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Ki67 and PIM1 expression predict outcome in mantle cell lymphoma treated with high dose therapy, stem cell transplantation and rituximab: a Cancer and Leukemia Group B 59909 correlative science study

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Abstract

The proliferation index in mantle cell lymphoma (MCL) has not been validated in the context of aggressive therapy regimens in the rituximab era. We assessed Ki67 and PIM1 (a cell cycle-related gene upregulated in blastoid MCL) expression by immunohistochemistry in a phase II study Cancer and Leukemia Group B 59909 of aggressive chemotherapy and rituximab followed by autologous stem cell transplantation plus rituximab in untreated MCL patients < 70 years of age. As a continuous variable or using a cutoff of 35%, higher image analysis (IA Ki67, $n = 52$) was associated with shorter progression free survival (PFS) ($P = 0.030$) and event free survival (EFS) ($P = 0.017$). PIM1 expression ($n = 50$) was associated with PFS ($P = 0.033$) and EFS ($P = 0.043$). Bivariate Cox models showed IA Ki67 and PIM1 were independent of clinical factors. High Ki67 (> 35%) is an important independent prognostic marker in aggressively treated MCL in the rituximab era. PIM1 expression predicts poor outcome and, given its potential role as a therapeutic target, deserves further study.

Keywords

Mantle cell lymphoma; prognosis; Ki67; PIM1; clinical trial; transplant

Introduction

Mantle cell lymphoma (MCL) is considered incurable with a median overall survival of 3–4 years using conventional chemotherapy [1-4]. MCL is characterised clinically by a pattern of continuous relapse following conventional chemotherapy. New therapies are needed and recent studies using high dose chemotherapy regimens with or without stem cell transplant as first line treatment have suggested benefit in terms of response and remission duration; however, whether overall survival is improved upon compared with conventional therapies remains to be proven [3,5-9].

Pathologically, MCL is composed of small, mature appearing lymphocytes with slight nuclear irregularities. The nodal infiltration is usually diffuse, although nodular and, rarely, mantle zone variants may be seen. The relatively aggressive nature is characterised by the frequent presence of mitotic figures, a feature unusual in other B-cell lymphomas of small lymphocytes. At the molecular genetic level, the vast majority of cases harbour an *IGH-CCND1* fusion as a consequence of the t(11;14)(q13;q32) resulting in overexpression of cyclin D1 protein, a diagnostic hallmark of this lymphoma. Only rare cases lack this abnormality and likely have abnormalities in other cyclins, such as cyclin D2 [10].

To elucidate molecular features of MCL, gene expression profiling has been performed in MCL and a recent seminal study has shown that a quantitative proliferation gene signature is a powerful predictor of outcome in MCL [11]. Several groups have investigated the significance of proliferation in MCL as measured by single markers such as Ki67 and have shown that high proliferative fraction was associated with poor outcome in the studied populations [12,13]. Indeed, other indicators of increased proliferation such as mitotic counts and blastoid morphology have been associated with poor prognosis in MCL [1,14]. These studies have been retrospective in nature and were performed on heterogeneously treated patients. Furthermore, prognostic significance of the proliferation index has not been examined in the context of combined high-dose therapy and rituximab.

Blastoid MCL is a variant of MCL with an extremely poor prognosis in which PIM1 has been found to be over-expressed [13,15,16]. *PIM1*, a serine/threonine kinase and protooncogene, has been implicated in lymphomagenesis with proliferative and anti-apoptotic effects [17-21]. It is a member of a family of proteins containing homologues PIM2 and PIM3, and is transcriptionally regulated by cytokines, mitogens and numerous growth factors [22-24]. The enzymatic substrates reported for PIM kinases are diverse (p100, Bad, NFATc1, p21/Cip1, Cdc25a, SOCS), suggesting that PIM kinases play a central regulatory role in numerous processes [25-30]. Elevated expression of PIM kinases has been described predominantly in murine and human leukemias and lymphomas [24,31-33]. Studies using either *PIM* transgenic or *PIM* knockout mice suggest that overlapping functions exist for *PIM1* and *PIM2*, and that the full transforming potential of PIM kinases cannot be realised *in vivo* without cooperation from other oncogenes, including *MYC* and

MYCN [33,34]. *PIM1* has been implicated in the development of leukemias, lymphomas, in late progression events and also in prostate cancer [35]. Therefore, *PIM1* is a target of drug development programs. With the promise of improved outcome in MCL patients treated with high dose therapy and rituximab, we have examined the prognostic importance of the proliferation marker Ki67 and PIM1 in predicting outcome in patients enrolled in Cancer and Leukemia Group B (CALGB) 59909 [36].

Methods

Patients

CALGB 59909 is a phase 2 trial for newly diagnosed MCL with planned intensive cytoreduction with rituximab plus methotrexate and augmented-dose CHOP, consolidation with high-doses of cytarabine and etoposide with rituximab added to *in vivo* purge mobilised autologous peripheral blood stem cells, and then autologous stem cell transplantation following high-doses of carmustine, etoposide and cyclophosphamide. Rituximab was also given post-transplant. The preliminary clinical results have been reported in abstract form [36]. Each participant signed an IRB-approved, protocol-specific informed consent in accordance with federal and institutional guidelines. Hematoxylin and eosin stained slides were scanned with the Aperio ScanScope system (Aperio, Vista, CA) at a 400 × microscope resolution at the CALGB Pathology Coordinating Office (PCO, Columbus, OH) and reviewed via the internet using ImageScope software (Aperio, ImageScope version 8.2.5.1263) with anonymised pathology reports. Two panel pathologists reviewed each case and a case was eligible for study if agreement in diagnosis was reached independently. In the case of disagreement, a third hematopathologist reviewed the case as arbitrator. Cyclin D1 immunohistochemistry (IHC) (clone SP1) was performed at the PCO using automated immunostainers (Ventana Benchmark, Tucson, AZ) and cyclin D1 expression was required for eligibility.

Fifty-two patients had available stained slides for image analysis (IA) Ki67, 49 were available for manual counting and 50 patients had PIM1 data available. Given that there were more patients available with IA Ki67 than manual Ki67, IA Ki67 forms the basis for this analysis.

Immunohistochemistry

Ki67 staining (rabbit monoclonal clone 30-9, Ventana Medical Systems, Tucson, AZ) was performed at the Cleveland Clinic using an automated immunostainer (Ventana Benchmark, Ventana). Antigen retrieval was performed using CC1 (Ventana) and primary incubation time was 16 min. Ki67 staining was evaluated by two methods: 1) manual counting # positive cells per high power field (hpf, mean of five fields) (Olympus BX40, Olympus, Center Valley, PA; 100 × immersion oil objective, 10 mm × 22 mm ocular) and 2) computerised IA. For IA, the entire histologic sections stained for Ki67 were scanned using an Aperio XT whole slide scanner (Aperio, Vista, CA) using a 20 × objective (performed at University of Los Angeles). Areas of the section selected as suitable for Ki67 quantitation were captured using the Aperio ImageScope viewer. Once images were acquired, Metamorph software (Molecular Devices, Downingtown, PA) was used to threshold the

images using a Hue Saturation Intensity algorithm. The parameters (area, intensity) were quantified using the Metamorph integrated morphometry analysis module. The measurements were exported to an Excel file. An average of 15,000 cells (average fields selected based on areas with technically adequate staining) were counted per case (range from 2000 to 47,000 cells depending on the size of the sections). Figures 1(A) and 1(B) show two examples of Ki67 staining.

PIM1 staining was performed at Cross Cancer Centre using standard techniques: whole tissue and microarray sections were deparaffinised and rehydrated with graded alcohol. Heat-induced epitope retrieval was performed using Tris buffer (pH 9.9; Dako, Mississauga, Ontario, Canada) and a rapid microwave histoprocessor (RHS, Milestone, Bergamo, Italy). Endogenous peroxidase activity was blocked by incubating samples in 3% H₂O₂ for 10 min, followed by a wash with water and then phosphate-buffered saline (PBS; pH 7.2). Tissue sections were then incubated with 1:25 dilution of polyclonal PIM-1 antibody (Biolynx INC San Diego, USA) overnight in a humidified chamber at 4°C. After two washes with PBS, tissue sections were incubated with anti-rabbit IgG and peroxidase (EnVision, Dako) for 30 min at room temperature. After two washes with PBS, tissue sections were incubated with 3,3'-diaminobenzidine/H₂O₂ (Dako) for colour development and hematoxylin was used as a counter stain. PIM1 expression was evaluated by two hematopathologists. For each case, a score of 0–3+ was given based on the intensity of staining that was present in the majority of the cells (0 = absent, 1+ = weak 2+ = moderate, 3+ = strong). Figures 1(C) and 1(D) show cases scored as 1+ and 3+, respectively.

Statistical analysis

Cox regression was used to determine associations between IHC markers and outcome. For a robust inference, the Cox regression model uses the rank of a marker value, instead of the raw value, as a covariate [37]. Progression free survival (PFS, time until progression, censoring deaths due to treatment or other causes) and event free survival (EFS, time until progression or death from any cause) were used as clinical endpoints. Kaplan–Meier and log rank testing was used for graphical representation of survival analysis. Wilcoxon rank-sum and Fisher's Exact testing was used for comparison of continuous and categorical data, respectively.

Results

Clinical characteristics and histology

Fifty two cases were eligible for inclusion based on histology review and expression of cyclin D1 and IA Ki67 data. Four cases (8%) had blastoid morphology. All patients received the planned autologous stem cell transplantation (ASCT) and rituximab. Median follow-up was 3.7 years with a range of 2.4–5.4 years among living patients. The 3 year PFS and EFS for this cohort were 0.57 (95% confidence interval (CI) 0.42–0.70) and 0.52 (95% CI 0.37–0.64), respectively. The baseline clinical features are summarised in Table I. Increased LDH was associated with higher Ki67 index ($P = 0.01$). Table I also shows the association of these characteristics with outcome. None of the clinical factors were significantly associated with outcome with the exception of elevated LDH (associated with PFS, $P = 0.046$), likely

due to the relative small number of patients in this study. Stage III/IV disease was also associated with shorter PFS; however, over 90% of patients had high stage disease. The IPI was also not associated with outcome. Although there were only four cases, blastoid morphology was associated with shorter PFS and EFS.

Ki67 expression and outcome

Ki67 staining showed the appropriate nuclear pattern in all cases (Figure 1). Expressed as a percentage of positive cells, we found substantial variability in IA Ki67 (range 0.01–84%, median 27.8%). Manual Ki67 data was available in 49 cases. Despite the different methods of evaluation, there was a high degree of correlation (Spearman correlation coefficient of 0.79, $P < 0.0001$) between IA and manual methods. When considered as a continuous variable, IA Ki67 was statistically associated with shorter PFS and EFS ($P = 0.013$ and $P = 0.007$, respectively, Cox proportional hazards) [37]. The importance of Ki67 as a marker of poor outcome was confirmed in analysis of the manual Ki67 data, in which increased Ki67 staining was also associated with poor outcome ($P = 0.02$ for PFS and $P = 0.005$ for EFS, Cox proportional hazards) [37]. For purposes of showing this relationship, a cutoff of 35% maximises the differences (Table II). Figures 2(A) and 2(B) show patients with a high IA Ki67 had a shorter PFS and EFS compared with those with low IA Ki67.

PIM1 expression and outcome

PIM1 IHC was available on 50 patients. Fourteen patients were negative for PIM1, 36 patients were scored as 1+, 19 patients were scored as 2+ and five patients as 3+ (Figure 1). There were no statistically significant relationships between clinical features noted in Table I and PIM1 score. There was no correlation between PIM1 expression and blastoid morphology. When considering PIM1 as a semiquantitative variable there was no correlation between PIM and IA Ki67 staining (Spearman correlation coefficient of 0.03, $P = 0.86$). There was a statistically significantly shorter PFS ($P = 0.033$) and EFS ($P = 0.043$) for patients with biopsies showing high expression (2+ or higher) compared to those with low PIM1 expression (Table II). Figures 2(C) and 2(D) show Kaplan–Meier curves illustrating this association.

Multivariable analysis

Although there were relatively few cases and events, we explored factors potentially confounding the associations seen with Ki67 and PIM1. Multivariate Cox models were fit including each of the baseline characteristics listed in Table I. None of the factors changed the relationship of Ki67 with outcome, i.e., IA Ki67 is an independent predictor of PFS and EFS, controlling for gender, marrow involvement and IPI risk factors, including LDH. Likewise, $PIM1 > 1+$ was also independent of factors in Table I.

Discussion

The clinical outcome for MCL is poor; however, substantial variability exists with some patients succumbing in less than a year while others may survive 10 years or longer [1,4,38,39]. Since its recognition as a distinct entity, numerous studies have been published identifying prognostic factors that have ranged from standard morphologic features such as

blastoid morphology or mitotic counts [14,40] to increased Ki67 index [12,13,41], P53 overexpression and other cell cycle protein expression abnormalities [42-44]. More sophisticated comparative genomic hybridisation abnormalities or gene expression profiles have also been shown to have prognostic value [11,45,46]. The importance of cell cycle and proliferation in determining the clinical behaviour of MCL has been confirmed by expression profiling and a quantitative proliferation signature has been proposed based on 20 genes that belonged to a larger set of proliferation signature genes that were highly variably expressed in MCL and associated with survival [11]. Although these latter developments are exciting and potentially useful, they are still impractical for routine use.

Most of the studies to date using conventional techniques such as IHC, have confirmed the prognostic value of Ki67 staining and, in fact, its use has been advocated in the new MCL international prognostic index [47]. Most studies evaluating this proliferation marker have been performed outside the context of a single clinical trial [12,13,41,48,49]. Investigators have also found Ki67 level to remain predictive of outcome in multivariate analysis [12,13,41,48]. However, patient populations were clinically heterogeneous and manual methods were used for evaluation of Ki67 (Table III).

A prognostic marker must be evaluated in the context of the treatment regimen of the study group, as novel treatment approaches may alter the utility of prognostic markers. Rituximab is now commonly used in MCL treatment. A single study has evaluated Ki67 in patients treated with rituximab-CHOP chemotherapy and reported that high Ki67 (30%) predicted poor outcome [50]. We have now demonstrated that high Ki67 remains a poor prognostic indicator in the setting of high dose chemotherapy/stem cell transplantation regimens containing rituximab. Of note, our cutoff of 35% is similar to the high level suggested by Determann [50]. To our knowledge, the current study is also the first to use IA to more objectively quantify Ki67 in MCL. Indeed, computerised methods of Ki67 quantitation have been shown to be reproducible within and between operators, with correlation coefficients over 0.9 [51,52], a prerequisite for widespread use outside the setting of controlled clinical trials. Indeed, a recent study showed problems with reproducible scoring of Ki67 when a semiquantitative visual estimate was used [53]. Thus use of IA is suggested in future practice. It is interesting that the proliferation signature defined by gene expression analysis did not include Ki67 [11]. This may be due to differences in protein and RNA expression or complex interactions of multiple genes serving to outweigh Ki67 as part of the model. Nevertheless, Ki67 as a single biomarker, correlated with proliferation signature in the small number of cases in which both were available [11]. It appears to be a robust MCL prognostic marker, both in conventionally treated patients and in patients receiving high dose therapy with rituximab.

Blastoid MCL is an aggressive variant of MCL and PIM1 has been found to be overexpressed in this variant, leading some to propose therapeutic targeting of this molecule [15,16,54]. We, therefore, also examined expression of PIM1 in these cases. PIM1 has been implicated in anti-apoptosis through the phosphorylation of p21^{cip1/waf1} [55], which is thought to mediate survival by binding to and preventing the activation of pro-caspase-3 [56]. Most recently, the PIM1 kinase has been found to promote inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser¹¹² gatekeeper site [57]. PIM1 is a

target of the gp130-mediated STAT3 signalling pathway [58]. It is stabilised by HSP-90 [59] and it phosphorylates the tyrosine phosphatase Cdc25 [60], which plays a critical role in G1/S cell cycle transition. In addition, PIM1 and PIM2 regulate rapamycin-resistant T cell growth and survival [61]. Thus, PIM kinases have defined biological roles in cell survival, proliferation and differentiation [24]. Consistent with these roles, PIM1 expression appears to be a marker of aggressive disease in MCL, even in the absence of blastoid morphology. Indeed, PIM1 was scored as > 1+ in 48% of cases and there was no association with blastoid morphology or Ki67.

Since blastoid histology is known to be associated with poor outcome, it may be that PIM1 expression also identifies cases with blastoid-like biologic features while not demonstrating overt “blastoid” cytologic features. Because of the small number of cases available for this study, additional study of PIM1 and other PIM kinases is warranted. The kinase activity of PIM1 is directly linked to the expression level of PIM1. But mutations in PIM1 have been reported in diffuse large B-cell lymphomas that result in altered function [62]. Perhaps measuring the kinase activity of PIM1 or phosphorylated substrates of PIM1 by IHC (as a surrogate of PIM1 activity) may be even more informative than the protein expression level.

New therapies are needed for MCL. The poor outcome and lack of standardised therapy has spurred numerous clinical trials. Aggressive treatment protocols with or without autologous stem cell transplantation have been attempted with promising results, including a relatively high rate of durable remissions [5,6,63,64]. CALGB 59909 follows in a similar vein [36]. As in prior studies with conventional therapies, Ki67 staining pattern inversely correlated with EFS and thus appears to remain an important biologic marker of outcome. Taken together, the evidence is now overwhelming in support of the Ki67 proliferative index as a prognostic marker in MCL. As shown in this study, this includes those patients treated with high dose therapies, stem cell transplantation and rituximab. Issues of technical standardisation remain to be completely resolved; however, Ki67 is a robust marker with control tissues commonly available such as hyperplastic lymph nodes or tonsil that can easily be evaluated for quality of stain and IA should make reporting more reproducible. In combination with IA, we believe it is time to develop prognostic models that incorporate this widely available biologic marker. In particular, we agree with the proposal to include the Ki67 index in prognostic scoring systems [47] with the goal of increasing our ability to stratify patients at diagnosis and select appropriate therapy.

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Figure 1.

MCL immunohistochemistry. (A) MCL with low Ki67 index of 3% (400 × original magnification); (B) MCL with Ki67 index of 80% (400 × original magnification); (C) Weakly positive PIM1 staining scored as 1+ (400 × original magnification); (D) Strongly positive PIM1 staining scored as 3+ (400 × original magnification). Insets show the scanned image of the biopsy at 1 ×.

Figure 2.

Kaplan–Meier curves for PFS (A) and EFS (B) showing a statistically significant shorter survival for patients with high Ki67 index (dotted line) compared to those with a low Ki67 index (< 35%, solid line). Kaplan–Meier curves for PFS (C) and EFS (D) showing a statistically significant shorter survival for patients with PIM1 scores of 2+ or 3+ (dotted line), compared to cases with low expression (solid line). *P* values reflect the log-rank test.

Table I

Baseline characteristics and association With PFS and EFS.

	KI 67	35% (n = 35)	KI 67 > 35% (n = 17)	All patients (n = 52)	P-value*	PFS HR (95% CI)	P-value*	EFS HR (95% CI)	P-value*
Age (median, range)	56 (37–68)	57 (39–68)	56 (37–68)	0.95					
Age > 60	9 (25.7%)	5 (29.4%)	14 (26.9%)	1.00		0.96 (0.38, 2.42)	0.924	1.27 (0.57, 2.80)	0.557
Male	28 (80.0%)	13 (76.5%)	41 (78.8%)	1.00		0.71 (0.28, 1.80)	0.470	0.62 (0.27, 1.40)	0.240
Performance status > 1	3 (8.6%)	2 (11.8%)	5 (9.6%)	1.00		1.96 (0.58, 6.60)	0.267	2.28 (0.79, 6.60)	0.118
Elevated LDH	8 (22.9%)	10 (58.8%)	18 (34.6%)	0.015		2.28 (0.99, 5.24)	0.046	1.96 (0.90, 4.25)	0.083
> 1 Extra-nodal site	18 (52.9%)	9 (52.9%)	27 (52.9%)	1.00		1.47 (0.64, 3.41)	0.362	1.11 (0.53, 2.33)	0.790
missing data	1	0	1						
Stage III/IV	34 (97.1%)	16 (94.1%)	50 (96.2%)	1.00		0.25 (0.06, 1.06)	0.042	0.30 (0.07, 1.27)	0.082
B symptoms	7 (20.0%)	8 (47.1%)	15 (28.8%)	0.06		0.81 (0.30, 2.18)	0.681	1.18 (0.52, 2.66)	0.699
Marrow involvement	25 (73.5%)	13 (76.5%)	38 (74.5%)	1.00		0.72 (0.30, 1.72)	0.454	0.56 (0.26, 1.22)	0.139
Missing data	1	0	1						
IPI				0.30		1.58 (0.69, 3.58)	0.273	1.52 (0.72, 3.21)	0.270
Low	7 (20.6%)	2 (11.8%)	9 (17.6%)						
Low/Int	14 (41.2%)	6 (35.3%)	20 (39.2%)						
Int/High	9 (26.5%)	4 (23.5%)	13 (25.5%)						
High	4 (11.8%)	5 (29.4%)	9 (17.6%)						
Missing data	1	0	1						
Blastoid morphology	2 (5.7%)	2 (11.8%)	4 (7.7%)	0.59		5.23 (1.51, 18.15)	0.004	5.84 (1.95, 15.50)	0.0004

IPI, international prognostic index; EFS, event free survival; PFS, progression free survival; HR, hazards ratio; CI, confidence interval; LDH, lactate dehydrogenase.

* Wilcoxon rank sum for continuous or ordered categorical variables, Fisher's exact for dichotomous variables.

Table II

Immunohistochemical markers and correlation with outcome.

	PFS HR (95% CI)	<i>P</i>-value[*]	EFS HR (95% CI)	<i>P</i>-value[*]
IA Ki67 > 35%	2.45 (1.06, 5.63)	0.030	2.46 (1.14, 5.31)	0.017
PIM1 > 1+	2.35 (1.05, 5.27)	0.033	2.10 (1.01, 4.37)	0.043

EFS, event free survival; PFS, progression free survival; HR, hazards ratio; CI, confidence interval.

*
Cox-regression analysis.

Table III

Ki-67 staining and prognosis in mantle cell lymphoma.

Study	N	Ki67 antigen antibody	Cutoff	Counting method	Treatment	Significant in univariate analysis (OS)	Significant in multivariable analysis (OS)
Raty et al. 2002	127	Polyclonal	26%	Manual	Variable SCT (5.5%)	Y	Y
Schrader et al. 2004	95	KiS5	10%	Manual	Variable SCT (0%)	Y	N
Katzenberger et al. 2006	134	MIB1	Tiered*	Manual	Variable SCT% not specified	Y	Y
Tienann et al. 2005	304	MIB1 or KiS5	Quartiles [†]	Manual	Variable SCT% not specified	Y	Y
Hui et al. 2005	48	Not specified	50%	Manual	Variable SCT% not specified	Y	Y
Determann et al. 2008	249	MIB1 or KiS5	10%, 30%	Manual	Variable, cohorts with CHOP ± rituximab	Y	Y (CHOP) N (CHOP + rituximab)
Current study	52	30-9	35%	Image analysis	SCT (100%) with rituximab	Y [#]	Y [#]

OS, overall survival; SCT, stem cell transplant.

* < 20%, 21–40%, 41–60%, > 60%;

[†] Also tested 10% and 40%.

[#] PFS and EFS.