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Increased expression of Ki-67 in mantle cell lymphoma is associated with de-regulation of several cell cycle regulatory components, as identified by global gene expression analysis

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A B S T R A C T

Background and Objectives. Mantle cell lymphoma (MCL) is an aggressive disease. Patients with this malignancy have a median survival of 3 years. To better understand disease progression, which is characterized by increased proliferation, we analyzed the gene expression of MCL with different proliferative indices, as determined by immunohistochemical staining for Ki-67. Furthermore, primary and relapsed tumors were compared to identify the possible growth advantages possessed by cells which persist after therapy and which might evolve into a tumor relapse.

Design and Methods. Twenty-one samples of MCL were analyzed, using the Affymetrix U95Av2 chip, containing probes for approximately 12,000 transcripts. Samples with a high versus low fraction of Ki-67^{+} cells were compared as were relapsed versus primary tumors. Immunohistochemistry was used to confirm the expression of some gene products.

Results. A distinct genetic signature, consisting of 32 genes, was found when comparing Ki-67^{high} with Ki-67^{low} MCL. The signature consisted of genes involved in cellular processes, such as mitotic spindle formation, gene transcription and cell cycle regulation, e.g. components of the p53 and retinoblastoma protein (pRb) pathways. Of note, *cyclin D1*, the hallmark of MCL, as well as *Ki-67* were up-regulated in the samples with a high proliferative index. Comparing primary vs. relapsed tumors, 26 individual genes were found, several involved in cell adhesion. Furthermore, increased expression of transferrin receptor was found in the relapsed tumors.

Interpretation and Conclusions. A genetic signature distinguishing Ki-67^{high} MCL from Ki-67^{low} was established. The generated signature was used to assign new MCL samples to the high proliferative group, validating the association between these genes and proliferation in MCL.

Key words: Ki-67, proliferative index, DNA microarray, B-cell lymphoma, mantle cell lymphoma, gene expression.

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Mantle cell lymphomas (MCL), a distinct subtype of non-Hodgkin's lymphoma, display the translocation t(11;14)(q13;32) that juxtaposes *cyclin D1* with the immunoglobulin heavy chain.[1,2] Most MCL overexpress cyclin D1. The increase of this cell cycle regulatory protein contributes to the pathogenesis of the disease, but alone is not sufficient to promote malignant transformation.[3] However, some MCL-like B-cell lymphomas lack cyclin D1 expression, but still have the same morphologic and immunophenotypic characteristics.[4] These cyclin D1-negative MCL-like lymphomas have been shown to have a better prognosis and should be considered as an entity separate from MCL.[4,5] Furthermore, MCL cells typically express CD5, CD19 and CD20, but lack expression of CD23.[6] Most MCL lack somatic mutations and have been proposed to derive from naïve B cells. However, in a recent study we proposed an activated B cell as the origin for MCL, since a distinct genetic signature resembling that of activated B cells was found.[7]

MCL account for 6-7% of all non-Hodgkin's lymphomas and have a poor prognosis due to resistance to treatment.[6] The median survival of patients with MCL is 3 years, but some patients survive more than 10 years, indicating that this is a clinically heterogeneous malignancy.[5] MCL display three different morphologic growth patterns: mantle zone, nodular and diffuse. These morphologic growth patterns can be further divided into typical and blastoid variants. Studies have demonstrated a better prognosis for patients with MCL showing the mantle zone[8] or the nodular growth pattern.[9] The blastoid features have been reported to be associated with a shorter survival[10,11] and 26-70% of MCL patients

have progression towards this variant during their life or it is found at autopsy.[9,12] Shorter survival among MCL patients has also been correlated with overexpression of *myc*,[13] p53,[11,14] CD38[14] and a high proliferative index as measured by nuclear expression of Ki-67 antigen.[11] The correlation between increased expression of p53 and decreased survival time in aggressive types of MCL is probably due to mutations of p53.[15] Recently, the proliferative gene expression signature has been demonstrated to be a quantitative predictor of survival in MCL, in that expression of only four genes identified groups of patients whose median survival differed by more than five years.[5] A few other studies have also analyzed differences in gene expression associated with non-physiological features, such as blastoid/classic cytomorphology.[16,17] These studies demonstrated the potential of global gene expression profiling in correlating genetic signatures with clinical characteristics, as well as with molecular and cellular events.

In this study, we compared the gene expression profile between tumors with different fractions of Ki-67$^+$ cells, as determined by histochemical staining. A high proliferative index, blastoid features and decreased survival time are strongly correlated, since increased Ki-67 antigen expression is suggested to predict for both blastoid transformation[18] as well as shorter survival.[11] The purpose of comparing tumors with different Ki-67 protein expression was to identify genes involved in increased cell cycling, since these genes might become targets for new therapeutic approaches. Furthermore, the genetic signatures of recurrent and primary tumors were determined to investigate the possible clonal selection of cells during relapse.

Design and Methods

Patients' material

The diagnostic material was obtained from lymph nodes (n=18), or from spleen (n=3). Immunophenotyping by immunohistochemistry (paraffin-embedded and/or frozen tissue sections) and/or by flow cytometry was performed in all cases showing a monoclonal B-cell population positive for CD5. The diagnosis of MCL was confirmed by cyclin D1 staining and/or interphase fluorescence *in situ* hybridization (FISH) analysis on imprints showing either overexpression of cyclin D1 or translocation t(11;14) in all cases.

Immunohistochemistry

Immunohistochemical staining for Ki-67 antigen (M7240, DakoCytomation, Glostrup, Denmark) was performed using an automated methodology (Enhanced DAB paraffin, IHC Staining Module, Ventana Medical Systems (VMA), Tucson, Arizona, USA). Evaluation of the proliferative fraction was performed as previously described.[19]

Immunohistochemical staining for cyclin F, HCK and thymidylate synthase was performed on 5 mm paraffin sections with antibodies against cyclin F (sc-952, dilution 1:400), hemopoietic cell kinase (HCK sc-72, dilution 1:400) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and thymidylate synthase (TYMS, ab 3145, dilution 1:20) (Abcam, Cambridge, UK). Incubation with primary antibodies for cyclin F and HCK was carried out for 24h at 4°C. Before staining for TYMS, an antigen retrieval procedure was applied by microwave cooking in citrate buffer pH 6.0 (10 min 750W + 10 min 450W) and incubation with anti-TYMS was carried out at room temperature for 90 min. Staining was visualized using the immunoperoxidase method with Strept. ABComplex/HRP Duet Mouse/Rabbit and diaminobenzidine as substrate (DakoCytomation).

Preparation of samples, mRNA and microarrays

Fresh MCL tumors (2 samples, designated MCL1 and MCL2) were cut into small pieces and suspended in RPMI 1640, containing 10% fetal calf serum (FCS). Cells were filtered through a cell strainer (BD Labware, Franklin Lake, New Jersey, USA) to remove tissue debris. The lymphocytes were then purified using Ficoll-Isopaque (Amersham Pharmacia Biotech, Uppsala, Sweden), and the T cells were depleted using CD3$^+$ dynabeads (Dynal A.S, Oslo, Norway), leaving CD5$^+$/CD19$^+$ tumor cells with >95% purity. The cells were pelleted and lysed in Trizol (Life Technologies, Gaithersburg, MD, USA). Frozen tumors (19 samples, designated MCL3-21) were homogenized (2×15 s) directly into Trizol, using an Ultra Turrax knife homogenizer (IKA-WERK, Tamro Med Lab, Mölndal, Sweden). All preparations of mRNA, *in vitro* transcription and hybridization of labeled cRNA to the U95Av2 arrays (Affymetrix, Inc., Santa Clara, CA, USA) were performed according to Ek *et al.*[7]

Microarray analysis and statistics

The expression level for each probe set is given as a signal value by the MicroArraySuite 5.0 software, provided by Affymetrix, Inc. The signal values were scaled in MicroArraySuite 5.0 against a target value of 500 to enable different arrays to be compared. Comparison files were created using the batch analysis tool.

The statistical algorithms are described in detail at *www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf*. Briefly, the detection algorithm describes whether the transcript was present (P), absent (A), or marginal (M). The change algorithm indicates the qualitative change (increase (I), decrease (D), marginal increase (MI), marginal decrease (MD) or no change (NC)) between two different arrays.

The signal log ratio is the quantitative change in expression level for a transcript between two different arrays. The change is expressed as the ^{2}log ratio.

The signal values were imported into Gene Spring 6.0 (Silicon Genetics, Redwood City, CA, USA) for further data analysis e.g. filtering, statistical calculations and hierarchical clustering. Hierarchical clustering was performed on scaled (*see above*) and normalized data (per gene: normalized to the median), using Spearman's confidence algorithm (the minimum distance was set at 0.001 and the separation ratio at 1). The *p*-values for HCK, cyclin F and thymidylate synthase were calculated using a non-parametric test (Mann-Whitney) for samples with >30% Ki-67$^+$ cells (n=4) compared to samples with <10% Ki-67$^+$ cells (n=7).

Differential Ki-67 expression

When comparing samples with different proliferative indices, samples having >30% Ki-67$^+$ cells (Ki-67^{high}, n = 2) were compared to samples having <10% Ki-67$^+$ cells (Ki-67^{low}, n = 7). This was done by creating 2×7 = 14 comparison analysis files. The genes on the U95Av2 array were then filtered for differential expression by adding the following three criteria: 11/14 (>75%) of the comparison analysis files had to have (i) an increase (I) or decrease (D) call in the change algorithm, (ii) a log ratio of more than 1 or less than –1, using the signal log ratio algorithm. Furthermore, (iii) 2/2 (Ki-67^{high}) or 5/7 (Ki-67^{low}) of the files in the up-regulated group (i.e. Ki-67^{high} for up-regulated genes or Ki-67^{low} for down-regulated genes), had to be called present by the detection algorithm. Genes that fulfilled all three criteria were considered significantly up or down-regulated, comparing Ki-67^{high} to Ki-67^{low} MCL samples. The 9 samples that were used to perform this analysis were all primary tumors from different patients.

Relapsed vs. primary MCL tumors

Samples from two patients, obtained at primary diagnosis and at relapse, were used to compare recurrent and primary tumors. An additional, third sample from a relapse was available and was used when filtering (present call) for up-regulated genes. The recurrent and primary tumors were compared for each of the 2 patients and the genes that differed in both of the comparisons were selected. Thus, in each comparison, genes with a present call in the relapsed tumor and an increase call and >1 signal log ratio, comparing primary to relapsed tumor, were considered to be up-regulated. In addition, the up-regulated genes had to be present in the sample from the third relapsed tumor. Filtering for down-regulated genes, the genes had to have a present call in the primary tumor, a decrease call and < -1 signal log ratio, comparing primary to relapsed tumors. The genes that were called up or down-regulated in both patients, according to the criteria described above, were combined and are listed in Table 2.

Results

A distinct genetic signature distinguishes patients with Ki-67^{high} expressing tumors from those with Ki-67^{low} expressing tumors. Samples displaying a high (>30% Ki-67$^+$ cells) or low (<10% Ki-67$^+$ cells) proliferative index were utilized to identify differentially expressed genes. Using our strict three-algorithm approach (*see Design and Methods*) a list of 32 genes that were differentially expressed in these two subsets of tumors was extracted (Table 1). To validate the genetic signature, 12 other MCL samples were used. Two of the validation samples were relapsed tumors from MCL with a high proliferative index. The remaining 10 tumor samples had 10-30% Ki-67$^+$ cells and could be divided into one group (n = 5) with 10-20% Ki-67$^+$ tumor cells and a second group (n=5) with 20-30% Ki-67$^+$ tumor cells. Tumors with 20-30% Ki-67$^+$ cells clustered (4/5) with tumors having >30% Ki-67$^+$ (4/4), ($p < 0.02$) (Figure 1). Furthermore, tumors with <10% (7/7) or 10-20% (4/5) Ki-67$^+$ cells clustered in four separate groups ($p \leq 0.01$) with 2-4 samples in each group. Thus, the genetic signature correlated well with elevated expression of Ki-67, in that 6/7 tumors from the validation set with >30%, or 20-30% Ki-67$^+$ cells were correctly stratified, with an overall statistical significance of $p \leq 0.02$.

When further analyzing the genetic signature it was clear that many of the de-regulated genes were involved in the control of cell proliferation, as for example *MKI67 (Ki-67), ZNF148 (ZBP-89),*[20] *protein phosphatase 2C (PPM1A),*[21] cyclin F and cyclin D1 (Table 1). *Cyclin D1* was 3-fold overexpressed and correlated with the proliferative index, as was recently suggested by Rosenwald *et al.*[5] *Ki-67* was 10-fold overexpressed and the mRNA expression correlated well with the histochemical staining of Ki-67 protein, which validated the stratification of patients into those with Ki-67^{high} and Ki-67^{low} expressing tumors. ZNF148 (ZBP-89), a zinc finger transcription factor, and PPM1A regulate growth by promoting the expression of p53. Cyclin F is important for G_2/M cell-cycle transition and the gene was >3-fold overexpressed.[22,23] The expression of cyclin F was further confirmed on tissue sections in which MCL with a high proliferative index showed positive staining for cyclin F (Figure 3), whereas MCL with a low proliferative index showed no reactivity for cyclin F (not shown). *TPX2 (C20orf1)*, *Bub1b* and *HEC* were up-regulated 3-5-fold in the Ki67^{high} tumors. These genes are all important for the mitotic machinery, where *TPX2* has a structural role in the spindle and is required for normal spindle morphology and centrosome integrity during cell division.[24]

Table 1. De-regulated genes comparing MCL with a high or low proliferative index.

Common Name	*Genbank no.*	*Description*	*Fold change*[#a]
Up-regulated			
HSPA6	X51757	Member 6 of the heat shock HSP70 family of molecular chaperones; may act in protein folding, translocation, and assembly into complexes	12.1
MKI67	X65550	Antigen identified by monoclonal antibody Ki-67	10.4
HCK	M16591	Hemopoietic cell kinase, protein-tyrosine kinase expressed in hemopoietic cells	5.7
C20orf1	AB024704	Chromosome 20 open reading frame 1. Proliferation-associated nuclear protein that associates with the spindle pole and mitotic spindle during mitosis.	5.0
CCNF	Z36714	Cyclin F, cell-cycle protein involved in the G2/M transitions.	3.5
TYMS	X02308	Thymidylate synthetase. Thymidylate synthase uses the 5,10-methylenetetrahydrofolate as a cofactor to maintain the dTMP (thymidine-5-prime monophosphate) pool critical for DNA replication and repair. The enzyme has been of interest as a target for cancer chemotherapeutic agents.	3.3
HMGA1	L17131	This gene encodes a non-histone protein involved in many cellular processes, including regulation of inducible gene transcription, and the metastatic progression of cancer cells.	3.3
HEC	AF017790	Highly expressed in cancer; may have a role in chromosome segregation during M phase	3.0
BUB1B	AF053306	BUB1b budding uninhibited by benzimidazoles 1 homolog beta (yeast); Protein kinase that is required for mitotic spindle checkpoint and normal mitotic progression	3.0
CCND1	M64349	Cyclin D1 (PRAD1: parathyroid adenomatosis 1); This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition.	2.9
TREX2	X99270	Exonuclease; catalyzes excision of nucleoside monophosphates from 3'-termini of DNA. Enzymes with this activity are involved in DNA replication, repair, and recombination.	2.7
HMGB2	X62534	High-mobility group box 2; The proteins of this family are chromatin-associated and ubiquitously distributed in the nucleus of higher eukaryotic cells. *In vitro* studies have demonstrated that this protein is able to efficiently bend DNA and form DNA circles. This protein was also reported to be involved in the final ligation step in DNA end-joining processes of DNA double-strand breaks repair and V(D)J recombination.	2.5
Down-regulated			
MEF2C	L08895	Myocyte enhancer factor 2C; Member of the MADS box family of transcription factors that regulates muscle-specific and mitogen-inducible genes	-8.3
C18orf1	AF009426	Chromosome 18 open reading frame 1	-6.7
NEB	X83957	Nebulin is a giant protein component of the cytoskeletal matrix that coexists with the thick and thin filaments within the sarcomeres of skeletal muscle.	-6.4
CLECSF2	X96719	This gene encodes a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell-cell signalling, glycoprotein turnover, and roles in inflammation and immune response.	-5.6
ADD3	U37122	Adducin 3 (γ); Belongs to a family of membrane skeletal proteins involved in the assembly of spectrin-actin network in erythrocytes and at sites of cell-cell contact in epithelial tissues.	-5.0
SVCT2	D87075	SVCT2 appears to account for tissue-specific uptake of vitamin C.	-3.7
SATB1	M97287	Special AT-rich sequence binding protein 1. SATB1 is a new type of gene regulator with a novel nuclear architecture, providing sites for tissue-specific organisation of DNA sequences and regulating region-specific histone modification.	-3.7
NCOA3	AF012108	Nuclear receptor coactivator 3. Interacts with nuclear hormone receptors to enhance their transcriptional activator functions.	-3.6
BS69	AA127624	cDNA clone, function unknown	-3.1
ALDH2	X05409	Aldehyde dehydrogenase 2 family (mitochondrial); second enzyme of the major oxidative pathway of alcohol metabolism	-2.9
PROL2	U03105	Proline rich 2, cDNA clone, function unknown	-2.8
MFHAS1	AB016816	Malignant fibrous histiocytoma (MFH) amplified sequence 1 is a potential oncogene. The primary structure of its product includes an ATP/GTP-binding site, three leucine zipper domains, and a leucine-rich tandem repeat, which are structural or functional elements for interactions among proteins related to the cell cycle.	a
PPM1A	AF070670	Protein phosphatase 1A is a serine/threonine protein phosphatase. Overexpression of thisphosphatase is reported to activate the expression of the tumour suppressor gene TP53/p53, which leads to G2/M cell cycle arrest and apoptosis.	a
ZNF148	L04282	Kruppel zinc-finger protein 148. Transcriptional regulatory proteins containing tandemly repeated zinc finger domains are thought to be involved in both normal and abnormal cellular proliferation and differentiation.	a
DYRK2	Y13493	Dual-specificity tyrosine-phosphorylation regulated kinase 2; belongs to a family of protein kinases whose members are presumed to be involved in cellular growth and/or development.	a

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Common Name	Genbank no.	Description	Fold change[#a]
Down-regulated			
CHAC	AB023203	Chorea acanthocytosis; The protein encoded by this gene may control steps in the cycling of proteins through the trans-Golgi network to endosomes, lysosomes and the plasma membrane.	a
ZNF135	U09413	Zinc finger protein 135. Transcriptional regulatory proteins containing tandemly repeated zinc finger domains are thought to be involved in both normal and abnormal cellular proliferation and differentiation.	a
AKR1C1	U05861	Human hepatic dihydrodiol dehydrogenase gene. This enzyme catalyses the reaction of progesterone to the inactive form 20-α-hydroxy-progesterone.	a
Sorcin	M32886	Binds to the α1 subunit of cardiac and skeletal muscle voltage-dependent L-type $Ca2^+$ channels and $Ca2^+$ release channel/ryanodine receptors. Sorcin gene overexpression is significantly associated with clinical multidrug resistance and prognosis, it is one of the indicators for predicting prognosis of acute myeloid leukemia patients	a
PIK3CG	X83368	Phosphoinositide-3-kinase, catalytic, γ polypeptide. In addition to its role in promoting assembly of adherens junctions, the protein is thought to play a pivotal role in the regulation of cytotoxicity in NK cells.	a

[#a] Fold change- average Intensity (Ki-67[high])/average Intensity (Ki-67[low]). For the downregulated genes the figure is presented as - 1/fold change. a - absent call in Ki-67[high]. Two samples with a high proliferative index (>30% Ki-67[+] cells) were compared to 7 samples with a low proliferative index (<10% Ki-67[+] cells). Briefly, the genes were filtered for an increase/decrease call, signal log ratio >1/<-1 in at least 11/14 comparisons and a present call in the group with highest expression (for a detailed description see Design and Methods).

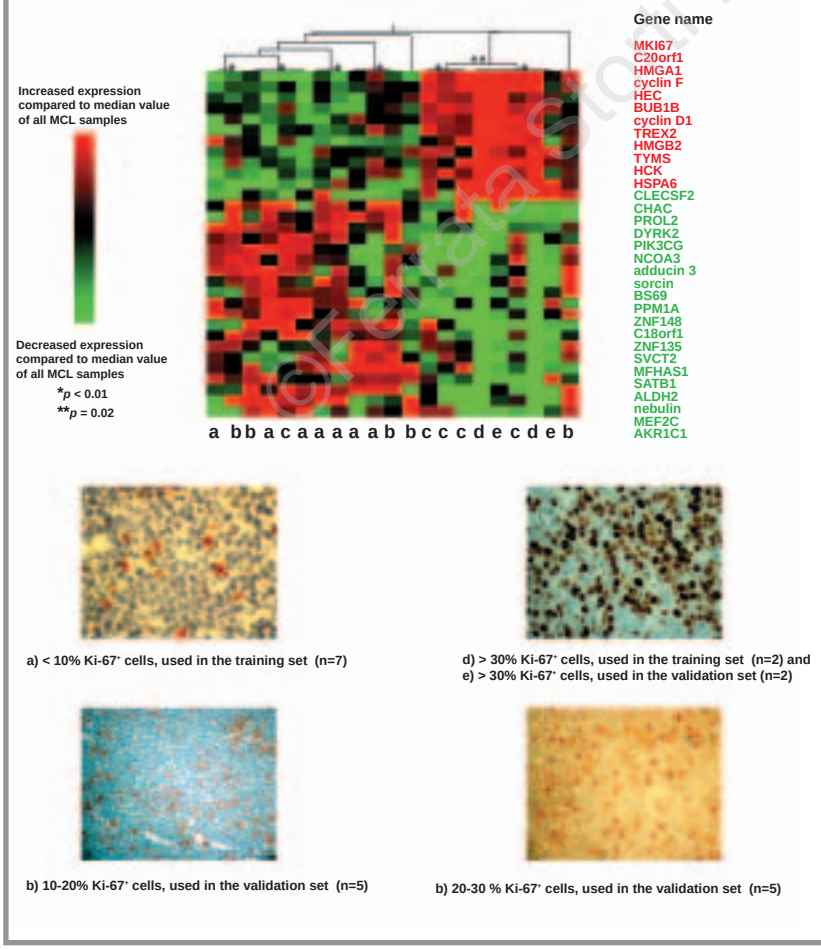

Figure 1. Hierarchical clustering of Ki-67[high] vs. Ki-67[low] samples. By comparing samples with a high proliferative index (>30% Ki-67[+] cells, a) and those with a low proliferative index (<10% Ki-67[+] cells, d) a gene list consisting of 32 genes was created. This list distinguished between tumors with different proliferative indices. In addition to these learning samples, 12 new samples were used to validate the *Ki-67 gene signature*. Two samples (e, relapsed tumors) with a high proliferative index, 5 tumors with 20-30% Ki-67[+] cells (c) and 5 tumors with 10-20% Ki-67[+] cells (b) were used for the validation. Both of the samples with a high proliferative index and 4/5 samples with 20-30% Ki-67[+] cells correlated ($p \leq 0.02$) with the samples with a high proliferative index in the learning group. The samples with <20 % Ki-67[+] cells had a more heterogeneous gene expression and clustered into 4 different groups together with the low proliferative samples in the learning group ($p<0.01$). Representative Ki-67 staining of samples with <10% (a), 10-20% (b), 20-30% (c) and >30% Ki-67[+] cells (d, e) are shown.

Table 2. De-regulated genes comparing relapsed vs. primary MCL tumors.

Common Name	*GenBank no.*	*Description*	*Fold change#b*
Up-regulated			
CD163	Z22971	Macrophage-associated antigen; putative member of the scavenger receptor superfamily	17.4
STAT1	M97935	This protein mediates the expression of a variety of genes, which is thought to be important for cell viability in response to different cell stimuli and pathogens.	6.2
COL1A2	J03464	pre-pro-α-2 type I collagen	6.0
PRSC1	D55696	Legumain; cysteine endoprotease that hydrolyses asparaginyl bonds	6.0
Fibronectin 1	X02761	Fibronectin is involved in cell adhesion and migration processes including metastasis.	5.0
CXCL10	X02530	Reported to be a potent inhibitor of angiogenesis in vivo. May also have chemotactic and mitogenic activity.	3.9
UBE2G	D78514	Ubiquitin-conjugating enzyme. The modification of proteins with ubiquitin is an important cellular mechanism for targeting abnormal or short-lived proteins for degradation.	3.6
EST	AL049963	cDNA DKFZp564A132, function unknown	2.9
TFRC	X01060	Transferrin receptor; binds and internalises the iron carrier transferrin	2.7
DYRK2	Y13493	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2. DYRK2 belongs to a family of protein kinases whose members are presumed to be involved in cellular growth and/or development.	b
osf-2/ periostin	D13666	Putative bone adhesion protein. Periostin secreted by epithelial ovarian carcinoma is a ligand for α(V)β(3) and α(V)β(5) integrins and promotes cell motility.	b
Down-regulated			
HBB	L48215	Hemoglobin β subunit; transports oxygen and carbon dioxide between the lung and tissues	-8.6
CXCL13	AF044197	B lymphocyte chemoattractant BLC. It preferentially promotes the migration of B, by stimulating calcium influx into, and chemotaxis of, cells expressing Burkitt's lymphoma receptor (BLR-1).	-4.2
EST	AL049390	cDNA DKFZp586O1318, unknown function	-3.4
KIAA0977	AB023194	mRNA for KIAA0977 protein, unknown function	-3.2
EST	AA522530	cDNA clone IMAGE:979127 3', unknown function	-3.1
BIRC3	U45878	Baculoviral IAP repeat-containing 3; The protein encoded by this gene is a member of a family of proteins that inhibits apoptosis by binding to tumour necrosis factor receptor-associated factors TRAF1 and TRAF2.	-3.1
TCFL5	AB012124	Transcription factor-like 5. May regulate transcription associated with growth and differentiation	-2.7
CD39	AJ133133	Ectonucleoside triphosphate diphosphohydrolase 1; plasma membrane-bound enzyme that hydrolyses extracellular ATP and ADP to AMP. B cell activation marker.	-2.6
ILT7	AF041261	Leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 4	-2.4
SLAP	D89077	Homo sapiens mRNA for Src-like adapter protein, complete cds. Involved in downregulating T and B cell-mediated responses.	-2.3
Cytochrome P450		U23942 monooxygenase which catalyse many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.	-1.9
Kell blood group	M64934	This gene encodes a type II transmembrane glycoprotein that is the highly polymorphic Kell blood group antigen. The encoded protein contains sequence and structural similarity to members of a family of zinc metalloglycoproteins with neutral endopeptidase activity.	c
TYRO3	U18934	Human receptor tyrosine kinase (DTK)	c
HSPA1L	M11717	member of the heat shock protein 70 family. Stabilises existing proteins against aggregation and mediates the folding of newly translated proteins in the cytosol and in organelles	c
E-cadherin	Z35402	The encoded protein is a calcium dependent cell-cell adhesion glycoprotein. Mutations in this gene are correlated with gastric, breast, colorectal, thyroid and ovarian cancer. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis.	c

#bFold change- average Intensity (relapsed tumors)/average Intensity (primary tumors). For the down-regulated genes the figure is presented as - 1/fold change. b - absent call in the primary tumors. c - absent call in the relapsed tumors. Recurrent and primary tumors from two patients were compared. Briefly, genes were sorted for increase/decrease call, >1/<-1 signal log ratio and present call in the relapsed/primary tumor. The genes shown overlapped between the two analyses (for detailed description see Design and Methods).

Bub1b (*BubR1*) is a protein kinase involved in the spindle checkpoint and its expression has previously been shown to correlate with that of Ki-67.[25] Hec is required for the recruitment of necessary complexes to the kinetochores, ensuring bipolar spindle attachment of the chromosomes before passing the spindle checkpoint.[26] Increased expression of these genes clearly facilitates cellular proliferation. In the MCL with a high prolifera-

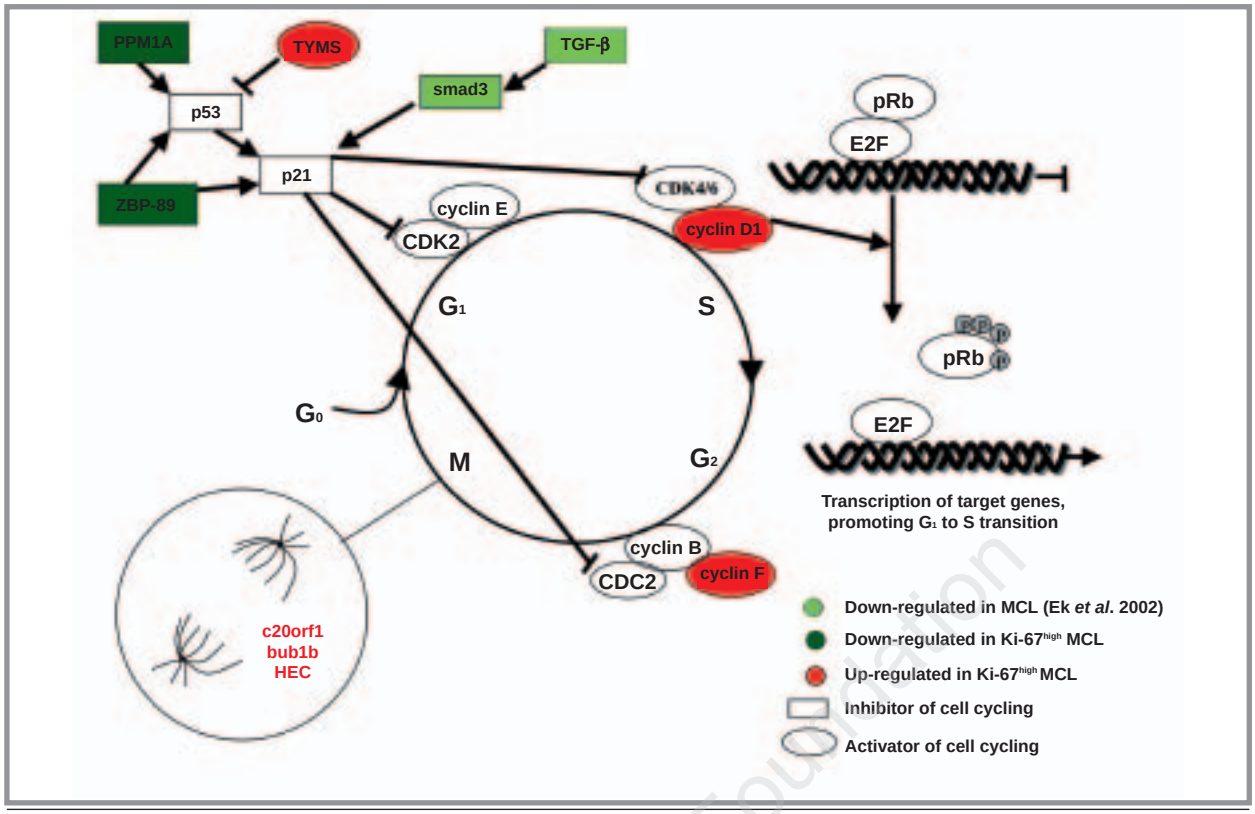

Figure 2. Schematic representation of genes identified to be involved in cell cycle regulation and mitotic spindle formation in MCL. Several genes involved in the regulation of the cell cycle were found to be de-regulated comparing MCL samples with different proliferative indices. *Thymidylate synthase* (*TYMS*) is up-regulated and is known to block the inhibitory effect of p53 on the G1 to S phase transition. *PPM1A* and *ZBP-89* are both down-regulated and are known to inhibit cell cycle progression when overexpressed. *Cyclin D1* and *cyclin* F are both overexpressed and promote the G1 to S and G2 to M phase transitions. Cyclin D1 is overexpressed in all MCL and promotes the release of the E2F transcription factor from the pRb. E2F release is necessary for the transcription of genes involved in the G1 to S phase transition. We have previously reported a decrease in *TGF-β* and *smad3* expression.[7] The proteins coded by these genes normally inhibit the G1 to S phase transition by a number of mechanisms.[42] Furthermore, several genes (*c20orf1, BUB1b, HEC*) involved in mitotic spindle formation were up-regulated.

tion index (Ki67^{high}), *thymidylate synthase (TYMS)* was also found to be more than 3-fold overexpressed. TYMS is a RNA binding protein that represses the expression of the p53 tumor suppressor gene and the myc family of transcription factors. The functional roles of these genes are summarized in Figure 2.[27] The expression of TYMS protein in MCL with a high proliferative index was confirmed by immunohistochemistry (Figure 3). Furthermore, *HCK*, a hematopoietic cell kinase with similarity to the proto-oncogene *c-src*[28] was found to be heavily (5.7-fold) overexpressed, which was also confirmed on a protein level by immunohistochemical staining (Figure 3).

In addition, several genes involved in DNA binding activities, such as the remodeling of chromatin, *SATB1*,[29] exonuclease activity, *TREX2*,[30] and transcription, *NCOA3*,[31] *HMGA1*,[32] *HMGB2*[33] and *MEF2C*,[34] were de-regulated. Of note, *HMGA1*, which we found to be more than 3-fold overexpressed, has been shown to be a c-Myc target gene and a potential oncogene.[32] *c-Myc* is up-regulated in MCL with a high proliferative index, but the difference was just below the stringent cut-off criteria and thus this gene is not included in Table 1.

Altered expression of genes coding for the transferrin receptor and integrin ligands in relapsed tumors

To determine any growth advantages associated with relapsed tumors, we compared recurrent and primary tumors from the same patients. Both primary and recurrent tumors used for this comparison showed a high fraction of Ki-67^{+} cells and the genetic signature obtained consisted of 26 differentially expressed genes. Of these, 11 genes were identified as up-regulated in the recurrent tumors, while 15 genes were down-regulated (Table 2). Interestingly, two integrin ligands, *periostin (osf-2)* and *fibronectin 1*, were found to be overexpressed in the relapsed tumors, as compared to in the primary ones. *Periostin*, which has been shown to be involved in tumor cell motility,[35] was qualitatively over expressed, i.e. it was completely absent in the primary

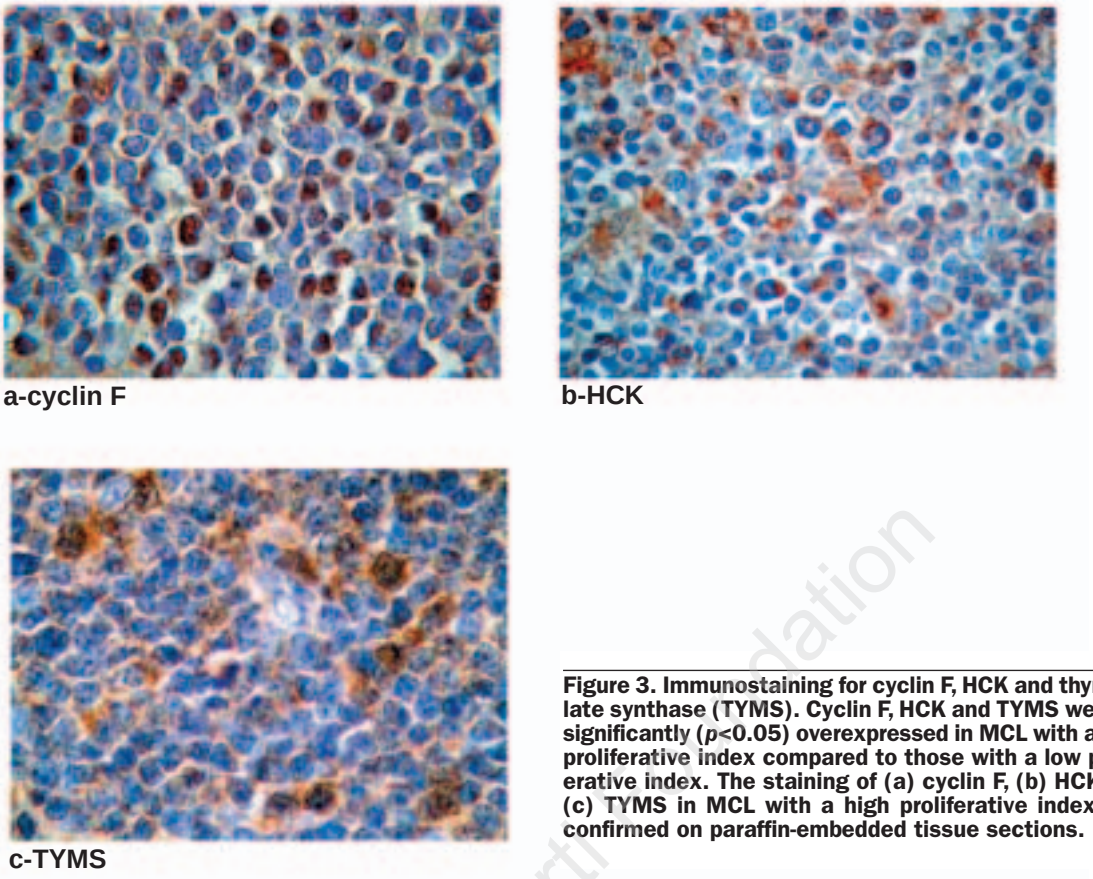

Figure 3. Immunostaining for cyclin F, HCK and thymidylate synthase (TYMS). Cyclin F, HCK and TYMS were all significantly ($p<0.05$) overexpressed in MCL with a high proliferative index compared to those with a low proliferative index. The staining of (a) cyclin F, (b) HCK and (c) TYMS in MCL with a high proliferative index was confirmed on paraffin-embedded tissue sections.

samples, whereas *fibronectin 1*, which has been shown to be involved in malignant transformation,[36] was 5-fold overexpressed.

The mRNA of transferrin receptor (TFRC), which mediates uptake of iron bound to transferrin by receptor-mediated endocytosis, was also 2.7-fold up-regulated in the recurrent tumors. This receptor has been previously reported to be overexpressed in several different types of cancers[37] and its levels have been found to be correlated with the severity of malignant lymphomas.[38]

Discussion

Global expression analysis, using 21 MCL tumors from 19 patients, was performed by comparing groups of samples with different Ki-67 protein expression. A distinct genetic signature consisting of 32 genes was established. This signature could stratify tumors into groups, characterized by a high or low proliferative index. A comparative analysis, using primary and recurrent tumors from the same patients was also performed and 26 genes, potentially associated with the growth advantage of relapsed tumor cells, were found. For the majority of these genes, the association with differential Ki-67 expression or tumor relapse in MCL had not been shown previously. Most autopsy samples from MCL patients display blastoid features, indicating that most tumors transform from the classical to the more aggressive blastoid morphology.[9,12] The blastoid features are associated with high proliferation[5,11,39] and the genes included in the signature characteristic for tumors with high proliferative index are likely to be involved in the transformation from classical to blastoid cytomorphology.

The comparative study of MCL samples with different proliferative indices identified genes encoding proteins involved in the mitotic machinery (Hec1, Bub1b, TPX2), binding to chromatin/DNA (HMGA1, HMGB2, NCOA3, TREX2, SATB1, thymidylate synthase, and MEF2C) or cell cycling/proliferation (ZNF148 (ZBP-89), PPM1A, cyclin D1, cyclin F, Ki-67) (Table 1). The deregulation of the cell cycle seems to involve down-regulation of the p53 pathway and up-regulation of cyclins involved in the G1 to S and G2 to M phase transition (Figure 2). ZNF148 (ZBP-89) and PPM1A, whose mRNA is down-regulated in MCL samples with a high proliferative index, regulate growth by interacting with p53 and may thus be involved in the altered regulation of the cell-cycle machinery in

MCL[20,21] (Figure 2). ZNF148 (ZBP-89) has an additional association with p53, since it is implicated in regulation of $p21^{waf1}$. This is of interest, since the loss of both $p21^{waf1}$ mRNA and protein expression has been associated with aggressive variants of MCL.[39,40] Furthermore, *thymidylate synthase* (TYMS), which is a RNA binding protein, functions as a translational repressor of both p53 and the *myc* family of transcription factors.[27] The expression of TYMS in MCL with a high proliferation index was confirmed on tissue sections (Figure 3).

The overexpression of *cyclin D1*, which promotes the transition from G1 to S phase, is a hallmark of MCL.[2] We here report an increased expression of *cyclin D1* in MCL with a high proliferative index. A similar correlation between proliferation and *cyclin D1* expression was also recently suggested by Rosenwald *et al.*[5] It is of interest to note that the *cyclin D1* expression is proportional to proliferation, which indicates that the overexpression of cyclin D1 is not only a major component of malignant transformation but is also involved in the disease progression of MCL. Cyclin D1 exerts its effect on the cell cycle in combination with CDK4, promoting phosphorylation of pRb and release of E2F transcription factor (Figure 2). The effect of cyclin D1 on the phosphorylaton of pRb is normally balanced by the blocking effect of the TGF-β/Smad3 pathway. We previously reported that this pathway is down-regulated in MCL, which most probably also contributes to increased cell cycling.[7] In addition, increased expression of cyclin F, which promotes the transition from G2 to M phase,[22] was also seen in $Ki67^{high}$ MCL and was further confirmed on tissue sections (Table 1, Figures 2 and Figure 3).

Our data correlate well with the proliferation signature, consisting of 20 genes, published by Rosenwald *et al.*[5] Ten out of 13 of these corresponding genes that were available on the U95v2 chip had highest expression in Ki-67^{high} tumors, although only one (HMGA1) was identified by us as significantly de-regulated, comparing Ki-67^{high} with Ki-67^{low} tumor samples. In an attempt to validate our 32-gene signature further, we demonstrated that the signature could be used to stratify new samples in a validation set of tumors (Figure 1), confirming the significance of the generated gene list. Using hierarchical clustering, 6/7 tumors with >20% Ki-67^{+} cells were correctly assigned ($p \leq 0.02$). However, the MCL samples with <20% Ki-67^{+} cells had more heterogeneous gene expression and formed four sub-groups ($p < 0.01$). It is known that the microenvironment and crosstalk from neighboring cells are crucial for most tumors and their progression. When comparing relapsed vs. primary tumors we found that two common integrin ligands, periostin and fibronectin, were overexpressed in the tumor tissue, which might affect the survival and metastatic potential of the relapsed tumors. Fibronectin is degraded, like other extra-cellular matrix (ECM) proteins, by matrix metalloproteinases which are often expressed by tumors. The degradation products from fibronectin have been shown to be involved in malignant transformation in a number of tumors.[36] In addition, periostin, which probably binds to αvβ3 and αvβ5 integrins, has also been shown to be secreted by ovarian carcinomas and to promote cell motility.[35]

Due to an increased frequency of cell cycling, most tumor cells increase their expression of growth-factor receptors to promote uptake of various nutrients. Among others, an increased uptake of iron is often necessary for high proliferation of tumor cells. Overexpression of transferrin receptor, here reported in relapsed MCL, has previously been reported in a number of different tumor types in which the expression has been shown to correlate to proliferation.[38,41] The serum level of soluble transferrin receptor has even been shown to be associated with the severity of disease in malignant lymphomas.[38] Increased expression of transferrin receptor in relapsed tumors would probably provide an additional survival advantage over that of the neighboring cells.

In summary, the genetic signature defining the Ki-67^{high} subset of MCL includes a number of genes directly involved in the de-regulation of cell cycle control, underpinning the genetic mechanisms of aggressive disease progression in MCL. It is clear that the transformational events affect several of the regulatory pathways, involving p53, pRb as well as cyclins, thus demonstrating the complexity of the de-regulation. Furthermore, genes providing growth advantages for MCL, such as the transferrin receptor, could be of interest as potential therapeutic targets.

SE designed the study, analyzed the data and wrote the manuscript. EB and AP-M were responsible for collecting the tumor material, as well as performing and interpreting the immunohistochemical staining. They were also involved in planning the project and revising the manuscript. MN was involved in the conception of the project and revision of the manuscript. CAKB was involved in the conception and design of the project, interpretation of the results and writing the manuscript. He takes primary responsibility for the paper. Responsibility for tables 1 and 2 and figure 1: SE; for figure 2: SE and CAKB and for figure 3: AP-M. The expert technical assistance of Ann-Charlotte Olsson and Margareta Waern is gratefully acknowledged. The authors reported no potential conflicts of interest.

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