Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia

J Malcikova, K Stano-Kozubik, B Tichy, B Kantorova, S Pavlova, N Tom, L Radova, J Smardova, F Pardy, M Doubek, Y Brychtova, M Mraz, K Plevova, E Diviskova, A Oltova, J Mayer, S Pospisilova, M Trbusek

Cite this article as: J Malcikova, K Stano-Kozubik, B Tichy, B Kantorova, S Pavlova, N Tom, L Radova, J Smardova, F Pardy, M Doubek, Y Brychtova, M Mraz, K Plevova, E Diviskova, A Oltova, J Mayer, S Pospisilova, M Trbusek, Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia, *Leukemia* accepted article preview 7 October 2014; doi: 10.1038/leu.2014.297.

Received 29 January 2014; revised 17 September 2014; accepted 18 September 2014; Accepted article preview online 7 October 2014

Detailed analysis of therapy-driven clonal evolution of *TP53* mutations in chronic lymphocytic leukemia

Jitka Malcikova[1,2], Katerina Stano-Kozubik[1], Boris Tichy[1,2], Barbara Kantorova[1], Sarka Pavlova[1,2], Nikola Tom[1,2], Lenka Radova[1], Jana Smardova[3], Filip Pardy[1], Michael Doubek[1,2], Yvona Brychtova[2], Marek Mraz[1,2], Karla Plevova[1,2], Eva Diviskova[2], Alexandra Oltova[2], Jiri Mayer[1,2], Sarka Pospisilova[1,2], Martin Trbusek[1,2#]

1 - Center of Molecular Medicine, Central European Institute of Technology, Masaryk University, Brno, Czech Republic

2 - Department of Internal Medicine – Hematology and Oncology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic

3 - Department of Pathology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic

[#] **Corresponding Author:**

Dr. Martin Trbusek

Address: Center of Molecular Medicine, Central European Institute of Technology, Kamenice 5, 625 00 Brno, Czech Republic. E-mail: mtrbusek@fnbrno.cz; Phone: +420 532 234 207; Fax: +420 532 234 623

Running title: Early Identification of *TP53* Mutations

Conflict of Interest statement: No conflict to disclose for any author.

Financial support: Supported by MZ CR grants NT13519-4 and NT13493-4, Central European Institute of Technology project CZ.1.05/1.1.00/02.0068 from the European Regional Development Fund, FP7-HEALTH-2012-INNOVATION-1 (NGS-PTL/2012-2015/no.306242), MSMT (2013-2015, no.7E13008), MUNI/A/0830/2013, SoMoPro II Programme – no. 4SGA8684 (M.M.) co-financed by the EU and the South-Moravian Region), and EHA Research Fellowship award (M.M), and the Czech Leukemia Study Group for Life (CELL).

Abstract

In chronic lymphocytic leukemia (CLL), the worst prognosis is associated with *TP53* defects with the affected patients being potentially directed to alternative treatment. Therapy administration was shown to drive the selection of new *TP53* mutations in CLL. Using ultra-deep next-generation sequencing (NGS) we performed a detailed analysis of *TP53* mutations´ clonal evolution. We retrospectively analyzed samples assessed as *TP53*-wild-type (wt) by FASAY from 20 patients with a new *TP53* mutation detected in relapse and 40 patients remaining *TP53*-wt in relapse. Minor *TP53*-mutated subclones were disclosed in 18/20 patients experiencing later mutation selection, while only one minor-clone mutation was observed in those patients remaining *TP53*-wt (n=40). We documented that (i) minor *TP53* mutations may be present before therapy and may occur in any relapse; (ii) the majority of *TP53*-mutated minor clones expand to dominant clone under the selective pressure of chemotherapy, while persistence of minor-clone mutations is rare; (iii) multiple minor-clone *TP53* mutations are common and may simultaneously expand. In conclusion, patients with minor-clone *TP53* mutations carry a high risk of mutation selection by therapy. Deep sequencing can shift *TP53* mutation identification to a period before therapy administration, which might be of particular importance for clinical trials.

Key words: Chronic lymphocytic leukemia; *TP53* mutation; early identification; deep sequencing; NGS

Introduction

In chronic lymphocytic leukemia (CLL), patients harboring *TP53* defects represent a major challenge concerning the effective treatment[1]. *TP53* mutation and/or 17p deletion severely impede response to chemotherapy[2, 3], and affected patients also manifest short clinical responses to its combination with rituximab[4, 5]. Although alemtuzumab is supposed to act independently on p53, the response rates in monotherapy are far from satisfactory in chemorefractory patients[6]. The inability of mutated p53 protein to induce apoptosis properly seems to be a primary reason for the observed resistance to treatment [7]. The p53 dysfunction is also the major cause of genomic instability in CLL cells[8], which leads to the acquisition of other genomic variants available for further selection.

TP53 gene defects have been observed as primarily subclonal events in CLL patients, often emerging at later disease stages[9]. The frequency of *TP53* defects at diagnosis or before first therapy is only between 5 to 15%[2, 3, 10, 11], but the proportion of affected patients is significantly higher after treatment and has been reported to be as high as 44% in a fludarabine-refractory cohort[12]. Clonal evolution of genetic abnormalities including *TP53* defects is well evidenced in CLL. Recent studies have illustrated the development of 17p and 11q deletions during the disease course, and associated clonal evolution of new 17p deletions with the presence of foregoing therapy[13, 14]. Concerning *TP53* mutations, well documented cases of their acquirement under the pressure of chemotherapy have also been reported by us and independently by others[15-18]. This led to the suggestion that *TP53* mutations should be investigated before each therapy in CLL patients[19].

Next-generation sequencing (NGS) technologies currently enable mutation analyses in cancer patients with previously unattainable sensitivity, reaching as far as fractions of percentages. The clinical significance of minor-clone *TP53* mutations has recently been demonstrated by Rossi et al[18]. Therefore, we utilized this powerful tool to study the clonal evolution of *TP53* mutations in detail. We used an amplicon ultra-deep NGS approach with a high coverage to reach maximum sensitivity, and we used a highly accurate proof-reading polymerase to minimize the sequencing errors. The aims of this NGS-based study were to disclose (i) whether minor *TP53*-mutated clones had already been present before the preceding therapy, and if yes, (ii) whether some patients, who are *TP53*-wt after therapy, harbor minor *TP53* mutations that are not selected. These two issues should address whether NGS is capable of identifying patients at risk of *TP53* mutation selection by treatment.

Materials and Methods

Patients´ cohort

The study was performed on CLL patients' peripheral blood samples at the University Hospital Brno (with written informed consent provided in accordance with the Declaration of Helsinki). Four common cytogenetic aberrations including 17p deletions were detected by Interphase fluorescent *in situ* hybridization (I-FISH) using probes from MetaSystems (Altlussheim, Germany), and were classified according to the hierarchical cytogenetic model[20]. *TP53* mutations were identified by yeast functional analysis (FASAY) coupled to Sanger sequencing of DNA templates from red colonies bearing non-functional p53[16]. Patient selection criteria for NGS analysis were: Cohort I: (i) *TP53* mutational status change from wild-type to mutated documented using FASAY; (ii) only one therapy applied between the last *TP53*-wt examination and new *TP53* mutation detection. This criterion was established to enable the tracking of clonal evolution during just one relapse; (iii) available DNA from the time when the sample was *TP53*-wt. Cohort II: (i) available results of consecutive FASAY analyses performed in relapse(s) with no *TP53* mutational status change; (ii) DNA available from the period preceding therapy (Supplementary Figure 1).

Statistical analyses

Fisher's exact test was used to assess the association between categorical variables. Mann-Whitney test was used to compare the continuous variables. Wilcoxon signed rank test was used for paired comparison of mutation numbers. Survival analysis and time to mutation detection were calculated using the Kaplan-Meier survival estimator. Overall survival was assessed from the date of diagnosis; only disease-related death was considered an event. Time to mutation detection was assessed from the date of diagnosis to the date of new *TP53* mutation detection (event) or the last *TP53*-wt examination (censored).

Median survival, time to mutation detection, differences between the curves, and hazard ratios were evaluated by the log-rank test using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Ultra-deep next-generation sequencing

NGS analysis was performed on MiSeq (Illumina, San Diego, CA, USA) using gDNA from cryopreserved peripheral blood separated CD19+ B-lymphocytes or mononuclear cells; the percentage of CLL cells (CD5+CD19+) was assessed using flow-cytometry and was >80% in all cases. 25 ng of patient DNA was amplified with highly accurate proof-reading Q5 Polymerase (New England Biolabs, Ipswich, MA, USA) using *TP53* exon-specific primers (Supplementary Table 1). The experimental design and reaction conditions followed the manufacturer recommendations. Briefly, PCR products were pooled, purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), and quantified using Qubit dsDNA HS Assay Kit (Life Technologies, Waltham, MA, USA). The purified amplicon mixes were diluted to a total amount of 1 ng. The indexed paired-end library was prepared with Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using MiSeq Reagent Kit v2 300 cycles (Illumina). To avoid cross-contamination, samples obtained from the same patient in different time periods were sequenced in separate runs. Amplicons and libraries for each run were prepared separately. The median coverage per base achieved was 31599 reads (range 2601-177021).

An in-house bioinformatics pipeline was established to call the sequencing variants. For read pre-processing and alignment we used CLC Genomic Workbench (Qiagen, Hilden, Germany). Variant calling was performed using the deepSNV R-package[21] with a statistical approach applying the shearwater algorithm to compute Bayes classifiers based on a betabinomial model[22, 23]. By the reproducibility test we disclosed that we were able to reliably distinguish point mismatches and ≥2nt insertions/deletions (indels) at the level of 0.2% of variant reads, and 1-nucleotide deletions at the level of 1% of variant reads as these may be artificially introduced during the sequencing and alignment process. For further details see Supplementary material. Moreover, to evaluate the established pipeline, 20 control samples (*TP53* exons 4-10) derived from healthy individuals were sequenced and no alteration in any sample was observed on the above mentioned detection limits.

Results

Consecutive TP53 mutational analysis confirms the prominent impact of newly acquired TP53 mutations on survival

Consecutive *TP53* mutation investigation using FASAY was performed in 330 patients in at least 2 serial samples. All patients harbored intact *TP53* gene at the time of the first analysis (for patients´ characteristics see Supplementary Table 2). Among 121 patients who did not receive any therapy during the follow up, new *TP53* mutations was observed in only one patient (median follow-up of the group 50 months). In contrast, analyses performed at the time of relapse after one or several therapy lines (n=209 patients; median follow up of the group 61 months) identified new *TP53* mutation(s) in 43 patients. Altogether, the risk of *TP53* mutation acquisition at 5 years after diagnosis was 1% in untreated vs. 17% in treated patients (hazard ratio 0.2504 [95% CI 0.13- 0.47; P<0.001]) (Figure 1).

To assess the importance of *TP53* mutation status change from wild-type to mutated, we used our cohort analyzed using FASAY and compared the overall survival from diagnosis in patients who acquired a new dominant mutation in relapse with patients who remained *TP53* wild-type in relapse, and patients who already harbored *TP53* mutations at diagnosis (Figure 2). The overall survival was significantly reduced in the group of patients who had selected *TP53* mutations compared to patients assessed as wild-type in relapse (P= 0.03). The shortest survival was noted for patients with *TP53* mutations already detected at diagnosis.

NGS analysis reveals the presence of minor mutated clones before their therapy-driven selection

In the first part of our retrospective study, we focused on 20 patients who had acquired a new *TP53* mutation in relapse, as assessed by FASAY coupled to Sanger sequencing (Cohort I; Sample 2). In these patients we used ultra-deep NGS to examine samples taken before the preceding therapy which showed *TP53*-wt status using Sanger sequencing and FASAY (Sample 1). In 10 patients these retrospective samples were treatment-naïve (Cohort IA), while the remaining 10 patients had already been pre-treated at the time of NGS analysis (Cohort IB) (Table 1). The schematic visualization of samples´ inclusion criteria is shown in Supplementary Figure 1. The mutations analyzed consisted of 16 missense mutations, 2 nonsense mutations, and 2 deletions and were hence representative of the p53 mutation profile in CLL[24]. In order to decipher *TP53* mutagenesis, we sequenced not only the affected regions but all commonly mutated exons 4-9[24] with a high median coverage for the positions containing mutations (25709 reads; range 5245 – 64979). We were able to detect minor-proportion mutations in 18/20 samples (90%), with a proportion of 0.20-3.71% of the reads showing mutations. In two of the 18 patients, we surprisingly detected other *TP53* mutations that had not undergone expansion. The results are summarized in Table 1, for details see Supplementary table 3.

Interestingly, in addition to the presumed retrospective mutations, we also identified other minor-proportion *TP53* mutations in both treatment-naive and pretreated samples (10/20 patients; 2-6 mutations per patient) (Table 1). It indicates that in a proportion of patients, there is a pool of *TP53* mutations available for therapy-driven selection.

We next intended to investigate whether the minor *TP53* mutated subclones detectable by NGS in pretreated samples and undergoing selection in subsequent relapse (Cohort IB) had already been present before first therapy. Therefore we used NGS in four available treatment-naïve samples (patients no. 149, 365, 542, and 1043) and confirmed the presence of respective mutation in one of them (patient no. 1043 – mutation c.844C>G p.R282G detected in 0.2% of NGS reads). This observation suggests that preexisting mutations may expand after the first but also after subsequent therapies at least in some patients.

Minor TP53 mutations detectable before therapy are rare in patients remaining TP53-wt at relapse

As the next step, we analyzed 40 samples taken before first treatment in patients showing wt-*TP53* status at relapse after one or several therapy lines (Cohort II). These cases were selected from the cohort of relapsing patients, and the inclusion criteria were chosen in order to collect the cohort with biological and clinical characteristics matching Cohort I (Table 2, Supplementary Figure 1). In this experiment, besides exons 4-9, exon 10 was also sequenced as it may occasionally harbor mutations[24].

We found *TP53* mutation in only one of the 40 patients (2.5%). Specifically, the mutation p.G266E; c.797G>A was detected in 0.55% (148/32973) sequencing reads, and its presence was verified by an independent NGS run. This mutation did not undergo a clonal expansion during the disease course despite several treatment lines – the patient was treated consecutively with 3 distinct therapy lines (FCR, Alemtuzumab, and Rituximab+Dexamethasone) and achieved two complete remissions. In the last available sample from the time of relapse after Rituximab+Dexamethasone treatment (follow up 47 months) the same mutation was present in 1.4% of reads.

Clonal selection frequently affects multiple TP53 mutations simultaneously

As emerged from the previous analyses, multiple minor-clone *TP53* mutations are commonly observed in CLL patients. To further explore this phenomenon in relation to the expansion of major mutations, we performed ultra-deep NGS of *TP53* gene in samples taken at relapse(s) (Sample 2 in Supplementary Figure 1). For this analysis we had chosen the following patients from Cohort I: (i) six patients with more than one mutation detected in sample 1; (ii) six patients with a single mutation detected in sample 1; (iii) two patients with no mutation detected in sample 1. Furthermore, the patient with a single non-expanding mutation from Cohort II was also included. An increase in the number of mutations compared to the preceding samples was observed in 13/14 patients from Cohort I (Table 3, Figure 3A). In the paired analysis restricted to samples taken before the first therapy and in the first relapse (Cohort IA) a significant increase in the number of mutations per patient was observed (mean number of mutations per patient 2.1 vs. 6.7; P=0.02). In the patient from Cohort II, only one mutation was found in both samples.

Regarding the evolution of individual subclones, the most frequently observed event (7/14 patients) was the clear expansion of one mutation from minor to dominant clone accompanied with the occurrence of additional minor *TP53*-mutated clones. In addition to that, we also observed other specific situations: (i) in one case the consecutive selection of two different dominant *TP53* mutations at the first and then the subsequent relapse was noted (one mutation replaced by the other) (patient no. 820; Figure 3B); (ii) in four patients there was not a prominent clonal expansion of one mutation, but multiple clones expanded simultaneously (patients no.8, 178, 354, and 485; Figure 3C); (iii) one patient underwent only a very slight expansion of a single minor-clone mutation in the first relapse (from 0.2 to 1,46% reads), in the second relapse the proportion of the mutation also increased only slightly (to 2.82% reads) and two other minor-proportion mutations appeared (patient no. 503; Table 3). The results summarizing the rise in the number of mutations in all performed NGS analyses are recapitulated in Supplementary Table 3.

Since the analysis of mutated patients disclosed an increased occurrence of minor *TP53* mutations after treatment, we further analyzed 15 randomly selected patients from Cohort II after 1-4 therapy lines using NGS. No *TP53* mutations were observed in any patient.

Molecular features of mutations

In total, we identified 148 mutations in 21 patients (Supplementary Table 3) in all the NGS analyses performed. The mutation profile is shown in Supplementary Figures 2 and 3. Compared with the reference study on *TP53* mutation profile in CLL[24] our results showed the following: (i) a similar proportion of missense mutations (79% vs. 74%; P=0.4) and nonsense mutations (both studies 4%); (ii) the same frequency of mutations at major hotspot codons (175, 179, 220, 248, 273, 281) (20% of all mutations in both studies); (iii) a significantly higher proportion of splice-site mutations (9% vs. 2%; P=0.005) and, on the other hand (iv) a significantly lower frequency of indel mutations (7% vs. 20%; P=0.0003). Concerning point mutations, transitions represented 61% with only 29% of them (17% of all mutations) occurring at CpG sites. The G-A transitions at CpG predominated C-T transitions (G-A:C-T ratio 2:1). The lower proportion of CpG transitions and the prevalence of G-A exchanges coincided with the reference study[24].

Comparison of *TP53* mutation profiles in cases with unmutated immunoglobulin heavy chain gene (*IGHV*; U-CLL) vs. mutated *IGHV* (M-CLL) showed no difference in mutation frequency within sequence motif (RGYW/WRCY) recognized by activation-induced cytidin deaminase (AID) in U-CLL vs. M-CLL (20% vs. 18% of point mutations P=0.8). In M-CLL, a significant prevalence of alterations in A:T pairs was found compared to U-CLL (56% vs. 27% of point mutations; P=0.0008). The A:T alteration predominance was the most prominent in case of A:T>C:G transversions (12% in M-CLL vs. 1% in U-CLL; P=0.009; Figure 4).

As the number of mutations increased after therapy, we also compared the molecular profile of mutations detected in pre-therapy samples only (n=24) with mutations that occurred exclusively after treatment (n=103) and we did not observe any significant differences regarding the proportion of hotspot mutations, transversion to transition ratio, proportion of transitions at CpG sites and G:C to A:T ratio (data not shown).

Discussion

The mechanisms leading to p53 mutation acquisition and accumulation in CLL are poorly understood. The direct induction of *TP53* mutations by DNA-damaging chemotherapy, namely alkylating agents, has been suggested[25]. In contrast, a large collaborative study involving 268 p53 mutations indirectly showed that the impact of therapy on *de novo* mutation induction is unlikely, as mutation spectra are similar in untreated and treated patients[24]. This observation, however, may not serve as definitive proof of the neutral impact of therapy on *TP53* mutagenesis, since similar mutations could evolve through different mechanisms. The current progress in highly sensitive techniques, specifically in ultra-deep next-generation sequencing, allows the possibility to explore whether therapy merely selects *TP53* mutations present in minor CLL clones before drug administration. Moreover, identifying *TP53* defects as early as possible during their evolution may represent a significant achievement in the clinical management of high risk CLL, since *TP53*-deffective patients could be offered alternative treatment[1]. The clinical impact of minor-proportion *TP53* defects is currently a matter of debate[18, 26, 27]; their relevance for relapse development is supported by the actual number of mutated cells. For instance, at common pre-therapy leukocytosis achieving 100 x 10e9 per liter with 90% CLL cells, a patient harbors approximately only 4.5 x 10e11 CLL cells in peripheral blood, not considering other organs like the spleen. In this case, a 1% *TP53* mutation corresponds to ~4.5 x 10e9 cells. Moreover, the clinical significance of small *TP53*-mutated clones under the detection limit of Sanger sequencing have very recently been manifested by the study of Rossi et al[18] showing their similar unfavorable prognostic impact compared with clonal *TP53* defects.

With this report, we focused on two principal issues: (i) exploration of *TP53*-mutated clone evolution, and (ii) assessment of NGS utilization in *TP53* mutation expansion prediction in clinical practice. Both these issues are important with respect to the clear negative impact of newly acquired *TP53* mutations on patients´ prognosis, which was evidenced by Rossi et al[28] using time-dependent Cox regression analysis, and is also confirmed here by survival analysis of patients with new mutations.

Concerning the clonal evolution, we documented that the risk of new *TP53* mutation acquisition at 5 years after diagnosis is 17% in patients requiring treatment, contrasting with 1% in untreated patients (P<0.001), and we confirmed that selection of pre-existing mutated clones by therapy is the predominant mechanism for *TP53* mutations´ accumulation. Moreover, we showed that mutations expanding during relapse are detectable before the preceding therapy in the majority of patients. Admittedly, based on our study we cannot entirely exclude that at least some *TP53* mutations are the consequence of DNA damaging drugs[25] since many minor-proportion mutations were undetectable before first therapy despite using ultra-deep NGS. Although we have not observed any profound difference in the mutation profile of these mutations compared to the mutations present before treatment, they could be induced by therapeutic agents or spontaneous mutagenesis during relapse. Alternatively, they may be present in a very low proportion of leukemic cells under the NGS detection limit.

Our study independently confirms two recent reports[18, 29] showing that in a proportion of patients there are multiple minor-clone *TP53* mutations (under the Sanger sequencing detection limit). These mutations may or may not accompany a major clonal mutation[29]. We noted the presence of multiple *TP53* mutations in patients with clonal selection of dominant *TP53* mutations, and also in patients with dominant *TP53* mutation detected at diagnosis (7/10 patients; data not shown). We further observed that selection may affect not only single *TP53*-mutated minor clone, but in some patients multiple mutations simultaneously. In fact, at least some cases without prominent expansion of one mutation underwent a slight selection of a burden of different *TP53*-mutated clones that are not detectable by Sanger sequencing. Using FASAY these patients were assessed as “mutation acquisition” since with this methodology the overall percentage of red colonies equals the sum of all mutations present.

The striking aspect of our study is the actual number of multiple *TP53* mutations, as according to our observation even tens of mutations may be present in individual patients. Our conclusion that these multiple alterations are true mutations and not NGS artifacts is supported by the following: (i) the point mutations present in ≥ 0.2% of NGS reads were confirmed in a reproducibility test; (ii) the same variants were often observed in consecutive samples, (iii) some of the minor-proportion mutations were also noted in individual colonies during FASAY analysis (Supplementary Table 3); this also shows that the mutations are present on separate alleles as FASAY is based on subcloning template molecules; (iv) only one mutation was detected in 56 samples from patients remaining *TP53*-wt throughout disease (40 pre-therapy and 16 relapsed samples from Cohort II), and no mutation was observed in any healthy control sample (n=20); and finally (v) the molecular profile of additional mutations was similar to that described for the reference cohort[24] with the common hotspots being the most prevalent mutations.

Despite the similarities between the mutation profile of additional mutations and the reference *TP53*-mutated CLL cohort, we noticed several specificities. The low number of indel mutations among additional mutations can likely be accounted to the NGS methodology itself as it is generally difficult to distinguish minor-proportion 1-nucleotide deletions from background. An interesting observation is the high number of minor-proportion splice-site mutations, predominantly in intron 6. These mutations are often present at subclonal level; however for a yet unknown reason they only rarely expand to dominant clone. Apart from this, we were not able to find any rule concerning a preferential selection of distinct mutation types. For instance, we recorded patients in which a truncating mutation outgrew the clone carrying hot-spot mutation with documented dominant-negative and gain-of-function effect. Therefore there should be other factors contributing to the preferential selection of particular *TP53*-mutated subclones, e.g. mutations in other genes or distinct stimulation by the microenvironment. In addition, an obvious important factor represents deletion 17p, since the wild-type allele absence may contribute to the selection advantage of a particular subclone. The new 17p deletion accompanying the new *TP53* mutation was found in 8/20 patients and in another two patients a new 17p copy-neutral loss of heterozygosity (cn-LOH) was noted. However, to determine the exact allele composition of minor subclones carrying different *TP53* mutations would require single-cell analysis, which was beyond the scope of this study.

The surprisingly large number of mutations led us to explore the mechanisms of *TP53* mutagenesis with regards to lymphoid-specific hypermutation machinery. No bias regarding mutations in sequence motifs recognized by cytidin deaminase AID was found. Interestingly, we observed a prevalence of mutations in A:T pairs in patients with mutated *IGHV* compared to unmutated *IGHV*, which was most prominent in A:T>C:G transversions. A similar disproportion was found in a whole-genome sequencing study[30] and is most likely to be attributed to the operation of error-prone polymerase eta [31].

The observation that the majority of new dominant mutations are already present before therapy offers the opportunity to predict their expansion later during the disease course and change the patients´ care strategy. The obvious prerequisite for such clinical utilization is that persisting minor proportion *TP53* mutations´ existence is not a common phenomenon among patients who do not undergo massive mutation selection. Our long-term observation based on sensitive FASAY analyses in consecutive samples indicates that minor *TP53* mutated clones may persist in occasional cases without significant expansion. Such a case was also documented here; in one patient we observed only a very slow increase of *TP53*-mutated subclone proportion in consecutive relapses. To explore the general incidence of non-selected mutations, we employed NGS and analyzed 40 pre-therapy samples from patients remaining wt after treatment line(s) and observed that non-selected mutations are in fact rare since 39/40 patients were devoid of any mutation.

When considering the applicability of highly sensitive NGS in diagnostics, it is important to bear in mind that (i) the original clone size may be variable and under the detection limit of any method, and (ii) the dynamics of the expansion process may vary among individual patients due to competition between CLL subclones and, potentially, also the type of therapy.

As we observed in our study, minor-clone mutations do not have to undergo selection after the first treatment. One may consider that the type of treatment could be critical for clonal selection, with the more intensive regimen being more likely to facilitate clonal selection as we indicated in previous studies[32, 33]. However, we document here that in some cases even administrating intensive chemo-immunotherapy resulting in complete remission does not necessarily result in clonal expansion. It is highly likely that there are other factors impacting the selection rate like other genomic defects present either in the *TP53*-mutated subclone itself or in the *TP53*-wt cells.

In conclusion, we show in our study that multiple *TP53* clonal evolution scenarios are possible, with some of them being more likely to occur (Figure 5). In cases when a minor-proportion *TP53*-mutated clone(s) is detected, the patient is at high risk of mutation selection by therapy in the first or subsequent relapse, and the presence of the new dominant mutation should be considered as a clearly negative factor impacting the patient´s outcome. Moreover, our detailed analysis of *TP53* mutations at subclonal level at different time points suggests that some patients are intrinsically prone to acquire *TP53* mutations and in the majority of these patients more than one clone carrying a different mutation with a different predisposition for expansion occur. Owing to deep sequencing, it is now technically possible to shift *TP53* mutation identification to time preceding therapy administration. It seems now especially interesting to explore whether similar rules drive the clonal evolution of other recurrently mutated genes in CLL.

Acknowledgements: We thank Lenka Jurackova and Jitka Kabathova for their technical help with experiments and Matthew Smith for text editing.

Conflict of Interest: The authors declare no conflict of interests.

Supplementary information is available at Leukemia website

References

1. Stilgenbauer S, Zenz T. Understanding and managing ultra high-risk chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program* 2010; **2010:** 481-488.

2. Zenz T, Eichhorst B, Busch R, Denzel T, Häbe S, Winkler D, *et al. TP53* mutation and survival in chronic lymphocytic leukemia. *J Clin Oncol* 2010 Oct; **28**(29)**:** 4473-4479.

3. Gonzalez D, Martinez P, Wade R, Hockley S, Oscier D, Matutes E, *et al.* Mutational status of the *TP53* gene as a predictor of response and survival in patients with chronic lymphocytic leukemia: results from the LRF CLL4 trial. *J Clin Oncol* 2011 Jun; **29**(16)**:** 2223-2229.

4. Byrd JC, Gribben JG, Peterson BL, Grever MR, Lozanski G, Lucas DM, *et al.* Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. *J Clin Oncol* 2006 Jan 20; **24**(3)**:** 437-443.

5. Badoux XC, Keating MJ, Wang X, O'Brien SM, Ferrajoli A, Faderl S, *et al.* Fludarabine, cyclophosphamide, and rituximab chemoimmunotherapy is highly effective treatment for relapsed patients with CLL. *Blood* 2011 Mar; **117**(11)**:** 3016-3024.

6. Stilgenbauer S, Zenz T, Winkler D, Bühler A, Schlenk R, Groner S, *et al.* Subcutaneous alemtuzumab in fludarabine-refractory chronic lymphocytic leukemia: clinical results and prognostic marker analyses from the CLL2H study of the German Chronic Lymphocytic Leukemia Study Group. *J Clin Oncol* 2009 Aug; **27**(24)**:** 3994-4001.

7. Pettitt AR, Sherrington PD, Stewart G, Cawley JC, Taylor AM, Stankovic T. p53 dysfunction in B-cell chronic lymphocytic leukemia: inactivation of ATM as an alternative to *TP53* mutation. *Blood* 2001 Aug 1; **98**(3)**:** 814-822.

8. Ouillette P, Fossum S, Parkin B, Ding L, Bockenstedt P, Al-Zoubi A, *et al.* Aggressive chronic lymphocytic leukemia with elevated genomic complexity is associated with multiple gene defects in the response to DNA double-strand breaks. *Clin Cancer Res* 2010 Feb; **16**(3)**:** 835-847.

9. Landau DA, Carter SL, Stojanov P, McKenna A, Stevenson K, Lawrence MS, *et al.* Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* 2013 Feb; **152**(4)**:** 714-726.

10. Rossi D, Cerri M, Deambrogi C, Sozzi E, Cresta S, Rasi S, *et al.* The prognostic value of *TP53* mutations in chronic lymphocytic leukemia is independent of Del17p13: implications for overall survival and chemorefractoriness. *Clin Cancer Res* 2009 Feb 1; **15**(3)**:** 995-1004.

11. Zainuddin N, Murray F, Kanduri M, Gunnarsson R, Smedby KE, Enblad G, *et al.* *TP53* Mutations are infrequent in newly diagnosed chronic lymphocytic leukemia. *Leuk Res* 2011 Feb; **35**(2)**:** 272-274.

12. Zenz T, Häbe S, Denzel T, Mohr J, Winkler D, Bühler A, *et al.* Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, *TP53* mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. *Blood* 2009 Sep; **114**(13)**:** 2589-2597.

13. Shanafelt TD, Witzig TE, Fink SR, Jenkins RB, Paternoster SF, Smoley SA, *et al.* Prospective evaluation of clonal evolution during long-term follow-up of patients with untreated early-stage chronic lymphocytic leukemia. *J Clin Oncol* 2006 Oct; **24**(28)**:** 4634-4641.

14. Stilgenbauer S, Sander S, Bullinger L, Benner A, Leupolt E, Winkler D, *et al.* Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival. *Haematologica* 2007 Sep; **92**(9)**:** 1242-1245.

15. Zenz T, Krober A, Scherer K, Habe S, Buhler A, Benner A, *et al.* Monoallelic *TP53* inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood* 2008 Oct 15; **112**(8)**:** 3322-3329.

16. Malcikova J, Smardova J, Rocnova L, Tichy B, Kuglik P, Vranova V, *et al.* Monoallelic and biallelic inactivation of *TP53* gene in chronic lymphocytic leukemia: selection, impact on survival, and response to DNA damage. *Blood* 2009 Dec; **114**(26)**:** 5307-5314.

17. Ouillette P, Saiya-Cork K, Seymour E, Li C, Shedden K, Malek SN. Clonal evolution, genomic drivers, and effects of therapy in chronic lymphocytic leukemia. *Clin Cancer Res* 2013 Jun; **19**(11)**:** 2893-2904.

18. Rossi D, Khiabanian H, Spina V, Ciardullo C, Bruscaggin A, Famà R, *et al.* Clinical impact of small *TP53* mutated subclones in chronic lymphocytic leukemia. *Blood* 2014 Apr; **123**(14)**:** 2139-2147.

19. Pospisilova S, Gonzalez D, Malcikova J, Trbusek M, Rossi D, Kater AP, *et al.* ERIC recommendations on *TP53* mutation analysis in chronic lymphocytic leukemia. *Leukemia* 2012 Jul; **26**(7)**:** 1458-1461.

20. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, *et al.* Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000 Dec 28; **343**(26)**:** 1910-1916.

21. R Core Team. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/. 2013.

22. Gerstung M, Papaemmanuil E, Campbell PJ. Subclonal variant calling with multiple samples and prior knowledge. *Bioinformatics* 2014 May; **30**(9)**:** 1198-1204.

23. Gerstung M, Beisel C, Rechsteiner M, Wild P, Schraml P, Moch H, *et al.* Reliable detection of subclonal single-nucleotide variants in tumour cell populations. *Nat Commun* 2012; **3:** 811.

24. Zenz T, Vollmer D, Trbusek M, Smardova J, Benner A, Soussi T, *et al. TP53* mutation profile in chronic lymphocytic leukemia: evidence for a disease specific profile from a comprehensive analysis of 268 mutations. *Leukemia* 2010 Dec; **24**(12)**:** 2072-2079.

25. Sturm I, Bosanquet AG, Hermann S, Guner D, Dorken B, Daniel PT. Mutation of p53 and consecutive selective drug resistance in B-CLL occurs as a consequence of prior DNA-damaging chemotherapy. *Cell Death Differ* 2003 Apr; **10**(4)**:** 477-484.

26. Catovsky D, Richards S, Matutes E, Oscier D, Dyer MJ, Bezares RF, *et al.* Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. *Lancet* 2007 Jul 21; **370**(9583)**:** 230-239.

27. Tam C, Shanafelt T, Wierda W, Abruzzo L, Van Dyke D, O'Brien S, *et al.* De novo deletion 17p13.1 chronic lymphocytic leukemia shows significant clinical heterogeneity: the M. D. Anderson and Mayo Clinic experience. *Blood* 2009 Jul; **114**(5)**:** 957-964.

28. Rossi D, Rasi S, Spina V, Bruscaggin A, Monti S, Ciardullo C, *et al.* Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood* 2013 Feb; **121**(8)**:** 1403-1412.

29. Jethwa A, Hüllein J, Stolz T, Blume C, Sellner L, Jauch A, *et al.* Targeted resequencing for analysis of clonal composition of recurrent gene mutations in chronic lymphocytic leukaemia. *Br J Haematol* 2013 Nov; **163**(4)**:** 496-500.

30. Puente XS, Pinyol M, Quesada V, Conde L, Ordóñez GR, Villamor N, *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2011 Jul; **475**(7354)**:** 101-105.

31. Zhao Y, Gregory MT, Biertümpfel C, Hua YJ, Hanaoka F, Yang W. Mechanism of somatic hypermutation at the WA motif by human DNA polymerase η. *Proc Natl Acad Sci U S A* 2013 May; **110**(20)**:** 8146-8151.

32. Panovská A, Smolej L, Lysák D, Brychtová Y, Šimkovič M, Motyčková M, *et al.* The outcome of chronic lymphocytic leukemia patients who relapsed after fludarabine, cyclophosphamide, and rituximab. *Eur J Haematol* 2013 Jun; **90**(6)**:** 479-485.

33. Trbusek M, Smardova J, Malcikova J, Sebejova L, Dobes P, Svitakova M, *et al.* Missense mutations located in structural p53 DNA-binding motifs are associated with extremely poor survival in chronic lymphocytic leukemia. *J Clin Oncol* 2011 Jul; **29**(19)**:** 2703-2708.

Figure Legends

Figure 1. Time from diagnosis to *TP53* mutation acquisition

Patients with *TP53*-wt status at first investigation were repeatedly tested. Time to mutation detection was assessed from the date of diagnosis to the date of new *TP53* mutation detection (event) or the last *TP53*-wt examination (censored). Patients treated during the follow up acquired new *TP53* mutation significantly more often than untreated patients.

Figure 2. Overall survival according to the *TP53* mutational status in relapse

Overall survival from diagnosis in patients who acquired a new *TP53* mutation at relapse (n=18; median survival 64 months) in comparison with patients who remained *TP53* wild-type at relapse (n=78; median survival undefined; pairwise comparison p=0.03), and patients who harbored *TP53* mutation already at diagnosis (n=49; median survival 39 months; pairwise comparison p=0.02). Only patients with *TP53* status examined at diagnosis or 12 months thereafter were included. All patients included in the analysis underwent treatment and patients having *TP53*-wt status at diagnosis were repeatedly tested for *TP53* mutation presence at subsequent relapse(s).

Figure 3. Kinetics of multiple mutations in subsequent samplings

A - Increase in number of mutations detectable using NGS during the disease course. All patients form Cohort I with repeated NGS analysis are shown (n=14). **B** - Dynamics of clonal evolution in patient no. 820. Clone bearing mutation p.249del that was detected at Relapse 2 using FASAY first expanded and was later outgrown by another mutation p.R175H. Splicing mutation c.375+2T>A slightly expanded and coexisted as a minor subclone. Subclonal dynamics of additional minor clones present below 2% is shown in detail. **C** -Examples of patients with no prominent expansion of one mutation shown. Proportion of variant reads in individual disease time points illustrated. Mutation detected using FASAY in the second sampling highlighted in bold.

Figure 4. Comparison of *TP53* mutation profile in patients with unmutated *IGHV* (U-CLL) versus mutated *IGHV* (M-CLL). Percentage from all point mutations shown.

Figure 5. Schematic representation of different scenarios of *TP53*-mutated subclones clonal evolution.

Table 1. Summary of NGS analysis in patients acquiring a new *TP53* dominant mutation after treatment

	Patient	Mutation name		FASAY		Ultra-deep NGS - Sample 1		Time from dg to NGS [mo]	Time between sample 1 and sample 2 [mo]	Therapy before sample 1	Therapy between sample 1 and sample 2	Cytogenetic aberrations	IGHV	Disease status
				Sample 1	Sample 2	[%]	Total number of mutations							
Cohort IA	126	c.559+33_54del		neg	pos (40%)	0.52	4	77	23	-	FC	NA → 13q-,17p-	unmut	Alive
	178	c.488A>G	p.Y163C	neg	pos (18%)	neg	0	45	66	-	FCR/R+D	neg → 13q-,17p-	unmut	Alive
	199	c.548C>G	p.S183*	neg	pos (24%)	0.57	1	1	81	-	FCR	neg	unmut	Dead
	227	c.536A>G	p.H179R	neg	pos (75%)	0.56	1	28	46	-	FC	11q-, 13q- → 13q-, 17p-	unmut	Dead
	286	c.736A>G	p.M246V	neg	pos (56%)	2.38	1	29	47	-	FCO	13q-	unmut	Alive
	503	c.838A>G	p.R280G	neg	pos (13%)	0.20	1	0	31	-	FCR	11q-, 13q-	unmut	Dead
	618	c.745A>T	p.R249W	neg	pos (14%)	neg§	1	113	32	-	FCR	11q-, 13q-	unmut	Dead
	812	c.817C>T	p.R273C	neg	pos (63%)	0.33	2	0	26	-	FCR	13q- → 13q-,17p-	mut	Alive
	820	c.743_745del	p.R249del	neg	pos (17%)	2.40	6	0	17	-	FCR	11q- → 11q-, cn-LOH 17p	unmut	Dead
	837	c.438G>A	p.W146*	neg	pos (17%)	0.85	6	0	24	-	FCR	11q- → 11q-, cn-LOH 17p	unmut	Alive
Cohort IB	8	c.527G>T	p.C176F	neg	pos (22%)	1.10	4	92	30	Clb, FCR	F/RCHOP	13q- → neg	mut	Dead
	149	c.814G>A	p.V272M	neg	pos (50%)	1.11	2	72	24	FCR	FCR	neg	unmut	Dead
	161	c.731G>A	p.G244D	neg	pos (18%)	neg	0	14	48	A	FCR	neg → 13q-,17p-	unmut	Dead
	280	c.818G>A	p.R273H	neg	pos (60%)	1.43	1	15	5	RCHOP	A	neg → 13q-,17p-	unmut	Dead
	322	c.524G>A	p.R175H	neg	pos (67%)	0.25	1	68	18	Clb	FC	neg → 13q-,17p-	unmut	Dead
	354	c.844C>T	p.R282W	neg	pos (22%)	0.76	1	77	18	FC	FCR	11q-, 13q-	unmut	Dead
	365	c.329G>T	p.R110L	neg	pos (25%)	3.71	4	48	9	FCR/FC	FCR	13q- → 13q-,17p-	unmut	Dead
	485	c.817C>T	p.R273C	neg	pos (18%)	neg§	2	115	33	Clb	FC	13q-	mut	Alive
	542	c.814G>A	p.V272M	neg	pos (35%)	0.76	2	12	22	FCR	FCR	neg → 13q-	unmut	Dead
	1043	c.844C>G	p.R282G	neg	pos (18%)	0.8	4	32	17	Clb	FCR	13q-	unmut	Alive

Sample 1 – sample showing *TP53*-wt status using FASAY; Sample 2 – new *TP53* mutation in relapse assessed by FASAY; Cytogenetic aberrations - in case of change cytogenetic aberrations are listed in the format: Sample 1 → Sample 2; dg – diagnosis; mo – month; neg – negative; pos – positive; mut – mutated; unmut – unmutated; § – other than the later expanding *TP53* mutation detected (for details see Supplementary Table 3); cn-LOH 17p – 17p copy-neutral loss of heterozygosity involving *TP53* gene detected by Cytoscan Affymetrix arrays

Therapy abbreviations: A – alemtuzumab; Clb – chlorambucil; C – cyclophosphamide; F – fludarabine; R – rituximab; FC – fludarabine + cyclophosphamide; FCR – FC + rituximab, FCO – FC + ofatumumab; CHOP – cyclophosphamide, doxorubicin, vincristine, prednisone; RCHOP – CHOP with rituximab; R+D – rituximab + dexamethason

Table 2. Clinical and biological characteristics of patients analysed using NGS

	Cohort I		Cohort II		
	Dominant *TP53* mutation acquisition		No *TP53* mutated dominant clone after therapy		
	number	%	number	%	P
Number of patients	20		40		
RAI stage at diagnosis					
0	6	30%	6	15%	0.3049
I-II	10	50%	23	58%	0.5853
III-IV	4	20%	11	28%	0.7529
Age at diagnosis					
Median	56.8		59.2		0.3828
Range	45-76		38-77		
Gender					
Male	15	75%	31	78%	1.0000
Female	5	25%	9	23%	
IGHV status					
Mutated	3	15%	4	10%	0.6763
Unmutated	17	85%	36	92%	
I-FISH# before treatment					
Del(17p)	0	0%	1	3%	1.0000
Del(11q)	6	30%	13	34%	1.0000
+ 12	0	0%	5	13%	0.1578
Del(13q)	7	35%	13	34%	1.0000
Normal	7	35%	8	21%	0.2199
Follow up† (months)					
Median	74.8		69.9		0.9808
Range	17-147		15-195		
Number of therapy lines during follow-up†					
Median	2		2		0.4768
Range	1-4		1-5		

According to the hierarchical cytogenetics[20]

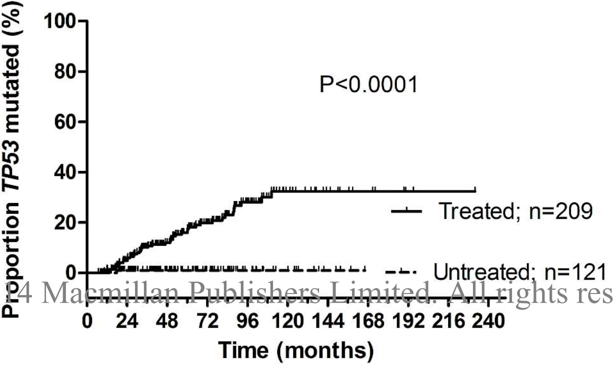
†Follow-up: Cohort I - from diagnosis to dominant *TP53* mutation detection; Cohort II - from diagnosis to the last FASAY investigation

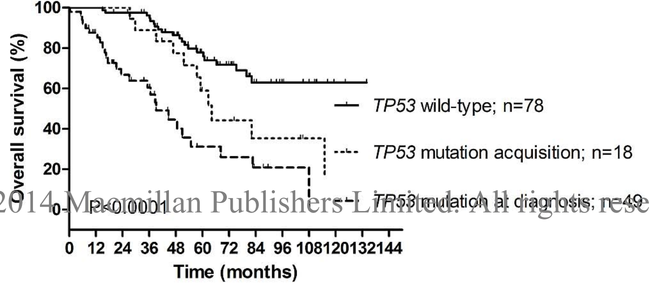
Table 3. Consecutive ultra-deep NGS analysis

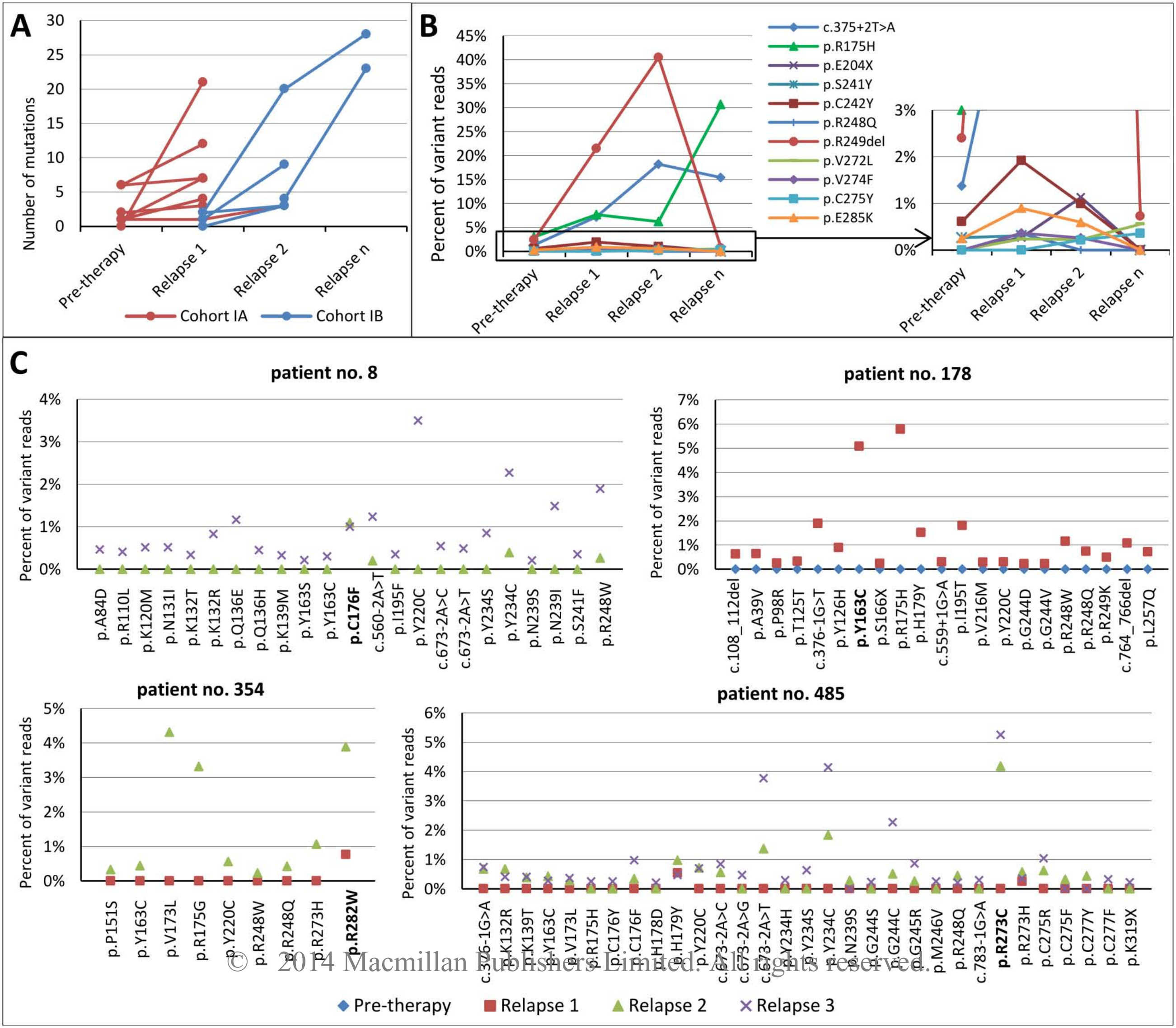
	Patient	FASAY		Mutation detected using FASAY - major mutation		Ultra-deep NGS						Time between samples 1 and 2 [mo]	Time between samples 2 and 3 [mo]	Therapy between samples 1 and 2	Therapy between samples 2 and 3
						Proportion of major mutation [%]			Total number of mutations						
		Sample 1	Sample 2			Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3				
Cohort IA	178	neg	pos (18%)	c.488A>G	p.Y163C	neg	5.08		0	21		66		FCR/R+D	
	199	neg	pos (24%)	c.548C>G	p.S183*	0.57	18.90		1	7		81		FCR	
	227	neg	pos (75%)	c.536A>G	p.H179R	0.56	81.50		1	4		46		FC	
	286	neg	pos (56%)	c.736A>G	p.M246V	2.38	48.70		1	1		47		FCO	
	503	neg	pos (13%)	c.838A>G	p.R280G	0.20	1.46	2.82	1	1	3	31	10	FCR	A
	618	neg	pos (14%)	c.745A>T	p.R249W	neg§	7.64		1	4		32		FCR	
	812	neg	pos (63%)	c.817C>T	p.R273C	0.33	75.90		2	3		26		FCR	
	820	neg	pos (17%)	c.743_745del	p.R249del	2.40	21.50	0.73	6	12	5	17	33	FCR	FCR, RCHOP, VAD
	837	neg	pos (17%)	c.438G>A	p.W146*	0.85	64.3		6	7		24		FCR	
Cohort IB	8	neg	pos (22%)	c.527G>T	p.C176F	1.10	1.00		4	23		30		F/RCHOP	
	354	neg	pos (22%)	c.844C>T	p.R282W	0.76	3.89		1	9		18		FCR	
	161	neg	pos (18%)	c.731G>A	p.G244D	neg	11.10		0	3		48		FCR	
	485	neg	pos (18%)	c.817C>T	p.R273C	neg§	4.18	5.25	2	20	28	33	18	FC	R+D
	542	neg	pos (35%)	c.814G>A	p.V272M	0.76	23.4		2	3		22		FCR	
Cohort II	311	neg	neg	c.797G>A	p.G266E	0.55	1.40		1	1		47		FCR, A, R+D	

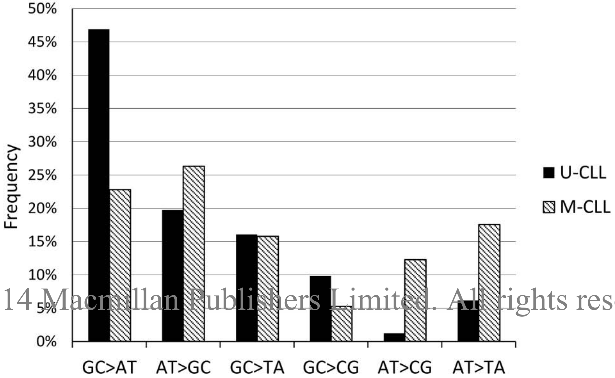
Sample 1 – sample showing *TP53*-wt status using FASAY; Sample 2 – new *TP53* mutation in relapse assessed by FASAY; Sample 3 – follow-up sample after mutation detection by FASAY; mo – month; neg – negative; pos – positive; mut – mutated; unmut – unmutated; § – other than the later expanding *TP53* mutation detected (for details see Supplementary Table 3)

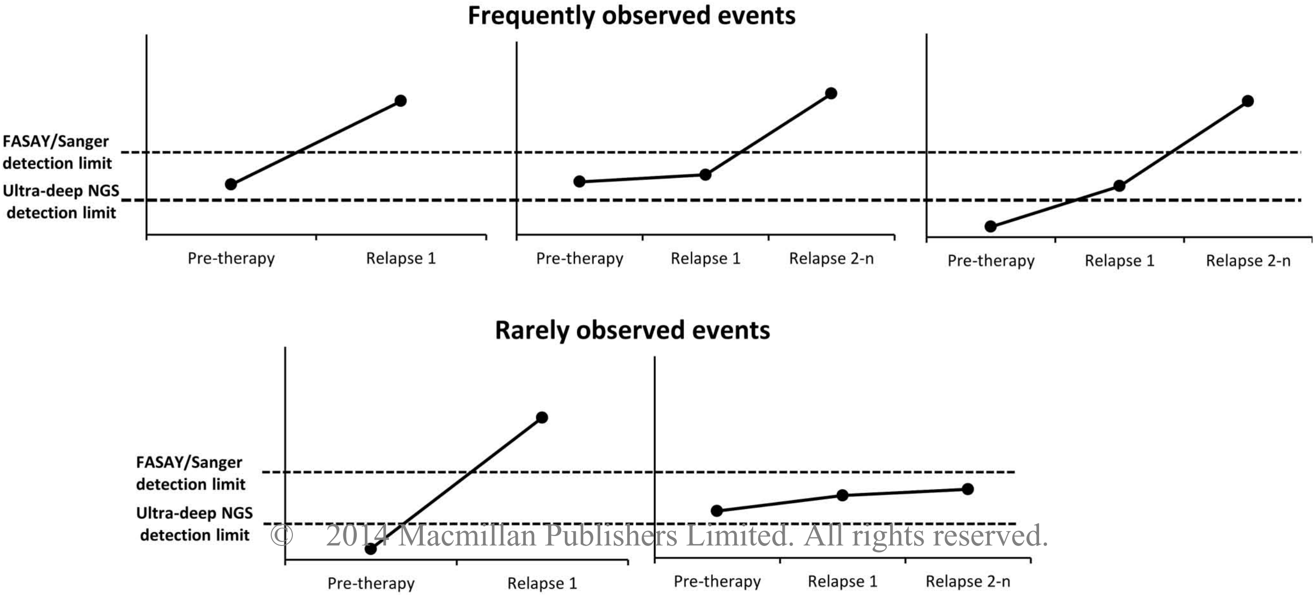
Therapy abbreviations: A – alemtuzumab; Clb – chlorambucil; C – cyclophosphamide; F – fludarabine; R – rituximab; FC – fludarabine + cyclophosphamide; FCR – FC + rituximab; FCO – FC + ofatumumab; CHOP – cyclophosphamide, doxorubicin, vincristine, prednisone; RCHOP – CHOP + rituximab; R+D – rituximab + dexamethasone, VAD - vincristine, adriamycin, dexamethasone


Proportion TP53 mutated (%)
100
80
60
40
20
0
P<0.0001
Treated; n=209
Untreated; n=121
0
24
48
72
96
120
144
168
192
216
240
Time (months)


100
80
60
40
20
0
Overall survival (%)
0
12
24
36
48
60
72
84
96
108
120
132
144
Time (months)
TP53 wild-type; n=78
TP53 mutation acquisition; n=18
TP53 mutation at diagnosis; n=49
P<0.0001


A
Number of mutations
30
25
20
15
10
5
0
Pre-therapy
Relapse 1
Relapse 2
Relapse n
Cohort IA
Cohort IB
B
Percent of variant reads
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Pre-therapy
Relapse 1
Relapse 2
Relapse n
c.375+2T>A
p.R175H
p.E204X
p.S241Y
p.C242Y
p.R248Q
p.R249del
p.V272L
p.V274F
p.C275Y
p.E285K
3%
2%
1%
0%
Pre-therapy
Relapse 1
Relapse 2
Relapse n
C
patient no. 8
Percent of variant reads
4%
3%
2%
1%
0%
p.A84D
p.R110L
p.K120M
p.N131I
p.K132T
p.K132R
p.Q136E
p.Q136H
p.K139M
p.Y163S
p.Y163C
p.C176F
c.560-2A>T
p.I195F
p.Y220C
c.673-2A>C
c.673-2A>T
p.Y234S
p.Y234C
p.N239S
p.N239I
p.S241F
p.R248W
patient no. 178
Percent of variant reads
7%
6%
5%
4%
3%
2%
1%
0%
c.108_112del
p.A39V
p.P98R
p.T125T
c.376-1G>T
p.Y126H
p.Y163C
p.S166X
p.R175H
p.H179Y
c.559+1G>A
p.I195T
p.V216M
p.Y220C
p.G244D
p.G244V
p.R248W
p.R248Q
p.R249K
c.764_766del
p.L257Q
patient no. 354
Percent of variant reads
5%
4%
3%
2%
1%
0%
p.P151S
p.Y163C
p.V173L
p.R175G
p.Y220C
p.R248W
p.R248Q
p.R273H
p.R282W
patient no. 485
Percent of variant reads
6%
5%
4%
3%
2%
1%
0%
c.376-1G>A
p.K132R
p.K139T
p.Y163C
p.V173L
p.R175H
p.C176Y
p.C176F
p.H178D
p.H179Y
p.Y220C
c.673-2A>C
c.673-2A>G
c.673-2A>T
p.Y234H
p.Y234S
p.Y234C
p.N239S
p.G244S
p.G244C
p.G245R
p.M246V
p.R248Q
c.783-1G>A
p.R273C
p.R273H
p.C275R
p.C275F
p.C277Y
p.C277F
p.K319X
Pre-therapy
Relapse 1
Relapse 2
Relapse 3


50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Frequency
■ U-CLL
▨ M-CLL
GC>AT
AT>GC
GC>TA
GC>CG
AT>CG
AT>TA


Frequently observed events
FASAY/Sanger detection limit
Ultra-deep NGS detection limit
Pre-therapy
Relapse 1
Pre-therapy
Relapse 1
Relapse 2-n
Pre-therapy
Relapse 1
Relapse 2-n
Rarely observed events
FASAY/Sanger detection limit
Ultra-deep NGS detection limit
Pre-therapy
Relapse 1
Pre-therapy
Relapse 1
Relapse 2-n