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Next Generation Sequencing-based Analysis of Immunoglobulin and T-cell Receptor Genes Rearrangements Repertoire in Haematological Malignancies

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Abstract

We established next generation sequencing (NGS)-based assays for the detection of rearranged immunoglobulin (Ig) and T-cell receptor (TR) genes (Ig heavy chain complete/incomplete, TR gamma and delta). We used these assays for minimal residual disease (MRD) detection and antigen repertoire analysis in children with acute lymphoblastic leukaemia (ALL).

We compared MRD levels in 210 paediatric ALL samples detected by NGS and by quantitative PCR (qPCR), which is a current gold standard for MRD detection. We showed that both methods provide highly comparable results and that NGS results are more specific for relapse prediction. The better specifity of NGS was also proven on 40 post-transplant samples, where qPCR can provide false positive MRD results. We showed that repertoire diversity at day 78 is an independent prognostic factor in paediatric ALL.

Besides the above mentioned advantages NGS-based MRD detection is cheaper and faster than qPCR. Before NGS-based MRD monitoring can safely replace qPCR the extensive standardization of the method must be performed, as well as the evaluation of its prognostic relevance on a larger group of patients.

Abstrakt

Zavedli jsme metodu pro sekvenování přestavěných genů pro imunoglobuliny (Ig) a T-buněčné receptory (kompletní/ inkompletní přestavby těžkých řetězců Ig, TR gamma a delta) založenou na sekvenování nové generace (NGS), kterou jsme použili pro detekci minimální reziduální nemoci (MRD) a k analýze antigenního repertoáru u dětí s akutní lymfoblastickou leukémií (ALL).

Srovnali jsme hladiny MRD ve 210 vzorcích dětí s ALL detekované pomocí NGS a kvantitativní PCR (qPCR), což je v současnosti používaná standardní metoda pro detekci MRD. Ukázali jsme, že obě metody poskytují vysoce srovnatelné výsledky a že výsledky získané pomocí NGS jsou specifičtější pro predikci relapsu. Lepší specifitu NGS jsme prokázali také na 40 post-transplantačních vzorcích, u kterých může docházet k detekci falešně pozitivních qPCR výsledků. Dále jsme ukázali, že diverzita antigenního repertoáru ve dni 78 je nezávislým prognostickým faktorem u dětských ALL.

Kromě výše zmíněných výhod je detekce MRD založená na NGS levnější a rychlejší než qPCR. Než však bude možno detekcí MRD pomocí NGS nahradit qPCR, bude nutné provést rozsáhlou standardizaci této metody a také zhodnotit její prognostický význam na rozsáhlejším souboru pacientů.

NGS-based Analysis of Immunoglobulin and T-cell Receptor Genes Rearrangements Repertoire in Haematological Malignancies

Introduction

Paediatric ALL is the most common childhood malignancy which represents approximately 25% of cancers diagnosed among children under 15 years of age. Survival rates for childhood ALL have improved significantly during last decades[1] and are currently reaching 90%[2] thanks to risk-adapted therapy and improved supportive care.

Actual paediatric ALL treatment protocols utilize risk-adapted therapy in order to reduce treatment toxicity in patients with the low risk of relapse whilst ensuring sufficiently aggressive treatment in children with the high relapse risk. The stratification into the risk groups is based on chromosomal abnormalities[3] and response to treatment[4,5].

Currently highly sensitive minimal residual disease (MRD) assays can evaluate response to treatment much more precisely than classical morphological screening. Leukemic cells have unique genetic and immunophenotypic signatures which can be detected by flow cytometry (FC) or quantitative PCR (qPCR).

Quantitative PCR-based detection of rearranged genes for immunoglobulins (Ig) and T-cell receptors (TR) is a laborious and time-consuming method, because it requires optimization of patient-specific assays. It can reach the sensitivity of 1 leukemic cell in 100,000 cells (10^{-5}).

NGS is a revolutionary technology producing up to billion reads within one sequencing run. The biggest advantage of NGS-based MRD detection over qPCR-based MRD detection is that NGS provides the information about the abundance of each leukemic clone (carrying the same Ig/TR rearrangement in all the cells) in the tested sample, whereas with qPCR we can only detect one clone, for which the assay is designed. Moreover it is faster and less laborious than qPCR and thus also cheaper.

Aims

I. To design, develop and validate NGS-based assay for the detection of rearranged Ig and TR genes

II. Use the developed assay for:

a. The screening of MRD targets in the diagnostic DNA samples from patients with haematological malignancies.

b. MRD detection in the DNA samples from paediatric B-precursor ALL patients and compare the MRD detected by NGS with the MRD detected by qPCR.

c. Sequencing of the entire immune repertoire in paediatric B-precursor ALL patients' DNA samples and assessing the repertoire diversity in these samples.

d. MRD detection in the samples from paediatric B-precursor ALL patients after hematopoietic stem cell transplantation (HSCT), which were low MRD-positive by Ig/TR – based qPCR and were not followed by relapse despite the fact that no additional chemotherapy was administered.

III. To assess the prognostic significance of NGS-MRD and the repertoire diversity in paediatric B-precursor ALL patients.

Hypothesis

We hypothesized that NGS for the antigen repertoire will have a number of benefits compared to current standard qPCR:

- NGS will be cheaper, less laborious and faster
- NGS can provide additional information about the number and abundance of all the clones in the sample, and thus enable us to monitor the immune reconstitution after chemotherapy or transplantation
- NGS will enable also the direct detection of subclones and biallelic rearrangements
- we expect that MRD detected by amplicon NGS will provide more reliable prediction of relapse
- highly specific MRD detection by amplicon NGS will confirm or disprove low qPCR positivities in regenerating post-transplant samples
- in ALL samples from patients with ongoing clonal evolution after malignant transformation NGS-MRD detection should be possible and reliable

Methods

We introduced the library preparation for NGS of Ig/TR (Ig heavy chain complete and incomplete, TR gamma and TR delta) rearrangements on Ion Torrent platform based on two-round PCR.

In the first PCR all possible V-J combinations (or D-J combinations in case of incomplete rearrangements) were amplified using primer mixes designed for IgH (complete or incomplete), TRG or TRD targets. Primers for the first round also contained universal M13 adaptors at their 5’ ends, which were used in the second PCR for the amplification of 1st PCR products.

After 1st PCR the products were purified by Agencourt AMPure XP beads (Beckman Coulter) using the beads:DNA ratio 1.5 and diluted 50 times prior to the 2nd PCR.

Primers for 2nd PCR contained M13 adaptors at 3’ ends and sequencing adaptors for the Ion Torrent sequencers with the sample-specific sequencing barcodes enabling for sequencing of many samples within one sequencing run at 5’ ends.

After the second round of PCR the quality and quantity of the resulting libraries were checked using Agilent High Sensitivity DNA kit. Libraries were then pooled equimolary prior the template preparation. The templates were sequenced on Ion Torrent PGM or Ion Proton sequencers.

For the evaluation of MRD results we developed our in-house bioinformatics algorithm using the fqgrep tool, which is a sequence pattern matcher optimized for fastq/fasta files.

The Assessment of repertoire diversity and the detection of diagnostic leukaemia-associated clones were performed using the online open-source program called Vidjil. Its algorithm is designed for NGS data and it quantifies the clonotypes abundances.

Results

We successfully used NGS of Ig/TR rearrangements for the detection of MRD targets in six patients with mantle cell lymphoma and in two paediatric ALL patients in whom the classical approach was unsuccessful. The prevailing rearrangement was identified for all patients and subsequently used for subsequent qPCR-MRD detection in samples from later time-points.

We sequenced 210 samples from 76 patients with childhood ALL with a median coverage of 729,842 reads per sample. The libraries were created from 450ng of bone marrow DNA (equivalent of ~ 70,000 cells) taken at days 15, 33 and 78 from the therapy initiation, which are an important stratification time-points at BFM protocols. We compared the MRD levels detected by NGS with MRD detected by qPCR for Ig/TR rearrangements, which is a current gold standard method. The overall correlation of NGS- and qPCR-MRD in all samples was satisfactory (R^2 = 0.72) with the majority of the differences in samples with MRD below the sensitivity of both methods.

NGS approach detected significantly higher MRD than qPCR at day 33 in patients who later relapsed (p=0.001), in other words NGS-MRD positivity at day 33 provides more accurate <u>prediction of relapse</u> than qPCR-MRD positivity (5-year relapse free survival, 5y RFS NGS-negative/positive: 90 ± 5% vs 53 ± 9%, P = 0.0003; PCR-negative/positive: 84 ± 6% vs 63 ± 8%, P =0.04). At day 78, the predictive value of NGS was comparable to qPCR.

Unlike qPCR, NGS provides the information about the entire immunological repertoire in tested samples. We performed the <u>assessment of repertoire diversity</u> in 69 samples from day 33 and 71 samples from day 78 using the bioinformatic tool Vidjil. The patients who subsequently relapsed had significantly lower IgH repertoire diversity at day 78. Even after removal of patients with NGS-MRD higher than 10^{-4}, the patients with lower clonal diversity had significantly worse 5-year RFS than other patients (P = .0005). We also showed that the repertoire diversity at day 78 has a prognostic significance. Patients with higher clones/reads ratio (>0.18) have a 5-year RFS 88±5%, whereas patients with clones/reads ratio <0.18 have 5-year RFS only 50±12% (p=0.0005).

We have previously shown that MRD detection by qPCR for clonal Ig/TR rearrangements can <u>yield false positive results in patients after HSCT</u> caused by massive regeneration of B-cells[6]. We detected MRD by NGS in 34 qPCR-low positive samples from 21 paediatric

BCP-ALL cases after HSCT, who remained in complete remission after HSCT with a median follow-up of 10.4 years. Only 2 of 34 (6%) follow-up samples from these patients were found to be MRD-positive by NGS. In ~80% of NGS-MRD negative samples we successfully identified the sequence responsible for the unspecific amplification leading to the false positive qPCR result. Six low-positive post-transplant samples from patients who later developed relapses were added as positive controls. Four (66%) of these samples were positive also by NGS.

Discussion

We showed that the Ig/TR target screening based on NGS works very efficiently: the costs of both methods are comparable, but NGS is much faster than classical approach and can identify qPCR targets also in patients where Sanger sequencing fails. It is also capable of detecting targets in subclonal populations which are present at the lower frequencies.

MRD quantification based on NGS is a reliable tool for monitoring the treatment response. The sensitivity of both NGS and qPCR is limited by the number of analysed cells and is comparable when similar numbers of cells are analysed. The correlation of MRD levels detected by NGS and by qPCR was high. The vast majority of the discrepancies were found among samples with MRD levels below 10^{-4} which is below the theoretical sensitivity of both methods. Looking at the outcome of MRD-negative patients at day 33 we proved that NGS is more specific in relapse prediction at this time-point.

We showed that NGS is also more specific in regenerating post-transplant samples, where qPCR can detect false positive results due to the unspecific amplification of rearranged Ig/TR genes. Four out of 6 (67%) qPCR positive samples from patients with subsequent relapse vs. only 2 out of 34 (6%) qPCR positive samples from patients without relapse were positive also by NGS (p=0.01).

At day 78 from the therapy initiation the repertoire diversity can predict the risk of relapse. Patients with higher repertoire diversity at this time-point had higher relapse-free survival than patients with lower repertoire diversity.

Conclusions

NGS is a highly useful tool not only for MRD detection, but also for the assessment of the entire antigen repertoire. It can overcome some of the limitations of qPCR, namely the time-consuming optimisation of patient-specific assays and the risk of false positive/negative MRD results. Moreover, it provides additional information about the entire immunological background during the leukaemia treatment, while being at least price-comparable to qPCR.

Before the implementation of NGS-based MRD detection into the routine clinical practice the intra- and inter-laboratory standardization of this method is necessary, as well as more extensive evaluation of its prognostic power. This is currently in progress (with contribution of our group) within the international EuroClonality-NGS consortium.

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Articles Related to the Topic of This Thesis

KOTROVA, M., MUZIKOVA, K., MEJSTRIKOVA, E., NOVAKOVA, M., BAKARDJIEVA-MIHAYLOVA, V., FISER, K., STUCHLY, J., GIRAUD, M., SALSON, M., POTT, C., BRÜGGEMANN, M., FÜLLGRABE, M., STARY, J., TRKA, J. and FRONKOVA, E. The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL. Blood. 2015. Vol. 126, no. 8, p. 1045–1047. DOI 10.1182/blood-2015-07-655159. IF: 10.45

Articles Not Directly Related to the Topic of This Thesis

1) FRONKOVA E, KLOCPERK A, SVATON M, NOVAKOVA M, **KOTROVA M**, KAYSEROVA J, KALINA T, KESLOVA P, VOTAVA F, VINOHRADSKA H, FREIBERGER T, MEJSTIKOVA E, TRKA J and SEDIVA A. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. PLoS ONE. 2014. Vol. 9, no. 12. DOI 10.1371/journal.pone.0114514. IF: 3.23

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Curriculum vitae

Education and Work Experience:

2006-2012**:** **2nd Faculty of Medicine, Charles University in Prague**
General Medicine, M. D.

2012-present: **Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol in Prague**
<u>M. D., Ph. D. programme:</u>
Ph. D. candidate in Molecular and Cell Biology, Genetics and Virology (CLIP Laboratory Centre),
M. D.: six months of clinical experience

Extracurricular activities:

2009-2013: **Redactor of „Scientific News“ on 2nd Faculty of Medicine webpage**

2013-present: **Associate Faculty Member of „Faculty 1000“**
Travel grant winner 2013
Travel grant winner 2014

Language skills:

Czech (native), English (fluent), German (beginner)

Courses:

3/2013: **Ion PGM system workflow Training**
Library preparation, Template preparation, Instrument operation, Data evaluation

9/2013 **5th Educational Workshop on Immunoglobulin Gene Analysis in Chronic Lymphocytic Leukemia**

4/2015: **Interpretation of Genome Data in Diagnostics**

Awards:

2015 - Audience Award at the 2nd Faculty of Medicine Scientific Conference
2016 - Albert Schweitzer price in medicine – 1st place

Publications:

The Predictive Strength of Next Generation Sequencing MRD Detection for Relapse Compared with Current Methods in Childhood ALL. Kotrova M, Muzikova K, Mejstrikova E, Novakova M, Bakardjieva-Mihaylova V, Fiser K, Stuchly J, Giraud M, Salson M, Pott C, Brüggemann M, Füllgrabe M, Stary J, Trka J, Fronkova E. Blood, 2015.

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Polyclonal, newly derived T cells with low expression of inhibitory molecule PD-1 in tonsils define the phenotype of lymphocytes in children with Periodic Fever, Aphtous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome. Dytrych P, Krol P, Kotrova M, Kuzilkova D, Hubacek P, Krol L, Katra R, Hrusak O, Kabelka Z, Dolezalova P, Kalina T, 1, Fronkova E. Molecular Immunology, 2015.

Oral and poster presentations:

April 2014:	**Childhood Leukemia symposium, poster**: Post-induction Minimal Residual Disease (MRD) Detection in Childhood Acute Lymphoblastic Leukemia (ALL) BFM Trial Using Immunoglobulin Gene Rearrangement Next Generation Profiling.
June 2014:	**EHA 19th Congress, oral presentation**: Minimal Residual Disease Stratification by Next Generation Sequencing Profiling of Immunoglobulin Gene Rearrangements as an Alternative to Classical qPCR-based Technique in Childhood ALL.
June 2014:	**6th Midsummer Meeting on Paediatric Haematology and Oncology, invited lecture:** Minimal Residual Disease Stratification by Next Generation Sequencing Profiling of Immunoglobulin Gene Rearrangements as

	an Alternative to Classical qPCR-based Technique in Childhood ALL.
December 2014:	**ASH meeting, poster:** Next Generation Amplicon Sequencing of Immunoglobulin Heavy Chain Gene Rearrangements for Minimal Residual Disease (MRD) Stratification in Childhood Acute Lymphoblastic Leukemia (ALL): A comparison with Classical qPCR-Based Technique.
June 2015:	**7th Midsummer Meeting on Paediatric Haematology and Oncology, invited lecture:** Next Generation Sequencing of Rearranged Immunoglobulin and T-cell Receptor Genes for Minimal Residual Disease Detection in Childhood Acute Lymphoblastic Leukemia.
June 2015:	**EHA 20th Congress, oral presentation**: The Predictive Strength of Next Generation Sequencing Minimal Residual Disease Detection for Relapse compared with current qPCR Technique in Childhood Acute Lymphoblastic Leukemia.
November 2015:	**4th ESLHO Symposium, oral presentation:** A Comparison of Minimal Residual Disease detection via Immunoglobulin Gene Rearrangements on Two Major Next-Generation Sequencing Platforms
December 2015:	**ASH meeting, poster**: Library Preparation Is the Major Factor Affecting Differences in Results of Immunoglobulin Gene Rearrangements Detection on Two Major Next-Generation Sequencing Platforms.

Six oral presentations at regional Czech/Slovak conferences /meetings.

Grants:

2014-2016:	**Grant Agency of Charles University**: Grant "Monitoring of Immune Reconstitution after Chemotherapy, Determined by Massively-Parallel Sequencing", main researcher.