

p53 and mdm2 in mantle cell lymphoma in leukemic phase

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Background and Objectives. In mantle cell lymphoma (MCL), abnormalities additional to t(11;14) including those affecting genes involved in the p53 pathway, are important for disease development and progression. This study aimed to assess the frequency, relationship and impact of p53 abnormalities and those of its inhibitor mdm2 in blastoid and non-blastoid MCL in leukemic phase.

Design and Methods. Isolated blood lymphocytes from 21 patients with MCL in leukemic phase, characterized by the presence of t(11;14), were analyzed by flow cytometry and by fluorescent *in situ* hybridization in order to investigate whether there is a correlation between overexpression and deletion of p53, overexpression of mdm2 and gain of chromosome 12. Results were also correlated with morphologic subtypes, proliferative activity assessed by expression of Ki67 and clinical outcome.

Results. Cells from 2/21 (10%) and 7/21 (33%) patients overexpressed p53 and mdm2, respectively. No single case expressed both proteins. Ten out of 19 (53%) patients had a hemizygous loss of 17p (p53) including the 2 patients (11%) overexpressing p53. Gains of chromosome 12 (mdm2) were found in only 2 cases with expression of mdm2 in one of them. Overall, p53 deletion and/or overexpression of mdm2 was found in 71% of cases. Ten of 19 patients had a blastoid MCL, including all 5 patients who were Ki67 positive, 6 of the 7 patients expressing mdm2 and one of the 2 patients expressing p53. There was no correlation between p53 deletion and morphologic subtypes. All patients with blastoid MCL have died after a median time of 25 months.

Interpretation and Conclusions. In MCL in leukemic phase there is a high frequency of p53 deletion and/or overexpression of mdm2. In contrast, overexpression of p53 is relatively rare. Overexpression of mdm2 is seen

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predominantly in blastoid MCL, the latter being characterized by a short median survival, and seems unrelated to a numerical gain of chromosome 12. It does not reflect a high proliferative rate but might indicate an alternative mechanism of inactivating p53 in prognostically adverse types of MCL.

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Key words: p53, mdm2, mantle cell lymphoma, flow cytometry, 17p deletion.

Mantle cell lymphoma (MCL) is an aggressive B-cell lymphoma which accounts for 5% of all non-Hodgkin's lymphomas (NHL). MCL affects mainly elderly patients with a slight predominance in males. It is characterized by distinctive clinical, morphologic, immunophenotypic, cytogenetic and molecular findings.^{1,2} A key event in the pathogenesis of MCL is the translocation t(11;14)(q13;q32) that takes place in a CD5⁺ B-lymphocyte leading to the juxtaposition of the cyclin D1 gene (BCL-1/CCND1/PRAD-1) on 11q13 to the immunoglobulin heavy chain enhancer elements on 14q32. This rearrangement results in an increased proliferative rate with constitutive cyclin D1 overexpression. However, additional abnormalities, including those affecting genes involved in the p53 pathway are important for disease development and progression. MCL is usually refractory to chemotherapy and median overall survival is estimated to be about 3 years.³

Several factors have been described to affect the prognosis of MCL. A high mitotic activity, blastoid morphology and leukemic presentation seem to be associated with a poorer outcome³⁻⁸ although the prognostic significance of peripheral blood involvement in MCL has been questioned.⁹ Blastoid MCL is a morphologically defined, aggressive variant, which has been associated with additional cell cycle deregulations such as abnormalities of p53,^{5,8,10,11} p21 and/or p16,¹²⁻¹⁴ mdm2¹⁵ and the occurrence of hyperploidy or tetraploidy.^{16,17} In addition, a high incidence of loss of 8p has recent-

ly been found in leukemic MCL with amplification of the MYC oncogene especially in the blastoid and large-cell subtypes.¹⁸ Irrespective of the morphologic subtype of MCL, p53 overexpression and trisomy 12 as well as more complex cytogenetic abnormalities have been shown to confer an adverse prognosis.^{19,20} p53 overexpression usually correlates with the presence of a mutated, dysfunctional p53 gene; alternatively, p53 function may be suppressed by mdm2. mdm2 (hdm2 in humans) is induced by wild-type p53 and forms an autoregulatory feedback loop by repressing p53 transcriptional activity and mediating its degradation.²¹ In addition, mdm2 is an oncoprotein that has p53 independent functions related to cell division and tumor phenotype.²² mdm2 overexpression might result from increased mRNA levels due to mdm2 gene amplifications, as found in human sarcomas but not in hematologic malignancies,^{23,24} or to enhanced translational efficacy as shown in Burkitt's lymphoma cell lines.²⁵ There are only few reports concerning abnormalities of mdm2 in MCL.^{15,26} In addition, most of the studies addressing cell cycle deregulation in MCL have dealt primarily with nodal MCL and only recently has attention been focused on the leukemic presentations of this lymphoma.^{8,18,27}

The aim of this study was to get further insight into the role of p53 and mdm2 in prognostically adverse leukemic forms of MCL. Therefore, the frequency of p53 and/or mdm2 overexpression in patients with well-characterized MCL in leukemic phase was assessed by flow cytometry and results were correlated with proliferative activity (Ki67 expression and cell cycle analysis), clinical data and morphologic subtypes, i.e. blastoid versus non-blastoid. As p53 deletions are thought to be a surrogate marker of a dysfunctional p53 (with or without overexpression of p53) and because the adverse prognosis attributed to trisomy 12 could be due to increased mdm2 expression (the mdm2 gene locus is on 12q14.3-15), results were correlated with the presence of p53 deletions and gain of chromosome 12, respectively.

Design and Methods

Patients

Twenty-one MCL patients followed at or referred to the Royal Marsden Hospital (RMH) formed the population for this study. The diagnosis was based on cell morphology, immunophenotype, cytogenetics, fluorescence *in situ* hybridization (FISH) and/or histology.

Samples

Mononuclear blood cells, isolated by density gradient centrifugation with Histopaque 1077 (Sigma, St. Louis, USA), were analyzed. Fresh cells from 4 patients and thawed cells from frozen samples (stored in liquid nitrogen) from 17 patients were washed three times with Hank's balanced salt solution. Cell integrity and viability were assessed by light scatter characteristics.

Fixation and permeabilization

The cells ($1.0\text{--}2.0 \times 10^6$) were incubated for 30 minutes at 4°C in 1 mL of 2% cold paraformaldehyde. The cells were centrifuged, the supernatant discarded and washed first with 0.02% Na₃N, 0.02% BSA, 0.01% EDTA (buffer A) and thereafter with 1.0% BSA, 0.1% Na₃N and 0.05% Tween 20 (buffer B). Next, 4 mL of 80% ice cold ethanol were added, the samples gently mixed and then stored at -20°C for at least 2 hours and up to 10 days. Finally, the cells were centrifuged, the ethanol discarded, and washed with buffers A and B as described above.

Immunostaining

Immunostaining was carried out as previously described elsewhere.²⁸ The monoclonal antibodies (MoAb) used were as follows: 5 µL of neat anti-p53 McAb recognizing amino acids 11 to 25 of both wild-type and mutant p53 (clone DO-1, IgG2a, Novo Castra, Newcastle, UK), 5 µL of neat anti-mdm2 (clone IF2, IgG2b, Calbiochem, San Diego, USA), 5 µL of neat anti-Ki67 (IgG1, Dako, Glostrup, DK) and corresponding amounts of isotypic control (IgG2a, IgG2b and IgG1, Dako, Glostrup, DK) aiming at an equal final immunoglobulin concentration. The MoAbs and the control, together with 50 µL 2% AB serum, were added to the cells and incubated at 4°C for 15 minutes (p53, DO-1) or 30 minutes (mdm2, Ki67). After washing with buffers A and B, 50 µL of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG F(ab')₂ fragment (ICN Biomedicals Inc., Aurora, USA) diluted 1/25 in PBS for p53, Ki67 and isotypic controls, and diluted 1:25 in PBS R-phycoerythrin (RPE)-conjugated rabbit anti-mouse IgG F(ab')₂ (Dako, Glostrup, DK) for mdm2 and the corresponding isotypic control were added and incubated at 4°C for 15 minutes (DO-1) and 30 minutes (mdm2, Ki67), respectively.

Flow cytometry

After washing twice with buffer A, cells were resuspended in Isotone and analyzed on a FACScan flow cytometer (Becton Dickinson, San José, USA) using Cellquest software. The cells of interest were gated according to light scatter characteristics. In

cases in which more than one cell population was identified according to size and granularity, markers were analyzed separately for each cell population and the mean values were calculated. A sample was considered positive when there was a clear shift in the fluorescence axis compared to that produced by the isotypic control and when more than 15% of the cells stained with the MoAb anti-p53 and anti-mdm2, and more than 10% of the cells were Ki67 positive. After acquiring for Ki67, 20 μ L RNase (10 mg/mL) and 30 μ L propidium iodide were added to the sample, incubated for 30 minutes in the dark and then processed again for DNA analysis to assign the cells to the 3 different phases of the cell cycle, i.e. G₀/G₁, S and G₂/M. A G₂/M-peak was considered significant when at least 5% of cells were assigned to the G₂/M-phase using linear amplification. The T-lymphoblastic-derived CEM cell line was used throughout as a positive control for p53, Ki67 and DNA analysis and the U2OS osteosarcoma-derived cell line was used as a positive control for mdm2. Mononuclear cells from 5 healthy donors (fresh or frozen, separated as described above) were examined simultaneously and used as negative controls by gating on the lymphocytes. To validate flow cytometric measurement of p53 and mdm2, Western blot analysis of the respective protein expression was performed on positive and negative controls. Whole-cell protein extracts were prepared by sonication for 5 min in 1% NP40 buffer and quantified spectrophotometrically (Bio-Rad, Glattbrugg, Switzerland). Total protein (50 μ g) was size-fractionated on 8% SDS-polyacrylamide gel and blotted to nitrocellulose (Bio-Rad). The membrane was blocked in 2% dry milk/TBS-T for 1 hour, incubated overnight at 4°C with a 1:1000 dilution of the McAb anti-p53 (Ab-6, clone DO-1, Calbiochem, San Diego, USA) and anti-mdm2 (clone IF2, IgG2b, Calbiochem, San Diego, USA), respectively, and visualized using the ECL system (Amersham, Zurich, Switzerland). Flow cytometry and Western blot analysis data of positive (CEM, U2OS) and negative (lymphocytes of healthy donors) controls for p53 and mdm2 correlated well (*data not shown*). Overexpression of p53 and mdm2 was defined as a result above the threshold for positivity in comparison to normal lymphocytes which were negative and the isotypic control as previously described.

Fluorescence in situ hybridization

Mononuclear cells were exposed to hypotonic potassium chloride and fixed in Carnoy's fixative (3:1 methanol:acetic acid). To analyze p53 dele-

tion, FISH was performed according to standard procedures using a directly labeled probe 17p13 LSI p53 gene (spectrum orange) and a probe for chromosome 17 centromere (CEP17-spectrum green). Trisomy 12 was evaluated with a centromeric probe for chromosome 12, CEP12 (spectrum green). Following hybridization the slides were washed and counterstained with mounting medium containing DAPI (4'-6-diamidino-2-phenylindole dihydrochloride-vector shield with DAPI vector). Interphase nuclei were captured using a CCD camera attached to a Zeiss axioplan fluorescence microscope connected to a computer using Vysis Quips FISH software. The cut-off level for confident detection of p53 deletion was taken as the mean plus 3SD of the percentages of cells scored with 1 allele missing. Normal controls comprised 10 healthy donors. Five hundred cells/slide were scored and the cut-off established for p53 deletion (5%) and for trisomy 12 (3%).

Statistics

Fisher's exact test was used to correlate categorical clinical and laboratory variables and χ^2 test to compare patient groups. The patients' survival was estimated by the Kaplan-Meier method and survival curves were statistically compared by the log-rank test. A *p* value less than 0.05 was considered statistically significant in all the tests.

Results

Patients' characteristics, therapy and survival

All patients presented with leukemia as assessed by morphology and immunophenotyping. There were 11 males and 10 females with a median age of 61 years (range 43-86) and a median leukocyte count of $97 \times 10^9/L$ (range: 5.2-658). Only one patient (case #6) had lymphocytosis $<6 \times 10^9/L$. Splenomegaly was present in 15 (79%), lymphadenopathy in 7 (37%) and hepatomegaly in 2 (11%). Other manifestations were pleural effusions in 4, CNS involvement in 2, bowel involvement in 1 and autoimmune hemolytic anemia in another. Ten out of 19 patients (53%) had a blastoid MCL based on the morphology of the peripheral blood smear, 9 had a typical (i.e. non-blastoid) form of MCL and in 2 the morphology was not available for review. Seventeen patients were treated, 14 of them with a range of chemotherapeutic regimens (including 2 patients with radiotherapy), 2 had a splenectomy only and in 1 patient there was no information on the treatment modality used. One patient (case #2)

received no therapy and information was not available for 3 patients. Flow cytometry and FISH analysis was performed on follow-up material on 7 patients (cases #1, 5, 7, 11, 12, 18, and 20) who had already been treated but still had obvious disease in peripheral blood. Twelve patients were untreated at the time of analysis and in 2 cases treatment status was unknown (cases #14 and 19) at time of analysis. Thirteen patients have died at 2 to 54 months from diagnosis, five patients are alive at 7 months to 18 years from diagnosis and the follow-up was lost in the remaining patients. Clinical details are summarized in Table 1.

Immunologic markers and FISH

Immunophenotyping by flow cytometry of the circulating lymphocytes showed a clonal B-cell population with moderate to strong surface immunoglobulin ($11\kappa^+$, $9\lambda^+$), $CD5^+$ (in 19/21 patients), $CD23^+$ (3/21) and all were $CD19^+$, $FMC7^+$, $CD22^+$, $CD79b^+$.

There were less than 25% T-lymphocytes, as assessed by CD2 staining. $t(11;14)(q13;q32)$ was detected in all 21 cases.

p53 expression

In 2 out of the 21 patients (10%) over half of the cells (57% and 59%) overexpressed p53 protein as estimated by the MoAb DO-1, which detects the wild type as well the mutant type of p53 (Figure 1). With one exception (case #11 with 11% $p53^+$ cells), cells from the other patients were clearly negative (<6% DO-1⁺ cells). In one p53 protein-positive patient and in 8 p53 protein-negative patients results were confirmed by another p53 directly conjugated FITC MoAb, DO-7. However, discrimination between negative and positive staining was better seen with DO-1 and therefore further analyses were performed with DO-1 only (Table 2).

mdm2 expression

A proportion of cells (median 34%, range 18-63%) from 7 out of 21 patients (33%) were positive for mdm2 (Figure 2). In 3 mdm2-positive cases, two populations of lymphocytes were identified by light scatter with an increased positivity in the large sized cell population. Overall, 12 out of 21 patients (57%) showed two different sized lymphoid populations. In one case (case #17), only the larger lymphocyte population fulfilled the criteria for positivity for mdm2 (18% positive cells).

Ki67 expression/cell cycle phase

Ki67 expression was shown by a substantial proportion of cells (median 21%, range: 11-26%) from 5 patients. In all three cases with two cell popula-

tions, according to size, expression of Ki67 was greater in the larger cell population. Both cases with a significant ($\geq 5\%$) DNA-peak in G2/M phase were also Ki67 positive (21% and 26%).

Correlations between p53/mdm2 and Ki67/cell cycle phase

The two patients whose cells overexpressed p53 were negative for both mdm2 and Ki67. Out of the 7 mdm2 positive cases, 3 were Ki67⁺ ($p=0.28$) and two of them had a significant DNA-peak in G2/M phase.

FISH analysis and correlation with protein expression

Monoallelic p53 deletion was present in 10 cases (53%) with additional biallelic loss of the p53 gene in 2 of them (biallelic loss in 6% and 10% of the cells). Trisomy 12 was found in 2 cases (11%). One case had, in addition, 19% of cells with tetrasomy 12 while the other had tetrasomy 12 in 70% of cells. The two patients expressing p53 had monoallelic deletion of p53 (one case with additional biallelic loss) but no gain of chromosome 12. Of the 7 mdm2 positive patients one had trisomy/tetrasomy 12 and two others had monoallelic loss of p53 (15% and 97% of the cells). There was no correlation between p53 deletion and expression of mdm2 ($p=0.17$) or between trisomy/tetrasomy 12 and expression of mdm2. Overall, p53 deletion by FISH and/or overexpression of mdm2 by flow cytometry was present in 15 out of 21 cases (71%).

Comparison of laboratory results between the pretreated and untreated group of patients at the time of analysis

There were no significant differences between the pretreated and untreated group before analysis (χ^2 test) for the following parameters: blastoid morphology ($p=0.44$), p53 expression ($p=0.14$), mdm2 expression ($p=0.19$), Ki67 expression ($p=0.08$), p53 deletion ($p=0.49$) and trisomy 12 ($p=0.75$).

Correlations between laboratory and clinical findings, morphology and disease outcome

Six out of seven patients expressing mdm2 (86%, $p=0.06$) and one of the two patients expressing p53 had blastoid MCL. Taken together, 70% of blastoid MCL expressed p53 or mdm2 ($p=0.07$). Of the two patients with trisomy/tetrasomy 12 one had a blastoid subtype (mdm2⁺) and in the other the morphologic type was unknown (mdm2⁻). p53 deletion occurred independently of the morphologic subtype. Out of the 10 patients with blastoid

Table 1. Patients' clinical details.

Case	Sex/ age (yr)	Hb (g/dL)	Plt (10 ⁹ /L)	WBC (10 ⁹ /L)	Morphology	Lymph nodes	Hepatomegaly	Splenomegaly	Other	Therapy/Survival
1	F/54	10.0	118	29	blastoid	no	no	yes		CHOP/+54m dead
2	M/86	9.2	166	422	blastoid	no	no	no		No therapy/+5m dead
3	M/50	11.0	175	209	typical	yes	yes	yes	Pleura	Spx ^R /CHOP/+? dead
4	M/55	14.0	210	28	typical	no	no	yes		Spx ^R /18y+ alive
5	M/67	11.0	132	208	blastoid	no	no	yes	Pleura, CNS	CNOP ^{NR} /+? dead
6	M/52	12.2	129	5.2	not reviewed	no	no	yes		Spx ^R /28m+ alive
7	M/60	5.4	120	-380	blastoid	no	no	yes		CHP/Rx/CDA/+2y dead
8	M/?	No INF	No INF	No INF	typical	No INF	No INF	No INF		No INF/ No INF
9	F/75	9.2	102	658	typical	no	no	yes		FDR ^{NR} /14m+ alive
10	M/75	13.7	218	218	blastoid	no	no	yes		Ther/+25m dead
11	F/62	8.9	69	54	typical	no	no	yes		Ch ^{NR} /Spx ^R /7m+ alive
12	F/55	9.2	111	109	typical	no	no	yes		Spx ^R /CHOP ^{NR} /8y+ ?
13	F/85	11.4	162	21	blastoid	yes	no	no		Ch ^{NR} /+2m dead
14	M/75	11.2	117	43	typical	yes	no	no		No INF/ No INF
15	F/74	9.0	239	14.6	typical	yes	no	yes		No INF/+19m alive
16	F/79	11.4	137	15.4	not reviewed	no	yes	yes		Etop/Pred/+6m dead
17	M/55	14.0	149	75	blastoid	no	no	yes		CHOP/+6m dead
18	M/48	14.0	150	15-300	blastoid	yes	no	yes	Pleura, CNS	Poly ¹ /Spx/+4y dead
19	F/43	9.0	20	97	blastoid	yes	no	no	Pleura	CHOP ^{NR} /+? dead
20	F/78	10.4	205	10	blastoid	yes	no	no	Colon	Poly ² /+4y dead
21	F/58	11.5	156	14-150	typical	no	no	yes		Ch ^{NR} /Spx/+7m dead

Hb: hemoglobin; Plt: platelets; WBC: white blood cells; Spx: splenectomy; Rx: radiotherapy; CDA: chlorodeoxyadenosine; Chl: chlorambucil; CNOP: as CHOP, but mitoxantrone instead of doxorubicin; Etop: etoposide; Pred: prednisone; FDR: fludarabine; Poly(therapy)¹: CHOP, FDR, MBACOP; Poly(therapy)²: surgery, CHOP, Rx; R: response, NR: no response; PR: partial response; TR: transient response; m: month; y: year; No INF: no information; Ther: therapy not specified.

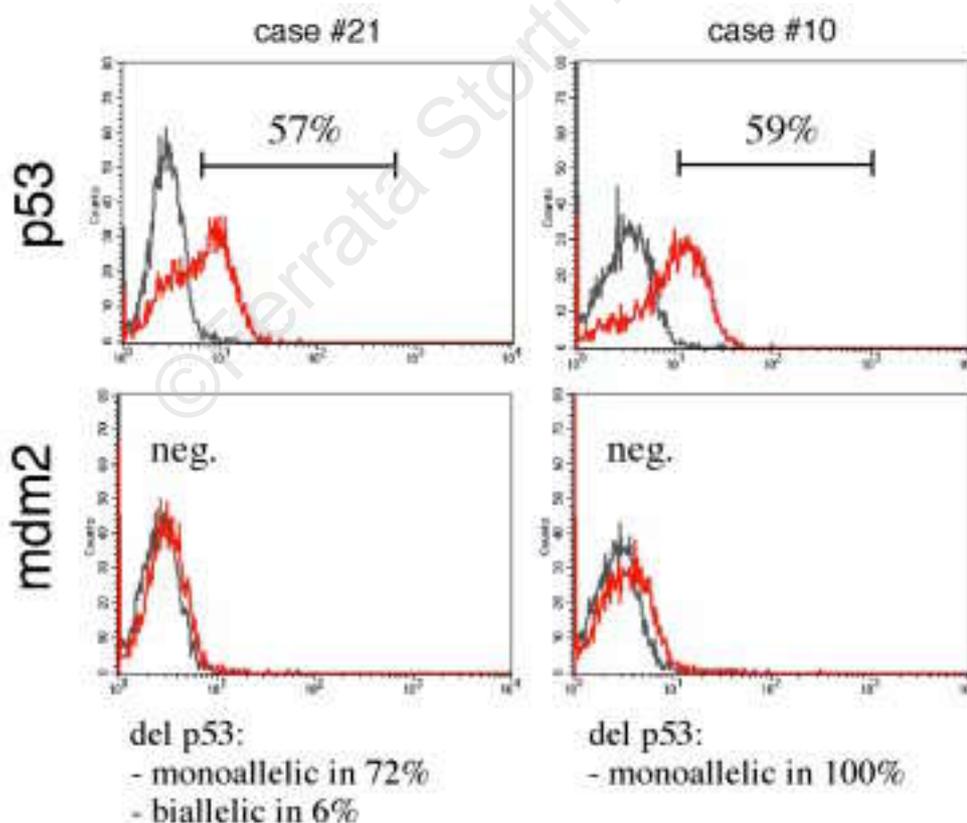


Figure 1. p53 overexpression in 2 out of 21 patients with MCL in leukemic phase in relation to mdm2 expression and allelic p53 status. Flow cytometric histograms (Ki67 negative in both cases).

Table 2. Summary of p53/mdm2/Ki67 overexpression (flow cytometry), FISH analysis and morphology/disease outcome.

Case	p53 expression	p53 alleles (deletion: -)	mdm2 expression	Trisomy/Tetrasomy 12	Ki67 expression	Morphology	Clinical outcome
1	neg.	+/+	pos.	no	pos.**	blastoid	dead
2	neg.	80%+/-; 10%-/-	neg.	no	pos.	blastoid	dead
3	neg.	95%+/-	neg.	no	neg.	typical	dead
4	neg.	13%+/-	neg.	no	neg.	typical	alive
5	neg.	15%+/-	pos.	no	pos.	blastoid	dead
6	neg.	+/+	neg.	27%/19%	neg.	not reviewed	alive
7	neg.	+/+	neg.	no	pos.	blastoid	dead
8	neg.	n.a.	neg.	n.a.	neg.	typical	No INF
9	neg.	100% +/-	neg.	no	neg.	typical	alive
10	pos.	100% +/-	neg.	no	neg.	blastoid	dead
11	neg.	n.a.	neg.	n.a.	neg.	typical	alive
12	neg.	10% +/-	neg.	no	neg.	typical	No INF
13	neg.	+/+	neg.	no	neg.	blastoid	dead
14	neg.	15%+/-	neg.	no	neg.	typical	No INF
15	neg.	+/+	pos.	no	neg.	typical	alive
16	neg.	+/+	neg.	no	neg.	not reviewed	dead
17	neg.	97%+/-	pos.*	no	neg.	blastoid	dead
18	neg.	+/+	pos.	no	pos.**	blastoid	dead
19	neg.	+/+	pos.	no	neg.	blastoid	dead
20	neg.	+/+	pos.	18%/70%	neg.	blastoid	dead
21	pos.	72% +/-; 6% -/-	neg.	no	neg.	typical	dead

* in the larger cell population only. ** ≥ 5% of cells in G2/M-phase.

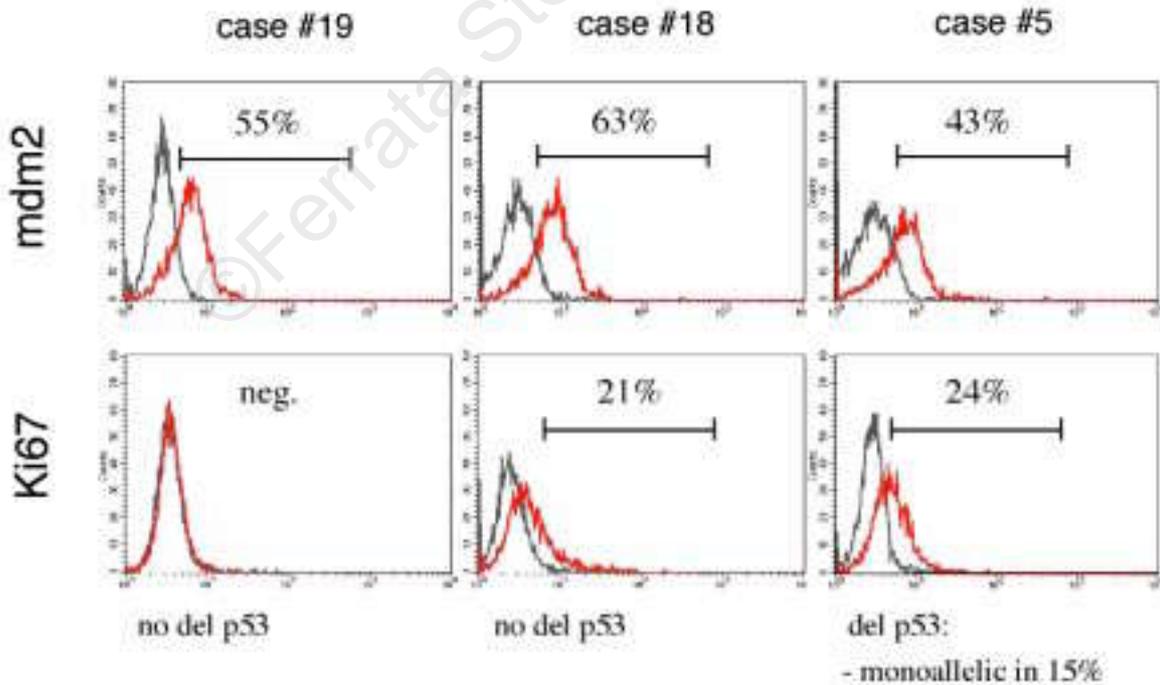


Figure 2. mdm2 overexpression in > 40% of cells in three out of 7 mdm2-positive patients with MCL in leukemic phase in relation to Ki67 expression and allelic p53 status. Flow cytometric histograms (no p53 expression in all 3 cases).

MCL, 6 had normal p53 alleles and 4 had p53 deletion. p53 deletion and/or overexpression of mdm2 occurred in more than 80% of blastoid MCL. All 5 patients expressing Ki67 had blastoid MCL ($p=0.03$), two of them with CNS involvement (cases #5 and 18), three with pleural effusions (cases #5, 18 and 19) and another with bowel involvement (case #20). There was no correlation between splenomegaly and/or other extranodal involvement and p53 deletion and mdm2 expression, respectively. All 10 patients with blastoid MCL have died after a median time of 25 months (range: 2 to 54 months, 77% of all deaths) whereas 2 out of 6 patients with non-blastoid MCL have died ($p=0.12$, log rank test of survival curves). Neither expression of mdm2, p53, Ki67 nor deletion of p53 nor gain of chromosome 12 correlated with survival. The results are summarized in Table 2.

Discussion

Several tumorigenic factors leading to cell cycle dysregulation in addition to cyclin D1 overexpression have been described in MCL. Abnormalities of p53 and mdm2, including protein overexpression, have been linked to disease progression, aggressive morphologic variants and poor prognosis^{10,11,15,19,26} as in other lymphoproliferative disorders with or without a leukemic presentation.^{26,28-33}

p53 is a tumor suppressor gene that plays a crucial role in cell cycle check point control (mainly at the transition of the G₀/G₁ to S-phase) and in the regulation of apoptosis in response to genotoxic stress and thus maintaining genetic stability of the cell. The p53 gene product mediates its function as a transcription factor for several genes such as p21, mdm2, cyclin G and Bax. p53 mutations are the most common genetic alteration in human cancers³⁴ and are often associated with loss of the remaining wild type p53 allele.³⁵ Most of the p53 mutations in neoplastic conditions are missense mutations changing the stability of the p53 protein, which may result in conformational change with loss of function as well as an increase of its half life that allows detection by immunologic methods.^{28,36} However, false negative and false positive results regarding p53 gene abnormalities are expected using immunohistochemistry or flow cytometry with monoclonal p53 antibodies such as DO-1, as this recognizes both the wild type and the mutant forms of p53. A false negative result will be found in cases with chain-terminating mutations of p53 and a false positive result may be found in high-grade lymphomas with wild type p53

and high proliferative activity.³⁷ Thus, p53 protein expression and the allelic status of the p53 gene may only be surrogate markers of dysfunctional, mutated p53.

In MCL, the highest proportion of p53 abnormalities have been described in aggressive histologic variants (i.e. anaplastic or large cell/blastoid).^{5,10,11} However, published information on cell cycle deregulation focusing on leukemic MCL, which is a prognostically adverse manifestation of the disease,^{3,6} is scarce. Gandini *et al.*³⁸ reported two patients with an aggressive MCL in leukemic phase with hyperdiploid karyotype, mutated p53 (chain-terminating in one patient) and additional trisomy 12 in one case. This is interesting because an alternative type of disruption of the p53 pathway, independently of deletion and/or mutation of p53, may result from the overexpression of its natural inhibitor mdm2, an oncoprotein which is coded for on chromosome 12q. Furthermore, mdm2 may act as an oncogenic factor in a p53-independent manner.^{22,39} Overexpression of mRNA or protein mdm2 in hematologic malignancies has been associated with unfavorable cytogenetics,²⁴ advanced disease²³ and poor response to chemotherapy in childhood ALL.⁴⁰ There are scanty reports regarding mdm2 expression in MCL. Møller *et al.*²⁶ documented mdm2 overexpression by immunohistochemistry in 2 out of 14 cases of MCL. Another report postulated a relationship between mdm2 overexpression with deletion of 12q and extranodal blastoid MCL.¹⁵ However, definition of what mdm2 overexpression means varied considerably in these studies.

Our results have shown that the incidence of p53 deletion in MCL in leukemic phase is high. Thus, half of the cases had monoallelic loss of 17p, a higher rate than reported so far. Cuneo *et al.*²⁰ found, in a relatively large series of newly diagnosed cases of MCL, loss of 17p in 29% and 21% of nodal and primary leukemic MCL, respectively, comparable to the findings of Schlette *et al.*⁸ who described abnormalities of 17p in 22% and overall abnormalities of chromosome 17 in 57% of MCL patients presenting primarily with leukemic involvement. In the latter study, abnormalities of chromosome 17 correlated with p53 expression on immunohistochemistry and occurred more commonly (although not statistically significantly so) in a large/blastoid subtype group. With regard to the time of analysis, 11/23 patients were untreated whereas in our study 12/19 patients had not been treated. In addition, we found no statistical differ-

ences in results between the treated and untreated groups of patients supporting the view that treatment status had no obvious impact on cytogenetics and protein expression. However, in our study there was neither a correlation of p53 deletion with p53 or mdm2 overexpression by flow cytometry nor with the blastoid subtype of the disease. In contrast to the findings of Schlette *et al.*,⁸ our p53 positive MCL cases were clearly positive (>50% of cells expressing p53) or negative (in the majority <6% of cells expressing p53), as has also been described by others examining p53 expression in MCL, albeit by immunohistochemistry.^{11,19} Considering the low proportion of cases with p53 overexpression in our study (10% using anti-p53, clone DO-7 and/or DO-1) compared to in other MCL cases with prognostically adverse morphology (19-50% using anti-p53, clone DO-7),^{10,11} it seems unlikely that the majority of cases with a deleted p53 were false negatives for p53 mutation due to chain-terminating mutations of the remaining p53 allele without p53 expression. The majority of p53 mutations in MCL are missense point mutations and therefore non-chain-terminating.^{10,11} On the other hand neither p53 protein positive cases with p53 deletion showed any signs of a high proliferation rate (Ki67 negative, no G2/M peak in cell cycle analysis) suggesting a non-chain-terminating mutation of the remaining p53 allele and not elevated expression of wild type p53 due to a generally high proliferative activity of the malignant cells. Taken together, our results add further evidence for a pathologic role of p53 abnormalities in the course of MCL and seem to confirm the occurrence of p53 mutations in prognostically adverse types of MCL (i.e. leukemic MCL), although the frequency of non-chain-terminating p53 mutations is assumed to be relatively low.

Overexpression of mdm2 occurred in a third of cases of which all but one showed a blastoid subtype, a ratio just failing to reach statistical significance ($p=0.06$) possibly because of the small number of cases. The blastoid subtype *per se* correlated significantly with a high proliferative activity and seems to be an aggressive variant as suggested elsewhere.^{2,8,27} There were no patients whose cells co-expressed p53 and mdm2, and the majority of the blastoid MCL cases which were mdm2 positive had normal allelic p53 status (4 out of 6 cases) suggesting a different mechanism of disease progression in this morphologic subtype. Because only two cases had gains of chromosome 12 (trisomy/tetrasomy 12) with expression of mdm2 in

one of them (case 20 with blastoid MCL and bowel involvement), it seems that gains of chromosome 12 are not the factor underlying an elevated expression of mdm2. Whether overexpression of mdm2 plays a pathologic role in MCL by disrupting the p53 pathway in an independent manner or represents an epiphenomenon in response to enhanced p53 wild type activity remains an open question.

Despite the high frequency of p53 and/or mdm2 abnormalities found in this study, follow-up indicated the blastoid subtype as the only potential factor predictive of an even shorter median survival in patients with leukemic MCL (not yet achieving statistical significance) and at the same time confirmed the dismal prognosis attributed to the blastoid subtype, whether in its leukemic^{8,27} or non-leukemic⁴¹ form.

In conclusion, our study shows that deletion of p53 and/or overexpression of mdm2 are frequent findings in MCL in leukemic phase, which is *per se* an adverse prognostic factor of the disease. Although p53 deletions were present in about half of the cases, overexpression of p53 occurred only in a minority (10%) suggesting a low frequency of non-chain-terminating mutation of the remaining p53 allele. In the blastoid subtype, elevated expression of mdm2 seems to be more frequent than p53 abnormalities. The reason why mdm2 is overexpressed in MCL remains open, but it seems neither to be attributable to a simple gain of chromosome 12 nor to merely reflect increased proliferative activity (Ki67⁺) of the malignant cells.

Overexpression of mdm2 might indicate an alternative mechanism of inactivating p53 in the progression of MCL. Our findings that p53 deletion and/or overexpression of mdm2 occurs in more than 70% of MCL in leukemic phase and in 80% of the blastoid subtype, the latter bearing an even worse prognosis, fit with the concept that progression in this lymphoma might be a consequence of a stepwise loss of cell cycle control and regulation of cell repair, through dysregulation of the p53 pathway.

Contributions and Acknowledgments

Order of authorship and contributions: 1) MS: conception and design, analysis and interpretation of intracellular flow cytometry results, draft, 2) EM: conception and design, analysis and interpretation of clinical data, morphology review, draft, revision, 3) VB-B: analysis and interpretation of FISH, draft, revision, 4) RM: technical advisor, performed flow cytometry for the diagnosis of MCL, revision, 5) DC:

morphology review, revision. MS: taking primary responsibility for the paper; EM: Tables 1 and 2; MS: Figures 1 and 2.

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Disclosures

Conflict of interest: none.

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PEER REVIEW OUTCOMES

Manuscript processing

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What is already known on this topic

The pathogenesis of mantle cell lymphoma is characterized by cyclin D1 gene activation by chromosomal translocation. Additional molecular lesions, frequently associated with disease progression, include mutations of the p53 tumor suppressor gene.

What this study adds

This study focuses on mantle cell lymphoma in leukemic phase. The results demonstrate that a large majority of leukemic mantle cell lymphomas associate with inactivation of p53, either through gene deletion/mutation or through overexpression of the p53 inhibitor mdm2.

Potential implications for clinical practice

This study identifies molecular markers for prognostic stratification of leukemic phase mantle cell lymphoma. These results also prompt the design of treatment strategies aimed at specifically interfering with the molecular machinery of the disease.

Gianluca Gaidano, Associate Editor (Novara, Italy)