Assessment of TP53 functionality in chronic lymphocytic leukaemia by different assays; an ERIC-wide approach

Chronic lymphocytic leukaemia (CLL) is characterized by an extremely variable clinical course, in which deletions of TP53 and ATM, regulators of the DNA-damage response (DDR)-pathway, are powerful predictors for adverse outcome and response to chemotherapy (Dohner et al, 2000). Deletions of chromosome 17p (TP53) and 11q (ATM) coincide with TP53 and ATM mutations respectively, however with markedly different frequencies (80% and 40% respectively; Malcikova et al, 2009; Navrkalova et al, 2013). Sole TP53 mutations and deletions usually lead to TP53 (p53) dysfunction (Mohr et al, 2011) and, in case of deletions in ATM, a mutation of the residual ATM allele determines whether there is complete ATM inactivation resulting in TP53-dysfunction (Navrkalova et al, 2013). Therefore, analysis of mutations in TP53 and ATM genes in addition to fluorescent in situ hybridization (FISH) analysis is of additional clinical value (Skovronska et al, 2012). However, mutational analysis of TP53 and ATM is currently not standardized and is challenging, especially for ATM due to extreme gene size with lack of well-characterized mutations (Navrkalova et al, 2013). Chemoresistance might additionally be a consequence of epigenetic and post-transcriptional factors or deregulations of other components of the DDR. Therefore, functional read-outs of the ATM/TP53 axis might add clinical relevant information on the actual DDR and chemo-sensitivity over FISH analysis, as well as important biological insight on the signalling capacity of the axis.

There are different approaches to explore the ATM/TP53 axis at the functional level; i.e. at baseline or following a TP53 stimulus (irradiation or chemotherapy) and by different read-outs; i.e. measuring expression of responsive targets (RT-PCR) of CDKN1A (p21) levels at baseline (Mohr et al, 2011; Mohr et al, 2011), (ii) RT-PCR/CDKN1A assesses RNA CDKN1A (p21) levels after irradiation (Mohr et al, 2011), (iii) reverse transcription multiplex ligation-dependent probe amplification (RT-MLPA) of CDKN1A (RT-MLPACDKN1A),

References


Fig 1. Robustness, reproducibility and correlation of different TP53-function assays. TP53-function assays were performed blindly on freshly frozen peripheral blood mononuclear cells (PBMCs) of 16 chronic lymphocytic leukaemia (CLL) samples (CD19/CD5 > 90%) which were exchanged between 5 different research groups. Each group provided 3 (one group 4) samples containing at least two samples harbouring a 17p and/or 11q deletion. (A) Each assay was performed in 2 different laboratories with ample experience with particular assay as indicated. RT-PCR CDKN1A in Amsterdam was performed as part of the RT-MLPA assay. Results of the same assays performed in different laboratories were compared. (B) Freshly frozen PBMCs of 5 CLL samples were again exchanged between the different research groups in a blinded fashion and results were compared with the results in the first exchange. For RT-MLPA the geometric mean of the gene induction of the 4 TP53-target genes in each sample was taken. (C). Results of the 4 different TP53-function assays were compared in relation to each other. In case one assay was performed in 2 facilities, the results of one representative assay were used for subsequent analyses. Comparisons are shown in a side-by-side table showing correlation coefficients \( r \)-values in a grey-scale scheme as depicted in the figure and \( P \)-values are indicated in the boxes; *0·01 ≤ \( P \) < 0·05, **0·001 ≤ \( P \) < 0·01 and *** \( P \) < 0·001. For (A–C) Spearman’s rank correlation. \( P \)-value <0·05 was considered statistically significant.
Fig 2. Performance of TP53-function assays in detecting TP53 and ATM aberrations. (A) Chronic lymphocytic leukaemia (CLL) samples are hierarchically clustered based on the detected level of expression of genes (or gene-sets in case of RT-MLPA). Each graph shows the results of one RNA-based assay. Each bar represents the results of one CLL sample. (B) Results of each RNA-based assay are shown in a standard false colour format. Data were normalized by subtracting the median of the results of all samples within one assay. The resulting matrix with positive and negative values was imported in the program MultiExperiment Viewer (www.tm4.org), and assigned green and red colours for negative and positive values, respectively. The CLL samples are grouped into 'TP53 aberrations', 'ATM aberrations' and 'WT' (wildtype). (C) Results of the FACS based assay is depicted as CDKN1A induction versus baseline TP53 levels and CDKN1A induction versus TP53 induction. Every dot represents 1 CLL sample. The cut-off values (depicted by dashed lines) are based on a previous study (Lin et al, 2012). (D) Results of the FACS-based assay are shown in a color format using MultiExperiment Viewer. Values lower than the previously established cut-off (Lin et al, 2012) for given parameters were assigned green. Values higher than previously established cut-off were assigned red. The CLL samples are grouped into 'TP53 aberrations', 'ATM aberrations' and 'WT' (wildtype). Types of defects are shown below the figure. Type A, B, C and D defects are determined based on earlier described discrimination, as summarized in Table SIII.
BAX, BBC3 and FAS, a multiplex RT-qPCR, measures the RNA level of these 4 different TP53 target genes following irradiation (Mous et al., 2009), and (iv) flow cytometry of TP53-CDKN1A (FACSp53-p21 assay) utilizes flow cytometry to determine basal protein TP53 level and protein TP53 and CDKN1A-induction following etoposide/nutlin-3a treatment or following irradiation (Table S1; Le Garff-Tavernier et al., 2011; Lin et al., 2012).

Thus far, these assays have independently shown their utility in detecting TP53 and, to a lesser extent, ATM aberrations. The present study aimed to study: (i) intra-assay reproducibility, (ii) inter-assay correlation and (iii) ability of the different available TP53-function assays to detect TP53 and ATM aberrations by a blinded comparative side-by-side analysis in those participating laboratories (n = 6) that previously developed these assays. In total, 16 different CLL samples were characterized for TP53 and ATM status using FISH, Sanger sequencing and functional analysis of separated allele in yeast (FASAY) (Malckikova et al., 2009; Navrkalova et al., 2013) (Tables SII and SIII). Seven patients carried TP53 aberrations (n = 6 del(17p) + TP53 mutation; n = 1 sole TP53 mutation), 5 ATM aberrations (n = 1 del(11q) + ATM mutation; n = 4 sole del(11q)) and 4 were wild type (WT) for both genes.

First, results of the same assay performed at different facilities were compared to study intra-assay correlation. RNA-based assays were highly reproducible (RT-PCR/MIR34A r = 0.96; RT-PCR/CDKN1A r = 0.91; Fig 1A). As the FACSp53-p21 assay generates 3 types of results, these data were analysed separately. Overall, FACSp53-p21 data showed lower correlation (basal TP53 level r = 0.51; TP53-induction r = 0.63, CDKN1A-induction r = 0.45; Fig 1A).

To study the reproducibility of the various assays within one centre, 5 of the above-used samples were again exchanged (blinded). Results of RT-PCR/MIR34A, RT-MLPA and RT-PCR/CDKN1A assays were highly comparable (r = 1.0) for each assay (Fig 1B). However, although the correlation coefficients for FACSp53-p21 between the results of the first and second exchange were comparable for basal TP53 level and TP53-induction (r = 0.72 and r = 1.0 respectively), they were low for CDKN1A-induction (r = -0.15; Fig 1B).

Next, the results of the 4 different assays were compared with each other to study inter-assay correlation. When one assay was performed in 2 facilities, the results of one representative assay were used for subsequent analyses. The RNA-based assays showed significant correlation in relation to each other and in relation to basal TP53 and TP53-induction, but no correlation to CDKN1A-induction of the FACS-based assay (Fig 1C).

Given that the RT-MLPA assay generates 4 and the FACSp53-p21 3 types of results, the correlation of these parameters within each assay was also compared, showing significant correlation, with correlation coefficients ranging between 0.64 and 0.96 for the RT-MLPA and 0.63 and 0.75 for FACSp53-p21 (Fig S1).

Finally, the performance of all assays in detecting TP53 and ATM aberrations was investigated. As expected, all WT patients showed clear upregulation of their respective genes in RT-MLPA and RT-PCR/CDKN1A after irradiation. Also, basal levels of MIR34A were relatively high in WT samples, as expected, and FACSp53-p21 correctly assigned all WT samples showing low basal levels of TP53 and upregulation of TP53 and CDKN1A after irradiation (Fig 2A–D). Altogether, all samples harbouring a complete disruption of TP53 (del (17p) + TP53 mutation) were assigned dysfunctional, whereas the one sample with a sole TP53 mutation was classified as functional by all assays (Fig 2D+ Table SII). Concerning the samples with an ATM aberration, all assays showed a defective response in the sample with both ATM mutation and deletion. Regarding the 4 samples with a sole (del11q), the RNA-based assays showed clearly defective and clearly functional TP53 responses in one sample each (samples 9 and 12, respectively). Results of the two remaining samples differed among the assays, whereas FACSp53-p21 showed defective responses in all samples that were mainly defined as type B.

In conclusion, this study demonstrates that RNA-based assays are robust and highly reproducible both within and between different centres. In contrast, the FACS-based assay was less reproducible both within and between different centres, specifically for CDKN1A-induction. Results of the different RNA-based assays strongly correlated with each other in case of WT samples, (del)17p + TP53 mutated and del(11q) + ATM mutated samples. However, labeling of samples harbouring a sole del(11q) varied between the assays. From a practical point of view RT-PCR/MIR34A is the easiest and fastest assay to perform, because activation of the DDR-pathway is not necessary.

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**Author contributions**

G.D.R. designed and performed experiments, analysed data and wrote the paper; I.M. performed mutational analysis of TP53 gene and reviewed the manuscript; M.M. performed the analysis of MIR34A expression and reviewed the manuscript, M.T. contributed to TP53 and ATM mutation analyses and reviewed the manuscript; M.G.F. and K.L., performed experiments and reviewed the manuscript; H.M.B, R.G., O.M., S.P., A.R.P., T.S., M.H.O., E.E., S.S. reviewed the
Conflicts of interest

The authors declare no conflicts of interest.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

- **Fig S1.** Correlation of different parameters of RT-MLPA and FACSp53-p21.
- **Table S1.** Different types of defects detected by FACSp53-p21.
- **Table SII.** Genotypic characteristics of 16 exchanged CLL samples.
- **Table SIII.** Types of identified mutations in TP53 and ATM.