APPLICATION OF TARGETED NEXT GENERATION SEQUENCING FOR THE MUTATIONAL PROFILING OF PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

PRIMENA CILJANOG SEKVENCIRANJA NOVE GENERACIJE U ANALIZI MUTACIONOG PROFILA PACIJENATA SA AKUTNOM LIMFOBLASTNOM LEUKEMIJOМ

Dragana Janić1,4, Jelena Perić2, Teodora Karan-Djurasevic2, Tatjana Kostić2, Irena Marjanovic2, Bojana Stanic2, Nadja Pejanovic2, Lidija Dokmanovic1,4, Jelena Lazić1,4, Nada Krstovski1,4, Marijana Virijević3,4, Dragica Tomin3,4, Ana Vidovic3,4, Nada Suvajdzic Vukovic3,4, Sonja Pavlovic2, Natasa Tosic2

1Department of Hematology and Oncology, University Children’s Hospital, University of Belgrade, Belgrade, Serbia
2Laboratory for Molecular Biomedicine, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia
3Clinic of Hematology, Clinical Center of Serbia, Belgrade, Serbia
4School of Medicine University of Belgrade, Belgrade, Serbia

Summary

Background: Acute lymphoblastic leukemia (ALL) is the most common cancer in children, whereas it is less common in adults. Identification of cytogenetic aberrations and a small number of molecular abnormalities are still the most important risk and therapy stratification methods in clinical practice today. Next generation sequencing (NGS) technology provides a large amount of data contributing to elucidation of mutational landscape of childhood (cALL) and adult ALL (aALL).

Methods: We analyzed DNA samples from 34 cALL and aALL patients, using NGS targeted sequencing TruSeq Amplicon – Cancer Panel (TSACP) which targets mutational hotspots in 48 cancer related genes.

Results: We identified a total of 330 variants in the coding regions, out of which only 95 were potentially protein-changing. Observed in individual patients, detected mutations predominantly disrupted Ras/RTK pathway (STK11, KIT, MET, NRAS, KRAS, PTEN). Additionally, we identified

List of abbreviations: cALL, childhood acute lymphoblastic leukemia; aALL, adult acute lymphoblastic leukemia; NGS, next generation sequencing; NFM mutations, nonsense, frameshift and missense mutations.
5 patients with the same mutation in HNF1A gene, disrupting both Wnt and Notch signaling pathway. In two patients we detected variants in NOTCH1 gene. HNF1A and NOTCH1 variants were mutually exclusive, while genes involved in Ras/RTK pathway exhibit a tendency of mutation accumulation.

**Conclusions:** Our results showed that ALL contains low number of mutations, without significant differences between cALL and aALL (median per patient 2 and 3, respectively). Detected mutations affect few key signaling pathways, primarily Ras/RTK cascade. This study contributes to knowledge of ALL mutational landscape, leading to better understanding of molecular basis of this disease.

**Keywords:** acute lymphoblastic leukemia, next generation sequencing, somatic mutations

**Introduction**

Acute lymphoblastic leukemia (ALL) is a heterogeneous malignant disorder resulting from the accumulation of aberrantly transformed B or T lymphoid progenitors at different developmental stages (1). ALL is the most common cancer in children, representing about 80% of acute leukemia, whereas it is less common in adults (20%). The heterogeneity of this disease originates from various clinical, morphological and immunological phenotypes, but also from the fact that ALL is a genetically complex entity (2).

Contemporary approach to ALL treatment implies precise stratification into different risk groups that is primarily based on specific clinical and genetic characteristics. Significant advances have been made in the treatment of ALL with the cure rates for childhood ALL approaching 90%. Still, survival in the adult ALL population is only about 40% and decreases with age (3).

It has been noted that the frequency of recurring cytogenetic abnormalities present in both childhood and adult ALL, differ between these groups of patients. This difference may be the basis for the reported discrepancy in the survival rates in these age groups. Moreover, the frequency of high risk T-ALL, is much higher in adults than among children. Childhood ALL is in 85% of cases B-cell type, characterized by the presence of high hyperdiploid (>50 chromosomes) and t (12; 21) (p13; q22) i.e. ETV6/RUNX1 rearrangement, both associated with favorable prognosis. Conversely, in adult leukemia, aberrations with poor prognostic significance, like the presence of hypodiploidy (30–39 chromosomes), translocations t (4; 11) (q21; q23) and t (9; 22) (q34; q11), i.e. MLL/AFF1 and BCR/ABL1 rearrangements, are much more frequent (4, 5).

Above-mentioned chromosomal rearrangements are common in ALL and are critical events in leukemogenesis. These, so called primary genetic events, usually affect lymphoid differentiation and proliferation processes, but for the induction of full-blown leukemia, multiple mutations are required. New technologies like next generation sequencing (NGS) offers great potential for variants identification and genomic profiling of ALL. Utilization of NGS has enabled detection of additional submicroscopic alterations in the genes involved in tumor suppression, apoptosis, and cell-cycle regulation, contributing to more comprehensive insight into leukemogenesis. And not only that, these new markers have been used in diagnosis, risk-stratification and targeted therapy application, leading to improvement of current protocols and patient management (6, 7).

In this study, we applied targeted next generation sequencing on MiSeq System for analyzing somatic mutations in groups of adult (aALL) and childhood (cALL) ALL patients, in order to facilitate recognition and better understanding of the genetic profile of the disease.

**Materials and Methods**

**Subjects**

Bone marrow samples from the 17 adult and 17 childhood ALL patients at diagnosis were collected. Adult ALL patients came from the Clinic of Hematology, Clinical Center of Serbia, and childhood patients came from the Department of Hematology, University Children Hospital in Belgrade. The study was approved by the Ethics Committee of the Clinical Center of Serbia. Research was conducted in accordance with the ethical standards of the World Medical Association’s Declaration of Helsinki. Informed consent was obtained from each patient or patient’s parent or guardian.

Mononuclear cells were separated by Ficoll density gradient centrifugation and cryopreserved until mutational analyses. Some clinical characteristics of the patients are listed in Tables I and II.
Table I Clinical characteristic of cALL patients.

| Patient No. | Sex | Age | immunophenotype           | karyotype                   | RT-PCR analysis |
|-------------|-----|-----|---------------------------|-----------------------------|-----------------|
| 1           | F   | 34  | B-ALL (common)            | NA                          | TEL/AML1        |
| 2           | F   | 30  | B-ALL (common)            | 46,XX [20]                 | neg             |
| 3           | M   | 46  | B-ALL (common)            | NA                          | neg             |
| 4           | F   | 18  | B-ALL (common)            | hyperdiploidy (51–55, XX)  | neg             |
| 5           | F   | 76  | B-ALL (common)            | hyperdiploidy (49–52, XX)  | neg             |
| 6           | M   | 97  | B-ALL (common)            | NA                          | TEL/AML1        |
| 7           | F   | 29  | B-ALL (common)            | NA                          | neg             |
| 8           | M   | 23  | pre-B-ALL                 | 46,XY [20]                 | neg             |
| 9           | F   | 30  | pre-B-ALL                 | 46,XX [20]                 | BCR/ABL         |
| 10          | M   | 61  | B-ALL (common)            | hyperdiploidy (55–60, XY)  | neg             |
| 11          | F   | 30  | B-ALL (common)            | 46,XX [20]                 | TEL/AML1        |
| 12          | F   | 125 | B-ALL (common)            | 46,XX [20]                 | neg             |
| 13          | F   | 77  | B-ALL (common)            | hyperdiploidy (47, XX)     | TEL/AML1        |
| 14          | M   | 63  | B-ALL (common)            | NA                          | neg             |
| 15          | M   | 213 | pre-B-ALL                 | NA                          | neg             |
| 16          | M   | 145 | pre-B-ALL                 | NA                          | TEL/AML1        |
| 17          | M   | 126 | B-ALL (common)            | NA                          | NA              |

M – male, F – female; NA – not available

Table II Clinical characteristic of aALL patients.

| Patient | Sex | Age (years) | immunophenotype | Karyotype | RT-PCR analysis |
|---------|-----|-------------|-----------------|-----------|-----------------|
| 1       | M   | 29          | pro B-ALL       | 46,XY [20]| ND              |
| 2       | F   | 26          | B-ALL (common)  | 46,XX [6] | MLL/AFF1        |
| 3       | M   | 37          | B-ALL (common)  | 46,XY, t(4,11)[q21;q23]) [2]/62–82,XY,t(4,11)(q21;q23) [18] | ND              |
| 4       | M   | 44          | B-ALL (common)  | 46,XY [20]| neg             |
| 5       | M   | 19          | B-ALL (common)  | 46XY [20] | neg             |
| 6       | F   | 64          | B-ALL (common)  | 46,XX [20]| neg             |
| 7       | M   | 40          | T-ALL           | 46,XY [13]| BCR/ABL         |
| 8       | F   | 24          | B-ALL (common)  | 46,XX,del(cq)[5]/46,XX,t(9;22)(q34;q11) [2]/46,XX [7] | BCR/ABL         |
| 9       | F   | 45          | B-ALL (common)  | NA        | BCR/ABL         |
| 10      | M   | 33          | B-ALL (common)  | 46,XY, t(8,22)(q24;q11) [20] | ND              |
| 11      | F   | 19          | B-ALL (common)  | 46 XX/46XX, -B, -C, +M1, +M2 | ND              |
| 12      | M   | 41          | B-ALL (common)  | 46XX [20] | ND              |
| 13      | M   | 28          | B-ALL (common)  | 46XY [20] | ND              |
| 14      | F   | 61          | B-ALL (common)  | 46,XX [20] | BCR/ABL         |
| 15      | M   | 43          | B-ALL (common)  | NA        | ND              |
| 16      | M   | 28          | T-ALL           | 46, XY [20]| ND              |
| 17      | F   | 35          | B-ALL (common)  | 46, XX [20]| ND              |

M – male, F – female; NA – not available; ND – not done
variant calling and filtration was done by UnifiedGenotyper and VariantFiltration tools (13, 14). To filter out low quality variants from VCF file, variants (including synonymous variants) in the coding regions, (median per patient: 9, range: 6–12; median per cALL: 9, range: 6–12; median per aALL: 10, range: 7–12) (Figure 1). In the non-coding regions, we found 9 different types of indels (in 9 cALL patients and in 8 aALL patients), while in the non-coding regions we found 9 different types of indels (in 9 cALL and in 8 aALL patients). We also identified 54 different single nucleotide variants-SNVs (in 18 cALL and in 20 aALL samples) in the coding and 26 different SNVs (in 19 cALL and in 22 aALL patients) in the non-coding regions.

Additionally, in all patients’ samples, we identified a total of 330 (157 in cALL, 173 in aALL) variants (including synonymous variants) in the coding regions, (median per patient: 9, range: 6–12; median per cALL: 9, range: 6–12; median per aALL: 10, range: 7–12) (Figure 1). In the non-coding regions, we found 429 (211 cALL, 218 aALL) (median per patient: 13 range: 10–15; median per cALL: 13, range: 10–14; median per aALL: 13, range 10–15) (Figure 1).

Only mutations located within the coding regions were considered for further analysis, and from those only protein-changing mutations (nonsense, frameshift and missense (NFM) mutations). The total number NFM mutations was 95 (45 in cALL, 50 in aALL), median per patient 2, range: 1–7 (median per cALL: 2, range: 1–6; median per aALL: 3, range: 1–5). The majority of patients had no more than 5 NFM mutations, whereas only one cALL patient (#4) had 6 (Figure 2).

Our analysis revealed that 21 different genes had at least one NFM mutation in the coding regions (17 in cALL, 15 in aALL). Out of these, we identified variants in 6 cALL-specific genes (CDKN2A, GNAQ, HRAS, PTPN11, AKT1, and ERBB2) and 4 genes containing NFM mutations only in aALL patients (NRAS, CSF1R, RET, and FLT3). Mutations identified in the coding regions of following targeted genes: KIT (5 cases), HNF1A, STK11 and KRAS were present in at least 4 cases (more than 10%), whereas substitution variants KDR Q472H and TP53 P72R were detected in at least 18 cases (more than 50%). The
Figure 1 Total number of mutations in coding and non-coding regions identified by targeted NGS in cALL and aALL patients.

Figure 2 Distribution of nonsense (N), frameshift (F), and missense (M), mutations in the coding regions of targeted genes per cALL and aALL patient.
In seven cALL patients and in eight aALL patients we detected 16 unreported NFM mutations in 10 genes (Table III and Table IV). The largest number of new mutations were detected in the STK11 gene with four, followed by ABL1 gene with three and NOTCH1 gene with two mutations. These mutations were prevalently substitutions – missense type, but we also detected three nonsense and three frameshift truncating mutations.

**Discussion**

Acute lymphoblastic leukemia represents hematopoietic malignancy whose main feature is its clinical heterogeneity reflecting the heterogeneity that exists on the genetic level. As the development of full-blown leukemia implies a multistep process of gradual accumulation of genetic and epigenetic alterations, ALL represents a mixture of the sub clones, characterized by a special combination of the mutations (17). Each mutation, characterized as «driver» or «passenger» mutation, in its own way contributes to complete leukemic phenotype and clinical characteristics. In order to study such a complex nature of the disease next generation sequencing (NGS) methodology was used, enabling the detection of new somatic mutations that are contributing to the pathogenesis of ALL.

In this study, the application of TSACP cancer panel to analyze the mutational pattern of childhood and adult ALL samples we have analyzed the role of genes previously described primarily in solid tumors. Moreover, by applying targeted re-sequencing method we have achieved a high accuracy in variant detection, with an average coverage of 2609× per amplicon. High coverage is required for detection of somatic mutations in the samples with large number of sub clones, characteristic for hematological malignancies.

We have detected 95 potentially protein-changing variants, (45 in cALL and 50 in aALL patients). Our finding of low number of mutations in both cALL (median per patient 2, range 1–6) and aALL (median per patient 3, range 1–5) is in accordance with previous studies with reported frequency of 0–7 mutations per patient (18, 19). Moreover, in comparison to other types of both adult and childhood cancers, acute leukemias were described as low mutation rate cancers (20, 21). In particular, in many of our patients we were not able to detect any of the mutations, excluding common germline polymorphism in TP53 and in KDR gene (Table III and Table IV). Polymorphism P72R in TP53 gene, characteristic for 70% of European population, was found in 32 patients, while eighteen out of 34 contained Q472H variant in KDR gene (22).

In this study, we have noticed that commonly mutated genes belong to Ras/RTK signaling pathway, which is in accordance with previously published data (18, 23). Deregulation of Ras signaling pathway is very common feature among all cancers, because activated RAS proteins affect multiple downstream pathways (Raf/MEK/ERK and PI3K/Akt), and thus deregulate many important cellular processes (24).

One of the main mechanism of Ras deregulation is through acquisition of oncogenic mutations in 3 RAS genes: NRAS, KRAS, and HRAS (25). In our study, we identified hotspot mutations in HRAS gene affecting Glycine at G48, in KRAS gene affecting Glycine at positions G12 and G13, and in NRAS gene the same amino-acid at position G12 and Alanine at A59. Identified mutations were mutually exclusive. This observation is in accordance with the traditional concept according to which only one
### Table III Mutations identified in childhood ALL patients using NGS.

| Sample No. | Mutation detected by MySeq | Mutation Status | dbSNP       | COSMIC     |
|------------|---------------------------|-----------------|-------------|------------|
| 1          | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 2          | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 3          | HRAS, c.142G>A, p.G48R    | Heterozygous    |             | COSM5555612 |
| 4          | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 5          | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 6          | KIT, c.1621A>C, p.M541L   | Heterozygous    | rs3822214   | COSM28026  |
| 7          | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 8          | MET, c.5029C>T, p.T1010I  | Heterozygous    | rs56391007  | COSM707    |
| 9          | KRAS, c.35G>A, p.G12D     | Heterozygous    | rs121913529 | COSM521    |
| 10         | PTPN11, c.205G>A, p.E69K  | Heterozygous    | rs397507511 | COSM13013  |
| 11         | MET, c.5029C>T, p.T1010I  | Heterozygous    | rs56391007  | COSM707    |
| 12         | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 13         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 14         | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 15         | STK11, c.769delG, p.G257fs*28 | Heterozygous |             | COSM149673 |
| 16         | HNF1A, c.864_865insC p.P292fs*25 | Heterozygous |             | COSM4611384 |
| 17         | HNF1A, c.864_865insC p.P292fs*25 | Heterozygous |             | COSM4611384 |
| 18         | GNAQ, c.842A>G, p.E281G   | Heterozygous    |             | unreported |
| 19         | ABL1, c.754C>A, p.Q252*   | Heterozygous    |             | unreported |
| 20         | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 21         | TP53, c.215C>G, p.P72R    | Heterozygous    | rs1042522   | COSM250061 |
| 22         | STK11, c.802G>A, p.G268R  | Heterozygous    |             | COSM4559384 |
| 23         | HNF1A, c.862G>T, p.G288W  | Heterozygous    | rs539507291 |             |
| 24         | SMO, c.1916T>C, p.V639A   | Heterozygous    |             | unreported |
| 25         | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 26         | TP53, c.215C>G, p.P72R    | Heterozygous    | rs1042522   | COSM250061 |
| 27         | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 28         | AKT1, c.666G>A, p.22*stop | Heterozygous    |             | unreported |
| 29         | KRAS, c.38G>A, p.G13D     | Heterozygous    | rs112445441 | COSM532    |
| 30         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 31         | KDR, c.1416A>T, p.Q472H   | Homozygous      | rs1870377   | COSM149673 |
| 32         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 33         | PTEN, c.64G>A, p.D22N     | Heterozygous    |             | unreported |
| 34         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 35         | ERBB2, c.2341C>T, p.R811W | Heterozygous    |             | unreported |
| 36         | STK11, c.1087A>G, p.T363A | Heterozygous    |             | unreported |
| 37         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 38         | HNF1A, c.864_865insC p.P292fs*25 | Heterozygous |             | COSM4611384 |
| 39         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 40         | HNF1A, c.864_865insC p.P292fs*25 | Heterozygous |             | COSM4611384 |
| 41         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 42         | KDR, c.1416A>T, p.Q472H   | Heterozygous    | rs1870377   | COSM149673 |
| 43         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| 44         | HNF1A, c.864_865insC p.P292fs*25 | Heterozygous |             | COSM4611384 |
| 45         | TP53, c.215C>G, p.P72R    | Homozygous      | rs1042522   | COSM250061 |
| Sample No. | Mutation detected by MySeq | Mutation Status | dbSNP       | COSMIC       |
|------------|---------------------------|----------------|-------------|--------------|
| 1          | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 2          | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 2          | KRAS, c.38G>A, p.G13D      | Heterozygous   | rs121913488 | COSM552      |
| 3          | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 3          | STK11, c.782G>A, p.W261*stop | Heterozygous | unreported  | unreported   |
| 4          | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 5          | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 5          | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 6          | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 6          | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 7          | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 8          | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 8          | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 8          | CSF1R, c.2862C>T, p.C954*stop | Heterozygous | unreported  | unreported   |
| 10         | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 11         | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 11         | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 12         | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 12         | CSF1R, c.2862C>T, p.C954*stop | Heterozygous | unreported  | unreported   |
| 13         | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 14         | STK11, c.1046A>G, p.E349G  | Heterozygous   | unreported  | unreported   |
| 15         | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 16         | KDR, c.1416A>T, p.Q472H   | Heterozygous   | rs1870377   | COSM149673   |
| 16         | NOTCH1, c.4729_4734delGTGGTG | Heterozygous | unreported  | unreported   |
| 17         | FLT3, c.2503G>T, p.D835Y   | Heterozygous   | rs121913488 | COSM783      |
| 18         | HNF1A, c.864_865insC       | Heterozygous   | COSM4611384 | COSM4611384 |
| 19         | TP53, c.215C>G, p.P72R     | Heterozygous   | rs1042522   | COSM250061   |
| 19         | ABL1, c.880A>G, p.K294E    | Heterozygous   | unreported  | unreported   |
mutation in one pathway is sufficient for disease development. In one model of ALL genesis, it was suggested that G12D variant in KRAS is a first genetic event responsible for malignant transformation of hematopoietic stem cells (26). Still, the majority of studies have focused on KRAS and NRAS mutant forms and suggest that oncogenic Ras alone is insufficient to drive leukemogenesis and cooperating genetic events are necessary for full-blown leukemia. It was found that mutations affecting RAS gene family and entire Ras pathway as well, were associated with other aberrations (27). In our cohort of patients, it was the case in MLL1/AF4–driven leukemogenesis.

Deregulation of Ras/RTK signaling pathway can also occur due to constitutive activation of protein tyrosine kinase located upstream of RAS (25). Many studies described receptor tyrosine kinases as a key regulator of the process of hematopoiesis, as well as leukemogenesis (28). In our cohort of ALL patients, we have found mutations of missense type in receptor tyrosine kinase genes FLT3 and ERBB2 (at positions D835Y and R781W, respectively). Additionally, we identified variants in non-receptor tyrosine kinase ABL1 gene including two stop gain mutations at position Q252* and W261*, as well as one substitution mutation K294E in one patient. All of these mutations are unreported, although mutations in ABL1 gene have been previously associated with ALL occurrence or therapy resistance (7). We have also detected one mutation in PTPN11 gene coding for non-receptor tyrosine phosphatase SHP2. SHP2 is a putative positive regulator of the Ras signaling pathway and mutations in PTPN11 gene have been described as a «driver» mutations in B-ALL development (19).

Mutations in HNF1A gene, encoding transcriptional factor affect both Ras/RTK and Notch1 pathways. We have detected six patients with mutations in this gene; five frameshift mutations (P292fs*25), and 1 missense variant (G288W). In our study, these frameshift mutations were associated with the presence of TEL/AML1 rearrangement in cALL, probably as a «second hit» mutation, as an additional genetic event required for development of full-blown leukemia (30, 31). Changes in the HNF1A gene are associated with liver/pancreatic tumors, not with hematological malignancies (32, 33).

Another gene whose mutations are not usually associated with hematological malignancies is STK11 (LKB1) gene. STK11 gene is encoding serine/threonine kinase protein, which has been involved in the cell cycle and apoptotic processes. It is assumed that this gene has the role of a classical tumor suppressor gene, because its loss-of-function somatic mutations lead to deactivation of the PI3K/Akt signaling pathway (34). Significant frequency of somatic mutations in STK11 gene were reported only in lung and cervical tumors, while in other types of human cancers, the occurrence of these mutations is a sporadic event (35, 36). In our study, it was one of the most mutated one with one frameshift (G257fs*28) and four missense mutations (G268R, L341F, E349G, and T363A) found in 6 patients. All of the detected STK11 mutations were previously unreported, and mutations in this gene are not specific for leukemias.

In NOTCH1 gene, we identified one missense and one frameshift mutation in 2 T-ALL patients. Activating mutations in Notch1 signaling pathway have been described as a crucial factor in T-ALL development and their identification could lead to prognostic marker discovery and therapy improvement (37, 38). In five patients, 2 cALL and 3 aALL, we identified M541L mutation in KIT gene that encodes proto-oncogene receptor tyrosine kinase, while in another receptor tyrosine kinase gene coding proto-oncogene MET, we detected T1010I mutation that was present exclusively among cALL patients. KIT gene belongs to receptor tyrosine kinase gene family involved in hematopoietic stem cells (HSCs) self-renewal and differentiation, suggesting that any activating mutation in the receptor could alter hematopoietic development. Mammalian cells transformed with KIT gene that contained activating mutations exhibited increased growth both in vitro and in vivo, suggesting important role of this mutation in leukemogenesis (39). Pathogenic mutations in KIT tyrosine kinase gene have been reported in various diseases. One of the recently published results emphasizes the role of M541L variant in the therapy response in chronic eosinophilic leukemia patients (40).

In conclusion, by using targeted NGS method in studying the mutational landscape of our cohort of ALL patients, we have found that low number of mutations are implicated in the pathogenesis of this disease. The impact of the detected mutations is focused on few key signaling pathways, primarily on Ras/RTK cascade. Our findings provide additional information for mutational portrait of ALL and the results could be used as a supplement to classical therapy stratification methods. In that way, we would be able to apply therapeutics that target specific signaling pathways in each individual patient. It is possible that better outcome among ALL patients of all ages could finally be accomplished through such personalized treatment approach.

Acknowledgments. This work was funded by the Ministry of Education, Science and Technological Development, Republic of Serbia (grant no. III 41004) and by the European Commission, EU-FP7-REGPOT-316088, 2013–2016. We thank colleagues from Seven Bridges Genomics for assistance with bioinformatics analysis.

Conflict of interest statement
The authors stated that they have no conflicts of interest regarding the publication of this article.
References

1. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet 2008; 371: 1030–43.

2. Mrozek K, Harper DP, Aplan PD. Cytogenetics and molecular genetics of acute lymphoblastic leukaemia. Hematol Oncol Clin North Am 2009; 23: 991–1010.

3. Milosevic G, Kotur N, Krstovski N, Lazić J, Žukić B, Stankovic B, Janic D, Katsila T, Patrinos GP, Pavlovic S, Dokmanovic L. Variants in TPMT, ITPA, ABCC4 and ABCB1 genes as predictors of 6-mercaptopurine induced toxicity in children with acute lymphoblastic leukaemia. J Med Biochem 2018; 37: 320–7.

4. Moorman AV, Chilton L, Wilkinson J, Ensor HM, Bown N, Proctor SJ. A population-based cytogenetic study of adults with acute lymphoblastic leukaemia. Blood 2010; 115: 206–14.

5. Toft N, Birgens H, Abrahamsson J, Bernell P, Griskevičius L, Hallbööök H, et al. Risk group assignment differs for children and adults 1-45 yr with acute lymphoblastic leukaemia treated by the NOPHO ALL-2008 protocol. Eur J Haematol 2013; 90: 404–12.

6. Mullighan CG. The molecular genetic makeup of acute lymphoblastic leukaemia. Hematology Am Soc Hematol Educ Program 2012: 389–96.

7. Gasic V, Stankovic B, Žukić B, Janic D, Dokmanovic L, Krstovski N, Lazić J, Milosevic G, Lucafo M, Stocco G, Decorti G, Pavlovic S, Kotur N. Expression pattern of long Non-coding RNA growth arrest-specific 5 in the remission induction therapy in childhood acute lymphoblastic leukaemia. J Med Biochem 2018; 38: 292–8.

8. www.bioinformatics.babraham.ac.uk/projects/fastqc. 2010; Available from: www.bioinformatics.babraham.ac.uk/projects/fastqc.

9. http://code.google.com/p/ea-utils. 2011.

10. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009; 25: 1754–60.

11. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 2010; 26: 589–95.

12. https://arxiv.org/abs/1303.5075. 2013.

13. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011; 43: 491–8.

14. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20: 1297–303.

15. McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GR, Thormann A, et al. The Ensembl Variant Effect Predictor. Genome Bio 2016; 17: 122.

16. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol 2011; 29: 24–6.

17. Warner JK, Wang JC, Hope KJ, Dick JE. Concepts of human leukemic development. Oncogene 2004; 23: 7164–77.

18. Ebrahimi-Rad M, Khatami S, Ansari S, Jalylfar S, Valadbeigi S, Saghir R. Adenosine deaminase 1 as a biomarker for diagnosis and monitoring of patients with acute lymphoblastic leukaemia. J Med Biochem 2018; 37: 128–33.

19. Mullighan CG. The genomic landscape of acute lymphoblastic leukaemia in children and young adults. Hematology Am Soc Hematol Educ Program 2014: 174–80.

20. Liu YF, Wang BY, Zhang WN, Huang JY, Li BS, Zhang M, et al. Genomic Profiling of Adult and Pediatric B-cell Acute Lymphoblastic Leukemia. EBioMedicine 2016; 8: 173–83.

21. Marjanovic I, Kostic J, Stanić B, Pejanovic N, Lucic B, Karan-Djurasevic T, et al. Parallel targeted next generation sequencing of childhood and adult acute myeloid leukaemia patients reveals uniform genomic profile of the disease. Tumour Biol 2016; 37: 13391–401.

22. Bodian DL, McCutcheon JN, Kothiжал P, Huddleston KC, Iyer RK, Vockley JG, et al. Germline variation in cancer-susceptibility genes in a healthy, ancestrally diverse cohort: implications for individual genome sequencing. PLoS One 2014; 9: e94554.

23. Ding LW, Sun QY, Tan KT, Chien W, Mayakonda A, Yeoh AEJ, et al. Mutational Landscape of Pediatric Acute Lymphoblastic Leukemia. Cancer Res 2017; 77: 390–400.

24. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res 1989; 49: 4682–9.

25. Shannon K. The Ras signaling pathway and the molecular basis of myeloid leukemogenesis. Curr Opin Hematol 1995; 2: 305–8.

26. Zhang J, Wang J, Liu Y, Sidik H, Young KH, Lodish HF, et al. Oncogenic Kras-induced leukemogenesis: hematopoietic stem cells as the initial target and lineage-specific progenitors as the potential targets for final leukemic transformation. Blood 2009; 113: 1304–14.

27. Stam RW. The ongoing conundrum of MLL-AF4 driven leukemogenesis. Blood 2013; 121: 3780–1.

28. Reilly JT. Receptor tyrosine kinases in normal and malignant haematopoiesis. Blood Rev 2003; 17: 241–8.

29. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood 2002; 99: 3472–5.

30. Yamamoto T, Isomura M, Xu Y, Liang J, Yagasaki H, Kamachi Y, et al. PTPN11, RAS and FLT3 mutations in childhood acute lymphoblastic leukemia. Leukemia Res 2006; 30: 1085–9.

31. Zuna J, Madzo J, Krejci O, Zemanova Z, Kalinova M, Muzikova K, et al. ETV6/RUNX1 (TEL/AML1) is a fre-
quent prenatal first hit in childhood leukemia. Blood 2011; 117: 368–9.

32. Luo Z, Li Y, Wang H, Fleming J, Li M, Kang Y, et al. Hepatocyte nuclear factor 1A (HNF1A) as a possible tumor suppressor in pancreatic cancer. PLoS ONE 2015; 10: e0121082.

33. Pilati C, Letouze E, Nault JC, Imbeaud S, Boulay A, Calderaro J, et al. Genomic profiling of hepatocellular adenomas reveals recurrent FRK-activating mutations and the mechanisms of malignant transformation. Cancer Cell 2014; 25: 428–41.

34. Gao Y, Ge G, Ji H. LKB1 in lung cancerogenesis: a serine/threonine kinase as tumor suppressor. Protein Cell 2011; 2: 99–107.

35. Sanchez-Cespedes M, Parrella P, Esteller M, Nomoto S, Trink B, Engles JM, et al. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. Cancer Res 2002; 62: 3659–62.

36. Wingo SN, Gallardo TD, Akbay EA, Liang MC, Contreras CM, Boren T, et al. Somatic LKB1 mutations promote cervical cancer progression. PLoS ONE 2009; 4: e5137.

37. Ferrando AA. The role of NOTCH1 signaling in T-ALL. Hematology Am Soc Hematol Educ Program 2009; 2009: 353–61.

38. Zhu YM, Zhao WL, Fu JF, Shi JY, Pan Q, Hu J, et al. NOTCH1 mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. Clin Cancer Res 2006; 12: 3043–9.

39. Hussain SR, Raza ST, Babu SG, Singh P, Naqvi H, Mahdi F, et al. Screening of C-kit gene mutation in acute myeloid leukaemia in Northern India. Iran J Cancer Prev 2012; 5: 27–32.

40. Iurlo A, Gianelli U, Beghini A, Spinelli O, Orofino N, Lazzaroni F, et al. Identification of kit (M541L) somatic mutation in chronic eosinophilic leukemia, not otherwise specified and its implication in low-dose imatinib response. Oncotarget 2014; 5: 4665–70.

Received: December 16, 2018
Accepted: March 22, 2019