Utility of multiplex ligation-dependent probe amplification in the copy number profiling of oncohematological diseases

Ph.D. Thesis

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Budapest
2020

1. IRODUCTION

Research and diagnostics of malignant oncohematological diseases have been at the forefront of discovering the genetic processes underlying cancer on several occasions. Thanks to comprehensive studies with rapidly evolving molecular genetic techniques, the genetic background of numerous haematological malignancies can be mapped in detail, identifying markers that may aid the diagnosis, subclassification, prognosis, selection of the most effective therapy, monitoring residual tumor mass and may even serve as targets for targeted treatments. Screening of key markers with molecular tests has now become an integral part of the day-to-day diagnosis and monitoring of oncohematological diseases, however, the identification of novel abnormalities of clinical significance remains the subject of extensive research.

Gene expression, genomic "array" -based, and next-generation sequencing (NGS) studies over the past decade have revealed a number of novel, clinically relevant genetic alterations at the whole exome and genome levels. In addition to point mutations and smaller deletions and insertions of a few base pairs, aberrations involving more extensive chromosomal regions often occur, including balanced as well as unbalanced subchromosomal aberrations. Unbalanced aberrations, i.e., DNA copy number aberrations (CNA), typically lead to copy number shortages (deletions) or copy number surpluses (gain). Incorporating the examination of newly identified clinically significant abnormalities into the diagnostic workflow requires fast and reliable methods to examine the aberrations of a given entity in the most comprehensive but cost-effective manner with minimal human effort.

The resolution, representativeness, scalability and / or throughput of the methodologies currently used in Hungarian practice are limited. There is a growing need for comprehensive detailed examination of CNAs, as they are present in 20-70% of hematological malignancies and in many cases have clinical relevance. Based on all this, a method by which a large number of loci can be examined simultaneously in high resolution with a short turnaround time could

effectively contribute to the diagnostic examination of the genetic background of oncohematological diseases.

Multiplex ligation-dependent probe amplification (MLPA) is a polymerase chain reaction (PCR) -based molecular genetic method that allows the detection of unbalanced aberrations with exon-level genomic resolution through targeted analysis of genomic DNA, as well as the detection of point mutations. The method gives results within 24 hours and DNA methylation patterns of specific loci can be examined with minimal modification of the protocol. In recent years, the so-called digital MLPA (dMLPA) procedure has also been developed, combining the capabilities of the Illumina NGS platform and the technical background of MLPA, allowing the simultaneous examination of an order of magnitude more loci than conventional MLPA in a targeted manner, but due to the large number of probes with significantly higher resolution.

There are several reports in the international literature on the use of conventional MLPA in oncohematological diseases, demonstrating the suitability of the method for genetic testing of malignant hematopoietic diseases. One of the earliest MLPA publications on oncohematology, published in 2006 by Buijs et al., identified known recurrent copy number abnormalities as well as additional deletions and chromosome aberrations not detected by iFISH in 54 patients with chronic lymphocytic leukemia (CLL). Subsequently, several other working groups also used the method to examine CLL, mostly to detect aberrations of prognostic significance. The cost-effectiveness, high throughput, and moderate labor input requirements of MLPA have been confirmed in several cases.

In the first study of unbalanced aberrations of prognostic and predictive significance of multiple myeloma (MM) with conventional MLPA, the method identified abnormalities in 65% of patients that were not detected by standard iFISH tests. However, since MLPA is not able to detect balanced genetic abnormalities, nor are the translocations of the IGH gene of major importance in MM, its combinaiton with iFISH is necessary in this case. Subsequently, the effectiveness of conventional MLPA for the study of MM was confirmed by other working groups.

The most intense publication activity regarding MLPA is seen in ALL. First in 2010, Schwab et al. applied the method to samples from patients with precursor B-cell ALL. Among other things, small deletions not detectable by iFISH were identified with MLPA and successfully validated by quantitative PCR. The added value of MLPA is also clearly indicated by the fact that the technique has since been used successfully in clinical trials involving hundreds, sometimes over a thousand patients. Because MLPA does not provide unique cell-level information on the distribution of each aberration, it does not appear to be an optimal tool for mapping the complex subclonal architecture specifically characteristic of some subtypes of ALL. However, comparing samples taken at the time of diagnosis and relapse, examining the extent of the loci affected by aberrations with multiple probes may allow the discovery of the clonal relationship of the two malignant cell populations dominant at different time points, thereby providing informative insights into subclonal evolutionary processes.

Overall, without intending to be exhaustive, the examples presented above clearly indicate that conventional MLPA is a versatile technique for the study of oncohematological diseases, including its use in the diagnostic workflow. However, before the routine clinicopathological application of conventional MLPA on a wider scale or the adaptation of the new dMLPA technique, it is worth conducting further studies to validate their efficacy and reliability.

2. OBJECTIVES

In our work, we aimed to directly compare the efficacy and applicability of conventional MLPA and dMLPA techniques with methods widely used in everyday routine diagnostics for the study of hematological malignancies. In our study, we examined samples from patients with various lymphoid malignancies, such as mature B-cell CLL and MM, and immature B- and T-cell pediatric ALL. We paid special attention to the detection of prognostic and predictive alterations of clinical significance, as well as to the mapping of the biological processes leading to the relapse of the studied diseases (e.g., clonal evolution).

Taking all these aspects into account, our specific objectives were:

- Setting up a conventional MLPA technique for the analysis of clinical samples from CLL patients and analyzing the reliability of the method compared to iFISH. Furthermore, we aimed to use the method to detect changes in DNA copy number over time in patients receiving ibrutinib therapy.

- Investigation of the efficacy of dMLPA method in MM in the determination of subchromosomal and whole chromosome CNAs compared to iFISH and conventional MLPA.

- Validation of the efficacy of the dMLPA method in a large study of disease-relevant CNAs in a group of patients with B and T-ALL, in which we evaluate the added value of the results in detecting clonal processes leading to relapse and classifying patients into prognostic groups.

3. METHODS

Samples

CLL samples

In our study, we included patients with at least one genetic abnormality detected by iFISH in a routine genetic study of treatment naïve CLL. We also examined the pre- and post-treatment CNA status of 5 patients receiving ibrutinib therapy and the CNAs in the peripheral blood and lymph node samples taken during progression in one ibrutinib resistant patient.

MM samples

In our study, we examined bone marrow samples from 56 MM patients at diagnosis. Following immunophenotyping by flow cytometry, in cases where plasma cell ratios below 20-30% were measured, samples were subjected to plasma cell enrichment with magnetic beads and the success of enrichment was verified by second round immunophenotyping.

Pediatric ALL samples

A total of 91 pediatric bone marrow samples were examined, 76 of whom had B-ALL and 15 had T-ALL. In addition to the diagnostic sample of 91 patients, a total of 14 relapse-time samples were available from 12 patients, dating from the time of the first or second relapse of the disease. An average blast ratio of 81% (35-100%) was measured in the samples by flow cytometry.

Conventional MLPA reactions

MLPA reactions, including the use of negative control samples, were performed according to the manufacturer's instructions. The CNA status of the samples for each locus was determined after normalization within and between samples, taking into account the tumor cell ratio determined by flow cytometry.

Probe mixtures of SALSA MLPA P037 CLL-1 and SALSA MLPA P038 CLL-2 provided by the manufacturer were used to examine CLL patient samples. For the examination of MM SALSA P425 v. A1 MLPA probe mixture was used, which contains 42 probes targeting the genomic locus often involved in MM. In the pediatric ALL study, one or more MLPA probe mixtures from P202-A1, P202-B2, P335-A4, P335-B2, and P383-A2 were used.

dMLPA reactions

Each dMLPA reaction was performed according to the manufacturer's instructions using 40 ng of DNA. For the MM study, we used the dMLPA D006 probe mix (lot X1-0613) developed by MRC Holland in 2017 and made available to the cooperating laboratories by the manufacturer for testing and validation. In the pediatric ALL study, dMLPA D007 (version D007-X2-0516) probe mixture was used.

Additional tests

iFISH reactions were performed according to the probe manufacturer's instructions. In each case, the signal pattern of 200 nuclei were analyzed with the involvement of two independent experts.

Detection and quantification of the V600E mutation in the *BRAF* gene was performed on a PyroMark Q24 system using 100 ng of DNA according to the manufacturer's instructions. The presence of the *BRAF* V600E mutation was also examined by digital droplet PCR (ddPCR), which exceeds the sensitivity of pyrosequencing. For the reaction, 50 ng of DNA and commercially available BRAF assays were used to detect wild-type and mutant targets according to the manufacturer's instructions.

Targeted amplicon sequencing of VDJ segments of the *IGH* gene was performed on pairs of patients with diagnosis and relapse at childhood B-ALL. During the reaction, primers binding to conserved regions of the VDJ regions of the *IGH* gene were subjected to multiplex polymerase chain reaction on 50-100 ng of genomic DNA.

Statistical analysis

The congruence between dMLPA, iFISH, and conventional MLPA data was examined by Fischer exact test. The correlation between the occurrence of IGH clonotypes identified by FR1 and FR2 assays in samples from patients with B-ALL was determined by the Pearson correlation coefficient. Event-free survival (EFS) was defined as the time interval from the start of treatment to relapse, second malignancy, or disease-related death in patients with B-ALL. Survival rates were estimated by Kaplan-Meier method and compared by log-rank test.

Ethical aspects

In all our research, we acted in accordance with the applicable legislation. Ethical Committee approvals were obtained for our studies study (ETT-TUKEB: 14383-2 / 2017 / EKU and 45371-2 / 2016 / EKU), which were conducted in accordance with the Declaration of Helsinki.

4. RESULTS

Application of MLPA in CLL

A total of 59 aberrations were detected by conventional MLPA in 18 patients with CLL who did not receive targeted therapy. During iFISH analysis of the samples, we successfully validated 38 aberrations identified with conventional MLPA. Together, the two methods detected 63 aberrations in 18 patients, with an average of 3.5 aberrations per patient.

Clonal evolutionary changes following ibrutinib therapy were studied in paired samples of 5 ibrutinib treated patients. In one patient, only 8q excess and 9p deficiency were detected before the start of treatment, while for the third month of treatment, in addition to the previous aberrations, 2p excess, 6q deficiency, 8p deficiency, 13q deficiency and 17p deficiency were also detected. Two patients had no detectable aberrations with MLPA at baseline, and this condition persisted in both cases after one year of treatment. One patient did not carry detectable aberration with MLPA prior to ibrutinib treatment, but 13q deficiency and 17p deficiency were detected in the 8-month sample. The last patient had 11q deficiency and 13q deficiency at diagnosis, but no aberration was observed in the 12-month sample.

In a high-risk patient treated with ibrutinib but showing signs of clinical progress, peripheral blood and lymph node samples were examined. Conventional MLPA examination of a DNA sample isolated from the enlarged lymph node containing tumor cells that underwent Richter's transformation according to the histological examination revealed 6q deficiency, 8q excess, and 13q deficiency, while examination of peripheral blood CLL cells revealed only 13q deficiency. These results supported our hypothesis, based on the results of other molecular genetic studies, that the therapeutic resistance and consequent relapse of the studied patient resulted from the complex convergent evolution of the disease based on spatial heterogeneity.

Examination of copy number aberrations in MM with dMLPA

The relative copy number of 371 genomic loci in each patient sample was examined by the dMLPA method. In our study, a total of 210 whole chromosome aberrations with dMLPA were detected in 47 patients (84%), of which 65 were feletions and 145 were excesses. The most common aberration was chromosome 13 monosomy, which occurred in the majority of cases in non-HD cases. Of the subchromosomal CNAs, 246 were detected, of which the 1q excess was the most frequently observed aberration. In each case, there was an average of 4.4 CNAs (range: 0–13), which averaged 3.7 in the hyperdiploid subgroup and 4.8 in the non-HD group.

The efficiency of the dMLPA method could be tested directly for 6 abberations compared to conventional MLPA and 4 aberrations compared to iFISH. Based on the comparison of the three methods, we obtained consistent results in 319/336 data points (56 patients x 6 examined differences) which corresponds to 95% congruence.

The point mutation c.1799T> A (p.V600E) affecting exon 15 of the *BRAF* gene was identified by dMLPA in two, however, its presence could only be validated by pyrosequencing in one sample. Because the mutation was also indicated by two independent dMLPA reactions in both patients, mutation analysis was also performed with ddPCR in the other patient, which confirmed the result obtained with the dMLPA assay due to its higher sensitivity.

Investigation of CNAs in pediatric ALL

With dMLPA, all 105 samples collected from patients with pediatric ALL (91 diagnostic and 14 relapse samples) were successfully analyzed. A dilution series was prepared from a sample of a patient with 7 mono- and 2 biallelic aberrations on their somatic chromosomes, and dMLPA reliably detected clonal copy number differences in the diluted samples up to a leukemic cell ratio of 30%.

In our study, we detected a total of 502 copy number aberrations in 87/91 diagnostic samples (96%). On average, 5.4 CNAs were detected per patient (B-ALL: 6, T-ALL: 2.6), while the mean incidence of subchromosomal abnormalities per patient was 2.8 (B-ALL: 2.9; T-ALL: 2 5). The 95% of total chromosome aberrations

were identified in patients with HD karyotype (3-14 chromosomes per patient affected). Subchromosomal CNAs were identified in 77 patients (B-ALL: 63, T-ALL: 14), a total of 218 in samples from B-ALL patients, and 33 in T-ALL cases. The most common aberration was CDKN2A / B defect, which occurred in 38% of B-ALL and 87% of T-ALL.

A total of 26 diagnosis and relapse sample pairs from 12 patients (B-ALL: 8, T-ALL: 4) were examined. Chromosomal and subchromosomal copy number differences were identified in 11 patients, and normal copy number was observed in 1 patient.

In a comparative study of diagnosis and relapse samples, and in exploring the clonal relationships between them, we identified three different patterns: (i) completely identical copy number status in a patient's diagnosis and relapse samples; (ii) all of the CNAs present in the diagnostic sample of six patients were also present at the time of relapse, however, new aberrations also appeared; (iii) in the relapse sample of four patients, in addition to the newly occurring aberrations, only a part of the differences in the diagnosis were detectable. The most likely explanation for this is that the subclone prevailing at the time of relapse and the cells dominant in the sample at the time of diagnosis evolved from a common ancestral clone by parallel evolution. Two patients also had samples available from 3 time points, so in these cases it was possible to examine the CNAs even at the second relapse of the disease. In one patient, the leukemic cell population appearing at the second relapse presumably developed from a previous ancestral cell population, while in the other patient, the CNA profiles seen during the first and second relapses suggest a direct clonal association.

Copy number differences detected with dMLPA were validated with 121 conventional MLPA reactions in 97/105 patient samples included in the study. Statistical comparison of the results of the two studies showed a high concordance of 936/949 (98.6%).

Based on the CNAs determined by dMLPA, each B-ALL patient was classified into a genetic risk group. In the group of patients we examined, we found a significantly shorter EFS in the case of *IKZF1* deficiency, regardless of the existence of criteria in the

IKZF1^{plus} group. Compared to conventional MLPA probe mixtures, dMLPA D007 also allows the study of a number of additional gene / genomic regions frequently involved in B-ALL, so we examined the possibility of establishing an alternative risk classification that is more detailed for B-ALL patients based on the CNA profile of disease-relevant regions. Considering the copy number differences detected by dMLPA alone, 4 different groups characterized by EFS were defined.

Similar to the UKALL risk assessment strategy, we integrated our CNA classification based on dMLPA data with cytogenetic risk groups, which were defined according to the UKALL classification. Following visual evaluation of event-free survival curves resulting from a combination of different copy number differences and cytogenetic risk groups, 4 combined genetic groups were identified that can be characterized by significantly different survival rates.

5. CONCLUSIONS

The conventional MLPA is a robust method in the identification of complex genetic alterations and in the profiling of copy number changes associated to ibrutinib therapy including alterations leading to treatment resistant disease via spatial heterogeneity.

To our knowledge we were first to examine the application of dMLPA in MM. Our results suggest this novel method's high sensitivity and specificity in detected whole chromosome aberrations, subchromosomal CNAs, the presence of certain intrachromosomal fusion genes and simultaneous detection of the *BRAF* V600E mutation.

In pediatric acute lymphoblastic leukemia by the comparative scrutiny of paired diagnostic and relapse samples we described three different patterns of clonal relationships with (i) identical copy number aberration profiles at diagnosis and relapse, (ii) clonal evolution with all lesions detected at diagnosis being present at relapse, and (iii) conserved as well as lost or gained copy number aberrations at the time of relapse, suggestive of the presence of a common ancestral cell compartment giving rise to clinically manifest leukemia at different time points during the disease course.

We determined four genetic subgroubs of B-ALL with distinct event-free survival rates with the combination of UKALL cytogenetic classifier and dMLPA-based CNA profiles.

6. PUBLICATIONS

Publications related to the thesis:

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7. ACKNOWLEDGEMENTS

Above all, I would like to express my gratitude to my parents for making my studies possible, loved and supported throughout. I thank my partner for her unceasing encouragement and for providing a supportive, loving environment.

I would like to thank Professor Dr. András Matolcsy for allowing me to complete my PhD studies at his department.

I would like to thank my supervisors, Dr. Donát Alpár and Dr. Csaba Bödör, for their years of working together, their professional advice and for helping me throughout my work.

Thanks to all members of the MTA-SE Momentum Molecular Oncohematology Working Group.

I am grateful to Cecilia Laczik for being able to turn to her for help at any time.

Thank you for the all staff of the 1st Deprtment of Pathology and Experimental Cancer Research work together.