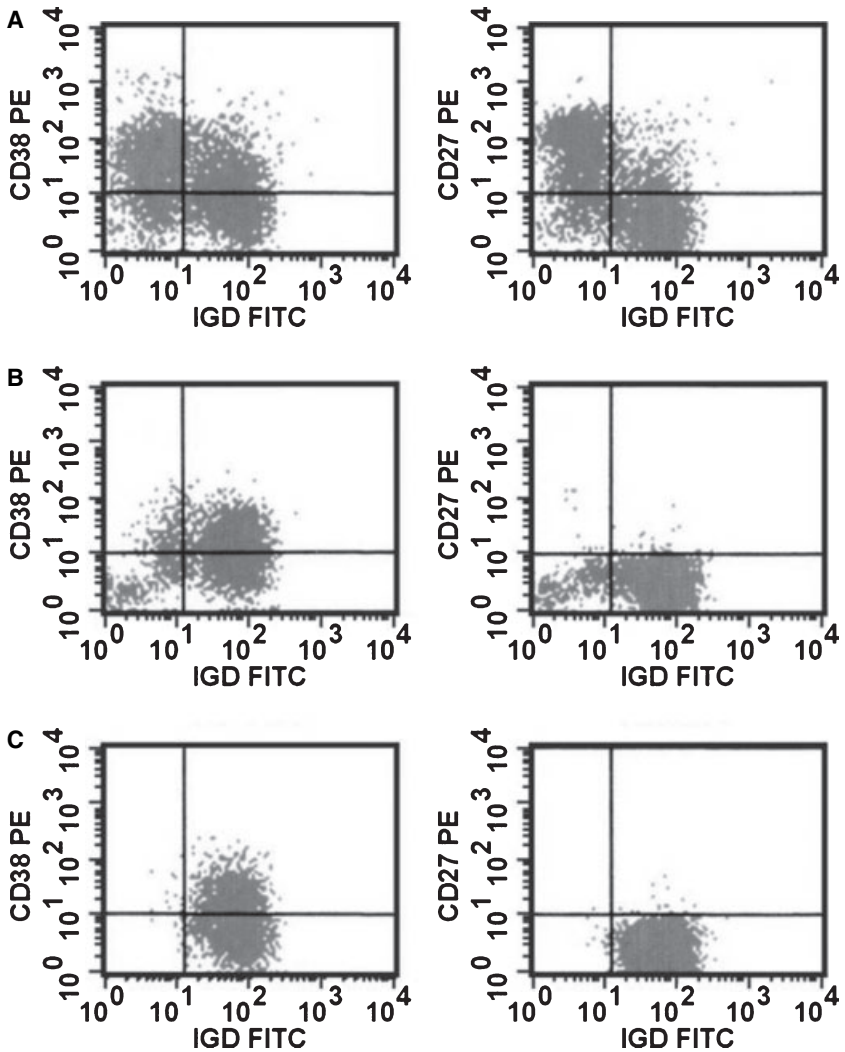

Fig 1. Flow cytometric profiles of tonsillar naive B cells isolation by magnetic cell sorting. Samples of cells from each step of the magnetic cell sorting were analysed by flow cytometry using anti-IgD-FITC, anti-CD38-phycoerythrin (PE) and anti-CD27-PE. Representative dot plots from the isolation procedures of one tonsil are illustrated. (A) Dot plots showing the tonsillar MNC after mincing and centrifugation on density gradient. (B) Dot plots showing tonsillar MNC after depletion of CD27, CD10 and CD14-positive cells. (C) Dot plots showing naive B cells positively selected by the expression of IgD on membrane.

total RNA and bacterial control RNA were reverse transcribed using T7 oligo(dT) primers, followed by synthesis of the second complementary DNA (cDNA) strand and purification of double-strand cDNA with QIAquick columns (Qiagen). Biotin-labelled cRNA was then generated by an *in vitro* transcription reaction using T7 RNA polymerase and biotin-11-UTP (Perkin Elmer, Boston, MA, USA). The cRNA was purified on an RNeasy column and 10 μg were fragmented by heating at 94°C for 20 min. The fragmented cRNA was hybridised to microarrays overnight at 37°C in a shaking incubator at 300 rpm. After posthybridisation washes, arrays were incubated with a Cy5-Streptavidin conjugate (Amersham Biosciences). Signal of the Cy5-dye was detected using a GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA) and images were analysed with the CodeLink Expression Analysis software (Amersham Biosciences). Expression values were normalised to the median expression value of the whole array spots, and the data were exported to worksheet files.

Real-time quantitative reverse transcription-PCR

In all samples, reverse transcription (RT) was performed with 2 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems) (Levy, 2003). For real-time PCR experiments, we used an ABI Prism 5700 Sequence Detection System (Applied Biosystems) device under standard thermal cycling conditions (Lossos *et al*, 2003). PCR reactions were prepared in replicates at a final volume of 20 μl, as described (Levy, 2003). The maximum coefficient of variation allowed between replicates was 2%, otherwise experiments were repeated. For quantitative analysis of all selected genes, we used commercially available TaqMan probes and primers (Assays-

on-Demand; Applied Biosystems), by comparing experimental levels with standard curves obtained by serial dilutions of cDNA from the Granta-519 cell line, which was also used as the calibrator. The normalisation factor was the geometric mean of the phosphoglycerate kinase 1 (*PGK1*) and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes (TaqMan PDARs; Applied Biosystems) (Vandesompele *et al*, 2002; Levy, 2003; Lossos *et al*, 2003).

Analysis of data

Data analysis of the microarray hybridisations was performed with the VECTORXPRESSION software (Informax, Bethesda, MD, USA). After filtering out genes with missing spots, expression values from duplicates were averaged and results from each MCL patient were normalised by each one of the three normal individuals. Genes were selected if either up or downregulated by at least threefold in each one of the nine normalisations generated by this analysis.

Aiming at identifying signalling pathways with aberrant pattern of gene expression in MCL, we used the PATHWAY-ASSIST software (Strategene, La Jolla, CA, USA) for data mining. In this case, we used less restrictive criteria and considered for analysis all genes displaying a fold change of 1·5 or more, and a $P < 0{\cdot}01$ (unpaired *t*-test).

Results

Gene expression profiling of MCL cells compared with naive B cells showed that 106 genes were differentially expressed in MCL (Fig 2); 63 upregulated (Table I) and 43 downregulated (Table II). To strengthen the confidence that the genes identified in this comparison were truly differentially expressed, a very stringent data analysis was performed. After exclusion of genes with missing spots, expression values from duplicates were averaged and only genes with a differential expression of at least threefold in all nine one-to-one possible normalisations of MCL cells by naive B cells were considered to be differentially expressed.

Reproducibility in microarray data can be assessed by hybridizing duplicate samples from the same RNA and defining coefficients of variation for expression intensities. We observed a mean (SD) coefficient of variation of 8·27% (0·80%) and 8·51% (0·52%) among replicates of MCL and naive B cells, respectively, attesting for high reproducibility of hybridisation data. In addition, results from replicated hybridisations enabled the calculation of the minimal detectible spot ratio that can be distinguished from platform noise. All replicate hybridisations, from both MCL cells and naive B cells, had 95% of the spot ratios within 1·3-fold, indicating that fold change ratios above this threshold most probably represent true biological variability.

To validate microarray results and extend the analysis to a larger set of clinical samples, we arbitrarily selected 10 genes and quantified their expression by real-time PCR in total PB or in the PB MNC fraction of 21 MCL patients. In addition, real-time PCR measurements were also performed in samples of purified MCL cells ($n = 6$) and naive B cells ($n = 4$). Figure 3 summarises the gene expression measurements of all validated genes by both microarrays and real-time PCR. We found with one exception, the *CCL4* gene, that both methods detected similar patterns of expression for the six upregulated and the four downregulated genes selected for validation. The fold change ratio of the *CCL4* gene in total PB was the only real-time PCR result in disagreement with microarrays, but the *CCL4* gene is activated in T-lymphocytes and monocytes from the PB (Lipes *et al*, 1988). Also of note, one of the genes selected for validation, the *TLR1*, had a differential expression of less than threefold as measured by microarrays (2·4-fold), but even in this case microarray results were confirmed by real-time PCR.

After confirmation of the results for all the genes selected for validation, we performed a data mining strategy aiming to identify specific signalling pathways altered in MCL cells compared with their normal counterparts. For this, we used a computer-assisted method and considered those genes that were up or downregulated at a fold change ratio of 1·5 or more, and with a *P*-value of <0·01, to be differentially expressed. Table III shows the genes from the signalling pathways mostly aberrantly expressed in MCL. Both the intrinsic and the extrinsic apoptotic pathways were shown to be altered in MCL. In addition, several genes from the PI3K/AKT (phosphatidylinositol 3-kinase-AKT), WNT and tumour growth factor β (TGFβ) signalling pathways were deregulated in MCL cells compared with naive B cells.

Discussion

The purpose of the present study was to investigate which genes and signalling pathways were abnormally expressed in purified MCL cells compared with their normal counterparts, the naive B cells. For this, we performed gene expression profiling of MCL cells isolated from the PB of patients with leukaemic MCL and of normal naive B cells isolated from normal tonsils.

Some of the genes we found to be differentially expressed have been previously shown to be altered on gene expression profiling of lymph node biopsies from MCL patients – e.g. *TNFRSF7*, *AHNAK*, *CDC25B* and *SLAMF1* (Ek *et al*, 2002; Islam *et al*, 2003; Martinez *et al*, 2003; de Vos *et al*, 2003). For some other genes, such as *TCF7*, controversial results have been obtained because it has been reported to be both up and downregulated in MCL (Ek *et al*, 2002; Islam *et al*, 2003). Although we have included only cases with leukaemic MCL, recent studies have shown that there is no clinical or biological characteristic capable of discriminating the leukaemic form as a non-MCL entity (Orchard *et al*, 2003; Letestu *et al*, 2004).

In our series, all genes selected for validation showed similar results for the microarrays and real-time PCR measurements, yet with different degrees of magnitude, from both purified

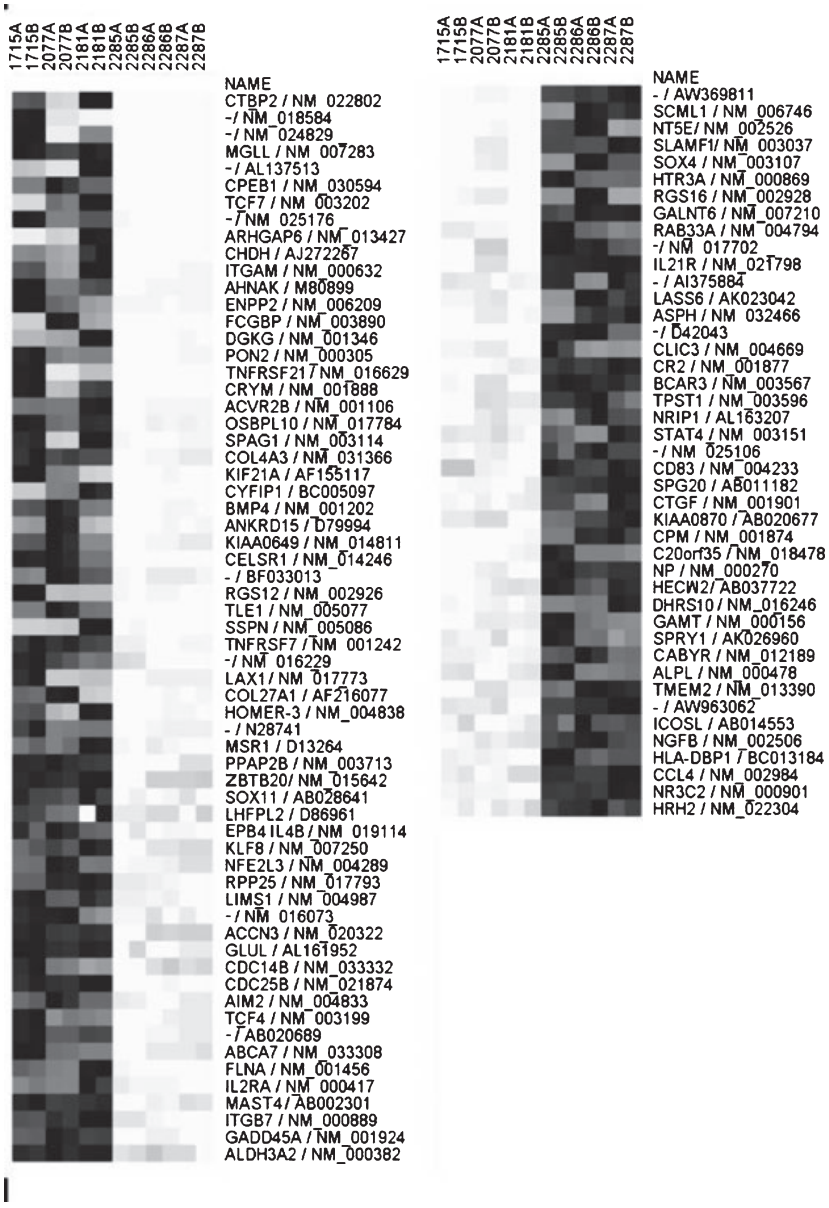

Fig 2. Genes differentially expressed in MCL cells compared with naive B cells as analysed by oligonucleotide microarrays. Heat maps depict the six hybridisations performed with MCL cells on the left half of each heat map, and the six hybridisations performed with naive B cells on the right. (Left) Genes upregulated in MCL cells. (Right) Genes downregulated in MCL cells.

and PB MCL samples. In addition, although cyclin D1 expression was not evaluated by the microarray platform we used, overexpression of this gene in PB of all MCL patients was confirmed by real-time PCR at diagnosis.

After validation of the microarray results, we looked for groups of aberrantly expressed genes functionally connected through the signalling pathways. Our results showed that several apoptosis-related genes were altered in MCL. The genes *BCL2* and *BID* were modulated in MCL. *BCL2*, a well-known inhibitor of apoptosis, was upregulated; whereas *BID*, which has pro-apoptotic activity and is responsible for the crosstalk between the intrinsic and extrinsic apoptotic pathways (Igney & Krammer, 2002), was downregulated. In addition, several pro-apoptotic genes from the extrinsic (death receptors) pathway are consistently downregulated in MCL, whereas *CFLAR*, an inhibitor of death receptor-mediated apoptosis, was upregulated. Hofmann *et al* (2001) and Martinez *et al* (2003), studying lymph node biopsies of MCL patients, have previously shown similar results, namely, upregulation of *BCL2* and downregulation of several pro-apoptotic mediators of the extrinsic pathway.

We observed that some genes from the PI3K/AKT signalling pathway were upregulated in MCL compared with naive B cells. The activity of this pathway is mediated by the presence of survival signals (hormones, growth factors, cytokines), which protect cells from 'death by neglect' (Igney & Krammer,

Table I. Genes upregulated in mantle cell lymphoma compared with naive B cells as analysed by oligonucleotide microarrays.

Access	Gene	Description	Map	Change
NM_022802	*CTBP2*	C-terminal binding protein 2	10q26·13	190·9
NM_018584	λ	Calcium/calmodulin-dependent protein kinase II	1p36·12	119·4
NM_024829	λ	Hypothetical protein FLJ22662	12p13·1	71·9
NM_007283	*MGLL*	Monoglyceride lipase	3q21·3	71·9
AL137513	λ	Hypothetical protein LOC150568	2q12·2	62·3
NM_030594	*CPEB1*	Cytoplasmic polyadenylation element binding protein 1	15q25·1	40·8
NM_003202	*TCF7*	Transcription factor 7 (T-cell specific, HMG-box)	5q31·1	37·3
NM_025176	λ	KIAA0980 protein	20p11·22-p11·1	34·6
NM_013427	*ARHGAP6*	Rho GTPase activating protein 6	Xp22·3	34·4
AJ272267	*CHDH*	Choline dehydrogenase	3p21·1	32·9
NM_000632	*ITGAM*	Integrin, alpha M (CD11b)	16p11·2	25·1
M80899	*AHNAK*	AHNAK nucleoprotein (desmoyokin)	11q12-q13	23·6
NM_006209	*ENPP2*	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	8q24·1	22·8
NM_003890	*FCGBP*	Fc fragment of IgG binding protein	19q13·1	22·7
NM_001346	*DGKG*	Diacylglycerol kinase, gamma 90 kDa	3q27-q28	18·8
NM_000305	*PON2*	Paraoxonase 2	7q21·3	17·6
NM_016629	*TNFRSF21*	Tumour necrosis factor receptor superfamily, member 21	6p21·1–12·2	16·6
NM_001888	*CRYM*	Crystallin, mu	16p13·11-p12·3	16·5
NM_001106	*ACVR2B*	Activin A receptor, type IIB	3p22	16·3
NM_017784	*OSBPL10*	Oxysterol binding protein-like 10	3p22·3	15·2
NM_003114	*SPAG1*	Sperm associated antigen 1	8q22	14·8
NM_031366	*COL4A3*	Collagen, type IV, alpha 3 (Goodpasture antigen)	2q36-q37	13·9
AF155117	*KIF21A*	Kinesin family member 21A	12q12	12·4
BC005097	*CYFIP1*	Cytoplasmic FMR1 interacting protein 1	15q11	11·9
NM_001202	*BMP4*	Bone morphogenetic protein 4	14q22-q23	11·8
D79994	*ANKRD15*	Ankyrin repeat domain 15	9p24·2	11·7
NM_014811	*KIAA0649*	KIAA0649	9q34·3	11·3
NM_014246	*CELSR1*	Cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)	22q13·3	11·0
BF033013	λ	601455990F1 Homo sapiens cDNA, 5′ end	λ	10·6
NM_002926	*RGS12*	Regulator of G-protein signalling 12	4p16·3	10·2
NM_005077	*TLE1*	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	9q21·32	9·9
NM_005086	*SSPN*	Sarcospan (Kras oncogene-associated gene)	12p11·2	9·9
NM_001242	*TNFRSF7*	Tumor necrosis factor receptor superfamily, member 7	12p13	9·8
NM_016229	λ	Cytochrome b5 reductase b5R.2	11p15·3	9·5
NM_017773	*LAX1*	Lymphocyte transmembrane adaptor 1	1q32·1	9·5
AF216077	*COL27A1*	Collagen, type XXVII, alpha 1	9q33·1	8·4
NM_004838	*HOMER3*	Homer homolog 3 (Drosophila)	19p13·11	8·3
N28741	λ	yx67e09.r1 Homo sapiens cDNA, 5′ end	λ	8·2
D13264	*MSR1*	Macrophage scavenger receptor 1	8p22	8·1
NM_003713	*PPAP2B*	Phosphatidic acid phosphatase type 2B	1pter-p22·1	7·6
NM_015642	*ZBTB20*	Zinc finger and BTB domain containing 20	3q13·2	6·8
AB028641	*SOX11*	SRY (sex-determining region Y)-box 11	2p25	6·8
D86961	*LHFPL2*	Lipoma HMGIC fusion partner-like 2	5q13·3	6·7
NM_019114	*EPB41L4B*	Erythrocyte membrane protein band 4·1 like 4B	9q22·1-q22·3	6·4
NM_007250	*KLF8*	Kruppel-like factor 8	Xp11·21	6·2
NM_004289	*NFE2L3*	Nuclear factor (erythroid-derived 2)-like 3	7p15-p14	6·2
NM_017793	*RPP25*	Ribonuclease P p25 kDa subunit	15q22·33	6·0
NM_004987	*LIMS1*	LIM and senescent cell antigen-like domains 1	2q12·2	5·8
NM_016073	λ	Hepatoma-derived growth factor, related protein 3	15q11·2	5·8
NM_020322	*ACCN3*	Amiloride-sensitive cation channel 3	7q35	5·7
AL161952	*GLUL*	Glutamate-ammonia ligase (glutamine synthase)	1q31	5·7
AI654054	*CDC14B*	CDC14 cell division cycle 14 homolog B (S. cerevisiae)	9q22·32	5·5
NM_021874	*CDC25B*	Cell division cycle 25B	20p13	5·3
NM_004833	*AIM2*	Absent in melanoma 2	1q22	5·3
NM_003199	*TCF4*	Transcription factor 4	18q21·1	5·2
AB020689	λ	KIAA0882 protein	4q31·1	5·0

Table I. (Continued).

Access	Gene	Description	Map	Change
NM_033308	*ABCA7*	ATP-binding cassette, sub-family A (ABC1), member 7	19p13·3	5·0
NM_001456	*FLNA*	Filamin A, alpha (actin binding protein 280)	Xq28	4·8
NM_000417	*IL2RA*	Interleukin 2 receptor, alpha	10p15-p14	4·7
AB002301	*MAST4*	Microtubule associated serine/threonine kinase family member 4	5q12·3	4·6
NM_000889	*ITGB7*	Integrin, beta 7	12q13·13	4·5
NM_001924	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	1p31·2-p31·1	4·2
NM_000382	*ALDH3A2*	Aldehyde dehydrogenase 3 family, member A2	17p11·2	4·1

Genes selected displayed at least threfold differences in expression in all nine one-to-one normalisations between MCL patients and normal subjects. Access, Entrez Gene or Entrez Nucleotide accession number; Gene, the gene symbol according to the HUGO database; Change, the mean expression value in the MCL group divided by the mean expression value in the naive B cells group; λ, not available.

Table II. Genes downregulated in mantle cell lymphoma compared with naive B cells as analysed by oligonucleotide microarrays.

Access	Gene	Description	Map	Change
AW369811	λ	IL0-BT0168-091199-139-e05 Homo sapiens cDNA	λ	−42·9
NM_006746	*SCML1*	Sex comb on midleg-like 1 (Drosophila)	Xp22·2-p22·1	−35·2
NM_002526	*NT5E*	5′-nucleotidase, ecto (CD73)	6q14-q21	−31·6
NM_003037	*SLAMF1*	Signalling lymphocytic activation molecule family member 1	1q22-q23	−29·5
NM_003107	*SOX4*	SRY (sex-determining region Y)-box 4	6p22·3	−22·3
NM_000869	*HTR3A*	5-hydroxytryptamine (serotonin) receptor 3A	11q23·1-q23·2	−19·2
NM_002928	*RGS16*	Regulator of G-protein signalling 16	1q25-q31	−15·3
NM_007210	*GALNT6*	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6)	12q13	−14·9
NM_004794	*RAB33A*	RAB33A, member RAS oncogene family	Xq26·1	−14·6
NM_017702	λ	Hypothetical protein FLJ20186	16q24·3	−13·2
NM_021798	*IL21R*	Interleukin 21 receptor	16p11	−12·3
AI375884	λ	tc14a01.x1 Homo sapiens cDNA, 5′ end	λ	−12·1
AK023042	*LASS6*	LAG1 longevity assurance homolog 6 (S. cerevisiae)	2q31·1	−11·3
NM_032466	*ASPH*	Aspartate beta-hydroxylase	8q12·1	−10·9
D42043	λ	Raft-linking protein	3p25·1	−10·7
NM_004669	*CLIC3*	Chloride intracellular channel 3	9q34·3	−10·3
NM_001877	*CR2*	Complement component (3d/Epstein Barr virus) receptor 2	1q32	−9·5
NM_003567	*BCAR3*	Breast cancer anti-estrogen resistance 3	1p22·1	−9·5
NM_003596	*TPST1*	Tyrosylprotein sulfotransferase 1	7q36·3	−9·4
AL163207	*NRIP1*	Nuclear receptor interacting protein 1	21q11·2	−8·2
NM_003151	*STAT4*	Signal transducer and activator of transcription 4	2q32·2-q32·3	−8·1
NM_025106	λ	SPRY domain-containing SOCS box protein SSB-1	1p36·22	−8·0
NM_004233	*CD83*	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	6p23	−7·7
AB011182	*SPG20*	Spastic paraplegia 20, spartin (Troyer syndrome)	13q13·1	−7·6
NM_001901	*CTGF*	Connective tissue growth factor	6q23·1	−7·4
AB020677	λ	KIAA0870 protein	8q24·3	−7·3
NM_001874	*CPM*	Carboxypeptidase M	12q15	−7·2
NM_018478	*C20orf35*	Chromosome 20 open reading frame 35	20q13·11	−6·9
NM_000270	*NP*	Nucleoside phosphorylase	14q13·1	−6·8
AB037722	*HECW2*	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	2q32·3	−6·6
NM_016246	*DHRS10*	Dehydrogenase/reductase (SDR family) member 10	19q13·33	−6·6
NM_000156	*GAMT*	Guanidinoacetate N-methyltransferase	19p13·3	−6·6
AK026960	*SPRY1*	Sprouty homolog 1, antagonist of FGF signalling (Drosophila)	4q28·1	−6·5
NM_012189	*CABYR*	Calcium-binding tyrosine-(Y)-phosphorylation regulated (fibrousheathin 2)	18q11·2	−6·1
NM_000478	*ALPL*	Alkaline phosphatase, liver/bone/kidney	1p36·1-p34	−6·1
NM_013390	*TMEM2*	Transmembrane protein 2	9q13-q21	−6·0
AW963062	λ	EST375135 Homo sapiens cDNA	λ	−5·4
AB014553	*ICOSLG*	Inducible T-cell co-stimulator ligand	21q22·3	−5·0

Table II. Genes downregulated in mantle cell lymphoma compared with naive B cells as analysed by oligonucleotide microarrays.

Access	Gene	Description	Map	Change
NM_002506	*NGFB*	Nerve growth factor, beta polypeptide	1p13·1	−4·6
BC013184	*HLA-DPB1*	Major histocompatibility complex, class II, DP beta 1	6p21·3	−4·4
NM_002984	*CCL4*	Chemokine (C-C motif) ligand 4	17q12	−4·1
NM_000901	*NR3C2*	Nuclear receptor subfamily 3, group C, member 2	4q31·1	−4·0
NM_022304	*HRH2*	Histamine receptor H2	5q35·3	−3·7

Definitions and explanations are as given in Table I.

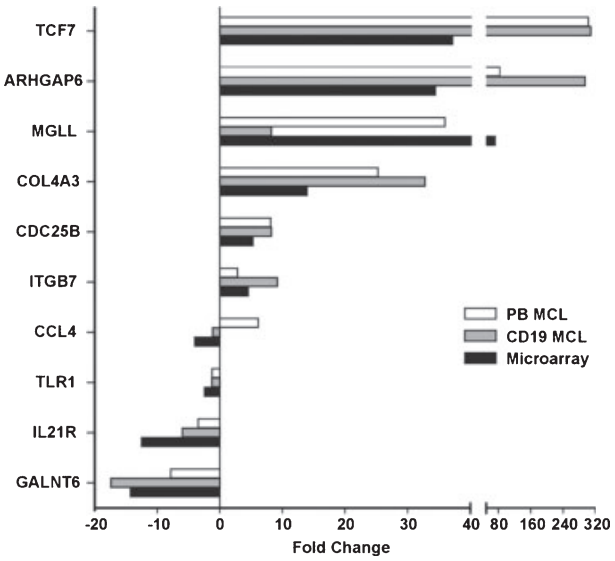

Fig 3. Correlation between gene expression measurements assessed by quantitative RT-PCR and oligonucleotide microarrays for 10 selected genes. The fold change ratios were determined by real-time RT-PCR in PB samples (PB MCL; $n = 21$) and purified MCL cells (CD19 MCL; $n = 6$), both normalised to naive B cells ($n = 4$). The fold change ratios for microarray expression levels were obtained from hybridisations performed in duplicate with purified MCL cells and naive B cells from three MCL patients and three normal individuals respectively. Note that *CCL4* expression in PB MCL was the only gene with dissimilar results between the methods. This apparent discrepancy occurred only because *CCL4* is abundantly expressed by activated T-lymphocytes and monocytes in PB samples.

2002). Survival signals confer protection from apoptosis by activation of the PI3K pathway, which recruits AKT and inhibits the intrinsic apoptotic pathway (Igney & Krammer, 2002). The activation of this pathway may be one of the mechanisms underlying apoptosis resistance in MCL with potential clinical relevance. In fact, Witzig *et al* (2004) have shown in a phase II trial that CCI-779, an inhibitor of the PI3K pathway, has substantial anti-tumour activity in relapsed MCL. In addition, Ghobrial *et al* (2005) recently found evidence of activation of the PI3K and AKT pathways in the proteomic analysis of MCL cells.

Several genes from the WNT signalling pathway were also predominantly upregulated in MCL cells. The activation of the WNT signalling leads to the recruitment of a complex composed by *AXIN1-APC-GSK3B* that stops the phosphorylation and degradation of β-catenin by *GSK3B* (glycogen synthase kinase 3), promoting β-catenin accumulation. High β-catenin levels will activate transcription factor/lymphoid-enhancer binding factor (TCF/LEF) transcription factors in the nucleus, which have cyclin D1 among their target genes (Tetsu & McCormick, 1999). The *TCF7* gene (or *TCF1*), whose strong upregulation was confirmed by real-time PCR in the PB of our patients, is also a target of WNT signalling (Roose *et al*, 1999). Our microarray experiments have revealed that there is a significant increase on transcript levels of many components of the WNT pathway in MCL cells, including *FZD7*, *LRP5*, *AXIN1*, *APC*, *DVL3*, *CREBBP* and *TCF4*. Some of these genes, like *FDZ7* and *CREBBP*, have also been shown to be transcription targets of the WNT signalling, which regulates several genes from its own circuitry (Willert *et al*, 2002).

As PI3K/AKT signalling also phosphorylates and inactivates *GSK3B* (Nicholson & Anderson, 2002), the *GSK3B* enzyme may constitute an important crosstalk between WNT and PI3K/AKT pathways in MCL cells. *GSK3B* activity includes a site-specific phosphorylation of cyclin D1, leading to its degradation via proteasome (Diehl *et al*, 1998). Therefore, inactivation of *GSK3B* by PI3K/AKT or WNT signalling may help to stabilise cyclin D1 levels in MCL.

A striking observation of our study was the aberrant expression of several genes from the TGFβ superfamily in MCL cells. The upregulated activin receptors *ACVR1*, *ACVR2A* and *ACVR2B* and the ligand *BMP4* are members of the TGFβ superfamily, which consists of TGFβ, activins, bone morphogenic proteins (BMPs) and others. Upon ligand binding, receptors from the TGFβ superfamily activate the SMADs, responsible for signal transduction, inducing anti-proliferative and pro-apoptotic responses and acting as tumour suppressors in early tumorigenesis. In advanced cancer, however, there is a loss of growth-inhibitory responsiveness downstream of the core TGFβ signalling pathway and it may be used as a tumour-progression factor by inducing immune suppression, angiogenesis, epithelial–mesenchymal transition and increased potential for metastasis (Derynck & Zhang, 2003; Siegel & Massague, 2003). Jung *et al* (2004) have recently shown that, in leukaemia and lymphoma cell lines, *TGFB1* inhibits FAS-mediated apoptosis by both downregulation of surface FAS receptors and upregulation of CFLAR, the inhibitor of death receptor-induced apoptosis, which we have shown to be

Table III. Genes aberrantly expressed in mantle cell lymphoma either related to apoptosis or from the PI3K/AKT, WNT and TGFβ signalling pathways.

Access	Gene	Description	Map	Change
Apoptosis-related genes				
NM_000633	*BCL2*	B-cell CLL/lymphoma 2, transcript variant alpha	18q21·3	4·5
NM_001226	*CASP6*	Caspase 6, apoptosis-related cysteine protease	4q25	2·3*
NM_003879	*CFLAR*	CASP8 and FADD-like apoptosis regulator	2q33-q34	1·6
NM_003809	*TNFSF12*	Tumour necrosis factor (ligand) superfamily, member 12	17p13	−1·5*
NM_003808	*TNFSF13*	Tumour necrosis factor (ligand) superfamily, member 13	17p13·1	−1·6
NM_033292	*CASP1*	Caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	11q23	−1·6*
NM_004347	*CASP5*	Caspase 5, apoptosis-related cysteine protease	11q22·2-q22·3	−1·9*
NM_003810	*TNFSF10*	Tumour necrosis factor (ligand) superfamily, member 10	3q26	−2·7*
NM_000595	*LTA*	Lymphotoxin alpha (Tumour necrosis factor superfamily, member 1)	6p21·3	−2·9*
NM_001196	*BID*	BH3 interacting domain death agonist	22q11·1	−3·8
NM_002546	*TNFRSF11B*	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	8q24	−4·3*
PI3K/AKT1 signalling pathway				
NM_006218	*PIK3CA*	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	3q26·3	3·1*
NM_002611	*PDK2*	Pyruvate dehydrogenase kinase, isoenzyme 2	17q23·3	2·7*
NM_002613	*PDPK1*	3-phosphoinositide dependent protein kinase-1	16p13·3	2,0
NM_005163	*AKT1*	v-akt murine thymoma viral oncogene homolog 1	14q32·32	1·7*
NM_003952	*RPS6KB2*	Ribosomal protein S6 kinase, 70 kD, polypeptide 2	11q12·2	1·7
NM_001455	*FOXO3A*	Forkhead box O3A	6q21	1·5*
AF086924	*PPP2R2C*	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	4p16	−1·6*
NM_002610	*PDK1*	Pyruvate dehydrogenase kinase, isoenzyme 1	2q31·1	−2·3*
WNT signalling pathway				
NM_003202	*TCF7*	Transcription factor 7 (T-cell specific, HMG-box)	5q31·1	37·3
NM_002335	*LRP5*	Low density lipoprotein receptor-related protein 5	11q13·4	6·7
NM_003199	*TCF4*	Transcription factor 4	18q21·1	5·2*
NM_001894	*CSNK1E*	Casein kinase 1, epsilon	22q13·1	3·5*
NM_003507	*FZD7*	Frizzled homolog 7 (Drosophila)	2q33	3·0*
NM_004423	*DVL3*	Dishevelled, dsh homolog 3 (Drosophila)	3q27	2·4*
NM_004380	*CREBBP*	CREB binding protein (Rubinstein-Taybi syndrome)	16p13·3	2·4*
AF009674	*AXIN1*	Axin	16p13·3	2·2*
NM_000038	*APC*	Adenomatosis polyposis coli	5q21-q22	1·8
NM_012242	*DKK1*	Dickkopf homolog 1 (Xenopus laevis)	10q11·2	−3·1
TGFβ signalling pathway				
NM_001106	*ACVR2B*	Activin A receptor, type IIB	3p22	16·3*
NM_001202	*BMP4*	Bone morphogenetic protein 4	14q22-q23	11·8*
NM_003244	*TGIF*	TGFβ-induced factor (TALE family homeobox)	18p11·3	4·0
NM_005901	*SMAD2*	SMAD, mothers against DPP homolog 2 (Drosophila)	18q21·1	3·4*
NM_001616	*ACVR2A*	Activin A receptor, type IIA	2q22·2-q23·3	2·9
NM_001105	*ACVR1*	Activin A receptor, type I	2q23-q24	2·5*
NM_005585	*SMAD6*	SMAD, mothers against DPP homolog 6 (Drosophila)	15q21-q22	−1·7

Genes selected displayed at least 1·5-fold difference in expression and had a $P < 0{\cdot}01$.
*$P < 0{\cdot}001$.

upregulated in MCL cells. Of particular interest for MCL, the cyclin D1/TGFβ double transgenic liver model in mice showed enhanced tumour formation when compared with its single transgenic littermates (Deane *et al*, 2004). Also of note, crosstalk between TGFβ and WNT signalling pathways has been identified in colon cancer cells, which require activated β-catenin for *BMP4* upregulation (Kim *et al*, 2002).

An alternative explanation for our findings could be that the signalling pathways we identified as altered in MCL cells in fact represent a different stage of activation or differentiation of these cells, but do not have an active role in the pathogenesis of MCL. However, studies on the gene expression profiling of the successive steps of B-cell differentiation have not identified significant differential expression of genes from the PI3K-AKT, WNT or TGFβ signalling pathways in any of the normal B-cell subpopulations or compartments in peripheral lymphoid organs (Klein *et al*, 2003; Shen *et al*, 2004).

In conclusion, this study identified several genes that are aberrantly expressed in MCL cells and suggests that the PI3K/AKT, WNT and TGFβ signalling pathways may play a significant role in the pathogenesis of MCL. Further investigation of these pathways as candidates for new therapeutic targets may shed new light on the way towards a specific therapy for MCL.

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