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p53 Expression by Immunohistochemistry is an Independent Determinant of Survival in Chronic Lymphocytic Leukemia Patients Receiving Frontline Chemo-Immunotherapy

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Abstract

Although chromosome 17p abnormalities and TP53 mutations are purported to be poor prognostic indicators in chronic lymphocytic leukemia (CLL), the significance of p53 expression in CLL has not been defined in patients uniformly treated with chemoimmunotherapy, nor has its utility in combination with other novel prognostic markers, such as IgVH mutation status (IgVH MS) or ZAP-70 expression, been evaluated. Therefore, we studied p53 expression by immunohistochemistry (IHC), using the bone marrow specimens from 222 CLL patients (pts) enrolled in the phase II evaluation of the fludarabine, cyclophosphamide and rituximab (FCR) regimen. ZAP70 expression and IgVH MS were assessed in 208 and 108 patients, respectively. 168 CLL pts had concurrent classical cytogenetic analysis. p53 expression correlated with abnormal karyotype ($p=0.002$) and adversely affected overall survival independent of ZAP70 expression and IgVH MS ($p<0.001$). p53(+)CLL pts were less likely to achieve complete remission, but patients who did achieve CR showed a durable response. In conclusion, p53 expression was an important determinant of complete remission and overall survival, but not remission duration, in CLL patients receiving chemo-immunotherapy.

Keywords

Chronic lymphocytic leukemia; p53; prognostic factors

Introduction

The TP53 gene, a tumor suppressor gene located on chromosome 17p, encodes the p53 protein, which acts as a transcriptional activator of proapoptotic genes such as NOXA, PUMA, and BAX. p53 can also directly bind to and sequester anti-apoptotic proteins (Chipuk and Green 2006). As CLL is a disease of cell survival due to the presence of anti-

apoptotic proteins such as BCL-2 and MCL-1, p53 plays a central role in coupling genotoxic stress induced by chemotherapeutic agents and activation of the intrinsic cell death pathway. These lines of evidence support a mechanistic rationale that places p53 function in the center of CLL sensitivity to cytotoxic therapy.

The adverse effect of abnormalities of TP53 on the survival and response to treatment of CLL patients have been reported in the literature (el Rouby, et al 1993), (Wattel, et al 1994), (Dohner, et al 1995), (Van Den Neste, et al 2007), (Zenz, et al 2007), (Xu, et al 2008). However, there are a number of methods to assess the integrity of the TP53 axis, reflecting how TP53 can be disrupted in different ways, and it is not known whether each of these disruptions carries the same prognostic significance. The best recognized mechanism of TP53 disruption is the deletion of a TP53 allele, as detected by fluorescent in-situ hybridization (FISH) (Dohner, et al 2000). In a randomized trial of three different DNA damaging chemotherapy regimens, TP53 deletion was associated with an inferior response rate, remission duration and overall survival (Catovsky, et al 2007). Another mechanism of TP53 disruption is the presence of point mutations in TP53, which had also been associated with resistance to DNA damaging agents (el Rouby, et al 1993). As the few studies that simultaneously evaluated for TP53 deletion and TP53 mutation in CLL had shown that the two do not necessarily overlap (Grever, et al 2007), the reliance on FISH detection of TP53 deletion (without a simultaneous assessment for mutations) may miss patients with prognostically important TP53 disruptions. These potential discrepancies give impetus to the development of novel functional assays which may provide a “pan-TP53” assessment of the entire TP53 axis (Best, et al 2008).

Until the wide availability of these functional assays, TP53 mutation detection requires direct sequencing which is available only in specialized research centers. The requirement for fresh or frozen tissue in direct sequencing means that TP53 mutation status cannot be performed retrospectively in archival material from completed trials with mature clinical outcomes. Furthermore, there is ongoing uncertainty about which TP53 mutations are physiologic (or silent), and which are pathologic. In this regard, the properties of p53 immunohistochemistry (IHC) make it an attractive alternative for TP53 mutation screening. IHC is widely available and can be performed in archival material that has undergone processing for histologic evaluation. It relies on the short half life of wild type p53 protein, which cannot normally be detected by IHC on tissue sections. However, mutated p53 protein has a prolonged half-life and becomes detectable by this method. In a large study of 128 patients with hematologic malignancies, p53 IHC showed a 96% concordance with TP53 mutation as detected by single-stranded conformation polymorphism analysis and direct sequencing (Lepelley, et al 1994). In CLL, 88% of patient samples positive for p53 IHC were subsequently shown to harbor a TP53 mutation on sequencing (Cordone, et al). Two studies had shown that a positive p53 IHC was significantly associated with poor treatment response and reduced survival in CLL patients receiving older chemotherapy regimens (Giles, et al 2003), (Cordone, et al 1998).

The impetus for the current study is three-fold. Firstly, despite the strong association between p53 IHC and important clinical outcomes, p53 IHC is infrequently employed in the clinical setting. We would therefore like to revisit this test in the context of modern practice.

Secondly, previous studies of p53 IHC were conducted in heterogeneous patient groups receiving a number of older regimens. This heterogeneity clouds the interpretation of the effect of p53 IHC on key clinical parameters including complete remission rate, remission duration and overall survival. Furthermore, as monoclonal antibodies may be able to activate cell death pathways independent of p53 (Cheson and Leonard 2008), we would like to evaluate whether TP53 disruptions remain prognostic in the era of combined chemotherapy and antibody therapy (chemoimmunotherapy). Lastly, the relevance of TP53 disruptions in the context of novel prognostic markers in CLL, including somatic hypermutation status (IgMS) and ZAP70 expression, has not been elucidated. In order to address these questions, we evaluated p53 IHC, conventional cytogenetics, IgMS and ZAP-70 status in a cohort of patients uniformly treated with the fludarabine, cyclophosphamide and rituximab (FCR) regimen, at a mature median follow-up of six years.

Methods

Study Group

Between July 1999 and January 2004, 300 patients with a diagnosis of CLL, confirmed at The University of Texas M.D. Anderson Cancer Center (MDACC), were enrolled in a phase II evaluation of the FCR regimen as initial therapy (Tam, et al 2008a). Treatment indications were according to the published NCIWG guidelines, and informed consent was obtained according to MDACC institutional guidelines (Cheson, et al 1996b).

All patients had a pretreatment evaluation including history, physical examination, CBC with differential, platelet count, liver and renal function tests, bone marrow (BM) aspiration and biopsy for morphologic evaluation and immunophenotypic analysis. Bone marrow specimens from 168 patients were assessed using conventional karyotyping (Glassman and Hayes 2005). fluorescence in situ hybridization (FISH) screening was not performed as the study pre-dated the widespread availability of these panels.

Therapy and Response Criteria

Details of the therapy schedule have been previously reported (Keating, et al 2005). Briefly, on day 1 of the first cycle of FCR, patients received 375 mg/m² of rituximab. The dose of rituximab on day 1 of subsequent cycles was 500 mg/m². On days 2, 3 and 4 in the first cycle and on days 1, 2 and 3 of the subsequent cycles, patients received fludarabine 25 mg/m²/d and cyclophosphamide 250 mg/m²/d i.v. A total of six cycles of treatment were planned (Borthakur, et al 2007). Response criteria were those defined by the NCIWG (Cheson, et al 1996a). BM evaluation was usually performed at the end of therapy, although it was not required for determination of PR. Computed tomography scans were not required to evaluate response. After completion of therapy, patients were re-evaluated at 3-month intervals with history, physical examination, and blood counts.

Immunohistochemistry

The archives of the UTMDACC Department of Hematopathology were searched and 222 formalin-fixed, paraffin-embedded BM biopsy or clot section specimens from the patients enrolled in the FCR protocol were available for p53 staining and 208 BM specimens for

ZAP70 staining. All study samples were collected four weeks or less before the first dose of chemotherapy. In addition, if the initial BM specimen was negative for p53 expression and the patient subsequently relapsed after treatment, relapse BM specimens were re-evaluated for p53 expression, if available.

Using immunohistochemical methods on paraffin-embedded tissue sections (3 or 4 µm thick), we evaluated p53 (DO-7, Dako Corporation) expression. Staining was performed on a Ventana Medical Systems automated slide stainer with Ventana detection kits. The anti-p53 stain is a nuclear stain. The stained slides were reviewed by two of the authors (ES and CT). The staining intensity of p53 varied, but any degree of expression by a cell was considered as positive. The percentage of p53(+) cells was determined by counting 300 to 400 cells in the tissue sections and determining a percentage of positive cells.

Cytoplasmic ZAP70 (clone 2F3.2 Upstate Cell Signaling, Lake Placid, NY) expression by IHC was also assessed, with a method similar to the p53 staining. The ZAP70 stained slides were reviewed by two of the authors (ES, JA). Only nuclear/cytoplasmic or cytoplasmic staining 20% of the lymphoid cells was considered positive for ZAP70 expression (Admirand, et al 2004). Nuclear staining of non-neoplastic, reactive T cells served as an internal control in each case.

Somatic hypermutation analysis

IgVH MS was determined in 108 CLL cases in pre-treatment bone marrow aspirate samples using methods previously described (Chen, et al 2002, Raval, et al 2005).

Statistical methods

Time to progression was calculated from the first day of FCR therapy until the date of disease progression or Richter (large cell) transformation. Survival was calculated from the first day of FCR therapy. Categorical variables were compared using the Fisher Exact or Chi-Square test, and continuous variables were compared using the Mann-Whitney test, as appropriate. Actuarial survival was calculated using the method of Kaplan and Meier, and compared in univariate analysis using the log-rank test. Multivariate modelling of factors independently determining survival was performed using Cox proportional hazards analysis. All p-values were two sided.

Results

Determination of p53 positivity

In order to determine what degree of p53 positivity was prognostically significant, the complete remission rate and overall survival of patients with varying proportions of p53 positive cells were analyzed. As the proportion of p53 positive cells varied between different areas of individual specimens (depending on the degree of normal cells intermixing with CLL cells and depending on the number of proliferation centers present), the following cut-offs were chosen by the reviewing authors (ES and CT) as being the most reproducible: <5%, 5–20%, 20–40% and >40% p53 positivity (figure 1). Using these categories, the

interobserver concordance was 88%, with all discordances spanning only one category (eg 5–20% vs 20–40%).

Patients with 40% p53 expressing cells (n=174) had similar survival and/or CR regardless of actual percentage of cells positive (Figure 2; $p>0.30$ for all comparisons), whereas the patients with >40% p53 positive cells (n=21) had a significantly inferior survival ($p<0.0001$) and lower probability of CR ($p=0.0001$).

Therefore, we chose 40% cell expression as the threshold for p53 positivity. Interestingly, patients with discordant readings had survival that was identical to that of patients with concordant readings 40% (Figure 2). This was likely due to the fact that 25 (93%) of discordances occurred in specimens with p53 positivity <40%, whereas only 2 (7%) specimens were discordant across the crucial 40% threshold.

Thus, 21 of 222 (10%) CLL cases were considered positive for p53 expression (>40% positive nuclei) and 201 were considered negative.

Correlation Between p53 Status and Baseline Characteristics

The baseline clinical and laboratory findings for p53 positive and negative patients are summarized in Table 1. There were no significant differences in age, performance status or Rai stage between p53(+) and p53(–) patients. However, p53(+) patients had significantly higher levels of β 2-microglobulin (median 4.3 vs 3.6 mg/L, $p=0.05$) and lactate dehydrogenase (692 vs 536 IU/L, $p=0.002$), and were more likely to have an abnormal karyotype (63% vs 24%, $p=0.002$); chromosome 17 abnormalities were present in 19% of p53(+) and <1% of p53(–) patients, respectively ($p=0.003$). ZAP70 was positive in 62% and 40% of p53(+) and p53(–) patients ($p=0.08$), respectively, and IgVH was unmutated in 50% and 67% of p53(+) and p53(–) patients ($p=0.44$), respectively.

p53 Status Determines Complete Remission Achievement, But Not Complete Remission Duration

Table 2 summarises the treatment outcome of p53(+) and p53(–) patients. Patients with p53(+) CLL were significantly less likely to achieve complete remission than p53(–) patients (33% vs 76%, $p=0.0001$). Responses in the remaining p53(+) patients were nodular PR in 10%, PR in 29%, and resistant disease in 29%. Complete remissions in the p53(+) population were durable with no relapses among 7 patients followed for up to 59 months (Figure 3). Of the 2 patients in nodular PR, one patient relapsed at 39 months, and the second died in remission at 30 months of lung cancer. Of the 6 patients in PR, 4 relapsed (at a median of 31 months), 1 remained in ongoing remission (at 49 months), and one patient died in remission at 2 months of progressive multifocal leuko-encephalopathy. All of the p53(+) patients without response developed disease progression at a median of 5 months. No p53(+) patient underwent histologic transformation.

Of the 201 p53(–)pts, 153 pts (76%) achieved CR, 17 (8%) achieved nodular PR, and 24 (12%) achieved PR. Five (2%) patients did not respond to FCR. Sixty (39%) of the 153 pts who achieved CR have relapsed, as have 10 of 17 patients in NPR and 16 of 24 patients in

PR. Complete remission durations were similar between p53(+) and p53(−) patients (Figure 3, $p=0.24$).

p53 Expression Predicts Inferior Survival Independent of ZAP70 or IgVH Mutation Status

Patients with p53(+) CLL had a significantly inferior survival than patients with p53(−) CLL (Figure 4; $p<0.0001$). This survival disadvantage was restricted to those patients who did not reach CR. Among patients who achieved CR, five year survival was not significantly different between p53(+) and p53(−) patients (100% vs 90%, respectively, $p=0.45$). In contrast, p53(+) patients in lesser categories of response had a significantly inferior five year survival rate than p53(−) patients (24% vs 58%, respectively, $p=0.02$).

On univariate analysis, significant ($p<0.05$) predictors of inferior survival in all patients were age ≥ 70 years, $\beta 2m$ $\geq$ twice upper limit of normal (2N), white cell count $\geq 150 \times 10^9/L$, p53(+), and ZAP70 (+). Advanced Rai stage (stages 3 & 4) and unmutated IgVH status were of marginal significance ($p=0.07$ and 0.06 respectively). Multivariate modelling of survival identified the following independent predictors of survival: age ≥ 70 years ($p=0.02$, hazard ratio (HR) 2.1), $\beta 2m \geq 2N$ ($p=0.01$, HR 2.0), p53 expression by IHC ($p=0.003$, HR 3.2) and LDH $\geq 2N$ ($p=0.02$, HR 4.5). Importantly, ZAP70 and IgVH MS did not impact significantly on survival after adjusting for p53 IHC in the multivariate model.

p53 expression after relapse

In order to determine if the development of aberrant p53 protein expression was a common mechanism of disease progression, we analyzed 31 BM samples from p53(−) patients at the time of relapse from CR. Of these samples, only 1 (3%) showed a conversion of p53 positivity. This BM sample also underwent concurrent FISH analysis, which did not show a deletion of the 17p13.1 locus.

Discussion

In this large cohort of uniformly treated patients with mature follow-up, p53 IHC positivity (found in 10%) was strongly associated with poor therapy response and short survival. p53 positivity also correlated with traditional markers of poor prognosis such as elevations in serum $\beta 2m$ and LDH, and with abnormalities in conventional karyotyping, including chromosome 17 aberrations. As abnormalities of chromosome 17 are postulated to disrupt the TP53 gene at 17p13, this association is expected and has been shown in other tumors (Erol, et al 2004), (Heselmeyer, et al 1998), (Eyfjord, et al 1995). Conventional cytogenetics is not a sensitive method of detecting 17p13 deletion, as highlighted by published data showing that 17p13 deletions are present in 7% of chemotherapy-naïve CLL patients by FISH, compared with 2% by classical cytogenetic analysis (Zenz, et al 2007). Therefore, had FISH been performed in this patient cohort, the association between p53 IHC positivity and chromosome 17 abnormalities may be even stronger. Interestingly, p53 IHC was not associated with IgVH mutation status and was only marginally associated with ZAP70 expression. The ability of p53 IHC to predict for inferior survival independent of ZAP70 and IgVH mutation in our study underscores its importance as a novel prognostic marker.

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An unexpected finding was that p53(+) patients who entered complete remission had favorable outcomes similar to their p53(-) counterparts, with no relapses or deaths in 7 p53(+) complete responders at up to five years of follow-up. None of these patients had chromosome 17 abnormalities by classical cytogenetic studies, which may have accounted for the relatively benign behaviour of their CLL. It has been established that the actual discovery of an abnormal karyotype by classical cytogenetics confers a worse outcome in CLL (Wierda, et al 2007), due to the nature of the test which depends upon generation of metaphases through stimulation of mitotic activity; therefore, patients with more proliferative and aggressive disease clones may be more likely to yield an abnormal karyotype. Other studies have also shown that the complexity of the karyotype is predictive of poor survival in patients with CLL (Han, et al 1987), (Oscier, et al 1988), (Crossen 1997), (Dierlamm, et al 1997), (Juliussen and Merup 1998).

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In order to gain insight into whether the acquisition of aberrant p53 protein expression is an important mechanism of disease progression in patients with p53(-) disease at baseline, we evaluated p53 expression in p53(-) CLL patients who had relapsed from CR. As only one of 31 (3%) patients acquired p53 expression at relapse, the current data does not support the acquisition of aberrant p53 expression as a major pathway of disease progression in this population. Interestingly, the one patient who became p53 positive did not have deletion of 17p13 on concurrent FISH testing, suggesting that the mechanism of TP53 disruption was due to a point mutation rather than deletion of an entire allele.

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Other studies have evaluated the impact of deletions or mutations of the p53 axis on treatment outcome in chemotherapy-naïve patients. In the UK CLL-4 trial comparing chlorambucil, fludarabine and fludarabine & cyclophosphamide, patients with over 20% p53 deletion as defined by FISH had inferior response rate and overall survival (Catovsky, et al 2007). The finding of a “dose effect” in this trial mirrors our observation that 40% positivity by immunohistochemistry was a prognostically significant threshold. In contrast to our observation, in the ECOG 2997 trial comparing F and FC (Grever, et al 2007), patients with 17p deletion did not have an inferior response rate compared with the non-17p deleted patients, although their progression-free survival was shorter. The complete response rates of F (5%) and FC (25%) in this trial were however substantially lower than that of FCR (72%), and the inferior response of patients with TP53 deletion may therefore not have been statistically apparent due to the low CR rates overall. This study also evaluated the impact of TP53 mutations as detected by denaturing gradient gel electrophoresis with confirmation by automated sequencing, and found no impact of TP53 mutations on response or progression-free survival. It is not clear however if all of the TP53 mutations found were pathogenic, and it is possible some of the apparent mutations were silent functionally. In contrast, using immunohistochemical techniques, the accumulation of p53 protein is always considered to be aberrant and IHC may therefore be a practical surrogate for TP53 mutation screening in the clinic.

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The FCR regimen is the most potent treatment protocol for patients with CLL, and sets a new benchmark for the initial therapy of symptomatic patients (Tam, et al 2008b). In this context, the finding that p53 IHC was strongly and independently prognostic in this population was important in confirming that TP53 disruptions remain relevant in the era of

chemoimmunotherapy. Our data demonstrate that p53 immunohistochemistry (or an equivalent method of screening for TP53 mutations) should form a part of comprehensive risk-assessment in patients with CLL.

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Figure 1.
p53 (+) CLL case (400×)

Figure 2.

Curves obtained when using different cut-off values for p53 expression. A significant difference in overall survival and complete remission is seen when 40% of CLL cells express p53, while lower values show no difference.

Figure 3.
Time to Progression in p53(+) CLL group

Figure 4.
Overall Survival in 222 CLL patients by p53 expression

Table 1

Patient characteristics

CHARACTERISTIC		p53(+) CLL (n=21)	p53(-) CLL (n=201)	p- value
Age		57 (39 – 86)	57 (24 – 86)	ns
Age > 70 years		2(10)	31 (15)	ns
Male Gender		141 (70%)	16 (76%)	ns
Performance Status >1		14(67)	124 (62)	ns
Rai Stage	Stage 0	0 (0%)	10(5%)	
	Stage I–II	11 (52%)	120(60%)	
	Stage III–IV	10 (48%)	71(35%)	ns
Hepatomegaly		5 (24)	42(21)	ns
Splenomegaly		14 (67)	106 (53)	ns
Hemoglobin		12.1 (8.9 – 15.7)	12.5 (6.1 – 18.7)	ns
White Cell Count		81.1 (13 – 552)	81.6 (4 – 408)	ns
Platelets		145 (36 – 226)	156 (8 – 406)	ns
β2-microglobulin		4.3 (2.3 – 7.4)	3.6 (1.6 – 16.4)	0.05
LDH		692 (449–1828)	536 (103–1722)	0.002
Abnormal Karyotype by classical cytogenetics		10/16 (63)	36/152 (24)	0.002
Chromosome 17 abnormalities		3/16 (19%)	1/152 (0.7%)	0.003
ZAP70 expression		13/21 (62%)	68/172 (40%)	0.08
IgVH MS	Unmutated	4 (50%)	67 (67%)	ns
	Mutated	4(50%)	33 (33%)	

Table 2

Response and remission based on p53 expression

	p53(+) CLL (n=21)	p53(-) CLL (n=201)	p-value
CR	7 (33%)	153 (76%)	<0.001
NPR	2 (10%)	17 (8%)	NS
PR	6 (29%) *	24 (12%)	0.045
No response to therapy	6 (29%) *	5 (2%)	<0.0001
Early Death	0	2	NS
5 year CR-TTP	100%	76%	0.24
5 year OS	48%	82%	<0.0001

*
2 PR and 1 NR patients had chromosome 17 abnormalities