bjh research paper

p53 Aberrations do not predict individual response to fludarabine in patients with B-cell chronic lymphocytic leukaemia in advanced stages Rai III/IV

Mikel Valgañón,[1] Pilar Giraldo,[2] Xabier Agirre,[3] María J. Larráyoz,[1] Araceli Rubio-Martinez,[2] Daniel Rubio-Felix,[2] María J. Calasanz[1] and María D. Odero[1,3]

[1]Department of Genetics, University of Navarra, Pamplona, [2]Fundación Estudio Hematología y Hemoterapia de Aragón (FEHHA), Zaragoza, and [3]Division of Oncology, School of Medicine, University of Navarra and Foundation for Applied Medical Research, Pamplona, Spain

Received 1 December 2004; accepted for publication 19 January 2005

Correspondence: María D. Odero, MD, PhD, Department of Genetics, University of Navarra, Irunlarrea 1, 31008-Pamplona, Spain.
E-mail: modero@unav.es

Summary

Abnormalities of *p53* have been associated with short survival and non-response to therapy in chronic lymphocytic leukaemia (CLL). We have evaluated the rate of response to fludarabine as first-line therapy in 54 patients with advanced stage CLL, analysing the cytogenetic profile, aberrations in *p53*, including the methylation status of its promoter, and the immunoglobulin heavy-chain variable-region (*IGVH*) mutation status. According to the advanced stage of the disease in this series, 75% of patients presented genetic aberrations associated with poor prognosis: del(17p) and/or del(11q), and no-mutated *IGVH* genes. Ten patients (18·5%) had methylation in the promoter region of p53. Eighty-three per cent of patients treated achieved a response, with a high rate of complete remission (47·6%). Although we found a significant correlation between failures and the presence of *p53* aberrations ($P = 0{\cdot}0065$), either with methylation ($P = 0{\cdot}018$) or deletion ($P = 0{\cdot}015$), 64% of the patients with aberrations in this gene responded to treatment (11/17), suggesting that fludarabine induces high remission rates, even in these patients. This is the first time that the significance of p53 promoter methylation status is described in this pathology, and our data support that this epigenetic phenomenon could be involved in the pathogenesis and clinical evolution of CLL.

Keywords: chronic lymphocytic leukaemia, fludarabine, *p53*, methylation.

In recent years, genetic factors have been established as important predictors of disease progression and survival in patients with chronic lymphocytic leukaemia (CLL), and they have implications for the risk-adapted clinical management of CLL, particularly in younger patients (Dohner *et al*, 2000; Krober *et al*, 2002; Lin *et al*, 2002; Stilgenbauer *et al*, 2002; Shanafelt *et al*, 2004). These biologic markers are currently being incorporated into clinical use as adjuncts to the classical staging systems. Genetic aberrations that modify the expression of *p53* (deletions and mutations) are independent poor prognostic factors in CLL (Stilgenbauer *et al*, 2002). Growing evidence has implicated aberrant promoter methylation in the molecular pathogenesis of several human cancers (Esteller *et al*, 2001). *In vitro* studies have demonstrated the expected correlation between hypermethylation in the promoter region of *p53* and a decrease in the transcription of this gene (Mass & Wang, 1997; Schroeder & Mass, 1997; Pogribny *et al*, 2000; Pogribny & James, 2002; Agirre *et al*, 2003a), however, only a few studies have been carried out in human malignancies. To date, the role of aberrant promoter methylation in B-CLL has not been investigated exhaustively, and widespread studies have been mainly restricted to the p15 and p16 genes (Baur *et al*, 1999; Rossi *et al*, 2004).

In many countries fludarabine is an approved first-line therapy treatment for CLL, which achieves superior remission rates when compared with traditional first-line therapies, such as the chlorambucil and anthracyline-based regimens. The drug is equally effective in early and advanced disease, and in younger and older patients with CLL (Keating *et al*, 1998; Rai *et al*, 2000; Leporrier *et al*, 2001; O'Brien *et al*, 2001; Leporrier, 2004; Montserrat, 2004). The presence of either mutations or deletions of the *p53* gene has been reported to predict

 doi:10.1111/j.1365-2141.2005.05405.x

non-response to therapy with purine analogues in two independent series of CLL patients. el Rouby *et al* (1993) analysed 53 B-CLL patients and found eight with *p53* mutations. Only two patients (2/53) were treated with fludarabine as first-line therapy, one had *p53* mutations and did not respond and the other, with no *p53* mutations, responded to treatment. In a retrospective study, Dohner *et al* (1995) analysed the presence of 17p deletions in 90 patients with CLL, and none of the 11 patients with del(17p) responded to second line therapy with fludarabine. They found that *p53* gene deletion was the strongest prognostic factor for survival after therapy. On the other hand, the clinical trials using fludarabine as first-line therapy treatment for CLL, do not include genetic analysis of *p53*. Thus, although *p53* mutations and/or deletions are widely associated with resistance to chemotherapy and short survival in CLL, there are few studies evaluating the association of *p53* aberrations and treatment with purine analogues, and none with fludarabine as first-line therapy. Indeed, in recent years, several reports have described the modest contribution of *p53* to the nucleoside-induced killing of CLL cells (Pettitt *et al*, 1999a, 2001), supporting the use of nucleoside therapy in patients with *p53* defects. Therefore, the efficacy of fludarabine as first-line therapy in patients with abnormalities on *p53* is still uncertain (Barnabas *et al*, 2001).

We have analysed the *p53* methylation status, cytogenetic aberrations with prognostic significance, including 17p deletions, and the presence of somatic hypermutation of immunoglobulin heavy-chain variable-region gene (*IGVH*), in patients with stage III/IV CLL, in order to assess the association of these parameters with rates of response to fludarabine as first-line therapy. We have confirmed in our series the high efficacy of fludarabine in CLL, even in patients with *p53* aberrations. The presence of abnormalities on *p53* was the only genetic factor related to non-response to fludarabine, confirming the importance of this event in CLL, but suggesting that *p53* aberrations are not specifically associated with non-response to purine analogue treatment.

Patients and methods

Patients

Patients were diagnosed with B-CLL based on standard morphologic and immunophenotypic criteria, and classified as high clinical risk category Rai stage III/IV. The median age at diagnosis was 68 years; the male to female ratio was 1·3. Table I summarizes the main clinical features of B-CLL patients.

Treatment was initiated for progressive disease in 42 patients with fludarabine as first-line therapy (25 mg/m^2/d for 5 d every 4 weeks). Criteria for progression and response were established using the Revised 1996 National Cancer Institute (NCI)-sponsored Working group Guidelines (Cheson *et al*, 1996) Samples were obtained after informed consent.

Table I. Clinical features of B-CLL patients at diagnosis.

Number of patients	54
Age (median/range)	68 (36–84)
Sex	
Male	31
Female	23
Rai stage	
III	29
IV	25
WBC (×10^9/l)	18187 (4·0–89800)
Haemoglobin (g/dl)	13·8 (8·5–17)
Platelets (×10^9/l)	257·0 (49–144)
Number of patients treated	
No	12
Yes	42
Number of cycles of therapy (mean/range)	6 (4–8)
Response	
Complete remission	20/42 (47·7%)
Partial remission	15/42 (35·8%)
Failure	7/42 (16·6%)

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) studies were performed using the probes LSI p53 (17p13), LSI D13S25 (13q14), and CEP 12 (Vysis, Downers, Grove, IL, USA). In order to detect aberrations of the *ATM* gene (11q22), we designed two BAC clones that cover the gene: RPCI-11 241D13 and RPCI-11 415G10 (http://genome.ucsc.edu and http://www.ncbi.nlm.nih.gov). FISH analysis was performed on fixed bone marrow (BM) 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated cultures as previously described (Odero *et al*, 2001).

Nucleic acid isolation

High molecular weight DNA and total RNA were extracted from mononuclear cells isolated on a Ficoll gradient (Lymphoprep™, Nycomed, Oslo, Norway) from peripheral blood (PB) and/or BM samples, using the QIamp® DNA mini-kit and the RNeasy® mini-kit (Qiagen, Hilden, Germany) respectively. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using SuperScript™ II Rnase H$^-$ *RT* (Invitrogen Life Technologies, Paisley, UK) with random hexamers. DNA from BAC and PAC clones was extracted using the QIAprep® Spin Miniprep kit (Qiagen).

Promoter methylation and mutational analysis of p53

A polymerase chain reaction (PCR) assay based on the inability of some restriction enzymes (HpaII, EcoRII) to digest methylated sequences, was used to analyse the methylation status of the *p53* promoter region as previously described (Agirre *et al*, 2003b). Positive results were considered after two independent digestions and two independent PCR. These assays detect methylation of the CpG dinucleotide located at position 883 of the *p53* promoter, and of the CC(A/T)GG sites located at

positions 756, 858 and 940 (Genbank accession no. X54156). Mutations of *p53* (5–8 exon) were studied with the SNPCaptureTM p53 Mutation Screening Kit (Panomics, Bayshore Road Redwood, CA, USA).

Mutation analysis of the IGVH genes

IGVH gene sequences were determined in patients' cDNA as previously described (Campbell *et al*, 1992). cDNA VH1 to VH6 gene family-specific PCR amplifications were performed using a mixture of 5′oligonucleotides specific for each leader sequence of the VH1 to VH6 *IGVH* gene families, together with 3′ primers complementary to the constant region (IgM or IgG). PCR products were purified with the Jetquick PCR purification spin kit (Genomed GmbH, Germany) or by gel excision using the Jetquick gel extraction spin kit (Genomed GmbH), and directly sequenced with the Abi-Prism^{TM} d-Rhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), in an Abi Prim^{TM} 377 DNA sequencer. Nucleotide sequences were aligned to publish germ line *IGVH* genes using the DNAPLOT (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) software and the V-BASE data library (http://www.mrc-cpe.cam.ac.uk/). Sequences with 2% or less deviation from any germline *IGVH* sequence were considered unmutated.

Statistical analysis

Association was studied using contingence tables, followed by Fisher exact test and mid-P. Mid-P is less conservative and more powerful than the Fisher exact test, and has some theoretical advantages (reviewed by Swinscow, 1997; Zojer *et al*, 2000) The Statistical Package for the Social Sciences (SPSS) macro was used to calculate a confidence interval for a difference between two proportions a/m and b/n based on unpaired data (independent groups), using an appropriate method (Zhu *et al*, 2001).

Results

Clinical evaluation

Genetic analysis of samples from 54 patients with CLL in stages III/IV at diagnosis was performed. Forty-two patients presented signs of progression and were treated. The response of these 42 patients was evaluated according to the modified NCI working group criteria for CLL (Cheson *et al*, 1996). Twenty patients (47·6%) achieved complete remission (CR), 15 (35·7%) partial remission (PR), and 7 (16·7%) were failures. Therefore, the overall response (OR) rate was 83·3%.

Fluorescence in situ hybridization

All 54 cases could be evaluated by interphase FISH cytogenetics; 44 (81%) had aberrations. Following the hierarchical model of chromosomal abnormalities proposed by Dohner *et al* (2000) 12 patients were classified in the del(17p) category, 13 del(11q), eight trisomy 12, nine normal, and 11 had del(13q) as the sole abnormality (Table II). One patient could not be included in any category because FISH analysis was normal but the G-banding karyotype was abnormal. All deletions were monoallelic, except for the 13q14 region: in two patients (2/33, 6%) there were concomitant monoallelic and biallelic deletions. In 22 patients (67%) del(13q) was accompanied by other aberrations, the most frequent being 11q deletions (15 cases).

Methylation status of the p53 promoter

We detected methylation of either one CpG dinucleotide or the three CC(A/T)GG motifs in the promoter region of *p53* in 10 patients (10/54, 18·5%). Three cases showed methylation in all cytosines analysed, three samples were only methylated on the CC(A/T)GG motifs, and four samples were only methylated in the CpG dinucleotide (Fig 1). Of the 10 patients, 3 (30%) also presented *p53* deletion. The mutation analysis of patients with *p53* methylation or deletion showed that only one deleted case also presented a nucleotide substitution, located at position 179 in exon 5 of the *p53* gene (Fig 2). Therefore, 35% of our patients presented *p53* aberrations, and del(17p) and methylation of the *p53* promoter, seemed to be independent factors (Table III). When we analysed the concomitant presence of *p53* aberrations and other genetic events, we only found a significant difference in the distribution of *p53* aberrations in patients with del(13q) ($P = 0{\cdot}0331$).

Mutational status of the IGVH genes

Forty-four patients could be analysed for the mutational status of the *IGVH* gene. Somatic mutations of the *IGVH* genes (<98% homology with the germ-line sequences) was found in 42% patients (18/43, including six with 95–96·3% homology), and 58% (25/43) presented >98% homology. We only found a significant correlation between presence of del(11q) and no mutated *IGVH* genes ($P = 0{\cdot}0194$) (Table IV).

Table II. Incidence of chromosomal abnormalities detected by FISH in 54 patients with CLL in advanced stages.

Chromosomal abnormality	Global distribution (%)	Hierarchical categories (proposed by Dohner *et al*, 2000) (%)
11q deletion	18 (33)	13 (24)
12 trisomy	11 (20)	8 (15)
13q deletion	33 (61)	11 (20)
17p deletion	12 (22)	12 (22)
Normal FISH	10 (18)	9 (17)

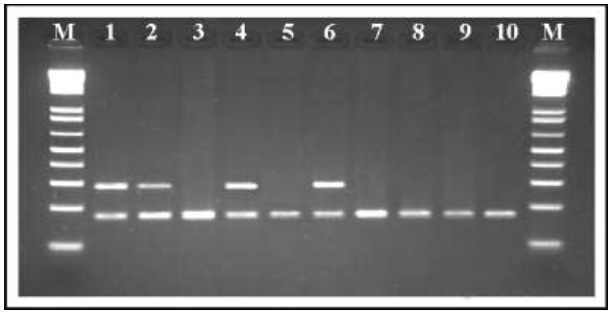

Fig 1. Methylation assay of non-methylated and methylated CLL patients. The lower band corresponds to *p53* exon 6 (used as internal control) and the upper band corresponds to the promoter region that encompasses the restriction sites analysed. Lane 1: PCR for patient sample without restriction enzyme added to the digestion reaction; lanes 2–5: PCR for patient sample after digestion with HpaII (lane 2), MspI (lane 3), EcoRII (lane 4), BstNI (lane 5). Lane 6: undigested control for the normal sample (no restriction enzyme added to the digestion reaction); lanes 7–10: PCR for the normal sample after digestion with HpaII (lane 7), MspI (lane 8), EcoRII (lane 9), BstNI (lane 10). Lanes M, molecular marker.

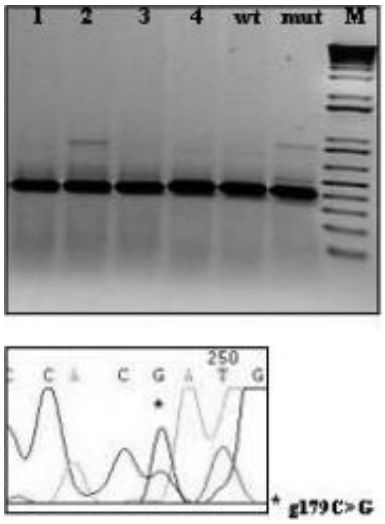

Fig 2. Mutation analysis of *p53* exon 5. (Top) Mutation analysis of four patients with CLL. Lanes 1, 3, 4: cases 3, 25 and 30, respectively, showing a normal pattern; Lane 2: case 23 showing an aberrant pattern, wt: normal DNA; mut: Mutant control, M: 1 Kb plus molecular weight marker. (B) Sequence analysis of case 23 confirming the mutation in this patient (g179C>G, wild type amino acid His, mutant amino acid Asp).

Correlation of genetic profiles with response rates

A significant correlation was found between non-response to treatment and the presence of *p53* aberrations ($P = 0{\cdot}0065$) (Table III). We found a significant correlation in both methylated ($P = 0{\cdot}018$) and deleted samples ($P = 0{\cdot}015$) when compared with non-clinical response. Age less than 60 years was also associated with non-response ($P = 0{\cdot}0090$).

Discussion

We analysed the presence of genetic aberrations with prognostic significance and their correlation with response rates to fludarabine as first-line therapy, in a series of 54 patients with CLL stages III/IV. Although we found a significant correlation between failures and the presence of p53 aberrations ($P = 0{\cdot}0065$), 64% of the patients with aberrations in this gene responded to treatment, suggesting that fludarabine induces high remission rates, even in these patients. This is the first time that *p53* aberrations, including the methylation status of its promoter, have been evaluated as markers for response to purine analogues in CLL.

We confirmed the high efficacy of fludarabine as first-line therapy in CLL. Our series had an OR of 83·3%, with 47·6% CR, which are similar to other extensive works in patients with advanced stages of CLL (Rai *et al*, 2000; Leporrier *et al*, 2001), however, neither of these works analysed the prognostic impact of genetic markers.

As expected, our patients with advanced CLL had a high percentage of genetic aberrations associated with poor prognosis. FISH high-risk categories del(17p) and del(11q) were present in 45% of the cases. Dohner *et al* (2000) found deletions on 17p13 in 18% of patients in III/IV stages, a result similar to the 22% reported in our series. This is consistent with the fact that *p53* deletion is usually considered as a marker of progression, related with advanced stages of the disease. Only 20% of our patients had del(13q) as a sole abnormality, which is considered a good prognostic factor. Interestingly, we found correlation between the presence of del(13q) and *ATM* deletions ($P = 0{\cdot}0138$), according to the hypothesis that *ATM* deletion is a secondary event in the clone with del(13q) (Dohner *et al*, 2000; Cuneo *et al*, 2002; Krober *et al*, 2002).

In recent years, the presence of *p53* aberrations has been confirmed as an independent poor prognostic factor in CLL (Gaidano *et al*, 1991; Fenaux *et al*, 1992; el Rouby *et al*, 1993; Gaidano *et al*, 1994; Dohner *et al*, 1995) and, more recently, several works have reported the prognostic and therapeutic value of the mutational status of the *IGVH* genes in individual patients (Damle *et al*, 1999; Hamblin *et al*, 1999). In fact, a hierarchical risk profile based on a combination of high-risk chromosomal abnormalities (17p- or 11q-) and IgVH mutational status has been proposed (Krober *et al*, 2002). In concordance with advanced stages of the disease, cases with no hypermutation in the *IGVH* genes were frequently observed in our series (58%), although we did not find the expected association between this variable and *p53* aberrations (Lin *et al*, 2002; Oscier *et al*, 2002). As previously described, no hypermutation (Krober *et al*, 2002) was related to *ATM* deletions ($P = 0{\cdot}0194$).

We have confirmed for the first time in a homogeneous series of advanced stage CLL patients that *p53* aberrations are associated with non-response to fludarabine ($P = 0{\cdot}0065$), although our data indicate that this cannot be considered as an individual marker of response. We found a high rate of

Table III. Univariate analysis of variables related to response to treatment with fludarabine in 42 CLL patients.

	Response	Non-response	Fisher exact test mid-P	95% confidence interval
Age (years)				
<60	4	4	0·0090*	−41·18 (−70·23; −9·38)
>60	31	3		
Sex				
Male	19	6	0·1546	18·12 (−6·41; 38·14)
Female	16	1		
Hierarchical FISH aberrations				
del(17p)				
Yes	5	4	0·0153*	35·35 (5·97; 64·85)
No	30	3		
del(11q)				
Yes	9	3	0·2755	14·37 (−9·33; 44·67)
No	26	4		
Trisomy 12				
Yes	6	0	0·4121	−19·44 (−35·03; 20·77)
No	29	7		
del(13q)				
Yes	8	1	0·8006	−7·07 (−25·67; 26·70)
No	27	6		
Methylation *TP53*				
Yes	7	3	0·2482	17·50 (−7·23; 48·74)
No	28	4		
Methylation *TP53* and no del(17p)				
Yes	1	3	0·018*	57·76 (9·61,80·36)
No	24	5		
TP53 aberrations				
Yes	11	6	0·0065*	31·29 (7·52; 54·93)
No	24	1		
IGVH status				
Mutated	14	1	0·4561	−9·12 (−31·58; 16·20)
Non-mutated	16	3		

Table IV. Incidence of genomic abnormalities according to the *IGVH* gene mutational status.

Categories	Mutated, 18 (43%)	Non-mutated, 25 (57%)	Mid-P
Sex (M/F)	8/10	16/9	
Age (years)	68·53	68·72	
FISH			
Normal	3	4	0·8401
del(17)(p13)	5	5	0·5989
del(11)(q22)	3	13	0·0194
Trisomy 12	5	5	0·5989
del(13)(q14)	11	16	0·6475
Methylation (yes)	4	5	0·8800

response even in patients with *p53* aberrations, in contrast to previously published works that associated the presence of *p53* aberrations with non-response to fludarabine (el Rouby *et al*, 1993; Dohner *et al*, 1995). 40% of our patients treated (17/42) presented *p53* aberrations and 11 responded to treatment. Interestingly, we found 18·5% of the patients had methylation in at least one of the four regions studied. All these patients needed to be treated, suggesting the poor prognosis associated with this epigenetic aberration, although this did not reach statistical significance, probably because of the small number of patients. Deletions in *p53* and methylation of the *p53* promoter seem to be independent factors ($P = 0{\cdot}5516$). Although the role of *p53* in the pathogenesis of CLL is still unclear, the study of the integrity and functionality of the *p53* pathway is acquiring importance. Hypermethylation could explain disruption of the *p53* pathway through an alternative mechanism to deletion and mutation. The reduction of *p53* gene dosage without inactivating mutation has been shown to be sufficient to promote tumorigenesis in *p53* heterozygous mice (Venkatachalam *et al*, 1998), therefore, this could suggest that a reduction in the expression of *p53* caused by methylation of the promoter region might be sufficient to promote the selection and expansion of pretumoural cells. The contribution of *p53* to the nucleoside-induced killing of CLL cells is controversial. Our study would support the hypothesis of Pettitt *et al* (1999a,b), which suggests that the poor clinical response to nucleoside therapy observed in CLL patients with

p53 abnormalities is unlikely to be due to nucleoside resistance at the time of diagnosis. The genomic instability associated with the *p53* dysfunction would facilitate the evolution during the course of the disease of CLL cell clones, which become resistant to nucleoside therapy for reasons not directly connected with *p53* (Pettitt *et al*, 1999a,b).

There are many questions regarding the pathogenesis of CLL that remain unanswered. Considering the heterogeneous evolution of CLL, the challenge is to continue to accurately identify the therapeutic strategy most likely to benefit individual patients. Cancer cells are exceptionally sensitive to reactivation of *p53* function and therefore, treatments that achieve this could be very effective (Lane, 2004). In conclusion, the presence of *p53* aberrations, including methylation of its promoter, was the only genetic factor predicting non-response to fludarabine as first-line therapy, confirming the importance of this event in CLL. However, a high percentage of patients with *p53* aberrations responded to fludarabine, suggesting that the poor clinical response to purine analogue therapy observed in CLL patients with *p53* abnormalities is unlikely to be because of resistance to this therapy at diagnosis. For individual risk-adapted therapy, global mechanism of *p53* inactivation should be evaluated in a larger group of patients.

Acknowledgements

This work has been partially supported by the Departamento de Educación del Gobierno de Navarra, and the RTIC Cancer C10/03 (FIS), Spain. We thank Marta Crespo for useful discussion, and Marta García-Granero for statistical support.

References

Agirre, X., Vizmanos, J.L., Calasanz, M.J., Garcia-Delgado, M., Larrayoz, M.J. & Novo, F.J. (2003a) Methylation of CpG dinucleotides and/or CCWGG motifs at the promoter of TP53 correlates with decreased gene expression in a subset of acute lymphoblastic leukemia patients. *Oncogene*, **22,** 1070–1072.

Agirre, X., Novo, F.J., Calasanz, M.J., Larrayoz, M.J., Lahortiga, I., Valganon, M., Garcia-Delgado, M. & Vizmanos, J.L. (2003b) TP53 is frequently altered by methylation, mutation, and/or deletion in acute lymphoblastic leukaemia. *Molecular Carcinogenesis*, **38,** 201–208.

Barnabas, N., Shurafa, M., Van Dyke, D.L., Wolman, S.R., Clark, D. & Worsham, M.J. (2001) Significance of p53 mutations in patients with chronic lymphocytic leukemia: a sequential study of 30 patients. *Cancer*, **91,** 285–293.

Baur, A.S., Shaw, P., Burri, N., Delacretaz, F., Bosman, F.T. & Chaubert, P. (1999) Frequent methylation silencing of p15 (INK4b) (MTS2) and p16 (INK4a) (MTS1) in B-cell and T-cell lymphomas. *Blood*, **94,** 1773–1781.

Campbell, M.J., Zelenetz, A.D., Levy, S. & Levy, R. (1992) Use of family specific leader region primers for PCR amplification of the human heavy chain variable region gene repertoire. *Molecular Immunology*, **29,** 193–203.

Cheson, B.D., Bennett, J.M., Grever, M., Kay, N., Keating, M.J., O'Brien, S. & Rai, K.R. (1996) National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood*, **87,** 4990–4997.

Cuneo, A., Bigoni, R., Rigolin, G.M., Roberti, M.G., Bardi, A., Cavazzini, F., Milani, R., Minotto, C., Tieghi, A., Della Porta, M., Agostini, P., Tammiso, E., Negrini, M. & Castoldi, G. (2002) Late appearance of the 11q22.3-23.1 deletion involving the ATM locus in B-cell chronic lymphocytic leukemia and related disorders. Clinicobiological significance. *Haematologica*, **87,** 44–51.

Damle, R.N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L., Buchbinder, A., Budman, D., Dittmar, K., Kolitz, J., Lichtman, S.M., Schulman, P., Vinciguerra, V.P., Rai, K.R., Ferrarini, M. & Chiorazzi, N. (1999) Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*, **94,** 1840–1847.

Dohner, H., Fischer, K., Bentz, M., Hansen, K., Benner, A., Cabot, G., Diehl, D., Schlenk, R., Coy, J. & Stilgenbauer, S. (1995) p53 gene deletion predicts for poor survival and non-response to therapy with purine analogs in chronic B-cell leukemias. *Blood*, **85,** 1580–1589.

Dohner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Krober, A., Bullinger, L., Dohner, K., Bentz, M. & Lichter, P. (2000) Genomic aberrations and survival in chronic lymphocytic leukemia. *New England Journal of Medicine*, **343,** 1910–1916.

Esteller, M., Corn, P.G., Baylin, S.B. & Herman, J.G. (2001) A gene hypermethylation profile of human cancer. *Cancer Research*, **61,** 3225–3229.

Fenaux, P., Preudhomme, C., Lai, J.L., Quiquandon, I., Jonveaux, P., Vanrumbeke, M., Sartiaux, C., Morel, P., Loucheux-Lefebvre, M.H. & Bauters, F. (1992) Mutations of the p53 gene in B-cell chronic lymphocytic leukemia: a report on 39 cases with cytogenetic analysis. *Leukemia*, **6,** 246–250.

Gaidano, G., Ballerini, P., Gong, J.Z., Inghirami, G., Neri, A., Newcomb, E.W., Magrath, I.T., Knowles, D.M. & Dalla-Favera, R. (1991) p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, **88,** 5413–5417.

Gaidano, G., Newcomb, E.W., Gong, J.Z., Tassi, V., Neri, A., Cortelezzi, A., Calori, R., Baldini, L. & Dalla-Favera, R. (1994) Analysis of alterations of oncogenes and tumor suppressor genes in chronic lymphocytic leukemia. *American Journal of Pathology*, **144,** 1312–1319.

Hamblin, T.J., Davis, Z., Gardiner, A., Oscier, D.G. & Stevenson, F.K. (1999) Unmutated Ig V (H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*, **94,** 1848–1854.

Keating, M.J., O'Brien, S., Lerner, S., Koller, C., Beran, M., Robertson, L.E., Freireich, E.J., Estey, E. & Kantarjian, H. (1998) Long-term follow-up of patients with chronic lymphocytic leukemia (CLL) receiving fludarabine regimens as initial therapy. *Blood*, **92,** 1165–1171.

Krober, A., Seiler, T., Benner, A., Bullinger, L., Bruckle, E., Lichter, P., Dohner, H. & Stilgenbauer, S. (2002) V (H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. *Blood*, **100,** 1410–1416.

Lane, D. (2004) p53 from pathway to therapy. *Carcinogenesis*, **25,** 1077–1081.

Leporrier, M. (2004) Role of fludarabine as monotherapy in the treatment of chronic lymphocytic leukemia. *Hematology Journal*, **5**(suppl. 1), S10–S19.

Leporrier, M., Chevret, S., Cazin, B., Boudjerra, N., Feugier, P., Desablens, B., Rapp, M.J., Jaubert, J., Autrand, C., Divine, M., Dreyfus, B., Maloum, K., Travade, P., Dighiero, G., Binet, J.L. & Chastang, C. (2001) Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. *Blood*, **98,** 2319–2325.

Lin, K., Sherrington, P.D., Dennis, M., Matrai, Z., Cawley, J.C. & Pettitt, A.R. (2002) Relationship between p53 dysfunction, CD38 expression, and IgV (H) mutation in chronic lymphocytic leukemia. *Blood*, **100,** 1404–1409.

Mass, M.J. & Wang, L. (1997) Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis. *Mutation Research*, **386,** 263–277.

Montserrat, E. (2004) Treatment options in chronic lymphocytic leukemia. *Hematology Journal*, **5**(suppl. 1), S2–S9.

O'Brien, S.M., Kantarjian, H.M., Cortes, J., Beran, M., Koller, C.A., Giles, F.J., Lerner, S. & Keating, M. (2001) Results of the fludarabine and cyclophosphamide combination regimen in chronic lymphocytic leukemia. *Journal of Clinical Oncology*, **19,** 1414–1420.

Odero, M.D., Carlson, K., Calasanz, M.J., Lahortiga, I., Chinwalla, V. & Rowley, J.D. (2001) Identification of new translocations involving ETV6 in hematologic malignancies by fluorescence in situ hybridization and spectral karyotyping. *Genes, Chromosomes and Cancer*, **31,** 134–142.

Oscier, D.G., Gardiner, A.C., Mould, S.J., Glide, S., Davis, Z.A., Ibbotson, R.E., Corcoran, M.M., Chapman, R.M., Thomas, P.W., Copplestone, J.A., Orchard, J.A. & Hamblin, T.J. (2002) Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood*, **100,** 1177–1184.

Pettitt, A.R., Sherrington, P.D. & Cawley, J.C. (1999a) The effect of p53 dysfunction on purine analogue cytotoxicity in chronic lymphocytic leukaemia. *British Journal of Haematology*, **106,** 1049–1051.

Pettitt, A.R., Clarke, A.R., Cawley, J.C. & Griffiths, S.D. (1999b) Purine analogues kill resting lymphocytes by p53-dependent and -independent mechanisms. *British Journal of Haematology*, **105,** 986–988.

Pettitt, A.R., Sherrington, P.D., Stewart, G., Cawley, J.C., Taylor, A.M. & Stankovic, T. (2001) p53 dysfunction in B-cell chronic lymphocytic leukemia: inactivation of ATM as an alternative to TP53 mutation. *Blood*, **98,** 814–822.

Pogribny, I.P. & James, S.J. (2002) Reduction of p53 gene expression in human primary hepatocellular carcinoma is associated with promoter region methylation without coding region mutation. *Cancer Letters*, **176,** 169–174.

Pogribny, I.P., Pogribna, M., Christman, J.K. & James, S.J. (2000) Single-site methylation within the p53 promoter region reduces gene expression in a reporter gene construct: possible in vivo relevance during tumorigenesis. *Cancer Research*, **60,** 588–594.

Rai, K.R., Peterson, B.L., Appelbaum, F.R., Kolitz, J., Elias, L., Shepherd, L., Hines, J., Threatte, G.A., Larson, R.A., Cheson, B.D. & Schiffer, C.A. (2000) Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. *New England Journal of Medicine*, **343,** 1750–1757.

Rossi, D., Capello, D., Gloghini, A., Franceschetti, S., Paulli, M., Bhatia, K., Saglio, G., Vitolo, U., Pileri, S.A., Esteller, M., Carbone, A. & Gaidano, G. (2004) Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. *Haematologica*, **89,** 154–164.

el Rouby, S., Thomas, A., Costin, D., Rosenberg, C.R., Potmesil, M., Silber, R. & Newcomb, E.W. (1993) p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood*, **82,** 3452–3459.

Schroeder, M. & Mass, M.J. (1997) CpG methylation inactivates the transcriptional activity of the promoter of the human p53 tumor suppressor gene. *Biochemical and Biophysical Research Communications*, **235,** 403–406.

Shanafelt, T.D., Geyer, S.M. & Kay, N.E. (2004) Prognosis at diagnosis: integrating molecular biologic insights into clinical practice for patients with CLL. *Blood*, **103,** 1202–1210.

Stilgenbauer, S., Bullinger, L., Lichter, P. & Dohner, H. (2002) Genetics of chronic lymphocytic leukemia: genomic aberrations and V (H) gene mutation status in pathogenesis and clinical course. *Leukemia*, **16,** 993–1007.

Swinscow, T.D.V. (1997) *Statistics at Square One*. BMJ Publishing Group, London.

Venkatachalam, S., Shi, Y.P., Jones, S.N., Vogel, H., Bradley, A., Pinkel, D. & Donehower, L.A. (1998) Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO Journal*, **17,** 4657–4667.

Zhu, Y., Loukola, A., Monni, O., Kuokkanen, K., Franssila, K., Elonen, E., Vilpo, J., Joensuu, H., Kere, J., Aaltonen, L. & Knuutila, S. (2001) Ppp2r1b gene in chronic lymphocytic leukemias and mantle cell lymphomas. *Leukemia and Lymphoma*, **41,** 177–183.

Zojer, N., Konigsberg, R., Ackermann, J., Fritz, E., Dallinger, S., Kromer, E., Kaufmann, H., Riedl, L., Gisslinger, H., Schreiber, S., Heinz, R., Ludwig, H., Huber, H. & Drach, J. (2000) Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. Multicenter Investigation of Bone Marrow Transplantation for Sickle Cell Disease. *Blood*, **95,** 1925–1930.