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ORIGINAL ARTICLE

TP53 mutation profile in chronic lymphocytic leukemia: evidence for a disease specific profile from a comprehensive analysis of 268 mutations

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The *TP53* mutation profile in chronic lymphocytic leukemia (CLL) and the correlation of *TP53* mutations with allele status or associated molecular genetics are currently unknown. We performed a large mutation analysis of *TP53* at four centers and characterized the pattern of *TP53* mutations in CLL. We report on 268 mutations in 254 patients with CLL. Missense mutations appeared in 74% of cases compared with deletions and insertions (20%), nonsense (4%) and splice site (2%) mutations. The majority (243 of 268) of mutations were located in the DNA-binding domain. Transitions were found in 131 of 268 mutations, with only 41 occurring at methylated CpG sites (15%), suggesting that transitions at CpGs are uncommon. The codons most frequently mutated were at positions 175, 179, 248 and 273; in addition, we detected a common 2-nt deletion in the codon 209. Most mutations (199 of 259) were accompanied by deletion of the other allele (17p–). Interestingly, trisomy 12 (without 17p–) was only found in one of 60 cases with *TP53* mutation (without 17p–) compared with 60 of 16 in the cohort without mutation ($P = 0.006$). The mutational profile was not different in the cohorts with and without previous therapy, suggesting that the mechanism underlying the development of mutations may be similar, independent of treatment.
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Introduction

p53 plays a central role in multiple essential functions of the cell including cell cycle arrest, apoptosis and senescence. It is through these functions that p53 mediates its tumor suppressor activity by inducing or repressing a multitude of p53 target genes. Its importance in carcinogenesis is easily appreciated simply based on the fact that *TP53* is the gene most frequently mutated in cancer. The current release (R14) of the *TP53* mutation database of the IARC (International Agency for Research on Cancer) and UMD (Universal mutation database) (http://p53.free.fr/) holds almost 29 000 somatic mutations. The mutations are generally located in the DNA-binding domain and different types of hot spot mutations have been described. Contact mutations remove essential DNA-contact sites (R248Q, R248W, R273H and R273C), whereas structural mutations affect residues that are essential for the overall architecture (R175H, G245S, R249S and R282W).[1]

Different cancers have been found to exhibit disease-specific mutation profiles, which sometimes help to gain clues on the carcinogenesis or progression.[2,3] In addition, *TP53* mutations have been associated with poor prognosis in numerous cancers including lymphomas and chronic lymphocytic leukemia (CLL).[4–8]

Mutations of *TP53* are found in 4 to 37% of patients with CLL, and unselected cohorts of untreated patients can be expected to show TP53 mutations in the order of 10%.[4–10] The highest incidence of *TP53* mutation is seen in patients with fludarabine refractory CLL and much of the heterogeneity in mutation prevalence is explained by different patient cohorts.[7] The presence of mutations in *TP53* has been associated with poor prognosis in a number of retrospective studies, but the association of 17p deletion and *TP53* mutation has led to the pooling of mutations with 17p deletion (usually the majority) and cases without 17p deletion. Therefore, the exact prognostic relevance of *TP53* mutations (alone and in relation to 17p deletion) had not been documented until very recently. In more recent studies, a comprehensive molecular genetic characterization with mature follow-up including fluorescence *in situ* hybridization and immunoglobulin heavy chain variable region (IGHV) mutation status defined the clinical impact of *TP53* mutations in CLL (4–6).[8]

Because of the relatively low frequency of *TP53* mutations, few studies to date have assessed the particular mutation profile in CLL. The studies that have been reported included 13−46 patients with mutations and therefore these results are subject to bias.[11–14] In addition, detailed analysis of clinical or genetic characteristics was not possible and is also not possible from an analysis of the databases.

In order to establish a precise description of *TP53* mutations in CLL, with particular emphasis on the genetic background and the relation to previous therapy, we have compiled a large set of *TP53* mutations from CLL samples from different centers in Europe. We compared this profile with the currently available data from the *TP53* mutation databases and also included a matching control CLL cohort lacking *TP53* mutations to search for differences in the genetic profile.

Materials and methods

Patient cohort

In our effort to compile a large set of patients with *TP53* mutations, we combined four cohorts where mutation analysis

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was available. The first cohort consisted of patients analyzed at the University of Ulm using denaturing high-performance liquid chromatography (DHPLC) and sequencing ($n=128$ with 134 mutations (single-center cases, trial cohorts CLL4, CLL2H))[4] and a p53 resequencing CHIP (Roche Molecular Systems, Pleasanton, CA, USA; $n=26$ with mutation; exons 2–11). The cohort was formed by patients from different clinical studies and also included selected cases with 17p deletion. The second cohort was from the University of Liverpool, which was tested in Ulm using DHPLC/sequencing ($n=14$ with 17 mutations (out of 104 samples); exons 4–10). The third cohort was from Brno and this set was investigated by functional analysis of separated alleles in yeasts (FASAY) and sequencing ($n=58$ with 63 mutations; codons 67–346).[15] Finally, cases from Bournemouth were included, which were analyzed by sequencing ($n=28$ with mutations; 5–9). The control cohort consisted of 463 patients, who were shown to not have a *TP53* mutation using DHPLC from Ulm.

Mutation detection

Genetic analyses. Fluorescence *in situ* hybridization analysis and IGHV sequencing were performed in all cases as previously described.[16] To define the cutoff level for the presence of the *TP53* gene deletion, hybridization experiments of blood specimens from probands were performed. The cutoff level was defined by the mean plus three s.d. of the frequency of control cells exhibiting only one *TP53* signal. A germ line homology of 98% was used as the cutoff between IGHV mutated and IGHV unmutated cases. The genetic characterization was performed at each center separately.

TP53 *sequencing analysis.* We analyzed all samples by automated fluorescent sequencing using Big Dye Terminator Kit and ABI 3100 sequencer (Applied Biosystems, Carlsbad, CA, USA; exons 2–11 and 4–10, respectively). The primer sequences are available upon request. The primers were designed to cover all coding exons and intron–exon boundaries. Cases with 17p deletion where no mutation was identified by DHPLC were sequenced in all coding exons to confirm the absence of a mutation.

DHPLC. DHPLC was used to identify samples containing mutations in exons 2–11 (coding region of p53; $n=151$). In the cohort from Liverpool, analysis was confined to exons 4–10, because previous DHPLC analysis of samples from Ulm had shown the absence of mutations in exons 2, 3 and 11. DHPLC analysis is based on the temperature-dependent differences in column retention time of PCR products generated from homoduplex (wild-type) and heteroduplex (mutated) DNA, resulting in the presence of distorted or additional peaks when mutations are present. Details have been previously reported.[4,7] All mutations were confirmed by an independent PCR which was used for sequencing.

Amplichip resequencing

The AmpliChip p53 test assesses exons 2–11 of *TP53* and 2 bp of intronic sequence at the exon/intron boundaries. Exons are amplified in two multiplex PCR reactions from genomic DNA, fragmented, 3′-end labeled with a fluoresceinated dideoxynucleotide and hybridized to the AmpliChip surface. For each nucleotide position of *TP53*, specific oligonucleotides represent the potential mutations on the AmpliChip surface. The research assay was performed according to the instructions of the manufacturer (Roche Molecular Systems Inc.).

FASAY

In this functional assay, the *TP53* gene is introduced into yeast cells, where *TP53* wild-type samples form large white colonies, whereas the colonies with the *TP53* mutations are small and red (fully inactive mutants) or pink (partially inactive mutants). The FASAY was performed as described by Flaman *et al.*,[15] with some modifications published earlier.[17] The split assay is a modified version of FASAY, allowing separate analyses of the 5′ and 3′ regions of the p53 complementary DNA. The assay was performed when the PCR product for FASAY was too faint,[18] using the protocol described by Waridel *et al.*[19] The appropriate vectors and yeast strain ADE2$^-$ carrying a reporter with a p53-binding site upstream of the *ADE2* gene were kindly provided by R Iggo.

We noted that FASAY was not able to detect the hot spot deletion (2 nt) at position 209. Therefore, the deletion was detected in Czech cohort by high-resolution melting analysis of exon 6 performed on genomic DNA.

Databases and references

For comparison with the data generated in the current study, we compiled a cohort of *TP53* mutations as reported in different publications and the databases. To the number of 148 mutations taken from the IARC TP53 Mutation Database (version R12, November 2007) and the UMD_TP53 Mutation Database (2007_R1 release curated version), we added 38 mutations from five recently published studies not mentioned in the databases. The compiled cohort consisted of 186 *TP53* mutations in CLL.[11,20–23]

The CLL cohort without *TP53* mutations was from the same cohorts (Ulm: single center, first line treatment trial, refractory CLL trial). Cases with wild type were included in the matching control.

Meta-analysis of p53 mutant loss of function in tumors

All mutations were collected and analyzed using MUT-TP53, a spreadsheet that automatically assesses the biological activity and likelihood of each *p53* mutant.[24] Previous studies have shown that *TP53* mutations could be very heterogeneous depending on the strategy used for the analysis. The UMD TP53 database also includes functional data of the majority of missense mutants that allows comparison of each publication.[25] For functional analysis, the mean and 95% confidence interval of the remaining biological activity of all mutants were calculated using the residual transactivational activity for each mutant p53 towards waf-1 promoter. The reference value corresponds to the mean and 95% confidence interval of all studies for the specific cancer.[25] For CLL analysis, we used all publications describing more than five *TP53* mutations. For comparison of CLL results with other cancers, we downloaded the curated data set and again removed the known polymorphisms. The statistical analyses were performed with PRISM software (graphPad Software Inc.) on a Mac OS X platform.

All other statistical analyses were performed using the statistical software environment R, version 2.9.2 (R Development Core Team 2009; R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2009.). For the analysis of contingency tables, Fisher's exact test was applied. A result was denoted as statistically significant at a *P*-value $<5\%$. No *P*-value adjustment owing to multiple testing was performed.

Results

For the current analysis, we have compiled a collection of 268 mutations in 254 patients with CLL (Table 1). In accordance

Table 1 Cohort of CLL patients with *TP53* mutation

	All mutations (n = 268)	
	Counts	*%*
Mutation type		
Transition (all)	131	48.9
At CpG	41	15.3
Transversion (all)	84	31.3
At CpG	15	5.6
Missense	198	73.9
Splice site	6	2.2
Nonsense	11	4.1
Inframe/frameshift		
Deletion	43	16.0
Insertion	10	3.7
IGHV status		
Mutated	51	21.1
Unmutated	191	78.9
NA	26	
Hierarchical cytogenetics		
Del 17p	199	76.8
Del 11q	14	5.4
Trisomy 12	1	0.4
Normal	21	8.1
Del 13q	24	9.3
NA	9	
Treatment		
Previous treatment	105	42.5
No previous treatment	142	57.5
NA	21	
Codons		
132	5	1.9
158	4	1.5
175	7	2.6
179	9	3.4
195	5	1.9
209	12	4.4
220	7	2.6
234	4	1.5
236	3	1.1
245	6	2.2
248	12	4.5
273	11	4.1
281	7	2.6

Abbreviations: CLL, chronic lymphocytic leukemia; NA, not available.

with the profound biological impact of a single *TP53* mutation, multiple mutations are very rare in CLL (14 patients with two mutations). The mutation profile showed mainly missense mutations (74%) but also a comparatively high incidence of insertions (4%) or deletions (16%; Figure 1) The mutations were mainly located in the DNA-binding domain and 90% of the mutations were located in exons 5–8 (Figure 2). The fraction of frameshift mutations was significantly higher in the non-DNA-binding regions (11 of 21 vs 42 of 239; $P = 0.0007$; Figure 2).

As shown in Figure 3, transitions were the most common events accounting for 49% of all alterations. Interestingly, transitions at CpG were relatively rare (41 of 131 (31% of all transitions)) compared with some other more common cancers such as colon (1518 of 2197 (69%; Fisher's exact test, $P < 0.001$)), lymphoma (206 of 492 (42%) $P = 0.04$) or breast cancer (542 of 1187 (45%) $P = 0.002$). The overall pattern of mutations was also different in comparison with these cancers ($P = 0.002$ (breast); $P = 0.04$ (non-hodgkin lymphoma); $P < 0.001$ (colorectal)).

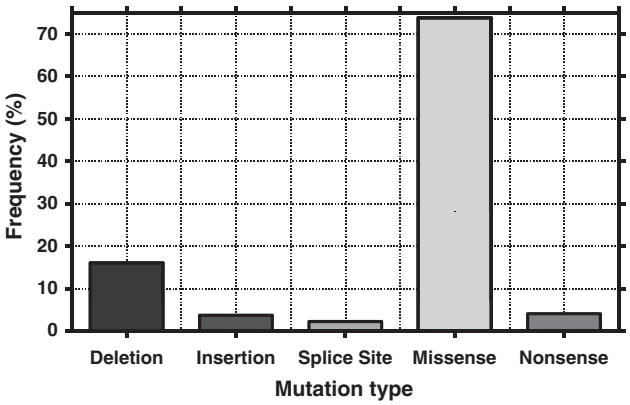

Figure 1 Mutation type profile in CLL ($n = 268$). The mutation profile of the cohort shows 73.9% missense, 4.1% nonsense and 2.2% splice site mutations (all single-base substitutions). The incidence of frameshift mutations was high with 16.0% deletions and 3.7% insertions.

The amino acids most frequently mutated were at positions 175, 179, 248 and 273. This indicates that the classical hot spots are also commonly mutated in CLL. Codons 175, 179, 220, 248, 273 and 281 made up for 53 of 268 mutations (20%). In addition, we identified one other commonly mutated codon at position 209 (in all instances 2-nt deletion). This alteration was—together with the known hot spot codons 248 and 273—the single most common mutation (Figure 4).

Comparison of DHPLC and FASAY cohort

We performed a detailed analysis of the results obtained by DHPLC and FASAY. Although both methods will have their respective shortcomings, DHPLC is generally considered an unbiased approach. In contrast, FASAY has a functional read out and can be expected to pick up only mutants affecting p53 transcriptional activity. In addition, it may not detect frameshift mutations if they lead to nonsense-mediated mRNA decay (see Material and methods section). Consequently, splice site mutations and nonsense mutations were rather rare in the FASAY cohort, as opposed to an incidence of 12 of 151 in the cases assayed by DHPLC. However, the overall mutation pattern of the FASAY cohort was not significantly different from that generated by DHPLC (data not shown).

In addition, an analysis of functional data was performed for each of the four sets of mutations described in Material and methods (Figure 5). The analysis shows that for all cancers, the mean activity was situated around −1.2 with a narrow 95% confidence interval, demonstrating an apparent homogeneity of p53 mutant activity for all of the mutations included in the database (Figure 5). This value corresponds to a residual transactivational activity of about 10% compared with wild-type p53. Comparison of the four sets of p53 mutations used in the present study shows that they display a homogeneous distribution with a 95% confidence interval, which includes the global mean value of CLL. Comparison with all publications describing *TP53* mutations in CLL shows that all but one study have a similar profile.

Comparison with published data/database

When we compared our data with what is available from different publications and databases, we observed a number of differences. We especially found a lower proportion of transitions at CpGs (41 of 131 transitions vs 47 of 87; $P = 0.001$)

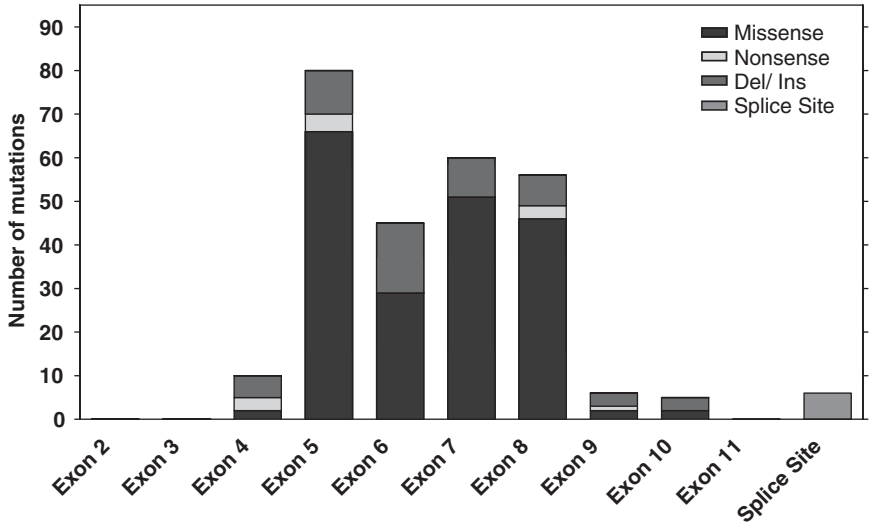

Figure 2 Exon distribution of *TP53* mutations in CLL. In the central core domain (exon 5–8), we found 89.9 of all mutations, mostly represented by missense mutations (80%). Outside this area (exon 2–4 and exon 9–11), nonsense (30 and 9.1%) and frameshift (50 and 54.5%) mutations were more frequent. del, deletion; ins, insertion.

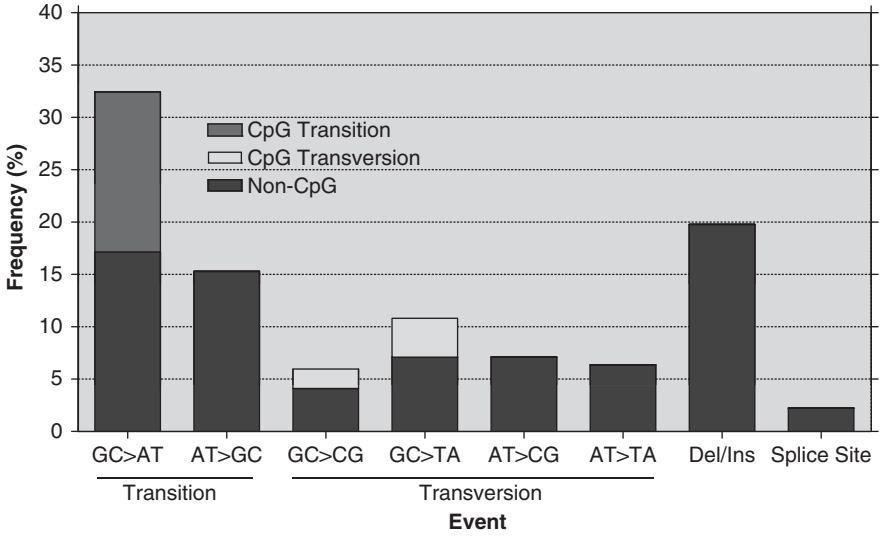

Figure 3 Mutational events in CLL. The most common events were transitions accounting for 48.9% of all events. Interestingly, transitions at CpG were relatively rare (41 of 131 (31% of all transitions)) compared with other more common cancers. In contrast, frameshift mutations are common in CLL. del, deletion; ins, insertion.

and fewer mutations at the hot spot codon 273 in our cohort (11 of 268 vs 20 of 186).

Comparison of the mutation profile in relation to the presence or absence of 17p deletion

In order to detect a potential difference in the *TP53* mutation profile of cases with and without 17p deletion, we studied the respective profiles (Supplementary Table 1). We found no difference in the proportion of transitions at CpGs in the two groups. There was an increased proportion of cases with insertions and deletions in the cohort with 17p deletion (23 vs 13%) and in turn a higher proportion of missense mutations in the cases without 17p– (82 vs 71%; Figure 6). A detailed comparison of mutational events showed no significant differences.

A comparison of the predicted residual activity of the mutated p53 towards a range of known targets showed no differences. In both groups, over 90% of all base substitutions were classified as non-functional. These findings suggest that the *TP53* mutations in CLL show a similar profile independent of the presence of 17p deletion.

Comparison of the mutation profile in relation to the presence or absence of previous therapy

TP53 mutations are more commonly observed with advanced disease and after previous therapy. Currently, we are lacking a precise picture of when these mutations occur during the disease and if the majority are selected or if *de novo* mutations occur more frequently after therapy. We therefore compared the mutation profile in our patients with ($n=105$) and without

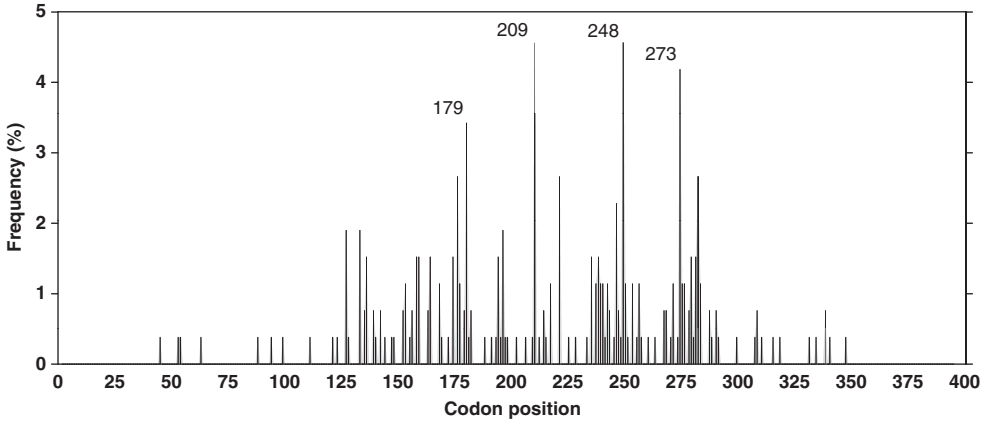

Figure 4 Codon distribution of *TP53* mutations in CLL. The amino acids most frequently mutated were at positions 175, 179, 209, 248 and 273. This indicates that the classical hot spots are also commonly mutated in CLL. Codons 175, 179, 220, 248, and 273, 281 made up for 53 of 268 of the mutations (20%), but we identified one other commonly mutated codon (209) that was—together with the known hot spot codon 248 and 273—the single most common mutation.

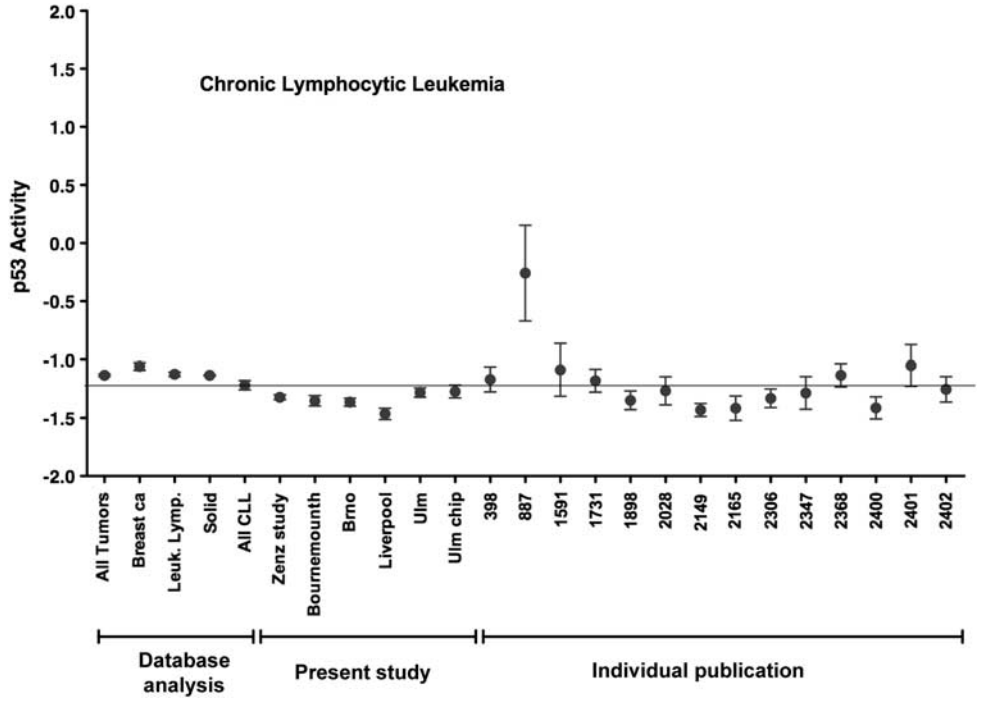

Figure 5 Analysis of p53 loss of function. Dot and bars; mean and 95% confidence interval (CI) of mean p53 activity as measured by transactivation with the waf promoter. The mean and 95% CI of p53 activity for all studies combined for a breast cancer and all cancer of the database is shown on the far left of the graph. The horizontal line shows the mean of the combined studies. The *y* axis corresponds to p53 transactivation activity, with a value of −1.5 for the negative control and a value of 2.5 for 100% of wild-type activity. All tumors: all tumors included in the database; breast ca: breast cancer; leuk lymp: all lymphoma and leukemia; solid: all non-hematological tumors; all CLL: all chronic lymphocytic leukemia; Zenz study: mutation from the present study; Bournemouth, Liverpool, Brno, Ulm and Ulm chip correspond to the various set of mutations described in Material and methods. Individual publication corresponds to each article describing *TP53* mutations in CLL.

($n=142$) treatment before mutation detection. The mutation profile and analysis of residual function showed no significant differences suggesting that the mechanisms underlying mutation acquisition are identical regardless of chemotherapy. Alternatively, one might conclude that mutations might be selected by chemotherapy rather than being caused by it.

Comparison of the genetic profile of CLL with and without TP53 *mutation*

When we assessed the cytogenetic profile (fluorescence *in situ* hybridization) of the cohort with *TP53* mutations, we found an overrepresentation of cases with 17p deletion (199 of 259 (77%) vs 25 of 441 in the control group (5%); $P<0.001$). Interestingly,

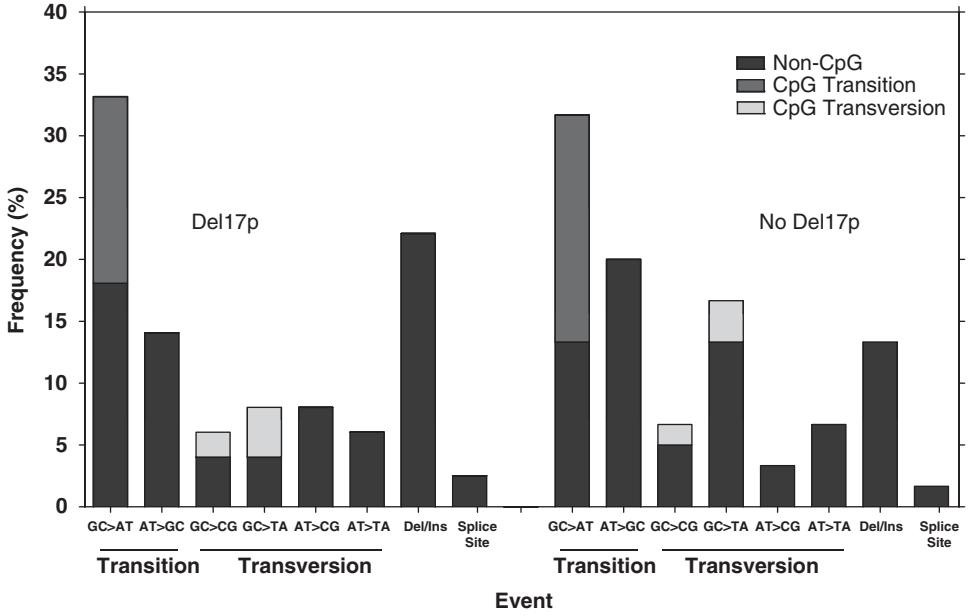

Figure 6 *TP53* mutation profile in CLL without ($n=60$) and with del17p ($n=199$). We found no difference in the proportion of transitions at CpGs in the two groups. There was an increased proportion of cases with frameshift mutations in the cohort with 17p deletion (22.6 vs 13.3%) and in turn a higher proportion of missense mutations in the cases without 17p– (81.7 vs 70.9%), without reaching statistical significance. del, deletion; ins, insertion.

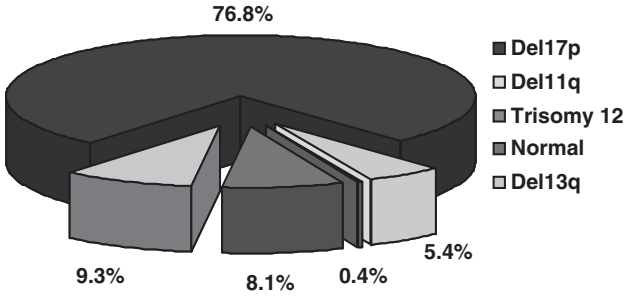

Figure 7 Hierarchical cytogenetics by fluorescence *in situ* hybridization in CLL with *TP53* mutation ($n=268$). In 76.8%, the mutations are accompanied by a deletion of the other allele (17p–). Deletion of 11q in the absence of deletion of 17p was found in 5.4% of the mutations. Deletion 13q in the absence of deletion 17p, 11q or trisomy 12 was seen in 9.3% of the cases. Trisomy 12 appeared just in one case in the absence of deletion 17p (0.4%).

the groups of patients with 11q deletion showed a similar frequency when excluding patients with 17p deletion (23 and 22% respectively). In contrast, trisomy 12 (in the absence of 17p deletion) was only seen once (1 of 60; 2%) in cases with *TP53* mutation compared with 60 of 416 (14%) when patients with 17p deletion were excluded ($P=0.006$; Figure 7).

The cases with *TP53* mutation mainly had an unmutated *IGVH* mutation status (191 of 242; 79%). There were five cases with V3-21 usage. The control cohort showed a significantly more balanced proportion of mutated and unmutated *IGVH* cases ($P<0.001$). An unmutated *IGVH* status was observed in 289 of 441 (66%) cases, whereas 152 of 441 (34%) had mutated *IGVH*. The *TP53* mutation profile did not differ in the groups with mutated or unmutated *IGHV*.

Discussion

Although the association of *TP53* mutations with poor prognosis has been proposed for many years,[16,21,26–31] the precise prognostic impact of *TP53* mutations in the absence of 17p deletion has only recently been shown.[4–6,8] Because of the profound clinical impact of *TP53* mutation in CLL, there is a continuing interest in its further characterization not only from a clinical and diagnostic but also a mechanistic perspective. In this respect, it is important to gain insight into the mutation profile of CLL. This has not been possible because of the limited size of previous studies. Although this problem may be approached by the compilation of mutation data in the IARC or other databases, the problem with the profile generated by databases is that it is usually compiled of different small and potentially biased studies.[25,32,33] The bias is usually imparted by different exon coverage, technique or patient cohort. Therefore, the correlation of *TP53* mutation results to database entries may be misleading.

The aim of our study therefore was to establish a reference dataset of *TP53* mutations in CLL. In order to achieve this, we have analyzed 268 *TP53* mutations from four different cohorts of CLL patients. Importantly, we have reanalysed the data comparing it to the current database information but also within the cohort by comparing the results from the DHPLC approach ($n=151$) and the results generated by FASAY ($n=63$). We intentionally avoided an analysis of clinical end points because the nature of recruitment into the cohorts/referral bias (clinical trial first line therapy, clinical trial refractory CLL, single-center cohorts) would have led to a bias.

The key findings of our study establish the disease-specific *TP53* mutation profile in CLL. Major outputs include the demonstration that the *TP53* mutation profile is independent of 17p deletion or previous therapy. These findings suggest that the *TP53* mutation, detectable after therapy, is selected rather than being caused by, for example, alkylating agents. The finding also supports the growing evidence that the clinical consequences of 17p deletion (and *TP53* mutation) are very similar to the *TP53* mutation in the absence of 17p–.

We found a low frequency of transitions at CpGs, a relatively high incidence of frameshift mutations (particularly in cases with 17p deletion) and confirmed the codon 209 frameshift mutation

as a 'hot spot' in CLL. Importantly, we did not find significant differences in the mutation profile of cases stratified by treatment history or 17p status suggesting that the underlying mechanisms are similar.

The first study investigating the mutation profile in CLL studied 42 mutations and found a CLL-specific profile when compared with the known *TP53* mutations in lymphoma at the time.[13] The study found a high incidence of codon 209 mutations (10%) and a high incidence of transversions in codon 273. This 'unusual' profile at CpG sites was suggested to implicate an exogenous carcinogen. In our study, we identify the codon 209 two base pair deletion as one of the most common mutations in CLL. Hot spot mutations are common in CLL. On the other hand, we found a comparatively small proportion of transitions at CpG sites compared with other cancers (for example, colon cancer). Interestingly, G→A and C→T transitions at the CpG sites were biased in favor of the G→A exchange in our study (27 vs 14; ratio 1.9:1). Similar data may be observed for CLL also in the IARC database, that is, 32 G→A mutations vs 15 C→T mutations (ratio 2.1:1). Other cancers showed a closer ratio (high grade lymphoma 128 vs 78 (ratio 1.6:1); breast cancer 335 vs 207 (ratio 1.6:1); colon cancer 864 vs 654 (ratio 1.3:1)). As the G→A transition detected at CpG site in the coding, non-transcribed DNA strand is considered to be a consequence of the cytosine deamination (and hence C→T mutation) on the opposite, transcribed DNA strand, it was suggested that these alterations are preferentially selected in quiescent non-dividing cells.[34]

There are a number of examples of disease-specific mutation profile of *TP53* in different cancers (reviewed in Soussi[3]). These include dinucleotide changes in UV-induced skin cancer, typical G→C to T→A transversions at codon 249[35] in liver cancer caused by aflatoxin B1 and, for example, a high incidence of G→T transversions (codon 157 of 158) in lung cancer.[36]

When we compared the genetic profiles of CLL cases with and without *TP53* mutation, some significant differences were observed. Although cases with 17p deletion show a lower incidence of concomitant 11q deletion (11%), the incidence of deletion 11q in cases with TP53 mutation only (23%) was not significantly different from the cohort without *TP53* mutation (22%). In contrast, there was a significant underrepresentation of trisomy 12 in the cohort of cases with *TP53* mutation in the absence of 17p deletion. Definite reasons for the imbalance are however currently unclear. Recent observations showing the very favorable outcome of patients with +12q after treatment with FC/R-FC may be partly imparted by the rarity of *TP53* mutations in the cohort.[37,38]

As the use of *TP53* mutational analysis to predict prognosis and define treatment strategies is likely to increase in CLL, the current study may serve as a reference point to the *TP53* mutation profile in CLL. Investigators assessing *TP53* mutations should preferentially cover exons 4–10 and detect the most common mutations including c.626_627delGA.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)