MOLECULAR GENETIC DIAGNOSIS OF PRIMARY IMMUNODEFICIENCIES IN THE REPUBLIC OF MOLDOVA

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Summary

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity, the majority of which present in infancy and result in significant morbidity and mortality. In Institute of Mother and Child, in Laboratory of Molecular Human genetics research on primary immunodeficiency has been started in 2015. Medium TREC (6565.2354 for 100000 cells) and KREC (8173.212 for 100000 cells) concentrations for presumably healthy newborns from Moldova were counted. We developed our proprietary TREC/KREC assay for newborn screening. A detailed analysis of the clinical profiles, biochemical and molecular-genetic investigations and outcome of the 3 children diagnosed with WAS in our laboratory during the period 2016-2019 was performed. During selective screening we found 9 cases of DiGeorge type I Syndrome and one case of familial Mediterranean fever (NGS and Sanger confirmed).

Molecular diagnosis of PID helps in genetic counseling and to make therapeutic decisions including the need for a stem cell transplantation.

Rezumatul

Imunodeficiențele primare (IDP) sunt un grup eterogen de erori înnăscute ale imunității, dintre care majoritatea se manifestă în copilărie și duc la o morbiditate și mortalitate semnificativă. În cadrul IMSP Institutul Mamei și Copilului, în Laboratorul de genetică moleculară umană, cercetările privind imunodeficiențele primare au început în anul 2015. Au fost cuantificate concentrațiile medii de TREC (6565.2354 pentru

100.000 de celule) și KREC (8173.212 pentru 100.000 de celule) pentru nou-născuții prezumtiv sănătoși din Republica Moldova. A fost dezvoltat un test propriu TREC/KREC pentru screening-ul nou-născuților. A fost efectuată o analiză detaliată a profilurilor clinice, investigațiilor biochimice și molecular-genetice, precum și prognosticul celor 3 copii diagnosticați în cadrul laboratorului cu sindromul Wiskott-Aldrich în perioada 2016-2019. În timpul screening-ului selectiv au fost identificați 9 pacienți cu sindromul DiGeorge tip I și un caz de febră mediteraneană familială (confirmate prin NGS și secvențierea Sanger). Diagnosticul molecular al IDP ajută la consilierea genetică și la luarea deciziilor terapeutice, inclusiv pentru necesitatea unui transplant de celule stem.

Introduction

In 1990 was created in frame of the department for hereditary patologies of the Scientific Research Institute of Mother and Child healthcare, First molecular genetics tests in Moldova (since 1992) were realized to detect mutations and analyze population aspects of the polymorphism of two genes determining monogenic pathologies – Duchenne muscular dystrophy (DMD) and hemophilia A and B, then phenylketonuria (PKU) and cystic fibrosis, spinal muscular atrophy [1]. In 1997, under the leadership of the professor Groppa St., the national program ”Improvement of the medical genetic service in Republic of Moldova. 1998-2005” was elaborated and later approved by the Ministry of Health, The program contained main strategies of regionalization and optimization of the medical genetic service structure, including the objective of the deepening of the molecular genetic investigations. As an entity in frame of the National Center of Reproductive Health and Medical Genetics, the laboratory of Human Molecular Genetics (LHMG) appeared in 2009.[1]

The LHGM began engage in research on primary immunodeficiency in the project “Studiul particularităţilor epidemiologice, clinico-imunologice şi molecular-genetice a

maladiilor imunodeficitare cu elaborarea procedeelor inedite pentru imunoreabilitare, inclusiv remedii autohtone" (cond. Doc. hab. Andries Lucia) coloboration with Nicolai Testimitanu State University of Medicine and Pharmacy, Republic of Moldova since 2015. The importance of this research consists in description of essential aspects related to primary immunodeficiency, including classification, prevalence in Europe and other countries, clinical features of frequent PID, characterization of some genes whose mutations cause immunodeficiency diseases, and also introducing of modern methods and techniques to diagnose patients with PID in Rep. of Moldova.

Material and Methods

Molecular genetic methods. The sample of genomic DNA received from 2.5 ml of venous blood taken from the participants of the research and placed into a test tube with citrate or K3 EDTA had been used for diagnostics and genotyping. Molecular genetic study had been held on basis of LMHG. The extraction of genomic DNA out of the nuclear cells had been performed via standard two stages method of phenol–chloroform extraction, method SALT-OUT and/or via use of Qiagen Genta Puregene Kit in accordance with the manufacturer's guidelines.

In order to examine mutations on the WAS gene direct sequencing was performed on the ABI 3500 DX Genetic Analyzer (Applied Biosystems) for all 12 coding regions of the WAS gene. Data were analyzed using the bioinformatic software "Sequencing Analysis Software v6.0".

For pilot TREC/KREC screening, DNA was extracted from dried blood spots. Both reagents for TREC/KREC quantitation and DBS DNA extraction were donated by our colleagues from Novosibirsk, Russia. Multiplex polymerase chain reaction in "real time" mode for quantitative analysis of DNA molecules TREC and KREC was carried out on the RealTime PCR 7500 (Applied Biosystems, SUA). The number of T-cell receptor excision circle (TREC)/kappa-deleting recombination excision circle (KREC) copies were quantified by qPCR and were related to the albumin control gene.

Results and disruption

Primary immune deficiency diseases (PIDDs) are rare, genetic disorders that impair the immune system. Without a functional immune response, people with PIDDs may be subject to chronic, debilitating infections, such as Epstein-Barr virus (EBV), which can increase the risk of developing cancer. Some PIDDs can be fatal. PIDDs may be diagnosed in infancy, childhood, or adulthood, depending on disease severity. There are more than 200 different forms of primary immune deficiency diseases (PIDDs). These rare genetic diseases may be chronic, debilitating, and costly.

Severe combined immunodeficiency (SCID) is one form of PID which is uniformly fatal without early, definitive therapy, and outcomes are significantly improved if infants are diagnosed and treated within the first few months of life. Screening for SCID using T cell receptor excision circle (TREC) analysis has been introduced in many countries worldwide. The utility of additional screening with kappa recombining excision circles (KREC) has also been described, enabling identification of infants with severe forms of PID manifested by T and B cell lymphopenia. Here, we review the early origins of newborn screening and the evolution of screening methodologies [2]. It has also been proposed to measure TREC concentration in combination with IL-7 level measurement, which should increase the screening specificity face to SCID [3].

A method for kappa recombining excision circles (KREC) quantification through PCR was proposed in 2007. KREC is formed during IGK locus rearrangement in the process of B-cells maturation, so its concentration level reflects the history of B-lymphocyte replication and can be used to track the process of restoring B-lymphocyte level after hematopoietic stem cell transplantation in patients with immunoglobulin deficiencies, such as CVID [4].

In 2011, it was demonstrated that measurement of KREC concentration allows detection of B-cell insufficiency in children by the example that KREC was absent in DNA isolated from dry blood spots of patients with Bruton agammaglobulinemia [5].

In 2015, Russia developed its own kit for the determination of TREC and KREC concentrations in the blood. Also, age norms for TREC and KREC content were established [6]

Due to collaboration with Russian scientists our laboratory start to utilized of TREC and KREC quantification for immune deficiencies research in Moldova. 75 newborns were analyzed during short pilot screening in 2017. Medium TREC (6565.2354 for 100000 cells) and KREC (8173.212 for 100000 cells) concentrations for presumably healthy newborns from Moldova were counted and they were compared with the concentrations reported by Deryabina in Russia (6419TREC and 1473 KREC for 100000 cells) Russia [7]. We also developed our proprietary TREC/KREC assay.

A detailed analysis of the clinical profiles, biochemical and molecular-genetic investigations and outcome of the 3 children diagnosed with WAS during the period 2016-2019 was performed [8].

Genetic analysis of WAS gene was performed in the first patient, and subsequently in the patient's mother. The direct sequencing analysis showed A-to-G transition at complementary nucleotide 274 (c.274-2 A>G), located in intron 2. Molecular analysis of the WAS gene performed in the patient's mother revealed no mutation. Thus, taking into consideration that family history analysis did not reveal the presence of relatives with clinical features of Wiskott-Aldrich syndrome, and DNA analysis of the patient's mother did not disclose any mutation, we can assume that the mutation found in the patient appeared de novo. The severe phenotype of the patient correlates with the presence of an aberrant protein [9].

In the second case, genetic analysis for the detection of a mutation of WAS gene showed a pathogenic mutation – c.391 G>A (p. E131K) in exon 3. Direct sequencing

performed in the patient’s mother confirmed that she is a heterozygous carrier of the same mutation. Family history analysis revealed the presence of a family member (the maternal uncle) with clinical signs similar to Wiskott Aldrich syndrome, who died in childhood [9].

In the third case, molecular genetic analysis performed through Sanger sequencing of the WAS gene revealed two mutations – c.57 G>T (p. Q19H) in the first exon, and c.136 C>A (p. L46M) in the second exon. The presumed impact on the patient phenotype was investigated on The Ensembl Variant Effect Predictor and as a result, c.57 G>T (p. Q19H) mutation has a severe phenotypic effect, while the impact of c.136 C>A (p. L46M) mutation is moderate. Considering the patient's age, the WAS clinical score, the immunological picture and the data of the molecular genetic analysis we can suggest the presence of XLT [9].

DiGeorge syndrome, also known as 22q11.2 deletion syndrome or Velo-cardio-facial syndrome (VCFS) is caused by a deletion of locus 11.2 at the long arm of chromosome 22. It affects genes involved in formation of 3rd and 4th brachial arches and causes different malformations like cardiac abnormalities, thymus hypo- or aplasia, cleft palate, hypoparathyroidism, intellectual disability etc. In 90% of cases it is caused by de novo deletion, in remaining 10% it is inherited from one of the parents. This health condition could severely affect quality of affected persons life and needs to be diagnosed as early as possible. Deletion causing this disease is higly variable in size, but includes a small conserved region consisting of about 14 genes present in about 100% of deletions. We aimed to develop a simple, fast and cost-efficient method for VCFS diagnostics. Knowing what one of the genes, presented in that region is CTP gene also known as SLC25A1, we developed primers and probe targeting it and used comparative expression technique with albumin as reference gene to assess 22q11.2 region copy number. As positive controls were used DNA of patients with VCFS diagnosis confirmed using ddPCR technique in Novosibirsk, Russia, by FISH in Kiev, Ukraine

and by aCGH in USA. During research we found three new VCFS cases, one of them being father of affected child diagnosed by ddPCR and FISH, another - a woman with miscarriage history and the third one - a newborn girl with thymus aplasia. All of them shared tetralogy of Fallout as a part of their clinical features. In present work we will describe these cases. We succeeded in development of test system with desired parameters. In our lab we developed qPCR assay for DiGeorge type I diagnostic [9].

During selective screening we found 9 cases of DiGeorge type I Syndrome, one case of familial Mediterranean fever (NGS and Sanger confirmed).

Conclusion

PID are very heterogeneous, making impossible to screen them by a single assay and necessitate a challenge of current newborn screening paradigms. In the genomic era, it is likely that this will involve up-front next generation sequencing, including whole exome sequencing and ultimately, whole genome sequencing. This approach must be evaluated in large, prospective trials prior to adopting this strategy in population-based newborn screening programs. Molecular diagnosis of PID helps in genetic counseling and to make therapeutic decisions including the need for a stem cell transplantation.

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