Cyclin D1–negative mantle cell lymphoma: a clinicopathologic study based on gene expression profiling

Kai Fu, Dennis D. Weisenburger, Timothy C. Greiner, Sandeep Dave, George Wright, Andreas Rosenwald, Michael Chiorazzi, Javeed Iqbal, Stefan Gesk, Reiner Siebert, Daphne De Jong, Elaine S. Jaffe, Wyndham H. Wilson, Jan Delabie, German Ott, Bhavana J. Dave, Warren G. Sanger, Lynette M. Smith, Lisa Rimsza, Rita M. Braziel, H. Konrad Müller-Hermelink, Elias Campo, Randy D. Gascoyne, Louis M. Staudt, and Wing C. Chan, for the Lymphoma/Leukemia Molecular Profiling Project

Cyclin D1 overexpression is believed to be essential in the pathogenesis of mantle cell lymphoma (MCL). Hence, the existence of cyclin D1–negative MCL has been controversial and difficult to substantiate. Our previous gene expression profiling study identified several cases that lacked cyclin D1 expression, but had a gene expression signature typical of MCL. Herein, we report the clinical, pathologic, and genetic features of 6 cases of cyclin D1–negative MCL. All 6 cases exhibited the characteristic morphologic features and the unique gene expression signature of MCL but lacked the t(11;14)(q13; q32) by fluorescence in situ hybridization (FISH) analysis. The tumor cells also failed to express cyclin D1 protein, but instead expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases). There was good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis. Using interphase FISH, we did not detect chromosomal translocations or amplifications involving *CCND2* and *CCND3* loci in these cases. Patients with cyclin D1–negative MCL were similar clinically to those with cyclin D1–positive MCL. In conclusion, cases of cyclin D1–negative MCL do exist and are part of the spectrum of MCL. Up-regulation of cyclin D2 or D3 may substitute for cyclin D1 in the pathogenesis of MCL. (Blood. 2005;106:4315-4321)

© 2005 by The American Society of Hematology

Introduction

Mantle cell lymphoma (MCL) is now recognized as an aggressive B-cell lymphoma with various growth patterns (mantle zone, nodular, or diffuse) and a broad range of cytologic features.[1-5] Most cases of MCL exhibit a characteristic phenotype (CD20^{+}, CD5^{+}, CD43^{+}, CD3^{−}, CD10^{−}, CD23^{−}) and have the t(11;14)(q13;q32) with overexpression of the cyclin D1 (CCND1) gene on chromosome 11q13.[6,7] Cyclin D1, a D-type cyclin that is not expressed in normal B lymphocytes, plays a key role in cell cycle regulation during the G_1 to S phase transition by binding to cyclin-dependent kinase 4 (CDK4) and CDK6, resulting in phosphorylation and inactivation of the retinoblastoma protein (RB).[8-10] The current World Health Organization guidelines for the diagnosis of MCL rely on morphologic examination and immunophenotyping, with demonstration of cyclin D1 protein overexpression and/or the t(11;14)(q13;q32) for confirmation.[11]

The existence of cyclin D1–negative MCL has been controversial and difficult to substantiate since cyclin D1 overexpression is believed to be essential in the pathogenesis of MCL. Most reported cases of cyclin D1–negative MCL have been attributed to suboptimal immunostaining, inadequate genetic or molecular analyses, or misdiagnosis. Nevertheless, in a recent study of 99 lymphomas that were morphologically consistent with MCL, we identified a small group of cases that lacked cyclin D1 mRNA expression by both quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and Lymphochip cDNA microarray analyses.[12] However, these cases had the characteristic MCL gene expression signature by cDNA microarray analysis and, therefore, were considered to be cases of cyclin D1–negative MCL. We have further refined the algorithm for diagnosing MCL using gene expression profiling and, herein, we report the clinical, pathologic, and genetic features of 6 cases of cyclin D1–negative MCL.

Patients, materials, and methods

Case selection

The lymph node biopsies from 6 patients without a previous history of malignancy are included in this study. All 6 cases were reviewed by a panel

of expert hematopathologists to confirm the diagnosis. Two of these cases were identified in our previous report.[12] This study was approved by the institutional review board at the University of Nebraska Medical Center. Informed consent was provided according to the Declaration of Helsinki.

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)

To measure cyclin D1 mRNA expression, 2.5-ng aliquots of mRNA were analyzed by quantitative RT-PCR using TaqMan reagents and a thermal cycler (Applied Biosystems, Foster City, CA).[12] The samples were run in triplicate and the β2-microglobulin transcript was used as a reference. Primers and probes for β2-microglobulin and the coding region of cyclin D1 have been previously described.[13]

Microarray gene expression profiling

Lymphochip cDNA microarrays containing 12 196 cDNA elements[14] were used to profile mRNA expression in the lymphoma samples. A set of MCL signature genes that can be used to distinguish MCL from other lymphoma subtypes has been described previously.[12] These cases were further analyzed for gene expression using Affymetrix Human U133 A/B microarrays (Affymetrix, Santa Clara, CA). The gene expression profiles of these cases were compared with those of other B-cell non-Hodgkin lymphomas, including the various subtypes of diffuse large B-cell lymphoma, follicular lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma, extranodal marginal zone lymphoma, mucosa-associated lymphoid tissue (MALT) type, and splenic marginal zone lymphoma. The distributions of the Bayesian predictor for each category were used to estimate the probability that any particular case belonged to that category by applying Bayes rule.[12,15] Specifically, for each lymphoma category we generated a Bayesian predictor between that category and cyclin D1–positive MCL samples, based on the 50 genes with the largest t-statistics between them. Only those cases for which all pair-wise predictors agreed that there was a more than 90% estimated probability of the case being MCL were considered to be cyclin D1–negative MCL samples. This set of models was applied to our data set in a leave-one-out, cross-validated fashion so that the models tested on a given case were based on a data set that excluded that case. Cases for which there was a less than 90% likelihood of being in any category were termed unclassified.

Histologic and immunologic studies

The lymph node specimens were fixed in 10% neutral buffered formalin and embedded in paraffin, and 4-μm sections were cut and stained with hematoxylin and eosin (H&E) for histologic evaluation. Immunohistochemical stains for cyclin D1 protein were performed on formalin-fixed, paraffin-embedded tissue sections. Briefly, after deparaffinization in xylene and rehydration in graded alcohols, endogenous peroxidase was blocked with hydrogen peroxide. Heat-induced antigen retrieval was performed using citrate buffer, pH 6.0.[16] After rinsing in phosphate-buffered saline,

mouse anti–cyclin D1 antibody was applied at a dilution of 1:200. The rabbit monoclonal antibody SP4 against cyclin D1 (Neomarkers, Fremont, CA) was also used, using the suggested procedure for antigen retrieval with minor modification (Table 1).[17] Antibodies against CD3, CD5, CD20, CD23, CD43, cyclin D2, cyclin D3, cyclin E, RB, and p27[kip1] were also used for immunohistochemical stains (Table 1). These stains were performed on a Ventana ES automated immunostainer (Ventana Biotek, Tucson, AZ) with a streptavidin-biotin peroxidase detection system. Positivity for the cyclins, RB and p27[kip1] was defined as a strong nuclear staining in more than 50% of the neoplastic cells.

Fluorescence in situ hybridization (FISH) analysis

Interphase FISH analysis was performed on cells left over from prior cytogenetic analyses or on formalin-fixed, paraffin-embedded tissue sections. For detection of the t(11;14)(q13;q32), a commercially available LSI IGH/CCND1 double-color, double-fusion probe was used (Vysis, Downers Grove, IL). For break-apart FISH assays for the *CCND1* (11q13), *CCND2* (12p13), and *CDKN1B/p27KIP1* (12p13) loci, appropriate bacterial artificial chromosome (BAC) clones flanking the respective genes were selected using bioinformatic resources available at the University of California at Santa Cruz (http://genome.ucsc.edu). All BAC clones were derived from the RPCI11 library and were obtained from Invitrogen/Research Genetics (Carlsbad, CA) or the Sanger Center (Hinxton, United Kingdom). The following clones were used: *CCND1* (pooled RP11-211G23/RP11-378E8 and pooled RP11-300I6/RP11-626H12), *CCND2* (RP11-578L13 and RP11-388F6), and *CDKN1B/p27KIP1* (RP11-180M15 and RP11-59H1). For each locus, centromeric and telomeric BAC clones were differentially labeled with Spectrum Orange or Spectrum Green (Vysis) and pooled for break-apart assays. Bacterial culture, BAC DNA isolation and labeling, probe preparation, and FISH on cytogenetic suspensions were performed as previously described.[18,19] The *CCND3* locus was investigated using a recently described break-apart assay.[20] Locus-specific interphase FISH was performed on paraffin-embedded tissue sections according to the manufacturer's instructions (Vysis) or recently described protocols[21] with minor modifications. Whenever possible, at least 100 cells were analyzed.

INK4a/ARF locus deletion analysis

To detect genomic loss of *INK4a/ARF* tumor suppressor locus in the specimens, we performed quantitative real-time PCR assays using genomic DNA as previously described.[12] The *REL* locus on chromosome 2p was chosen as the reference gene, and a cutoff ratio of *INK4a/ARF* locus copy number relative to *REL* locus copy number was used to assess tumor DNA for genomic deletions. A tumor DNA sample that yielded an *INK4a/ARF*-to-*REL* ratio below the cutoff ratio was considered to have a genomic deletion of the *INK4a/ARF* locus. The primers and probe sets for the *INK4a/ARF* and the *REL* loci have been described previously.[22,23]

Table 1. Antibodies and methods for immunohistochemical stains

Antibody	Clone	Source	Dilution	Retrieval
CD3	PS1	Ventana Medical Systems, Tucson, AZ	Neat	A
CD5	4C7	Novocastra, Newcastle upon Tyne, United Kingdom	1:20	A
CD20	L26	DAKO, Carpinteria, CA	1:200	A
CD23	BU38	The Binding Site, San Diego, CA	1:5	B
CD43	L60	Ventana Medical Systems	Neat	None
Cyclin D1	DCS-6	DAKO	1:200	A
Cyclin D1	SP4	Neomarkers	1:100	C
Cyclin D2	Polyclonal	Santa Cruz Biotech, Santa Cruz, CA	1:500	D
Cyclin D3	DCS-22	Neomarkers	1:100	E
Cyclin E	13A3	Novocastra	1:10	E
RB	Rb1	DAKO	1:10	A
p27[Kip1]	SX53G8	DAKO	1:20	A

A indicates 10 mM citrate buffer, pH 6.0, 30 minutes, water bath (95°C); B, protease I enzymatic digestion, 8 minutes; C, 1 mM EDTA (ethylenediaminetetraacetic acid, pH 8.0), 30 minutes, water bath (95°C); D, 1 mM EDTA, pH 8.0, 60 minutes, water bath (95°C); and E, 10 mM citrate buffer, pH 6.0, 10 minutes, pressure cooker (115°C).

Statistical analysis

The clinical characteristics of the cyclin D1–negative and cyclin D1–positive cases were compared using Fisher exact test. Overall survival was calculated as the time from initial diagnosis to the date of death or last follow-up, and those alive at last follow-up were treated as censored. The Kaplan-Meier method was used to estimate the overall survival distributions of the 2 groups, and the log-rank test was used to compare the distributions.

Results

Case selection

In our previous study,[12] we identified 7 cyclin D1–negative cases by quantitative RT-PCR and cDNA microarray analysis among 99 cases of lymphoma with MCL morphology. Subsequent studies revealed that 1 of these 7 cases had the characteristic t(11;14)(q13;q32) by FISH analysis and expressed cyclin D1 protein by immunohistochemical staining (data not shown). Thus, this case was thought to be a false-negative case and was reclassified as a cyclin D1–positive MCL. During the interim, we performed additional gene expression profiling analysis using Affymetrix U133A/B microarrays and refined the algorithm for diagnosing MCL. Using the refined algorithm, 4 of the other 6 original cases were regarded as unclassifiable B-cell lymphomas and, thus, were excluded from the current study. Recently, we identified 4 additional cases of MCL that were negative for cyclin D1 by both immunohistochemistry and quantitative RT-PCR analysis. All 6 cases in the current study (2 from the previous study and 4 newly identified) exhibited the characteristic gene expression signature of MCL, which is distinct from the other common types of B-cell non-Hodgkin lymphoma including the various subtypes of diffuse large B-cell lymphoma, follicular lymphoma, small lymphocytic lymphoma/chronic lymphocytic leukemia, extranodal marginal zone lymphoma, MALT type, and splenic marginal zone lymphoma (Figure 1 and Table S1, which is available on the *Blood* website; see the Supplemental Materials link at the top of the online article). By applying Bayesian analysis, all 6 cases were predicted as MCL in all pair-wise models with 99.99% confidence. On the other hand, none of the cases from the other categories were predicted as MCL with more than 20% confidence in their respective cross-validated, pair-wise comparisons. Therefore, these 6 cases are considered to be bona fide cases of cyclin D1–negative MCL.

Histologic and immunologic features

All 6 cases exhibited a nodular or diffuse growth pattern and consisted of tumor cells with typical mantle cell cytology (Table 2, Figure 2A, and Figure S1). Immunophenotypic analysis of the tumor cells demonstrated a B-cell phenotype in all cases. Expression of CD5 antigen was noted in all 6 cases. The tumor cells in 1 of the 6 cases were weakly positive for CD23 antigen. Expression of CD43 antigen was noted in 5 of the 6 cases (Table 2).

Detection of cell cycle regulatory proteins

Immunostains for cyclin D1 and p27[kip1] were performed on all 6 cases, and stains for cyclins D2, D3, E, and RB were performed on 5 cases with available formalin-fixed, paraffin-embedded tissue (Table 2). All 6 cases were negative for cyclin D1 by using the mouse monoclonal antibody DCS-6 (Figure 2B). Four of the 6 cases were also studied using the newly available rabbit monoclonal antibody SP4, and all 4 cases were negative for cyclin D1 (data
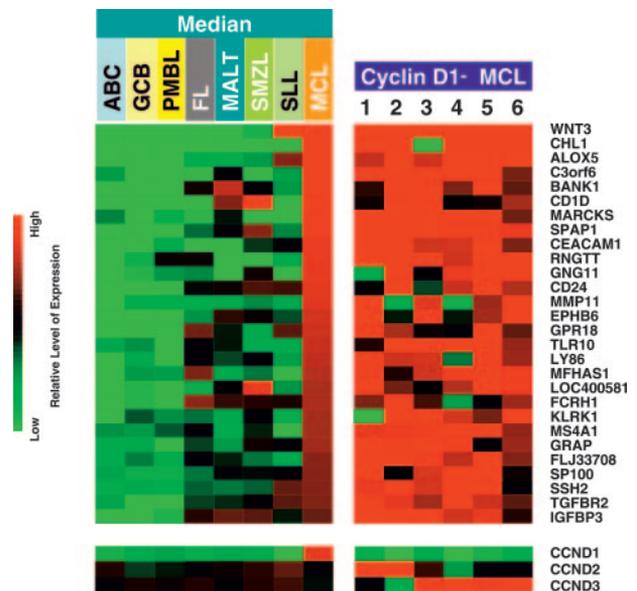
Figure 1. Expression profiles of mantle cell lymphoma (MCL) signature genes in 6 cases of cyclin D1–negative MCL using Affymetrix U133 A/B arrays. These expression profiles are compared with 22 cases of cyclin D1–positive MCL, 78 cases of activated B-cell–like (ABC), 85 cases of germinal center B-cell–like (GCB), and 33 cases of primary mediastinal (PMBL) variants of diffuse large B-cell lymphoma, 193 cases of follicular lymphoma (FL), 14 cases of extranodal marginal zone lymphoma, MALT type (MALT), 6 cases of splenic marginal zone lymphoma (SMZL), and 14 cases of small lymphocytic lymphoma (SLL) (median expression levels of the MCL signature genes in these entities are shown). In the 6 cases of cyclin D1–negative MCL, each column represents a single lymphoma specimen and each row represents the level of expression of a single gene in the MCL signature. Red squares indicate increased expression and green squares indicate decreased expression relative to the median expression level, according to the color scale shown over a 4-fold range. In the bottom panel, the gene expression levels of the D-type cyclins in the various entities and the 6 cases of cyclin D1–negative MCL are shown according to the color scale over a 16-fold range. For microarray data of all cases, refer to Table S1.

not shown). Two cases (cases 1 and 2) demonstrated overexpression of cyclin D2 by immunostaining (Figure 2C), which correlated well with the increased cyclin D2 mRNA levels detected by microarray analysis (Figure 1, lower panel). Both of these cases were negative for cyclin D3. Three cases (cases 3-5) exhibited overexpression of cyclin D3 by immunostaining (Figure 2D), which correlated well with the increased cyclin D3 mRNA levels detected by microarray analysis (Figure 1, lower panel), and these cases were negative for cyclin D2. Case 6 also showed upregulation of cyclin D3 mRNA by microarray analysis (Figure 1, lower panel), but the tissue block from this case was not available for immunostains.

All of the cases studied were negative for cyclin E, but showed positive immunostaining for RB (Table 2). The expression levels of RB protein were similar in all of the cases and were comparable with that seen in cases of cyclin D1–positive MCL (data not shown). Down-regulation of p27[kip1] protein expression was observed in all 6 cases with the intensity of nuclear staining much weaker than that seen in reactive T lymphocytes (Table 2).

Genetic features

FISH analysis for the t(11;14)(q13;q32) was performed on all 6 cases and none displayed the *IGH/CCND1* fusion. FISH studies with a locus-specific probe were also negative for variant translocations or amplifications involving the CCND1 locus at band 11q13 in all of the 6 cases (Table 2). Conventional cytogenetic analysis was also performed on case 6 and did not reveal a chromosomal alteration affecting band 11q13. FISH analysis using break-apart

Table 2. Pathologic and genetic features of 6 patients with cyclin D1-negative MCL

	Case no. 1	Case no. 2	Case no. 3	Case no. 4	Case no. 5	Case no. 6
Pathologic features						
Growth pattern	Nodular	Diffuse	Nodular	Diffuse	Nodular	Nodular
Cytology	Typical	Typical	Typical	Typical	Typical	Typical
CD20	+	+	+	+	+	+
CD3	−	−	−	−	−	−
CD5	+	+	+	+	+	+
CD23	−	−	−	+ (w)	−	−
CD43	+ (w)	+	−	+	+	+
Cyclin D1	−	−	−	−	−	−
Cyclin D2	+	+	−	−	−	NA
Cyclin D3	−	−	+	+	+	NA
Cyclin E	−	−	−	−	−	NA
RB	+	+	+	+	+	NA
p27^{Kip1}	−	−	−	−	−	−
Genetic features						
t(11;14)(q13;q32)	−	−	−	−	−	−
11q13 (cyclin D1)	Normal*	Normal	Normal	Normal	Normal	Normal
12p13 (cyclin D2)	Normal	Normal	Normal	Normal	Normal	Normal
6p21 (cyclin D3)	Normal	Normal	Normal	Normal	Normal	Normal
12p13 (p27^{kip1})	Normal	Normal	Normal	Normal	Normal	Normal

+ indicates positive; −, negative; + (w), weakly positive; and NA, not available.

*Normal indicates no split or amplification.

probes for the *CCND2* (12p13), *CCND3* (6p21), and *CDKN1B/p27KIP1* (12p13) loci did not reveal any evidence of chromosomal translocation or amplification in these cases (Table 2). We also used a quantitative PCR assay to evaluate the genomic loss of one or both alleles of the *INK4a/ARF* locus, which encodes the tumor suppressor proteins p16^{INK4a} and p14^{ARF}. No *INK4a/ARF* locus deletions were detected in any of the 6 cases of cyclin D1–negative MCL.
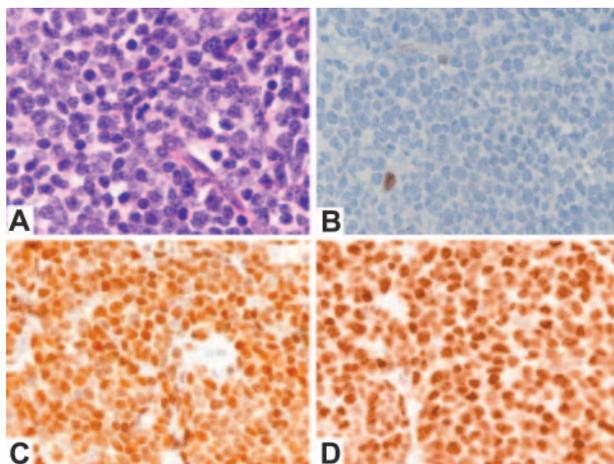
Clinical features

The clinical features of the 6 patients are summarized in Table 3. The patients consisted of 5 males and 1 female, with a median age of 61 years (range, 54-77 years). All patients presented with stage IV disease. Lymphadenopathy was the most common presentation

Figure 2. Cytologic features and expression of D-type cyclins in cyclin D1–negative MCL. (A) Typical MCL cytology (case 1) (hematoxylin and eosin stain; original magnification, × 500); (B) cyclin D1 protein, showing only a rare nontumor cell with nuclear staining (case 1); (C) cyclin D2 protein, showing strong nuclear staining of the tumor cells (case 2); (D) cyclin D3 protein, showing strong nuclear staining of the tumor cells (case 4). Panels B-D are immunoperoxidase stains; original magnification, × 400. Figure S1 provides high-power photos of cytomorphology of other 5 cases.

and extranodal sites were involved by lymphoma in all 6 patients. Five patients received combination chemotherapy initially, but none of these patients achieved a complete clinical response. One patient (case 1) was not treated initially and developed gastrointestinal involvement 26 months after the initial diagnosis. At the time of last follow-up, one of the patients had died and the other 5 were alive with disease.

Ninety-three cases (92 cases in the original study plus the false-negative case) of cyclin D1–positive MCL[12] were used for comparison. The median follow-up for these patients was 26 months (range, 7-166 months). The clinical features including age and sex distribution, stage, presence of B symptoms, serum lactate dehydrogenase (LDH) levels, extranodal sites, International Prognostic Index (IPI) scores, types of treatment, and clinical responses were similar between the cyclin D1–positive and cyclin D1–negative groups (data not shown). At the time of last follow-up, 65 of 93 patients with cyclin D1–positive MCL had died, with a median overall survival of 31 months. No significant difference in overall survival between the cyclin D1–positive and cyclin D1–negative groups was identified.

Discussion

In the current study, we have confirmed and extended our previous findings in which we identified a new variant of MCL, which we designated cyclin D1–negative MCL.[12] All 6 cases in this study were negative for cyclin D1 mRNA expression by both quantitative RT-PCR and gene expression analysis. These cases also lacked the characteristic IGH/CCND1 fusion by FISH analysis and were negative for cyclin D1 protein expression by immunostains. Nevertheless, all of the cases exhibited the characteristic pathologic features of MCL and, more importantly, shared the characteristic MCL gene expression profile by microarray analysis. Therefore, these cases are regarded as bona fide cases of cyclin D1–negative MCL. The existence of such cases sheds new light on the pathobiology of MCL and challenges the idea that cyclin D1 overexpression is essential in the pathogenesis of MCL. We have

Table 3. Clinical features of 6 patients with cyclin D1-negative MCL

	Case no. 1	Case no. 2	Case no. 3	Case no. 4	Case no. 5	Case no. 6
Age, y/sex	54/F	61/M	61/M	60/M	54/M	77/M
B symptoms	−	+	−	+	−	−
Serum LDH level	Normal	High	Normal	High	Normal	Normal
Extranodal sites	BM, PB	BM	BM	BM, spleen	BM	BM, lung, GI
IPI score	2	3	2	3	2	3
Initial therapy	None	R-CHOP	CHOP	COP	CHOP	COP
Response	NA	PR	PR	PR	PR	PR
Progression	+	−	+	+	+	+
Follow-up, mo	38	5	88	19	70	30
Status	AWD	AWD	DOD	AWD	AWD	AWD

All patients were at Ann Arbor stage IV.

LDH indicates lactate dehydrogenase; IPI, International Prognostic Index; BM, bone marrow; PB, peripheral blood; GI, gastrointestinal tract; R, Rituxan; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; COP, cyclophosphamide, vincristine, and prednisone; NA, nonapplicable; PR, partial response; AWD, alive with disease; and DOD, dead of disease.

also demonstrated that patients with cyclin D1–negative MCL have clinical and pathologic features similar to those with cyclin D1–positive MCL. In particular, tumors in both groups had the similar growth patterns and common cytologic and immunohistochemical features. Similar age and sex distribution, stage, serum LDH levels, extranodal sites, IPI scores, response to initial treatment, and overall survival were seen in the cyclin D1–positive and –negative groups.

Yatabe et al[24] previously reported 151 cases of lymphoma with the morphologic features of MCL. Among these, they identified 23 cases (15%) that were negative for cyclin D1 protein expression by immunohistochemical staining performed on formalin-fixed, paraffin-embedded tissue. Conventional cytogenetics was performed on only 3 of these 23 cases, and all 3 were negative for the t(11;14)(q13;q32). However, FISH or quantitative RT-PCR analysis was not performed on any of these cases. Compared with their group of cyclin D1–positive MCL (n = 128), the cyclin D1–negative cases had a significantly better overall survival. In another report, Hashimoto et al[25] identified 3 cases of apparent cyclin D1–negative MCL by immunostains and compared them with 14 cases of typical cyclin D1–positive MCL. They also suggested that cyclin D1–negative MCL is a more indolent form of MCL. However, both of these studies failed to provide convincing evidence that their cases of cyclin D1–negative MCL were actually true cases of MCL. In fact, Yatabe et al[24] noted in their paper that some of their cyclin D1–negative cases might have been marginal zone B-cell lymphomas or atypical small lymphocytic lymphomas. In contrast, we have clearly demonstrated the characteristic MCL gene expression signature in all of our cases of cyclin D1–negative MCL, and we did not find any significant differences in the clinical features between our 2 groups of MCL. However, since there are only 6 patients in our cyclin D1–negative group, with some having relatively short clinical follow-up, additional studies are warranted to confirm our findings.

The pathogenic mechanisms involved in the development of the cyclin D1–negative MCL are currently unknown. Since the oncogenic effect of overexpressed cyclin D1 is considered to be cell cycle dysregulation, we examined other proteins involved in cell cycle control, especially during the G_1 to S phase transition. The D-type cyclins, D1, D2, and D3, are all positive promoters of cell cycle progression from the G_1 to S phase. The D-type cyclins are similar in structure and biochemical function,[26] but are expressed in a lineage-specific manner.[27] There is considerable redundancy in the growth-promoting function of the D-type cyclins, since only limited phenotypic consequences due to the absence of either cyclin D1, D2, or D3 are seen in gene knock-out mice.[28-31] In

nonneoplastic lymph nodes and tonsils, cyclin D2 is found mainly in interfollicular T cells, whereas cyclin D3 is found in centroblasts in lymphoid follicles and in scattered B cells and T cells of the interfollicular areas.[32] However, cyclin D1 is not expressed in nonneoplastic T cells or B cells.[6,33] In low-grade B-cell malignancies, overexpression of cyclin D2 mRNA was observed by Northern blot analysis in 29 of 34 cases of chronic lymphocytic leukemia and in all 7 cases of lymphoplasmacytic lymphoma, but not in 2 cases of MCL.[34] Cyclin D3 appears to be expressed more ubiquitously in B-cell malignancies, including follicular lymphoma, marginal zone lymphoma, and diffuse large B-cell lymphoma,[30] but is usually not expressed in lymphoid malignancies with either cyclin D1 or D2 overexpression.[35-37] In the current study, overexpression of either cyclin D2 or D3 was observed in all 6 cases of cyclin D1–negative MCL, indicating an important substitute role for these cyclins in the pathogenesis of cyclin D1–negative MCL. However, the mechanism of cyclin D2 or D3 up-regulation in our cases remains unclear. We did not find any chromosomal translocations or gene amplifications involving the cyclin D2 or D3 gene loci by FISH analysis in these cases. Our findings are consistent with several previous studies that suggested that deregulation of cyclin D2 or D3 expression is often due to epigenetic mechanisms.[38-40]

Dysregulation of other genes or factors important in cell cycle control could also play a role in the pathogenesis of such cases. These may include dysregulation of p27[kip1], up-regulation of cyclin E, inactivation of the RB gene, and deletion of the p16[INK4a]/p14[ARF] locus, as well as involvement of other genes. The p27[kip1] protein regulates cellular progression from G_1 into S phase by inhibiting the cyclin E/CDK2 complex.[41] Regulation of p27[kip1] occurs primarily through posttranscriptional mechanisms, including sequestration by cyclin D1 or cyclin D3,[42,43] or proteasomal degradation.[44] In a prior study, the expression of p27[kip1], as assessed by immunostains, was noted in only 5 of 40 cases of typical MCL, but was found in 8 of 10 cases of blastic MCL.[45] In the current study, down-regulation of p27[kip1] protein expression was seen in all 6 cases, similar to that seen in typical cyclin D1–positive MCL.

The E-type cyclins, including cyclin E1 and E2, are also important in the G_1 phase of the cell cycle. When combined with CDK2, cyclin E promotes the hyperphosphorylation of RB protein and, thereby, facilitates the entry of cells into S phase.[46] None of our cases, however, was positive for cyclin E expression, arguing against a role for cyclin E in the pathogenesis of cyclin D1–negative MCL. Inactivation of the RB tumor suppressor gene has been implicated in the development of various types of human malignancy. However, RB protein expression was identified in all

of our cases and the expression levels were similar to those seen in cyclin D1–positive MCL. Our findings are consistent with a previous study in which the authors concluded that RB protein appears to be normally regulated in MCL.[47] We also investigated whether deletions of the tumor suppressor genes p16[INK4a] and p14[ARF] play a role in cyclin D1–negative MCL. p16[INK4a] regulates the G_1/S phase transition by forming binary complexes with CDK4 and CDK6, thereby preventing these subunits from association with D-type cyclins.[48] Deletion of p16[INK4a] or cyclin D1 overexpression may therefore promote the G_1/S phase transition by the same mechanism. An important function of p14[ARF] is to augment p53 function by antagonizing murine double minute 2 (MDM2), and loss of p14[ARF] function may contribute to the enhanced proliferation in tumor cells.[48] As we have shown previously, INK4a/ARF locus deletions occur in up to 21% (18/85) of cases of MCL and are preferentially observed among the more proliferative cases.[12] However, deletion of the INK4a/ARF locus was not identified in any of the 6 cases, arguing against a role for p16[INK4a]/p14[ARF] in the pathogenesis of cyclin D1–negative MCL.

Careful morphologic examination, with knowledge of the full spectrum of pathology in MCL, is critical in order to suspect a diagnosis of cyclin D1–negative MCL. This diagnosis may be challenging, particularly since some cases are weakly positive for CD23 or fail to express CD43. However, all 6 cases in our study were positive for CD5, which should suggest the diagnosis of MCL. In such cases, positive immunostains for cyclin D2 or D3 are supportive of this diagnosis. Nevertheless, careful examination to rule out other types of low-grade B-cell lymphoma, which may also be positive for cyclin D2 or D3, is essential. In the near future, gene expression profiling, or a panel of immunostains based on the MCL signature, will provide the means to confirm this diagnosis. Gene expression studies may also shed further light on the pathogenesis and biology of cyclin D1–negative MCL.

Acknowledgments

Appendix

The Lymphoma/Leukemia Molecular Profiling Project is an international consortium of the following institutions: the University of Nebraska Medical Center, the National Cancer Institute, the University of Arizona Health Sciences Center, the University of Oregon Health Sciences Center, the University of Rochester Medical Center, Cleveland Clinic, the British Columbia Cancer Agency, University of Würzburg in Germany, University of Barcelona in Spain, Norwegian Radium Hospital in Oslo, and St. Bartholomew's Hospital in London.

References

1. Weisenburger DD, Armitage JO. Mantle cell lymphoma: an entity comes of age. Blood. 1996;87:4483-4494.

2. Swerdlow SH, Zukerberg LR, Yang WI, Harris NL, Williams ME. The morphologic spectrum of non-Hodgkin's lymphomas with BCL1/cyclin D1 gene rearrangements. Am J Surg Pathol. 1996;20:627-640.

3. Campo E, Raffeld M, Jaffe ES. Mantle-cell lymphoma. Semin Hematol. 1999;36:115-127.

4. Ott G, Kalla J, Ott MM, et al. Blastoid variants of mantle cell lymphoma: frequent bcl-1 rearrangements at the major translocation cluster region and tetraploid chromosome clones. Blood. 1997;89:1421-1429.

5. Weisenburger DD, Vose JM, Greiner TC, et al. Mantle cell lymphoma: a clinicopathologic study of 68 cases from the Nebraska Lymphoma Study Group. Am J Hematol. 2000;64:190-196.

6. Rosenberg CL, Wong E, Petty EM, et al. PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. Proc Natl Acad Sci U S A. 1991;88:9638-9642.

7. Banks PM, Chan J, Cleary ML, et al. Mantle cell lymphoma: a proposal for unification of morphologic, immunologic, and molecular data. Am J Surg Pathol. 1992;16:637-640.

8. Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ, Kato JY. D-type cyclin-dependent kinase activity in mammalian cells. Mol Cell Biol. 1994;14:2066-2076.

9. Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol Cell Biol. 1994;14:2077-2086.

10. Mittnacht S, Lees JA, Desai D, Harlow E, Morgan DO, Weinberg RA. Distinct sub-populations of the retinoblastoma protein show a distinct pattern of phosphorylation. EMBO J. 1994;13:118-127.

11. Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization Classification of Tumors: Pathology and Genetics of Tumors of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001.

12. Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. Cancer Cell. 2003;3:185-197.

13. Bijwaard KE, Aguilera NS, Monczak Y, Trudel M, Taubenberger JK, Lichy JH. Quantitative real-time reverse transcription-PCR assay for cyclin D1 expression: utility in the diagnosis of mantle cell lymphoma. Clin Chem. 2001;47:195-201.

14. Alizadeh A, Eisen M, Davis RE, et al. The lymphochip: a specialized cDNA microarray for the genomic-scale analysis of gene expression in normal and malignant lymphocytes. Cold Spring Harb Symp Quant Biol. 1999;64:71-78.

15. Wright G, Tan B, Rosenwald A, Hurt EH, Wiestner A, Staudt LM. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. Proc Natl Acad Sci U S A. 2003;100:9991-9996.

16. Brynes RK, McCourty A, Tamayo R, Jenkins K, Battifora H. Demonstration of cyclin D1 (bcl-1) in mantle cell lymphoma: enhanced staining using heat and ultrasound epitope retrieval. Appl Immunohistochem. 1997;5:45-48.

17. Cheuk W, Wong KO, Wong CS, Chan JK. Consistent immunostaining for cyclin D1 can be achieved on a routine basis using a newly available rabbit monoclonal antibody. Am J Surg Pathol. 2004;28:801-807.

18. Schlegelberger B, Harder S, Zühlke-Jenisch R, Zhang Y, Siebert R. Classical and molecular cytogenetics of tumor cells. In: Wegner RD, ed. Diagnostic Cytogenetics. Berlin, Germany: Springer-Verlag; 1999:151-185.

19. Martin-Subero JI, Chudoba I, Harder L, et al. Multicolor-FICTION: expanding the possibilities of combined morphologic, immunophenotypic, and genetic single cell analyses. Am J Pathol. 2002;161:413-420.

20. Sonoki T, Harder L, Horsman DE, et al. Cyclin D3 is a target gene of t(6;14)(p21.1;q32.3) of mature B-cell malignancies. Blood. 2001;98:2837-2844.

21. Ye H, Liu H, Attygalle A, et al. Variable frequencies of t(11;18)(q21;q21) in MALT lymphomas of different sites: significant association with CagA strains of H pylori in gastric MALT lymphoma. Blood. 2003;102:1012-1018.

22. Labuhn M, Jones G, Speel EJ, et al. Quantitative real-time PCR does not show selective targeting of p14(ARF) but concomitant inactivation of both p16(INK4A) and p14(ARF) in 105 human primary gliomas. Oncogene. 2001;20:1103-1109.

23. Goff LK, Neat MJ, Crawley CR, et al. The use of real-time quantitative polymerase chain reaction and comparative genomic hybridization to identify amplification of the REL gene in follicular lymphoma. Br J Haematol. 2000;111:618-625.

24. Yatabe Y, Suzuki R, Tobinai K, et al. Significance of cyclin D1 overexpression for the diagnosis of mantle cell lymphoma: a clinicopathologic comparison of cyclin D1-positive MCL and cyclin D1-negative MCL-like B-cell lymphoma. Blood. 2000;95:2253-2261.

25. Hashimoto Y, Nakamura N, Kuze T, Abe M. The evaluation of the biological behavior and grade among cases with mantle cell lymphoma. Leuk Lymphoma. 2002;43:523-530.

26. Inaba T, Matsushime H, Valentine M, Roussel MF, Sherr CJ, Look AT. Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. Genomics. 1992;13:565-574.

27. Sherr CJ, Kato J, Quelle DE, Matsuoka M, Roussel MF. D-type cyclins and their cyclin-dependent kinases: G1 phase integrators of the mitogenic response. Cold Spring Harb Symp Quant Biol. 1994;59:11-19.

28. Sicinski P, Donaher JL, Parker SB, et al. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell. 1995;82:621-630.

29. Sicinski P, Donaher JL, Geng Y, et al. Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature. 1996;384: 470-474.

30. Ciemerych MA, Kenney AM, Sicinska E, et al. Development of mice expressing a single D-type cyclin. Genes Dev. 2002;16:3277-3289.

31. Sicinska E, Aifantis I, Le Cam L, et al. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell. 2003;4:451-461.

32. Teramoto N, Pokrovskaja K, Szekely L, et al. Expression of cyclin D2 and D3 in lymphoid lesions. Int J Cancer. 1999;81:543-550.

33. Yang WI, Zukerberg LR, Motokura T, Arnold A, Harris NL. Cyclin D1 (Bcl-1, PRAD1) protein expression in low-grade B-cell lymphomas and reactive hyperplasia. Am J Pathol. 1994;145:86-96.

34. Delmer A, Ajchenbaum-Cymbalista F, Tang R, et al. Overexpression of cyclin D2 in chronic B-cell malignancies. Blood. 1995;85:2870-2876.

35. Ott MM, Bartkova J, Bartek J, et al. Cyclin D1 expression in mantle cell lymphoma is accompanied by downregulation of cyclin D3 and is not related to the proliferative activity. Blood. 1997;90:3154-3159.

36. Doglioni C, Chiarelli C, Macri E, et al. Cyclin D3 expression in normal, reactive and neoplastic tissues. J Pathol. 1998;185:159-166.

37. Suzuki R, Kuroda H, Komatsu H, et al. Selective usage of D-type cyclins in lymphoid malignancies. Leukemia. 1999;13:1335-1342.

38. Andreasson P, Johansson B, Arheden K, et al. Genomic amplification of CCND2 is rare in non-Hodgkin lymphomas. Cancer Genet Cytogenet. 1998;102:81-82.

39. Pruneri G, Fabris S, Fasani R, et al. Immunoreactivity for cyclin D3 is frequently detectable in high-grade primary gastric lymphomas in the absence of the t(6;14)(p21.1;q32.3) chromosomal translocation. J Pathol. 2003;200:596-601.

40. Bergsagel PL, Kuehl WM. Critical roles for immunoglobulin translocations and cyclin D dysregulation in multiple myeloma. Immunol Rev. 2003; 194:96-104.

41. Polyak K, Kato JY, Solomon MJ, et al. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 1994;8:9-22.

42. Quintanilla-Martinez L, Davies-Hill T, Fend F, et al. Sequestration of p27Kip1 protein by cyclin D1

in typical and blastic variants of mantle cell lymphoma (MCL): implications for pathogenesis. Blood. 2003;101:3181-3187.

43. Lin Z, Lim S, Lim MS. Growth regulation by p27Kip1 is abrogated by multiple mechanisms in aggressive malignant lymphomas. Br J Haematol. 2003;121:739-748.

44. Chiarle R, Budel LM, Skolnik J, et al. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. Blood. 2000;95:619-626.

45. Quintanilla-Martinez L, Thieblemont C, Fend F, et al. Mantle cell lymphomas lack expression of p27Kip1, a cyclin-dependent kinase inhibitor. Am J Pathol. 1998;153:175-182.

46. Sherr CJ. Cancer cell cycles. Science. 1996;274: 1672-1677.

47. Jares P, Campo E, Pinyol M, et al. Expression of retinoblastoma gene product (pRb) in mantle cell lymphomas: correlation with cyclin D1 (PRAD1/CCND1) mRNA levels and proliferative activity. Am J Pathol. 1996;148:1591-1600.

48. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell. 2002;2:103-112.