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TP53 mutations at codon 234 are associated with chlorambucil treatment in chronic lymphocytic leukemia

Grégory Lazarian[1], Floriane Theves[2], Myriam Hormi[1], Rémi Letestu[1], Virginie Eclache[1], Audrey Bidet[3], Pascale Cornillet-Lefebvre[4], Frédéric Davi[5], Eric Delabesse[6], Marie-Hélène Estienne[7], Pascaline Etancelin[8], Olivier Kosmider[9], Sophy Laibe[10], Laurence Lode[11], Marc Muller[12], Nathalie Nadal[13], Dina Naguib[14], Cédric Pastoret[15], Stéphanie Poulain[16], Pierre Sujobert[17], Lauren Veronese[18], Samia Imache[1], Valérie Lefebvre[1], Florence Cymbalista[1], Fanny Baran-Marszak[1], Thierry Soussi[19,20]. On behalf of the French Innovative Leukemia Organisation (FILO)

1 Service d'hématologie biologique, CHU Hopital Avicenne, Bobigny, 93000, France
2 Service d'hématologie, CHU Hopital St Louis, Paris, 75010, France
3 Service d'hématologie biologique, CHU Bordeaux-Haut Lévêque, Bordeaux, 33000, France
4 Service d'hématologie biologique, CHU Hopital Robert Debré, Reims, 51100, France
5 Service d'hématologie biologique, CHU Pitié Salpétrière, Paris, 75013, France
6 Service d'hématologie biologique, CHU Toulouse, Toulouse, 31000, France
7 Service d'hématologie biologique, CHRU Tours, Tours, 37044, France
8 Departement d'onco-génétique, Centre Henri Becquerel, Rouen, 76038, France
9 Service d'hématologie biologique, CHU Hopital Cochin, Paris, 75014, France
10 laboratoire Bio-Santis, Cavaillon, 84300, France
11 Laboratoire d'oncobiologie, CHU Montpellier, Montpellier, 34000, France
12 Laboratoire de génétique médicale, CHU Nancy Hôpitaux de Brabois, VANDOEUVRE-LÈS-NANCY, 54511, France
13 Service de génétique chromosomique et moléculaire, CHU Dijon, Dijon, 21000, France
14 Service d'hématologie biologique, CHU Caen, Caen, 14033, France
15 Service d'hématologie biologique, CHU Rennes, Rennes, 35000, France
16 Service d'Hématologie Cellulaire, CHRU Lille, Lille, 59000, France
17 Service d'Hématologie Cellulaire, Hospices civils de Lyon, Lyon, 69002, France
18 Service de cytogénétique médicale, CHU Clermont-Ferrand, Clermont-Ferrand, 63000, France
19 Centre de Recherche des Cordeliers, Sorbonne Université, Inserm, Cell Death and Drug Resistance in Lymphoproliferative Disorders, F-75006, Paris, France.
20 Dept of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden

Correspondance:

Corresponding Authors: Fanny Baran-Marszak Service d'hématologie biologique, CHU Hopital Avicenne, Bobigny, 93000, France ; email fanny.baran-marszak@aphp.fr or Thierry Soussi, Dept of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden : email : thierry.soussi@sorbonne-universite.fr or thierry.soussi@igp.uu.se

Members of the French Innovative Leukemia Organization (FILO):

Thérese Aurran, Charles Herbaux, Guillaume Cartron, Caroline Dartigeas, Alain Delmer, Jehan Dupuis, Marie Sarah Dilhuydy, Emmanuelle Ferrant, Pierre Feugier, Franck Genevieve, Sophie De Guibert, Romain Guieze, Véronique Leblond, Vincent Levy, Stephane Leprêtre, Fatiha Merabet, Anne-Sophie Michallet, Florence Nguyen-Khac, Anne Quinquenel, Sophie Raynaud, Daniel Re, Catherine Thieblemont, Olivier Tournillhac, Xavier Troussard, Malgorzata Truchan-Graczyk, Lise Willems, Loic Ysebaert, Jean-Marc Zini.

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Author contributions

Conception and design: Florence Cymbalista, Fanny Baran-Marszak and Thierry Soussi.

Acquisition of data (acquired and managed patients, provided facilities, etc.): Grégory Lazarian, Floriane Theves, Myriam Hormi, Rémi Letestu, Virginie Eclache, Audrey Bidet, Pascale Cornillet-Lefebvre, Frédéric Davi, Eric Delabesse, Marie-Hélène Estienne, Pascaline Etancelin, Olivier Kosmider, Sophie Laibe, Laurence Lode, Marc Muller, Nathalie Nadal, Dina Naguib, Cédric Pastoret, Stephanie Poulain, Pierre Sujobert, Lauren Veronese, Samia Imache, Valérie Lefebvre, Florence Cymbalista, Fanny Baran-Marszak and the members of the French Innovative Leukemia Organization

Development of methodology: Flora Doffe

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Grégory Lazarian, Floriane Theves, Florence Cymbalista, Fanny Baran-Marszak and Thierry Soussi.

Writing, review, and/or revision of the manuscript: Grégory Lazarian Florence Cymbalista, Fanny Baran-Marszak and Thierry Soussi.

Study supervision: Florence Cymbalista, Fanny Baran-Marszak and Thierry Soussi.

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Conflict of Interest

"The authors declare no potential conflicts of interest."

Data Availability

All data are provided in the manuscript & supplementary files.

Letter to the editor

TP53 aberrations, including somatic mutations of *TP53* gene and 17p deletion, are a major predictive factor of resistance to fludarabine based chemotherapy in chronic lymphocytic leukemia (CLL) and remain an adverse prognostic factor in the chemofree era. The detection of deletion 17p and *TP53* gene mutations has become an integral part of routine diagnostic and should be performed before any administration of treatment [1]. TP53 mutations typically occur all along the DNA binding domain of the p53 protein. However, the functional role or the effect of each *TP53* variant remains elusive. In this study, we aimed at characterizing the profile of the *TP53* variants in CLL, their distributions and a correlation with clinical data. To this end, we retrospectively analyzed a large collection of 568 CLL-associated *TP53* variants in 336 patients compiled from centers affiliated with the French Innovative Leukemia Organization-CLL (FILO). Fluorescence in situ hybridization (FISH) analysis for del(17p) status was available for 207 patients of which 108 (52%) harbored a 17p deletion. Based on the *IGHV* mutation status, most patients belong to a high risk group as 73% (172/236) were *IGHV* unmutated (**Supplementary Table S1**).

TP53 mutational status was assessed from blood samples using NGS technologies (either Illumina or Ion Torrent technologies) covering exons 2 to 11, with a sensitivity allowing the detection of subclones with variant allele frequency (VAF) >1%. Both amplicon- and capture-based approaches available in commercial kits or custom gene panels including the *TP53* gene were used. Polymorphisms were carefully excluded using the gnomAD database and the new *TP53* SNP data included in the most recent version of the UMD_TP53 database [2]. Classification of *TP53* variant pathogenicity was performed in accordance with ACMG criteria and based on population data and *TP53*-specific functional information previously described [3].

Among the 568 *TP53* somatic mutations, the majority were missense variants (n=429, 75%) (**Supplementary Table S2**). Using ACMG criteria from the UMD_TP53 database, 518 variants were classified as pathogenic, 42 were likely pathogenic and 8 were VUS (Variants of Unknown Significance). As expected, *TP53* variants were predominantly located in the DNA-binding domain, with classical CpG-related hot spots at codons 175, 248 and 273. (**Fig. 1A**). Nevertheless, an unusual mutation hotspot occurring at codon 234 was identified. A total of 25 single nucleotide variants at codon 234 was observe in 24 patients with one patient harboring 2 mutations: 22 mutations p.Tyr234Cys (c.701A>G), 2 mutations

p.Tyr234Ser (c.701A>C) and 1 mutation p.Tyr234His (c.700T>C). It was the second most common missense variant of our cohort (25/568; 4.4%) and ranked fifth in the entire CLL subset of the UMD_*TP53* database (**Fig. 1A, and Supplementary Figure S1A**). Mutations at codon 234 were found predominantly at a subclonal level (median of variant allele frequency VAF=11% [1.8%-51%]) but with VAFs distributed in the same range as those observed for other hotspot codons (**Fig. 1B**). Analysis of the UMD_TP53 database showed that the frequency of mutations occurring at hotspot positions 175, 248 and 273 were similar to that observed in other cancers, while the frequency of mutations at codon 234 reached 4% in CLL and was very low (less than 1%) in the other malignancies (**Fig. 1C and Supplementary Figure S1B**), suggesting that this mutation is highly specific of CLL cases.

Both *in silico* predictive algorithms and structural studies suggested that variant p.Tyr234Cys was deleterious, which was confirmed by our functional analysis [4]. Indeed, the overexpression of p.Tyr234Cys by transfection in the p53 null H1299 cell line was unable to induce growth arrest, similar to p.Arg175His, while wild-type p53 or p.Thr312Ser (a variant without loss of function) lead to a profound reduction of colony number (**Fig. 1D)**. On the other hand, in a luciferase assay, p.Tyr234Cys was unable to transactivate a reporter gene with the *CDKN1* response element (**Fig. 1E**). In line with these results, the large-scale analysis performed by Kotler et al. showing the relative fitness score (RFS) for each TP53 variant (defining its capacity to induce growth arrest in H1299 cells) indicates that the majority of TP53 variants at position 234 are dysfunctional (**Supplementary Figure S2**) [5]. Altogether, these results indicate that TP53 variant p.Tyr234Cys is devoid of any transactivating and anti-proliferative activities.

In the present study, CLL patients frequently harbored multiple subclones with different *TP53* mutations. A single *TP53* variant was detected in 66% (n=222) of the patients (SM-patients), whereas two, three, or more TP53 variants were found respectively in 17% (n=57), 10% (n=33) and 7% (n=24) of the patients (polymutated patients: PM-patients) (**Supplementary Table S2** and **Supplementary Figure S3A**). Interestingly, while mutations at classical *TP53* hot-spot positions (codons 175, 248 or 273) were observed both as single and associated mutations, we noticed that variants at codon 234 were found mostly in polymutated patients (82% cases), highlighting an important intratumoral heterogeneity in cases harboring this mutation (**Fig. 2A**). Strikingly, analysis of the variants associated with mutations at codon 234 reveals a strong association with a splicing mutation c.673-2A (p<0.0001, Fischer test) (**Fig 2B**). The frequency of this mutation was 2.6% (15/572) in the

entire cohort and rose up to 34% (9 cases) in the 24 patients with mutation at codon 234. In contrast, it was associated with mutation at codon 248 or codon 273 in only 1 case. Consistently, the reanalysis of sequencing data from published cohorts gathering altogether 272 *TP53* mutated patients showed a frequency of 3.3% of mutation at codon 234 (9 cases), among them 5 were associated with the mutation at position c.673-2A (62.5%) [6–9]. Mutation at position c.673–2A at the splice acceptor site in exon-7 generates a p53 truncated protein [10] which functionally and molecularly resembles the naturally occurring alternative p53 splice variant, p53-psi. Shirole et al. have shown that "p53-psi like" proteins lacks canonical p53 tumor suppressor activities but promote cancer cell proliferation, survival, and metastasis [11]. These mutants can localize to the mitochondria where they promote tumor phenotypes by binding and activating the mitochondria inner pore permeability regulator, Cyclophilin D (CypD). However, the functional relationship between mutation at position c.673–2A>G and p.Tyr234Cys or whether one of these mutations promote the acquisition of the other remains elusive.

Clinical data were available for 222 patients. *TP53* sequencing was performed before treatment for 81 patients (37%). The type of treatment significantly impacted the nature of the *TP53* variant and the number of associated mutations in patients. Indeed, variants at codons 175 and 248 were observed in both treated and untreated patients whereas 100% of patients who harbored a variant at codon 234 had been treated (**Fig. 2C**). Moreover, while most treatment-naïve patients had a single mutation (n=73/81; 90%), nearly half of previously treated patients (n=66/141; 47%) had more than one *TP53* variant (from 2 to 11 mutations) ($p<0.0001$), showing that the exposition to a therapy promote the clonal evolution of *TP53*. (**Supplementary Figure S3B and S3C)**. Fifty nine percent (53/89) of patients who received one line of chemoimmunotherapy (fludarabine, cyclophosphamide and rituximab (FCR) or bendamustine and rituximab (BR)) had a single mutation while those who received only continuous chlorambucil treatment (CLB) were mostly polymutated (32/44; 73%, $p=0.0012$ with Yates correction), suggesting that CLB has a stronger capacity to drive clonal diversification (**Fig. 2D**). Furthermore, variants at codon 234 were found exclusively in treated patient (24/141; 17%) and in 43% of patients treated with CLB (19/44). In contrast, only 5.7% of patients treated with FCR and/or BR harbored this mutation (5/89) (**Fig. 2E**). Eventually, considering the association between mutation at codon 234 and c.673-2A, the 9 patients displaying the 2 mutations were all treated with CLB while this co-occurrence were not found in patients treated with fludarabine and/or BR. The co-occurence of the 2

mutations was significantly associated with CLB treatment ($p < 0.0001$) with an Odd Ratio of 26 [IC95%; 10.7 – 69.3].

Taken together, our data support a role of the alkylating agent CLB in the appearance and/or the selection of mutations at codon 234 and possibly the truncating mutation c.672-2a. No pre-CLB treatment sample was available for patients with Tyr234Cys mutations to test for *TP53* mutation status. However, in contrast to the other hot-spot mutations such as those at codon 175 or 248, the absence of variants at position 234 in any of the untreated patients in our database strongly supports the possibility that this mutation is associated with the mutagenic effect of CLB. Furthermore, the absence of this variant in a recent cohort of CLL patients in which none received CLB argues against the hypothesis of the specific selection of this mutation in CLL. This finding is reminiscent of the association between exposure to certain carcinogens (aflatoxin B1 in hepatocellular carcinoma and benzo(a)pyrene in lung cancer) and *TP53* hot-spot variants in codon 249 or 157 [12]. Whether or not chlorambucil adduct occurs at codon 234 or the splicing site 6.732-2A of the *TP53* gene remains to be tested.

References

Legends

Figure 1: Identification of TP53 mutation hot spots in CLL in codon 234.

A: Distribution of mutations at each codon of the TP53 protein. Only single nucleotide substitutions are analyzed. Classical TP53 hot spot mutations are indicated in blue with codons 175, 248 and 273 found in every type of cancer. The CLL-specific hot spot at codon 234 is shown in red. TP53_UMD: all cancers included in TP53_UMD; CRC: colorectal carcinoma: breast ca: breast carcinoma; CLL_UMD: CLL patients included in the UMD_TP53 database excluding FILO data: CLL_FILO: patient from the FILO cohort. **B:** VAF is similar for the various TP53 hot spot variants. Kruskal-Wallis Test was conducted to examine VAF differences among the various hot spot variants. No significant differences were observed (p=0.1106). **C:** p.Tyr234Cys is more frequent in CLL. Frequency of p.Arg248Gln, a classical hotspot variant and p.Tyr234Cys in various types of cancer. Dark blue: CLL_FILO cohort; Light blue: CLL_UMD database; Red: other cancers; Green: cancers known to be strongly associated with carcinogen exposure. **D:** Overexpression of TP53 in the p53 null H1299 cell line was performed to check the antiproliferative activity of the various variants. Although, wt TP53 or p.Thr312Ser (a variant that does not display any loss of activity) leads to a profound reduction of colony number, p.Tyr234Cys and the hot spot variant p.Arg175His are unable to induce growth arrest. T: untransfected cells. H1299 cells were plated into 6-well plates and transfected on the following day with Lipofectamine 2000 (Life Science). Twenty-four hours after transfection, cells were dissociated and plated at a density of 5,103 cells per well in 6-well plates in selective media with G418 at a concentration of 1 mg /ml. Cells were then stained after 14 to 16 days with crystal violet. **E:** p.Tyr234Cys is unable to transactivate a reporter gene with the WAF1 response element. Luciferase assay was performed in H1299 cells plated in 96-well plates (2,000 cells per well). After 48 hours, cells were transfected using Lipofectamine 2000. Seventy-five nanograms of reporter gene and 5 ng of p. 53 plasmid were used for each well. The luciferase activity was tested 24 hours after transfection. Each assay was performed in triplicate and TP53 variants were tested at least 4 times in separate experiments.

Figure 2 : TP53 variant p.Y234C is found predominantly in polymutated patients who received Chlorambucil.

A: Variant number for each hot spot variant from the FILO cohort is shown on the y-axis. SM: patients with a single TP53 mutation; DM: patients with two TP53 mutations; MM3 to MM11: patients with 3 to 11 mutations. **B:** Association between c.673-2A and p.Y234C variants. **C:** Variant number for each hot spot variant from the FILO cohort is shown on the y-axis. TT0: no treatment; TT1 to TT7: patients who received 1 to 7 lines of treatment. **D:** Distribution of PM-patients according to treatment. FCR: fludarabine, cyclophosphamide and rituximab; BR: bendamustine and rituximab; CLB: chlorambucil. SM: Patients with a single *TP53* variant; DM: patients with two *TP53* variants; MM3: patients with three *TP53* variants; MM4+: patients with four or more *TP53* variants. **E:** Distribution of mutations in codon 234 according to treatment with or without chlorambucil.

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