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The Molecular Signature of Mantle Cell Lymphoma Reveals Multiple Signals Favoring Cell Survival

Nerea Martínez,[1] Francisca I. Camacho,[1] Patrocinio Algara,[3] Antonia Rodríguez,[1] Ana Dopazo,[2] Elena Ruíz-Ballesteros,[3] Paloma Martín,[4] Jose A. Martínez-Climent,[5] Javier García-Conde,[5] Javier Menárguez,[6] Fernando Solano,[7] Manuela Mollejo,[3] and Miguel A. Piris[1]

[1]Molecular Pathology Program and [2]Biotechnology Program, Centro Nacional de Investigaciones Oncológicas, Madrid; [3]Department of Genetics and Pathology, Hospital Virgen de la Salud, Toledo; [4]Department of Pathology, Hospital Ramón y Cajal, Madrid; [5]Department of Hematology and Medical Oncology, Hospital Clínico, University of Valencia, Valencia; [6]Department of Pathology, Hospital General Universitario Gregorio Marañón, Madrid; and [7]Department of Hematology, Hospital Nuestra Señora del Prado, Talavera de la Reina, Toledo, Spain

ABSTRACT

Mantle cell lymphoma (MCL) is a prototypical neoplastic disease in which a common cytogenetic alteration, t11;14, leading to cyclin D1 overexpression, is associated with other changes that need to be considered in an explanation of the clinical, morphological, and molecular variability of this disease. Using a cDNA microarray (Oncochip-CNIO) containing clones for 6386 cancer-related genes, we have analyzed the expression profiles of a series of 38 cases. After normalization with the expression profiling of sorted mantle zone lymphocytes, we have related the findings to conventional clinical and molecular variables, including immunoglobulin variable heavy chain somatic mutation, blastoid cytology, increased proliferation, and long-term survival.

MCL signature (446 genes) includes genes involved in apoptosis, cell cycle, signal transduction, and cell structure. Especially striking was the presence of multiple concurrent alterations in the tumor necrosis factor and nuclear factor κB pathway, and the overexpression of *IL10R* and *SPARC* genes. We also identified a molecular signature for the presence of immunoglobulin variable heavy chain somatic mutation, which includes a number of genes potentially relevant in cancer (*CDC14A*, *ras*, and others). Signatures for proliferation and blastoid cytology were also found.

An integrated analysis of these data yields a gene-expression based survival predictor (26 genes grouped into two clusters), which distinguishes half of the patients with a survival probability of 52% at 5 years. The predictive model has been confirmed by cross-validation.

In conclusion, MCL seems to combine a disease-specific signature and different sets of genes of which the expression is associated with key clinical, molecular, and immunophenotypical events.

INTRODUCTION

MCL[8] is a paradigm of a molecularly defined neoplasm in which virtually all of the cases carry a chromosomal translocation t(11;14)(q13;q32), resulting in the juxtaposition of the cyclin D1 gene (*CCND1*) to transcriptional control elements from the immunoglobulin heavy chain locus (1–3).

Despite the presence of this common molecular marker, experiments with transgenic mice overexpressing CCND1 show that this protein alone cannot induce lymphomas and requires other oncogenic factors, such as *c-myc* or others (4). Thus, different mechanisms must be required for the development and progression of MCL (5). The loss of the chromosomal fragment 11q22–23, where the ataxia-telangiectasia mutated gene (*ATM*) is located, has been identified recently (6). This deletion affects almost 50% of MCLs, and it has been proposed that loss of ATM function may occur early in the malignant transformation of follicle mantle cells. Nevertheless, there must be currently unknown mechanisms that act in conjunction with these already characterized alterations in MCL. For instance, the knowledge of the molecular changes acquired in MCL cells is insufficient to explain fully the resistance to apoptosis that characterizes this tumor, in which treatment resistance to the commonly used drugs seems to be a cardinal feature.

Different clinical and molecular observations taken together highlight the molecular heterogeneity of MCL. Although it was initially claimed that MCL cells were derived from naive pre-GC B cells, recent studies have shown that around 25–30% of MCLs carry mutations in the IgV_H (7, 8). This is a characteristic feature of post-Germinal Centre cells and may indicate that a subset of MCLs originates from B cells exposed to the Germinal Centre environment. Whether the presence of IgV_H somatic mutations is merely an epiphenomenon or is related to some underlying molecular variability is still unresolved.

A more comprehensive knowledge of the underlying molecular alterations in MCL should also permit their correlation with clinical course in these patients, and, specifically, the identification of subsets of patients with relatively indolent or highly aggressive courses.

Here we have chosen to explore the constellation of molecular abnormalities in MCL through expression profiling, and to correlate these profiles with a number of molecular (IgV_H somatic mutation), immunohistological (increased proliferation fraction), and clinicopathological features of the tumors. This study supplements the data published previously of expression profiling in series of MCL, and joins a large enough series of cases with detailed molecular and immunophenotypical studies that allow for the proposal of molecular signatures for survival, blastoid variant, and IgVH somatic mutation.

MATERIALS AND METHODS

Patients and Tissue Samples. Our study featured a series of 38 MCL consecutive cases from the centers participating in this study: Hospital Clínico, Valencia; Hospital Virgen de la Salud, Toledo; Hospital Gregorio Marañón, Madrid; and the Tumor Bank of Centro Nacional de Investigaciones Oncológicas, Madrid, Spain. The period of recruitment of patients was from 1998 to 2002. Diagnostic criteria were based on those described in the WHO Classification (11), including, in addition to a morphology consistent with the diagnosis, the demonstration of cyclin D1 expression for the cases diagnosed in paraffin-embedded tissue samples, or the presence of t(11;14) in the cases diagnosed in peripheral blood or bone marrow.

For the expression profile analysis, only frozen tissues from lymph node biopsies collected at the moment of diagnosis were used. Mantle B cells isolated from reactive tonsillectomy specimens were used as controls.

Patient medical records were reviewed to gather information on age, gender, localization, and stage of disease at diagnosis, International Prognostic Index,

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Requests for reprints: Miguel Angel Piris, Molecular Pathology Program, Centro Nacional de Investigaciones Oncológicas, C/Melchor Fernández Almagro 3, E-28029 Madrid, Spain. Phone: 34-91-224-69-00; Fax: 34-91-224-69-23; E-mail: mapiris@cnio.es.

[8] The abbreviations used are: MCL, mantle cell lymphoma; IgV_H, immunoglobulin variable heavy chain; OS, overall survival; TNF, tumor necrosis factor; NFκB, nuclear factor κB; IL, interleukin.

and disease course. All of the patients received chemotherapy for curative purposes.

Isolation of Mantle B Cells. Tonsil specimens were obtained from patients undergoing routine tonsillectomy for chronic tonsillitis. Tonsillar cell suspensions were obtained by mincing the tissue in ice-cold culture medium (RPMI 1640) and leaving it to sediment for 5 min. Mononuclear cells were isolated by Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

B-cell isolation from mononuclear cell fraction was performed by immunomagnetic bead selection with a CD19 multisort kit (Miltenyi Biotech, Auburn, CA) following the manufacturer's instructions. B-cell purity of >90% homogeneity was confirmed by three-color flow cytometry using CD19/CD27/CD45.

CD27-negative/CD19-positive/CD45-positive cells were selected as the negative fraction, after incubation with CD27 microbeads (Miltenyi Biotech) conjugated with monoclonal mouse antihuman CD27 antibodies. CD19-positive, CD27-negative cells were incubated with rabbit antihuman IgD FITC-FITC (DAKO) and with anti-FITC microbeads, and the positive cells were collected.

The purity of the tonsillar mantle cells (CD19+, $CD27^-$, and IgD+) obtained by this method was 84%, as confirmed by flow cytometry (FAC Sort; Becton Dickinson, San Jose, CA) using the Paint a Gate program (Becton Dickinson).

RNA Extraction and Amplification. Total RNA was isolated in two steps using TRIzol (Life Technologies, Inc., Grand Island, NY) followed by RNeasy (Qiagen Inc., Valencia, CA) purification. The integrity of RNA was verified by electrophoresis. Double-strand cDNA was synthesized from 4 μg of total RNA using the Superscript System for cDNA synthesis (Life Technologies, Inc.). *In vitro* transcription was carried out using the T7 Megascript *in vitro* transcription kit (Ambion, Austin, TX). The quality of the amplified RNA produced was checked by electrophoresis and its concentration measured.

Microarray Procedures. Five μg of amplified RNA from each sample was directly labeled with cyanine 3-conjugated dUTP (Cy3), whereas 5 μg of aRNA from the Universal Human Reference RNA (Stratagene) was labeled with cyanine 5-conjugated dUTP (Cy5) as reference. For all of the microarray studies the CNIO OncoChip was used (12). Hybridizations were performed as described (12). After washing, the slides were scanned using a Scanarray 5000 XL (GSI Lumonics Kanata, Ontario, Canada) and images were analyzed with the GenePix 4.0 program (Axon Instruments Inc., Union City, CA).

Data Analysis and Clustering. Data obtained from each hybridization were stored in a database for analysis. The Cy3:Cy5 ratios were normalized to the median ratio value of all of the spots in the array. After normalization, spots with intensities for both channels (sum of medians) less than that of the local background were discarded. The ratios of the remaining spots were log-transformed (base 2), and duplicated spots on the OncoChip were averaged to the median. In addition, any gene of which the data were missing from >30% of the samples was not included in subsequent analyses. For supervised prediction, missing values for the remaining spots were replaced by the average value of the nearest K patterns (K = 17; Ref. 13). To obtain the expression profile of each tumor we referred the ratios of the tumors to the control. The cluster analysis was done with the Gene Cluster program (14).

Supervised Prediction. Classes were defined with respect to several variables: somatic mutation status, proliferation level, and OS. For somatic mutation and proliferation signatures marker genes were identified using Student's *t* test. These procedures are available for inspection.[9]

IgV_H Study. IgV_H mutational analysis was performed on 35 of the 38 MCL samples. A detailed description of the IgV_H findings in these cases has been published separately (8). Rearranged IgV_H genes were amplified using a seminested PCR method, as described previously (15). A sequence was considered mutated when there was <98% homology with the closest germ-line V_H genes.

Immunohistochemistry. An immunohistochemical analysis was carried out to evaluate some of the cDNA-microarray results at the protein level. It was carried out on a Tissue-Microarray containing samples of 32 MCL cases included in this series. The *Bcl*II, Bcl6, cyclin D1 (DAKO, Copenhagen, Denmark) ,and p18 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used for this purpose.

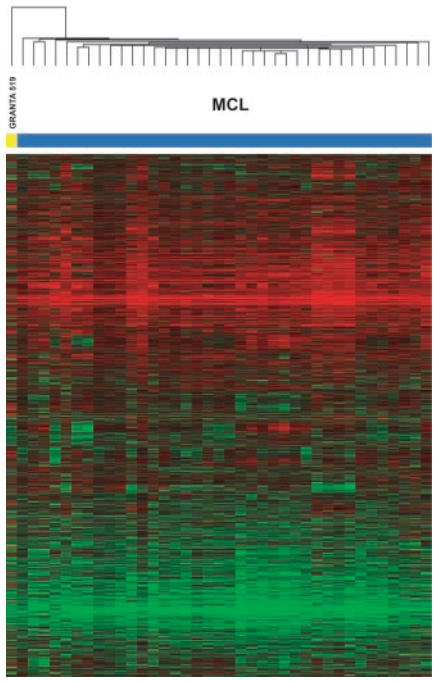


Fig. 1. Expression profile of MCL series, demonstrating the homogeneity of this type of lymphoma. *Red* indicates high level of expression in tumors *versus* mantle B cells, and *green* indicates low-level expression.

The proliferation index used to construct the "increased proliferation fraction signature" was quantitatively evaluated using MIB1 antibody (DAKO). At least 200 cells were counted in every case. The cutoff was 50% of positive cells; thus, a low proliferation index was ≤50% of positive cells and a high proliferation index was >50%.

Statistics. OS curves were derived using the Kaplan-Meier method. All of the deaths were attributed to the tumor. Statistical significance of associations between individual variables and OS was determined using the log-rank test. Cox's univariate proportional hazard analysis was also performed independently for each variable. Multiple testing and the random permutation test validated results. To build the gene expression-based predictive model, genes with values of $P < 0.05$ in the Cox's univariate analysis were grouped by their expression profile using the SOTA program.[10] Nine groups of predictor genes were obtained. The average of the expression ratio, in each cluster, was used to perform a conditional backward Cox's multivariate analysis.

The result of the Cox's multivariate analysis was then validated using a prediction model derived by discriminant analysis. In this model, patients were stratified by the median survival in the series: short- *versus* long-term survival (< *versus* >27 months, respectively). For patients who remained alive, only those with >27 months of follow-up were included. General applicability of the model was also tested by leave-one-out cross-validation.

Statistical analyses were performed using the SPSS (SPSS Inc., Chicago, IL) program and the tools for random permutation tests available.[10]

RESULTS

Clinical Features. Thirty-eight patients meeting the criteria for MCL diagnosis were enrolled: 31 patients were male and 7 were female. The median age was 64 years (range, 40–86 years). Thirty of them were at stage III to IV at diagnosis. Nine cases presented splenomegaly at diagnosis. Waldeyer's ring or gastrointestinal tract involvement was present in 5 and 2 patients, respectively. Peripheral blood and/or bone marrow infiltration was observed in 18 cases. The International Prognostic Index group distribution was: 5 high-risk

[9] Internet address: http://bioinfo.cnio.es/cgi-bin/tools/multest/multest.cgi.

[10] Internet address: http://bioinformatica.cnio.es/.

Table 1 *Number of genes deregulated in MCL (>2-fold up or downregulated), grouped by described functions*

Function	Expression ratio	*n* of genes
Apoptosis	<2-fold	6
	>2-fold	17
Cell cycle control	<2-fold	27
	>2-fold	1
DNA/RNA processing	<2-fold	8
	>2-fold	1
Signal transduction	<2-fold	25
	>2-fold	33
Transcription factors	<2-fold	17
	>2-fold	11
Stress response	<2-fold	6
	>2-fold	3
Ubiquitin pathway and chaperones	<2-fold	7
	>2-fold	2
Cytokines and immune response	<2-fold	5
	>2-fold	23
Differentiation and development	<2-fold	6
	>2-fold	1
Drug response	<2-fold	0
	>2-fold	14
Metabolism and transport	<2-fold	29
	>2-fold	23
Cell structure, cytoskeleton, and cell adhesion	<2-fold	26
	>2-fold	21
T cell	<2-fold	5
	>2-fold	8
B cell	<2-fold	1
	>2-fold	9
Others	<2-fold	11
	>2-fold	11
Function not known	<2-fold	37
	>2-fold	65

(stages 4,5) patients, 20 intermediate-risk (stage 2, 3), and 5 low-risk (stage 0, 1) cases. The median follow-up period for the entire series was 19 months (range, 1–106).

MCL Displays a Relatively Homogeneous Profile. Thirty-eight MCL samples and one MCL cell line (Granta 519) were analyzed by cDNA microarray technology. Unsupervised clustering revealed a relatively homogeneous expression profile, grouping all of the clinical samples in one main cluster (Fig. 1). The MCL cell line Granta 519 was grouped separately, probably because of all of the intrinsic molecular alterations that this cell line carries. To confirm the reproducibility of the technique, we repeated the hybridization of four of the samples and analyzed them together. Different hybridizations corresponding to the same patient clustered together using unsupervised clustering, demonstrating that the expression profiles obtained were reproducible.

From the 5217 clones, corresponding to 2945 known genes and 1854 expressed sequence tags, that were suitable for analysis after filtering steps, respectively, 233 and 213 genes were found to be >2-fold up-regulated and down-regulated relative to mantle zone B cells in at least 50% of the samples (Table 1). As expected, *CCND1* was up-regulated in the whole series, and so contributed to validate the data of the expression profiling. A large set of genes involved in important cellular processes, like apoptosis, cell cycle control, or signaling, was found to be consistently deregulated in the majority of MCL samples.

Thus, 23 genes described as being involved in apoptosis were found to be deregulated in at least 50% of the samples (Table 2). The most remarkable were the antiapoptotic genes *BCL2* and *TOSO*, of which the expression levels were 5.8- and 4.6-fold higher in lymphomas than in the control. An important number of genes related with TNF and NFκB pathways were found up-regulated in our series of MCL, including the activator of *NFkB*, *IKBKB*, *TNFRSF7*, *TNFS4*, *TANK*, *TRAF5*, *TRAF6*, and *DED*. On the other hand, the proapoptotic genes *CSE1L*, *CASP7*, *STK17A*, and *STK17B* were lost in our tumor series.

It is noteworthy that, with the exception of *CCND1*, of which the overexpression in MCL tumors is >4.9-fold, in the mantle B cells, there was no other up-regulated cell cycle gene in this series (Table 1). All of the alterations found in this group of genes were losses, thus being consistent with the relatively low rate of proliferation that characterizes this tumor. Thus, the CDK inhibitor *p18* was expressed at a very low level in our series. Some cyclins, *CCNH*, *CCNG2*, *PCNA*, *CCNA2*, *CCNB1*, and *CCNB2*, and several cell cycle-division proteins, cdc25a, cdc25c, cdc6, cdc45-l, and *CHEK-1*, were also lost (see supplementary data[11]).

The largest functional group deregulated in this series is the signal transduction-related group of proteins, with 25 and 33 genes >2-fold down- and up-regulated, respectively. Other remarkably deregulated genes involving different cell functions are described in Table 3, and in the supplementary data.[11] The most relevant were *SPARC* and *IL10R*, because of their very high level of expression in the series of MCL (11.5- and 9.1-fold, respectively).

Validation of Results. All of the studied (32 of 38 paraffin-embedded) cases in the series displayed a cyclin D1-positive, *Bcl*II-positive, Bcl6-negative, and p18-negative immunophenotype (Fig. 2), thus validating the cDNA microarray results. Only 1 case presented positive expression of p18 in the paraffin-embedded tissue, the MCL case having a stronger signal in the cDNA microarray.

MCL Profile Variability. Supervised clustering was performed to determine molecular signatures for biologically and clinically relevant features in MCL, such as somatic mutation, proliferation index, or blastoid cytology.

IgV_H Somatic Mutation Signature. To derive the molecular signature for somatic mutation, we did a Student's *t* test and selected the 15 genes with the lowest *P* (Fig. 3*A*). MCL cases with >2% somatic mutations showed up-regulation (compared with those cases without somatic mutation) of some genes involved in DNA and RNA processing, such as the gene encoding for the DNA replication-related protein RPA1, the helicase *SMARCA5*, and the splicing factor *SFRS3*. *K-ras2* was up-regulated, as was *FNTA*, a post-transcriptional modifier of Ras and other G proteins. The levels of the transcription factor *EGR2* were also higher in MCL cases with mutation. Finally, *CDC14A*, a regulator of p53 function, was found to be overexpressed in this group.

Blastoid Variant *versus* Classic. Five cases in this study correspond to the blastoid variant of MCL. Fifteen genes were selected by Student's *t* test (Fig. 3*B*). Blastoid cases had a lower expression of caspase 7, but an increased expression of TOP1 and CDK4 (with a lower significance).

Increased Proliferation Fraction Signature. The proliferation rate of the tumors was examined using Ki-67 expression, as detected by immunohistochemistry (MIB1 antibody). Fourteen cases presented high expression of Ki-67 (>50% positive cells), and the other 24 had low levels of Ki-67. The most relevant finding was the down-regulation of BIK, a BCL2-interacting proapoptotic protein, observed in the group of cases with higher proliferation.

Survival Analysis. A number of 137 genes were found to predict OS probability using Cox's univariate analysis. These genes were clustered into nine groups according to their expression profile. The average ratio of expression for each group was then calculated, and a

[11] Internet address: http://redlinfomas.cnio.es/publications/molecular_signature.

Table 2 *Apoptosis-related genes deregulated in MCL*

Gene symbol	Gene description	*n* fold (average)	
BCL2	B-cell CLL/lymphoma 2	5.855	Upregulated
TOSO	Regulator of Fas-induced apoptosis	4.588	
PIK3CD	Phosphoinositide-3-kinase, catalytic, δ polypeptide	3.525	
IKBKB	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase β	3.259	
TNFRSF7	Tumor necrosis factor receptor superfamily, member 7	2.995	
TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4	2.878	
TRAF5	TNF receptor-associated, factor 5	2.814	
FIP2	Tumor necrosis factor α-inducible cellular protein	2.675	
TNFSF13	Tumor necrosis factor (ligand) superfamily, member 13	2.420	
LTB	Lymphotoxin β (TNF superfamily, member 3)	2.410	
TANK	TRAF family member-associated NFκB activator	2.325	
TRAF6	TNF receptor-associated factor 6	2.321	
ASC	Apoptosis-associated speck-like protein containing a CARD	2.250	
CFLAR	CASP8 and FADD-like apoptosis regulator	2.152	
DED	Apoptosis-antagonizing transcription factor	2.111	
TNFAIP3	Tumor necrosis factor, α-induced protein 3	2.075	
CASP1	Caspase 1, apoptosis-related cysteine protease	2.027	
CASP7	Caspase 7, apoptosis-related cysteine protease	0.464	Downregulated
STK17A	Serine/threonine kinase 17a (apoptosis-inducing)	0.422	
CSE1L	Chromosome segregation 1 (yeast homolog)-like	0.406	
BIRC2	Baculoviral IAP repeat-containing 2	0.394	
STK17B	Serine/threonine kinase 17b (apoptosis-inducing)	0.388	
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	0.360	

backward Cox's multivariate analysis identified two clusters (containing 26 genes), which independently predict survival (see supplementary data[11]). The patients were ranked according to their score and divided into two groups of similar size (16 and 15 patients). Their survival probability was then estimated by the Kaplan-Meier method. The first group, with the lowest score, displayed a survival probability of 52% at 5 years (Fig. 4).

The results were validated using cross-validation discriminant analysis. There was a 75% correct classification rate for this model, 9 of 12 in the short-term survival group (OS <27 months), and 9 of 12 in the long-term survival group (OS >27 months).

DISCUSSION

The series analyzed here shows substantial homogeneity, confirming that MCL, taken as a whole, is a distinct entity.

The comparison with the expression profile of normal mantle zone B cells allows it to be recognized that, in addition to the increased cyclin D1 expression, there is a marked deregulation of 446 genes, present in at least 50% of the cases. This signature includes genes described as playing critical roles in apoptosis, cell cycle, signal transduction, cell structure, cytoskeleton, and so forth. The overexpression of cyclin D1 in this series was observed in all of the series of cases, thus being independent of the normalization with mantle zone cells.

Especially informative is the deregulation of a significant number of genes involved in apoptosis control. Thus, there is a simultaneous loss of a large set of proapoptotic genes including *CASP7*, *STK17A*, *STK17B*, and *CSE1L*. Caspase 7 is a well-characterized executioner caspase (16), whereas STK17A and STK17B, also named as DRAK1 and DRAK2, are kinases involved in the initiation of apoptosis (17). CSE1L is a nuclear transport factor that was first cloned while searching for genes that rendered breast cancer cells resistant toward toxin-induced apoptosis (18). On the other hand, some antiapoptotic genes, such as *BCL2* and *TOSO* (see Table 2), are up-regulated in our series. BCL2 is known to block apoptosis, regulating the permeability of the outer mitochondrial membrane (19), whereas TOSO inhibits Fas-mediated apoptosis in lymphoid cells, resulting in the inhibition of caspase activity (20). Another antiapoptotic gene considerably up-regulated is phosphatidylinositol 3′-kinase catalytic, δ polypeptide (p13K), a class I P13K (21), related with drug resistance mechanisms in leukemic cells (22).

In the same context, the simultaneous deregulation of multiple genes implicated in the regulation of the NFκB, and more specifically in the routes that interlink TNF with NFκB, is very remarkable. Thus, it appears to be an important up-regulation of the main genes of this pathway, such as *TNFRSF7*, *TNFS4*, *TANK*, *TRAF5*, *TRAF6*, *DED*, and others. This is also detectable for *IkBkB*, of which the product activates NFκB. It has been demonstrated recently that IkBkB, besides the central role that it plays in the antiapoptotic pathway controlled by NFκB, can also suppress p53 protein stabilization through up-regulation of Mdm2 (23).

Taking these findings together, the amount and relevance of apo-

Table 3 *Genes expressed with the highest expression level in the series of MCL*

Function	Gene symbol	Description	Median of change
Cell structure, cytoskeleton, and cell adhesion	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	11.503
Cytokines and immune response	IL10RA	Interleukin 10 receptor, α	9.109
Metabolism and transport	CTSS	Cathepsin S	6.008
Apoptosis	BCL2	B-cell CLL/lymphoma 2	5.855
Ubiquitin pathway and chaperones	UBD	Diubiquitin	5.576
Genes implicated in drug response	MIG	Monokine induced by gamma interferon	5.533
Signal transduction	CSF1R	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homologue	5.503
Cell cycle control	CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	4.943
Stress response	HSPB2	Heat shock 27kD protein 2	3.898
Transcription factors	ZNFN1A1	Zinc finger protein, subfamily 1A, 1 (Ikaros)	3.803
Differentiation and development	TGIF	TGFB-induced factor (TALE family homeobox)	2.618
DNA/RNA processing	DCTD	dCMP deaminase	2.058

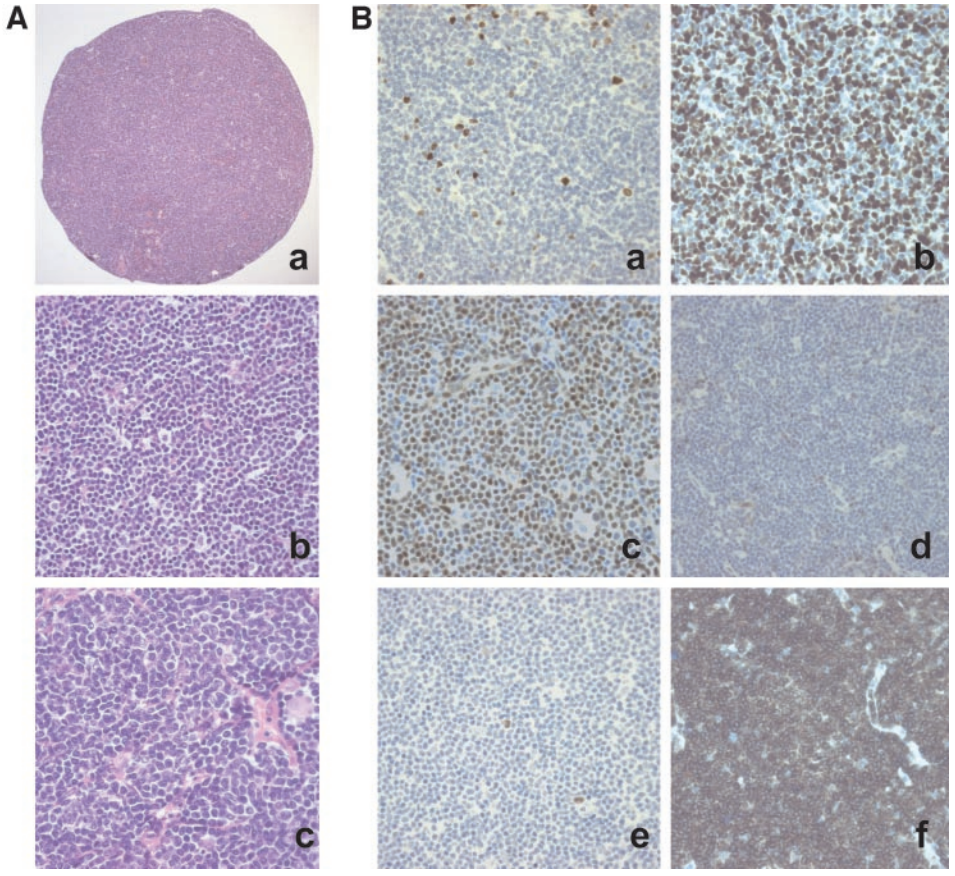
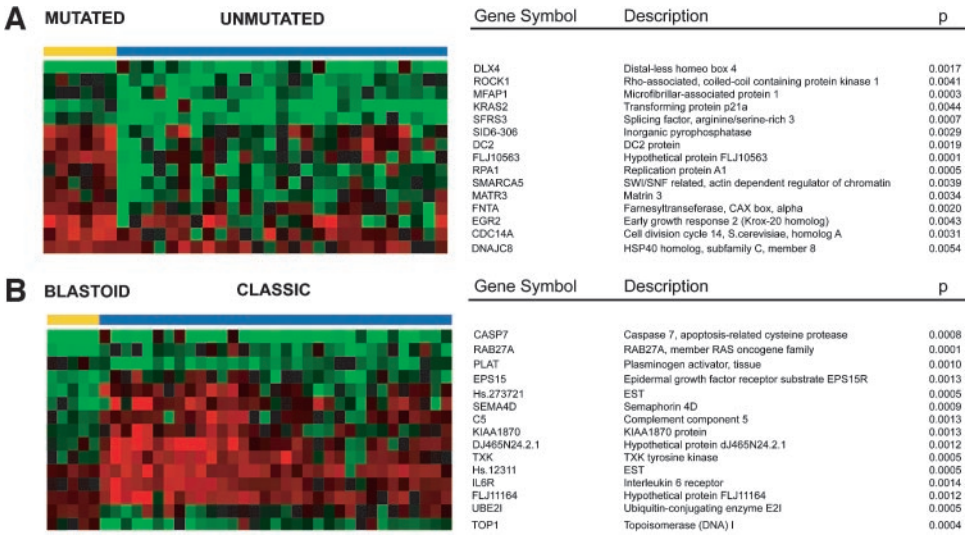


Fig. 2. *A, panel a,* H&E of a 1-mm-diameter cylinder of a MCL case (original magnification ×100). *Panel b,* H&E of a classic MCL case (×600). *Panel c,* H&E of a blastoid MCL case (×600). *B, panel a,* low proliferation index and *b,* high proliferation index measured by Ki67 expression (×600). *Panel c,* cyclin D1-positive expression (×600). *Panel d,* negative expression of p18 protein (×600). *Panel e,* negative expression of bcl6 protein (×600). *Panel f,* Bcl-2-positive expression (×600).

ptosis-regulator genes found to be altered in MCL, including the mitochondrial apoptotic pathway (up-regulation of *BCL2*) and the TNF and NFκB pathways, probably accounts for the low sensitivity of MCL tumor cells to chemotherapy, and the subsequent poor outcome of MCL patients. This is consistent with previous observations (24) that have already emphasized the noticeable accumulation of alterations in apoptosis-regulator genes.

In addition to these significant findings in relation to apoptosis, strong up-regulation of *IL10R* and *SPARC* found in MCL constitutes a potentially important observation. IL-10 is an autocrine growth factor, important for B-cell survival, and needed for the expansion of malignant B cells (25, 26). It has been demonstrated to be up-regulated in human peripheral B cells as a consequence of CD5 expression. Thus, CD5 supports the survival of B cells by stimulating IL-10 production, and by concurrently exerting negative feedback on B-cell receptor (BCR)-induced signaling events that can promote cell death (27). Up-regulation of IL-10R in MCL has been detected recently by expression profiling in a smaller series of seven cases, but at a lower level (1.9-fold *versus* 9.1-fold in our study; Ref. 10). The increased expression of IL-10 could be also related with the diminished presence of inflammatory T cells in MCL samples, because IL-10 has been demonstrated to be a potent immunosuppresser (28).



Gene Symbol	Description	p
DLX4	Distal-less homeo box 4	0.0017
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	0.0041
MFAP1	Microfibrillar-associated protein 1	0.0003
KRAS2	Transforming protein p21a	0.0044
SFRS3	Splicing factor, arginine/serine-rich 3	0.0007
SID6-306	Inorganic pyrophosphatase	0.0029
DC2	DC2 protein	0.0019
FLJ10563	Hypothetical protein FLJ10563	0.0001
RPA1	Replication protein A1	0.0005
SMARCA5	SWI/SNF related, actin dependent regulator of chromatin	0.0039
MATR3	Matrin 3	0.0034
FNTA	Farnesyltranseferase, CAX box, alpha	0.0020
EGR2	Early growth response 2 (Krox-20 homolog)	0.0043
CDC14A	Cell division cycle 14, S.cerevisiae, homolog A	0.0031
DNAJC8	HSP40 homolog, subfamily C, member 8	0.0054

Gene Symbol	Description	p
CASP7	Caspase 7, apoptosis-related cysteine protease	0.0008
RAB27A	RAB27A, member RAS oncogene family	0.0001
PLAT	Plasminogen activator, tissue	0.0010
EPS15	Epidermal growth factor receptor substrate EPS15R	0.0013
Hs.273721	EST	0.0005
SEMA4D	Semaphorin 4D	0.0009
C5	Complement component 5	0.0013
KIAA1870	KIAA1870 protein	0.0013
DJ465N24.2.1	Hypothetical protein dJ465N24.2.1	0.0012
TXK	TXK tyrosine kinase	0.0005
Hs.12311	EST	0.0005
IL6R	Interleukin 6 receptor	0.0014
FLJ11164	Hypothetical protein FLJ11164	0.0012
UBE2I	Ubiquitin-conjugating enzyme E2I	0.0005
TOP1	Topoisomerase (DNA) I	0.0004

Fig. 3. Gene expression signature for mutated MCL cases (*A*) and blastoid variant (*B*).

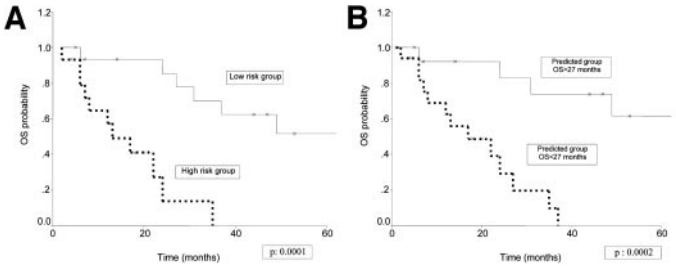


Fig. 4. Kaplan-Meier plot of OS probability. *A*, gene expression based survival predictor model identifies half the patients with 52% survival probability at 60 months in this series. No patient in the high-risk group reaches 60 months. Only gene cluster 3 (ExpB: 29.091), and cluster 9 (ExpB: 0.143) were considered significant in the Cox multivariate analysis. *B*, cross-validation of the survival predictor confirms that low-risk patients had significantly improved long-term survival, when compared with high-risk patients ($P = 0.0002$, log rank test). Twenty-seven months represents the median of OS.

Moreover, it has been shown that diffuse large B-cell lymphomas expressing CD5 are more aggressive than their CD5-negative$^-$ counterparts (29). Consistently with these observations, the data obtained here also show an increased expression of CD5 mRNA, as might be expected, because a majority of MCL cases regularly express CD5.

SPARC has been also found to be strongly up-regulated in this series (11.5-fold, on average). The product of *SPARC*, also known as osteonectin and BM-40, is a secreted, multifunctional, extracellular matrix glycoprotein, which inhibits cell adhesion and activates MMP2. SPARC is involved in diverse biological processes related with angiogenesis, and tumor cell migration and invasion (30), and has been observed to be overexpressed in multiple myeloma (31).

In addition to increased cyclin D1 expression, alterations in cell-cycle machinery mainly involve loss of relevant genes in control of cell cycle progression. A notable finding is the loss of *p18*, observed in most cases, which confirms a previous report of alterations of this CDKI in 2 MCL cases (32). More recently, other authors (33), based on *p18* induction assays in multiple myeloma cell lines, showed the importance of this protein in the induction of tumor regression dependent of apoptotic cell death.

This study has also revealed the presence of a characteristic signature associated with IgV_H somatic mutation, independent of those identified for blastoid cytology, survival, or proliferation. Recent studies have demonstrated that a significant fraction of MCL cases carry >2% of IgV_H mutations (7, 8), although this finding lacks any prognostic significance. Cases included in this series bearing these IgV_H somatic mutations display changes in the level of expression of a set of genes that includes *CDC14A*, a phosphatase of which the deregulated expression abolishes the centrosome division cycle and leads to chromosome missegregation, thus implying a role in multistep tumorigenesis and promotion of chromosomal instability (34). The biological relevance of IgV_H somatic mutation is given additional weight by the observed association with increased *K-ras* and farnesyl transferase expression, both of which are indicative of ras activation. Nevertheless, the exact role of somatic mutation in MCL needs additional investigation in a larger set of cases, to clarify whether it is merely an epiphenomenon, or whether indeed the presence of somatic mutation is pathogenically associated with critical genetic changes.

This analysis has also identified a molecular signature associated with blastoid cytology. Thus, the 5 cases analyzed differentially express a large set of molecules, including some associated with proliferation (gain of *TOP1*), apoptosis (loss of caspase 7), and vesicular transport (loss of *RAB27A*). Significantly, a striking increase of CDK4, an oncoprotein of which the amplification has been described in blastoid MCL (35), has been observed here, thus confirming previous observations (36).

We also attempted to obtain a molecular signature characteristic for a group of MCL with a high proliferation rate. As expected, we found a higher level of expression of several cyclins and other proliferation markers in the high proliferation rate group. One unexpected finding was the presence of a striking loss of the expression of *BIK* in the high proliferation rate group. This is an apoptosis promoter, downstream target, and effector in the E1A-induced p53 cell death pathway, and canonical member of the BH3-only family, which interacts with the cellular survival-promoting proteins, Bcl-2 and Bcl-xL (37).

Despite the fatal prognosis of MCL, different observations coincide to highlight the presence of a group of patients with a more favorable course and better response to chemotherapy. The vast amount of data collected here allows a survival predictor to be derived that identifies a subset of patients with a 52% probability of 5-year survival. If confirmed in a blind set of cases, these data would allow MCL patients to be stratified according to their individual risk and would eventually enable the use of risk-tailored treatment protocols. The information accumulated should also facilitate the search for therapeutic targets, which could eventually contribute to a more rational treatment and improved prognosis in this lymphoma subtype. The predictor here proposed differs from that described recently, based on a gene expression signature related with proliferation (9). Here, although a group of genes related with proliferation were present in the set of predictor genes (8 of 137 genes), the restriction to this proliferation cluster in the survival analysis reduces the capacity of the predictor (data not shown). Additionally, in our series cyclin D1 expression did not appear as a significant prognosis predictor.

In summary, this work demonstrates the existence of a constellation of gene-expression differences in MCL compared with normal mantle B cells. As well as the already known alterations, such as the increase in cyclin D1 expression, these data confirm that MCL cells are subject to a profound deregulation of multiple genes and pathways, singularly those in charge of regulation of apoptosis. Additionally, these data allow us to recognize some underlying molecular events associated with the presence of blastoid cytology, IgV_H somatic mutation, and to identify a subset of genes of which the expression is associated with longer-term survival. Experimental and clinical validation in a larger series of cases could allow confirmation of these data, facilitating a more rational approach to the diagnosis and risk-tailored treatment of MCL cases.

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