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

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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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 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 childhood acute lymphoblastic leukemia (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 9 variants that previously have not been reported in the relevant databases, including 2 stop-gain variants, *ABL1* p. Q252* and *AKT1* p. W22*, one frameshift, *STK11* p. G257fs*28, and 6 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

САЖЕТАК

Увод/Циљ Секвенцирање нове генерације (СНГ) омогућило је геномско профилисање сваког болесника. Нова сазнања у области фармакогеномике омогу-ћавају примену података добијених овом методом у циљу откривања нових потенцијалних генетич-ких 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 потенцијалних молекуларних мета за циљану терапију.

Методе 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 19 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 продукт 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, потенцијални 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

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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 last decade, collaborative

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clinical trials with advances in multiagent treatment protocols and risk stratification, based on early therapy response, clinical characteristic 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 target for new therapeutics (molecular-targeted therapy) and identification of genes and gene allelic variants that might influence response to drug that has already been used in therapy.

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

Various mutations in signalling pathways, that are 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 specific individualised therapy [5].

Next generation sequencing (NGS) has revolutionised genetic research allowing us to sequence a large number of genes or even entire genome in very short period of time [6]. Targeted NGS has made systematic studies of cancer genome possible and widely available. NGS gives us opportunity to define the genetic profile of any cancer. It could lead to 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 that were diagnosed with cALL at University Children's Hospital, Belgrade. The study was performed with consent of patients' parents or legal guardians. Ethics Committee of University Children's Hospital has approved this research.

Genomic DNA from mononuclear cells was extracted using Qiagen Blood Mini kit (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

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was performed on MySeq desktop system (Illumina Inc., San Diego, CA, USA). The average coverage of high-quality sequences was 2609 x per amplicon.

Genetic and bioinformatic analysis

FASTQ files produced upon library sequencing were processed in 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, additional quality control, variant calling and filtration performed using GATK UnifiedGenotyper and VariantFiltration tools, as previously described in Balint-Todorovic et. al and Marjanovic et al [9, 10].

In order to predict the effects of novel variants we used following software tools SIFT [11], PolyPhen-2 [12], and PROVEAN [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 9 variants that previously have not been reported in the relevant databases, including 2 stop-gain variants, *ABL1 p.* Q252* and *AKT1 p.* W22*, one frameshift, *STK11 p.* G257fs*28, and 6 missense variants. The full list of novel variants and their functional impact on protein structure are given in table 1.

Table 1. Novel genetic variants identified in cohort of cALL patients.

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

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 gene *STK11* were identified in our study. The impact of newly discovered genetic variants on biological function of encoded proteins was labeled as damaging or

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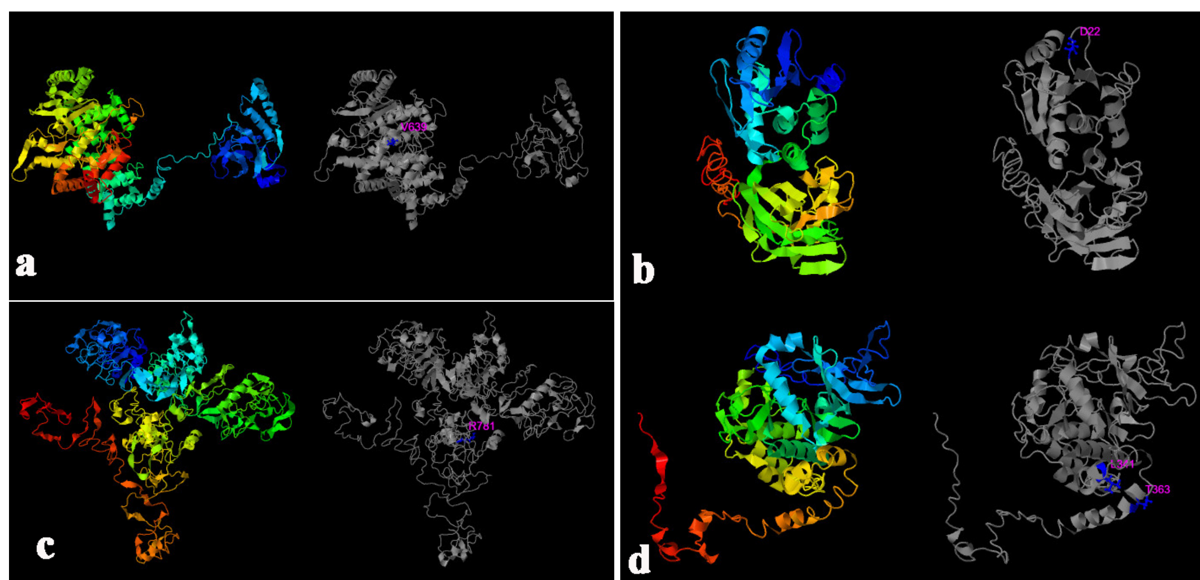

Figure 1. Three-dimensional protein structure with novel variants: a) SMO (wt left) SMO p.V639A (right), b) PTEN (wt left) PTEN p.D22B (right), c) ERBB2 (wt left) ERBB2 p.R781W (right), and d) STK11 (wt left) STK11 p.L341F and p.T363A. Protein structure modification caused by amino-acid substitution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

probably damaging/deleterious for one variant, neutral or tolerated for second variant and was unknown for third variant. Thus, we can expect that *STK11* c.1023G>T/ p.L341F variant, that was estimated as damaging/deleterious, could have some role in pathogenesis of cALL and maybe be a target for specific treatment.

In the genes *AKT1, ABL1 and ERBB2* we have identified one novel genetic variant per gene. The functional impact of these three variants was estimated as unknown for *AKT1* gene, damaging/deleterious or probably damaging for *ERBB2* and unknown or deleterious for *ABL1*. Hence, the variant in *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 *GNAQ* gene. The effect of variant c.842A>G/ p.E281G 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.

DISCUSSION

In our study, we have found nine, previously unreported, mutations, in seven different genes. Most of 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].

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Gene *STK11* was the most mutated gene in our study, with three novel genetic variants identified in our study. This gene is also called *LKB1* and is encoding tumor suppresor enzyme, called serine/theonine 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 malignances [18-20]. T363 residue is one of the major autophosphorilation site of STK11 controlling enzyme catalytic activity through the interaction with STRAD protein, suggesting that any variant at this position could alter protein-protein interaction leading to modulated function of protein [21].

Constitutive activation of tyrosine kinase receptors, that are 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) that has revolutionized targeted therapy [22]. *ABL1* gene provides information for making a protein that works as a kinase and if mutated can affect receptors for Ras pathway, thus leading to malignant transformation of cell (acute leukemias, chronic myeloid leukemia) [23]. The same applies for *ERBB2* gene that encodes protein tyrosine kinase receptor and is associated with numerous cancers such as lung cancer, neuroblastoma, glioma and gastric, breast and ovarian tumors [24]. V781 residue of ERBB2 protein resides at one of the three shell residues named Sh1 (αC- β4 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 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 *AKT1* gene, which is encoding another kinase protein (AKT1 kinase) is also related to lung and ovarian cancers and Proteus syndrome (overgrowth of different tissues, specially bones and skin) [26, 27].

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

G protein-coupled receptor is encoded by *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 intracellular domain (ICD) of SMO protein which interacts with various molecules [30]. Substitution change affecting this position is a potential event that impacts molecular interactions. *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 PTEN tumor suppressor, frequently described events in various tumors, occur in both phosphatase and

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C2 domain of the molecule. D22 residue, mutated in one sample in our study, is a part of a loop at N-terminal phosphatase domain of 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].

All of mutations that we have found in aforementioned genes haven't 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 to 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 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 application of gene-engineered chimeric antigen receptor expressing (CAR) T cells. In August 2017, the US Food and Drug Administration (FDA) 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 second or later relapse. CARs target antigens that are expressed on ALL cells, but they have limits to detect a range of 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

Pharmacogenenomic 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.

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