

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Next generation sequencing as a tool for pharmacogenomic profiling – nine novel potential genetic markers for targeted therapy in childhood acute lymphoblastic leukemia

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SUMMARY

Introduction/Objective Next generation sequencing (NGS) technology has enabled genomic profiling of each patient. Growing knowledge in pharmacogenomics makes it possible to use NGS data for discovery of novel potential genetic markers for targeted therapy of many diseases, especially cancers. The aim of this study was to use targeted NGS to make a genetic profile of childhood acute lymphoblastic leukemia (cALL) in order to evaluate potential molecular targets for targeted therapy.

Methods We analyzed DNA samples from 17 cALL patients using NGS targeted sequencing. Advanced bioinformatic analysis was used to identify novel mutations in analyzed genes and to predict their effect and pharmacogenomic potential.

Results We identified nine variants that have not been previously reported in relevant databases, including two stop-gain variants, *ABL1* p.Q252* and *AKT1* p.W22*, one frameshift, *STK11* p.G257fs*28, and six missense variants. We created three-dimensional models of four proteins harboring novel missense variants. We analyzed pharmacogenomic potential of each variant and found that two of them, *STK11* c.1023G>T/ p.L341F and *ERBB2* c.2341C>T/ p.R781W, are suitable candidates for targeted therapy.

Conclusion Most new variants detected in this study were found in the genes associated with Ras signaling pathway, which is frequently mutated in cALL patients. Pharmacogenomic profiling of each cALL will be indispensable for novel therapy approaches. Detection and initial analysis of novel variants, presented in this study, will become a standard procedure for the design and development of individualized therapies for children with ALL, leading to improved patient outcomes.

Keywords: pharmacogenomics; next generation sequencing; acute lymphoblastic leukemia; molecular targeted therapy

INTRODUCTION

Childhood acute lymphoblastic leukemia (cALL) is the most common cancer in children and it comprises approximately one third of all pediatric malignant disease [1]. In the last decade, collaborative clinical trials with advances in multiagent treatment protocols and risk stratification, based on early therapy response, clinical characteristics of patients and genetic features of the leukemia cells, have led to substantial increase in survival [1]. However, 10–20% of patients are refractory to treatment or develop relapse following therapy. For this group of patients, better risk stratification using new diagnostic methods, novel approach in treatment, and new therapeutic strategies are essential for better outcome [2, 3].

Pharmacogenomics is a major keystone of personalized medicine. Pharmacogenomics establishes guidelines for using therapeutics according to the individual's genomic, epigenomic, and transcriptomic profile [4]. Pharmacogenomic research comprises identification

of specific genes and gene products correlated with different diseases, which could represent a target for new therapeutics (molecular-targeted therapy), and identification of genes and gene allelic variants that might influence response to a drug that has already been used in therapy.

Molecular targeted therapy is a new approach in cancer treatment and it has been in focus of many researches as the best example of accurate, causal therapy, since the disease-causing molecular defect is the target of a drug.

Various mutations in signalling pathways, confirmed to be a part of cancer pathogenesis, result in appearances of mutated or overexpressed oncoproteins that can be potential targets for new therapeutic agents. Better understanding of mutational landscape of cALL could give us better understanding of disease pathogenesis and more potential targets for a specific, individualized therapy [5].

Next generation sequencing (NGS) has revolutionized genetic research, allowing us to sequence a large number of genes or even the entire genome in a very short period of time

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Table 1. Novel genetic variants identified in a cohort of childhood acute lymphoblastic leukemia patients

Gene	Genetic variant nucleotide / amino-acid change	Mutation status	PolyPhen-2	PROVEAN Prediction	SIFT
<i>STK11</i>	c.1023G>T/ p.L341F	Heterozygous	Probably damaging (1.00)	Deleterious (-3.78)	Damaging (0.003)
<i>GNAQ</i>	c.842A>G/ p.E281G	Heterozygous	NA	NA	NA
<i>ABL1</i>	c.754C>A/ p.Q252*	Heterozygous	NA	Deleterious (-14.39)	NA
<i>STK11</i>	c.769delG/ p.G257fs*28	Heterozygous	NA	NA	NA
<i>SMO</i>	c.1916T>C/ p.V639A	Heterozygous	Probably damaging (0.984)	Neutral (-1.38)	Damaging (0.027)
<i>AKT1</i>	c.66G>A/ p.W22*stop	Heterozygous	NA	NA	NA
<i>PTEN</i>	c.64G>A/ p.D22N	Heterozygous	Possibly damaging (0.893)	Neutral (-2.08)	Tolerated (0.407)
<i>ERBB2</i>	c.2341C>T/ p.R781W	Heterozygous	Probably damaging (1.00)	Deleterious (-5.27)	Damaging (0.00)
<i>STK11</i>	c.1087A>G/ p.T363A	Heterozygous	Possibly damaging (0.558)	Neutral (-1.12)	Tolerated (0.183)

[6]. Targeted NGS has made systematic studies of cancer genome possible and widely available. NGS gives us an opportunity to define the genetic profile of any cancer. It could lead to the discovery of new genetic events that could be used for risk stratification and could help us to identify potential targets for molecular therapy [7, 8].

The aim of our study was to use targeted NGS to make genetic profile of cALL in order to evaluate potential molecular targets for targeted therapy.

METHODS

Diagnostic bone marrow samples were collected from 17 patients diagnosed with cALL at the University Children's Hospital, Belgrade, Serbia. The study was performed with consent of the patients' parents or legal guardians. Ethics Committee of the University Children's Hospital has approved this research.

Genomic DNA from mononuclear cells was extracted using Blood Mini Kit (Qiagen, Valencia, CA, USA). The samples were examined using TruSeq Amplicon – Cancer Panel, TSACP (Illumina Inc., San Diego, CA, USA) targeting mutational hotspots in 48 cancer-related genes. The experiment was performed on MySeq desktop system (Illumina Inc.). The average coverage of high-quality sequences was 2,609 x per amplicon.

Genetic and bioinformatic analysis

FASTQ files produced upon library sequencing were processed in the following stages: basic quality control (FastQC) and trimming of low quality bases (FastqMc), the indel realignment and preprocessing were performed using RealignerTargetCreator and IndelRealigner from GATK (Broad Institute, Cambridge, MA, USA), additional quality control, variant calling, and filtration performed using GATK UnifiedGenotyper and VariantFiltration tools, as previously described in Marjanović et al. [9] and Balint-Todorović et al. [10].

In order to predict the effects of novel variants, we used the following software tools: SIFT, PolyPhen-2, and PROVEAN [11, 12, 13]. For protein structure stability and modeling upon single point mutations, we used STRUM method and i-TASSER server [14, 15].

RESULTS

We analyzed 48 oncogenes using TruSeq Amplicon Cancer Panel (TSACP) that covers 212 amplicons in 17 cALL. Among 72 different protein changing variants we identified nine variants previously unreported in the relevant databases, including two stop-gain variants, *ABL1* p.Q252* and *AKT1* p.W22*, one frameshift, *STK11* p.G257fs*28, and six missense variants. The full list of novel variants and their functional impact on protein structure are given in Table 1.

Using the adequate algorithms for protein modeling, we created three-dimensional models of four proteins harboring novel missense variants represented in Figure 1.

Pharmacogenomic potential of novel variants

Three novel genetic variants in the *STK11* gene were identified in our study. The impact of newly discovered genetic variants on biological function of encoded proteins was labeled as damaging or probably damaging/deleterious for one variant, neutral or tolerated for the second variant, and was unknown for the third variant. Thus, we can expect that *STK11* c.1023G>T/ p.L341F variant, which was estimated as damaging/deleterious, could have some role in the pathogenesis of cALL and may be a target for specific treatment.

In the *AKT1*, *ABL1*, and *ERBB2* genes, we identified one novel genetic variant per gene. The functional impact of these three variants was estimated as unknown for the *AKT1* gene, damaging/deleterious or probably damaging for *ERBB2*, and unknown or deleterious for *ABL1*. Hence, the variant in the *ERBB2* gene (c.2341C>T/ p.R781W) is the best candidate for a genetic marker involved in pathogenesis of cALL and a target for molecular therapy.

One novel genetic variant was discovered in the *GNAQ* gene. The effect of the c.842A>G/ p.E281G variant in this gene was labeled as unknown regarding its functional impact on protein structure, and therefore is not a good candidate for targeted therapy.

Two novel genetic variants in *SMO* and *PTEN* genes are estimated to have damaging to neutral/tolerated function impact on encoded proteins, making them not suitable candidates for further investigations.

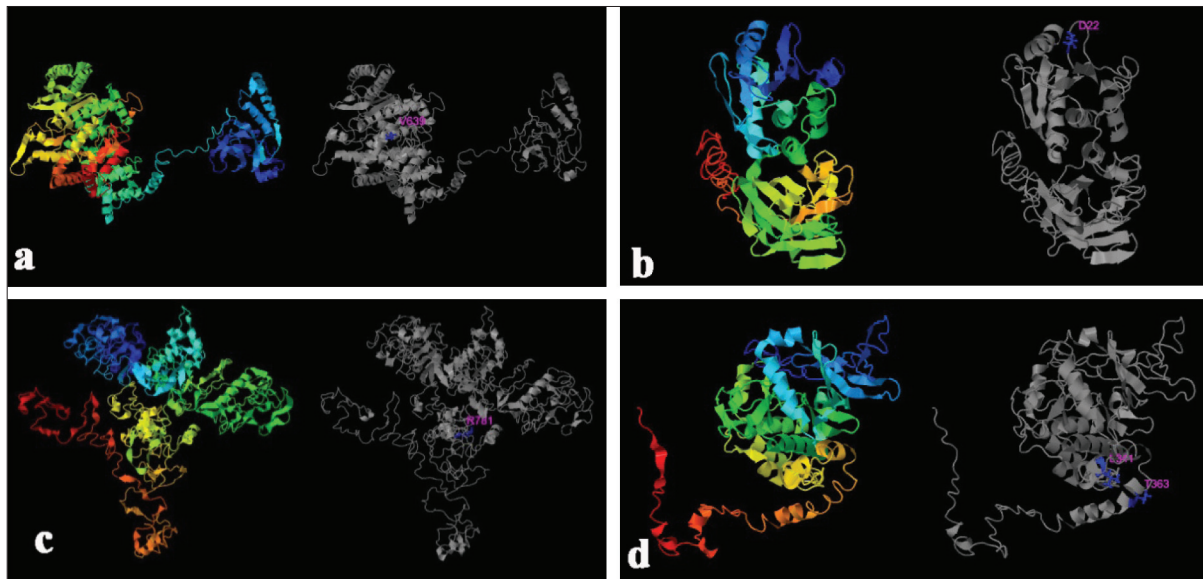


Figure 1. Three-dimensional protein structure with novel variants: a) SMO (wild-type left), SMO p.V639A (right); b) PTEN (wild-type left), PTEN p.D22B (right); c) ERBB2 (wild-type left), ERBB2 p.R781W (right); d) STK11 (wild-type left), STK11 p.L341F and p.T363A; protein structure modification caused by amino acid substitution is colored blue and labeled by the number corresponding to the position of the amino acid in the protein

DISCUSSION

In our study, we have found nine, previously unreported, mutations, in seven different genes. Most of the mutated genes belong to Ras signaling pathway, which is important in regulating many vital cellular processes (differentiation, proliferation, and cell survival) [16]. Mutations in these genes affect cell cycle and are a common feature of almost all malignancies [17].

The *STK11* gene was the most mutated gene in our study, with three novel genetic variants identified in our study. This gene is also called *LKB1* and it encodes a tumor suppressor enzyme, called serine/threonine kinase 11 protein. This protein is very important in cell cycle, apoptosis, and cell orientation in tissues. Mutation in this gene is related to lung cancer, cervical cancer, and Peutz–Jeghers syndrome (hamartomatous polyps in gastrointestinal tract and increased risk for intestinal cancer). Mutation has not been related to hematological malignancies [18, 19, 20]. T363 residue is one of the major autophosphorylation sites of the *STK11* controlling enzyme catalytic activity through the interaction with the STRAD protein, suggesting that any variant at this position could alter protein–protein interaction, leading to modulated protein function [21].

Constitutive activation of tyrosine kinase receptors, located upstream of Ras signaling pathway, cause dysregulation of entire Ras signaling pathway. BCR/ABL fusion protein is already recognized as a marker of poor prognosis in ALL, but also as a perfect target for molecular tailored therapy (imatinib), which has revolutionized targeted therapy [22]. The *ABL1* gene provides information for making a protein that works as a kinase and if mutated can affect the receptors for Ras pathway, thus leading to malignant transformation of cells (acute leukemias, chronic myeloid leukemia) [23]. The same applies to the *ERBB2* gene, which encodes receptor tyrosine kinases protein and

is associated with numerous cancers such as lung cancer, neuroblastoma, glioma, and gastric, breast, and ovarian tumors [24]. V781 residue of the ERBB2 protein resides at one of the three shell residues named Sh1 (α C- β 4 loop) responsible for enabling or disabling molecule approach to the protein. Mutations in this region lead to altered catalytic activity of this enzyme, suggesting that the V781A variant detected in our study could be important in protein kinase activity. Additionally, the results obtained using predictive algorithms suggest damaging effect of this variant on protein function [25].

Mutations in the *AKT1* gene, which encodes another kinase protein (AKT1 kinase), is also related to lung and ovarian cancers and Proteus syndrome (overgrowth of different tissues, especially bones and skin) [26, 27].

The *GNAQ* gene encodes a protein called guanine nucleotide-binding protein, which is one of the G complex proteins and has a role in activating and inactivating proteins of Ras signaling pathway. Several mutations in the *GNAQ* gene are described, and for at least one of them there is an association with Sturge–Weber syndrome and another with uveal melanoma [28].

G-protein-coupled receptors are encoded by the *SMO* gene, and mutation in this gene has been related to basal cell carcinoma, craniofacial malformation, and Curry–Jones syndrome (multisystem lesions, including skin, brain, head, and gastrointestinal tract) [29]. V639 residue, mutated in one case in our study, is a part of the intracellular domain of the SMO protein, which interacts with various molecules [30]. Substitution change affecting this position is a potential event that impacts molecular interactions. The *PTEN* gene functions as a tumor suppressor, and mutation in this gene has already been related to breast, lung, and prostate cancer, as well as with some rare syndromes [31]. Mutations in the PTEN tumor suppressor,

frequently described events in various tumors, occur in both phosphatase and the C2 domain of the molecule. D22 residue, mutated in one sample in our study, is a part of a loop at the N-terminal phosphatase domain of the PTEN molecule. This variant has not been previously described as an important site for modulation of enzyme activity and its interaction with other molecules, which is in accordance with predictive analyses that we obtained [32].

None of the mutations that we have found in the aforementioned genes have been previously reported. At this point, we can only speculate whether these mutations are important in pathogenesis of ALL and if any of them can be used as a potential target for new agents.

So far, numerous efforts have been made to exploit mutations in Ras signaling pathway as potential targets for specific and individualized therapy. Most of these attempts have reached a dead-end due to alternative pathways that can circumvent effects of targeted therapy [33]. However, discoveries of new mutations in various signaling pathways could help us in better risk stratification of cALL patients and in identifying the best targets for specific agents. In this way, we could apply pharmacogenomics in personalized therapy of ALL and other malignant diseases.

cALL is a complex genetic disease. Each cALL instance possesses its own unique characteristics in terms of genetic make-up. It is proposed that preleukemic and leukemic stem cells gain somatic molecular changes, which accumulate in a sequential fashion [34]. Genetic somatic mutations, as well as somatic epigenetic changes, are incorporated into this multistep leukemogenesis scheme. Identification of the specific genetic and epigenetic features

of cALL cells significantly contributes to the discovery of potential agents that can exclusively eradicate leukemia cells. More recently, unexpected positive clinical results in cALL have been achieved by the application of a gene-engineered chimeric antigen receptor (CAR) expressing T cells. In August 2017, the US Food and Drug Administration has approved the first CAR-T therapy, for the treatment of patients up to 25 years of age with B-cell precursor ALL that is refractory or in the second or later relapse. CARs target antigens expressed on ALL cells, but their detecting range is limited to specific markers (for example mutant oncogenes and translocations) [35]. However, other cALL immunotherapy approaches represent promising treatment opportunity, offering a possibility that specific genetic and epigenetic markers can be targeted.

CONCLUSION

Pharmacogenomic profiling of each cALL case will be indispensable for novel therapy approaches. Detection and initial analysis of novel variants, presented in this study, will become a standard procedure for the design and development of individualized therapy for children with ALL.

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Секвенцирање нове генерације као метода за фармакогеномско профилисање – девет нових потенцијалних генетичких маркера за циљану терапију акутне лимфобластне леукемије код деце

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САЖЕТАК

Увод/Циљ Секвенцирање нове генерације (СНГ) омогућило је геномско профилисање сваког болесника. Нова сазнања у области фармакогеномике омогућавају примену података добијених овом методом у циљу откривања нових могућих генетичких маркера за циљану терапију многих, посебно малигних болести.

Циљ овог истраживања је био да се применом СНГ одреди генетски профил акутне лимфобластне леукемије (АЛЛ) код деце у циљу процене могућих молекуларних мета за циљану терапију.

Метод Анализирали смо ДНК узорке 17 болесника оболелих од АЛЛ дечјег доба користећи циљано СНГ. Напредне биоинформатичке методе су коришћене да идентификују нове мутације у анализираним генима и да предвиде њихов утицај и фармакогеномски потенцијал.

Резултати Идентификовали смо девет генских варијанти које до сада нису описане у релевантним базама података. У наведеним варијантама идентификоване су две „бесмислене“ варијанте, *ABL1 p.Q252** и *AKT1 p.W22**, једна варијанта која помера оквир читања, *STK11 p.G257fs*28*, и шест несинонимних варијанти. Креирали смо тродимензионални модел за четири протеина који би били производ нових несинонимних варијанти. Анализирали смо фармакогеномски потенцијал сваке варијанте и открили да су две, *STK11 c.1023G>T/p.L341F* и *ERBB2 c.2341C>T/p.R781W*, могући кандидати за циљану терапију.

Закључак Нове варијанте откривене у овој студији припадају углавном генима повезаним са *Ras* сигналним путем, који је често захваћен мутацијама у АЛЛ код деце. Фармакогеномско профилисање сваке дечје АЛЛ биће незаменљиво за нове терапијске приступе. Детекција и иницијална анализа нових генских варијанти, која је представљена у овој студији, постаће стандардна процедура за дизајнирање и развој индивидуализоване терапије за децу оболелу од АЛЛ.

Кључне речи: фармакогеномика; секвенцирање нове генерације; акутна лимфобластна леукемија; молекуларна циљана терапија