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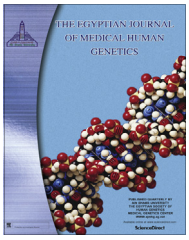

ORIGINAL ARTICLE

Detection of *TET2*, *KRAS* and *CBL* variants by Next Generation Sequencing and analysis of their correlation with *JAK2* and *FLT3* in childhood AML

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KEYWORDS

Childhood acute myeloid leukemia;
Mutation;
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Abstract *Background:* Acute myeloid leukemia (AML) is a heterogeneous clonal disorder in terms of cytogenetic and molecular aberrations. Ten-Eleven-Translocation 2 (*TET2*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and Casitas B-cell lymphoma (*CBL*) have an important role pathogenesis of acute myeloid leukemia (AML) and their activated mutations confer proliferative and survival signals.

Aim: In this study, we aimed to find possible genetic markers for molecular analysis in childhood AML by screening hot-spot exons of *TET2, KRAS*, and *CBL* using Next Generation Sequencing (NGS) analysis. In addition, association between found variants and mutations of Januse Kinase-2 (*JAK2*) and Fms-Related Tyrosine Kinase (*FLT3*) were analyzed which are important prognostic risk factors for AML.

Methods: Eight patients who were diagnosed with pediatric AML at Losante Pediatric Hematology–Oncology Hospital were included to the study. Hot-spot exons of *TET2, KRAS* and *CBL* genes were screened using the NGS method. Furthermore, *FLT3*-Internal Tandem Duplicate (*FLT3-ITD*) and *JAK2*-V617F were analyzed by Real Time Polymerase chain Reaction (Real Time-PCR).

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Results: In total, we identified 20 variants in studied genes by NGS. In our patient group, 16 variants in the *TET2* (seven novel, seven missense and two silent), two variants in the *KRAS* (one missense and one intronic) and two variants in the *CBL* (two novel) were found. All of AML patients were found negative for JAK V617 F. Three of the eight patients (37.5%) showed mutations of both FLT3-ITD and *TET2, KRAS, CBL*.

Conclusion: We found novel mutations for *TET2, KRAS*, and *CBL*. The detected variants in this article seem to be the first screening results of genes studied by NGS in childhood AML patients. Our results also showed some degree of association between FLT3-ITD and *TET2, KRAS, CBL* mutations.

1. Introduction

AML is a heterogeneous clonal neoplasm characterized by accumulated genetic aberrations, which causes enhanced proliferation, maturation arrest, increased survival of the leukemic blast cells and variable response to therapy [1–5].

During the past decades, a number of recurrent cytogenetic and molecular genetic abnormalities have been identified in AML such as *t*(8;21), inv(16), *FLT3, NPM1, CEBPA, TET2, KRAS*, and *CBL*.

TET family gene members (*TET1, TET2*, and *TET3*) have functions mostly in hematopoietic differentiation. The TET oncogene family member 2 (*TET2*) gene located at chromosome band 4q24 catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine [1,6,7]. *TET2* mutations are frequently detected during progression of MPN (myeloproliferative neoplasms) or MDS (myelodysplastic syndromes) to AML [1]. *TET2* mutations may contribute to leukemogenesis by altering epigenetic regulation of transcription via DNA methylation. The incidence of *TET2* mutations is approximately 10–20% in AML [2].

RAS (Rat Sarcoma Virus) proteins including *HRAS* (Harvey Rat Sarcoma Viral Oncogene Homolog), *KRAS* and *NRAS* (Neuroblastoma RAS Viral V-Ras Oncogene Homolog) are members of the small GTPases superfamily [8]. Abnormal RAS function is related to hyperproliferative developmental diseases and cancers [2]. *RAS* mutations, especially *KRAS*, represent about 90% of cancer-associated mutations. RAS proteins play a major role in cell signaling pathway of cell proliferation, differentiation, and survival [9]. The RAS mutations are the most common mutations in AML which are seen in approximately 25–44% of patients. Among RAS mutations, *KRAS* mutations are the most frequently seen and found in 10–15% of these patients [10].

CBL, CBL-B, and CBL C/3 are the members of the *CBL* which is localized on human chromosome 11 q23 containing several functional domains [11]. *CBL* is a mammalian gene encoding the protein CBL which is an E3 ubiquitin-protein ligase involved in cell signaling and protein ubiquitination. Mutations of this gene have been implicated in a number of human cancers, particularly in AML. These mutations have also been observed in 1% of AML. *CBL* mutations have been reported in myeloid malignancies and uniformly affect either the linker region or the RING finger domain. Loss of ubiquitination of activated receptor tyrosine kinases is thought to contribute to the transforming potential of leukemia-associated mutant CBL proteins [12].

JAK2 (located in chromosome 9p24) encodes a cytoplasmic tyrosine kinase. JAK2V617F presents a somatic point mutation (including exon 12 of the *JAK2*) resulting in the substitution of valine by phenylalanine amino acid at codon 617. This mutation, which causes JAK homology 2 (JH2) negative regulatory domain, derails JAK2 kinase regulatory activity which effects cytokine independent proliferation of hematopoietic cells [13]. The JAK2 V617F mutation is found in 1.8–28% of patients with AML [14].

FMS-like tyrosine kinase 3 (*FLT3*) is a member of the receptor tyrosine kinase (RTK) III subfamily. The *FLT3* receptor gene (located in chromosome 13q12) encodes a 993 amino acid protein. This protein is expressed in bone marrow, thymus and lymph nodes [15]. *FLT3* plays a major role in cell survival, proliferation, and differentiation of hematopoietic stem cells [16]. *FLT3-ITD* is found in about one quarter of newly diagnosed AML patients. This mutation causes the main activation of the receptor tyrosine kinase activity in the absence of ligand [17].

In our study, we aimed to screen whole *TET2, KRAS*, and *CBL* by NGS analysis, evaluating the association of mutations of JAK2 and FLT3 which is known as prognostic risk factors and finding possible genetic markers for molecular leukemia analysis. The NGS method is a powerful tool to discover novel disease mutations and candidate biomarkers. Therefore we chose NGS as a screening method for our study.

2. Subjects and methods

2.1. Subjects

The study population consisted of eight patients aged between one and 15 years who were admitted to Losante Hospital for Children with Leukemia with the diagnosis of AML. An informed written consent was obtained from all the patients' parents. Patient characteristics of the eight pediatric AML cases are shown in Table 1.

2.2. Cytogenetic techniques

Bone marrow samples were collected with Heparin-containing tubes, and chromosome analysis was performed using G-banding. After the slide preparation, G-banding using Giemsa-staining was carried out according to the standard procedures. On each slide 20 metaphases were analyzed by a light microscope (Nikon, JAPAN). Karyotypes were described according to ISCN [24].

Table 1 Demographic characteristics and *TET2*, *KRAS*, and *CBL* variants of children with AML.

No	Gender/ age/	Diagnosis	FAB	Risk group	Cytogenetics-Molecular abnormalities	TET2				CBL				KRAS				FLT3 ITD
						Rs number	Nucleotide change	Localization	Amino acid change	Rs number	Nucleotide change	Localization	Amino acid change	Rs number	Nucleotide change	Localization	Amino acid change	
1	M/13	AML	M4	HR	46, XY, monosomy, Trisomy 14	Novel	5284 A > G	Exon 11	Leu-Val	–	–	–	–	–	–	–	–	Negative
2	M/13	AML	M1	HR	*t*(9;22)	Rs 12498609	86 G > C	Exon 3	Pro-Arg	Rs 17851045	182 A > T	Exon 3	Gln-Leu	–	–	–	–	Negative
										Rs 111836509	310 A > C	Exon 3	Tyr-Ser					
3	F/8	AML	M5	SR	46, XX	Rs 17253972	1088 C > T	Exon 3	Pro-Leu	–		–	–	Novel	1347 T del	Exon 8	Gly- Gly	Negative
						Novel	1842 G del	Exon 3	Gly-Gly									
						Rs 142173406	5162 T > G	Exon 11	Ser-Ser									
						Novel	46 A del	Exon 4	–									
						Novel	730 A del	Exon 3	Trp-His									
4	F/3	AML + T-ALL	M4	HR	47, XX (+21) c 21 der(14) (14q1,2→q 3,2ii 1q21 → q43),der(19)(19qtel) → p13.3::11q13)11qtel [9]. *t*(9,22)	Rs 200327850	2286 C > T	Exon 3	Pro-Leu	Rs 111836509	310 A > C	Exon 3	Tyr-Stop	Novel	1347 T del	Exon 8	Gly-Gly	Positive
						Rs 112576862	2386 G > C	Exon 3	Ser-Ser									
						Rs 17253972	1088 C > T	Exon 3	Pro-Leu									
						Novel	1842 G del	Exon 3	Gly-Gly									
						Rs 142173406	5162 T > G	Exon 11	Ser-Ser									
						Novel	46 A del	Exon 4	–									
						Novel	730 A del	Exon 3	–									
5	M/3	AML	M4	SR	46, XY, monosomy 7	Novel	1842 G del	Exon 3	Gly-Gly	Rs 111836509	310 A > C	Exon 3	Tyr-Stop	Novel	1347 T del	Exon 8	Gly- Gly	Positive
						Novel	46 A del	Exon 4	–									
						Novel	730 A del	Exon 3	–									
						Rs 144386291	2599 T > C	Exon 3	His-Arg									
						Novel	5343 A > G	Exon 11	His-Val									
						Rs 372499795	5164 C > T	3' UTR	Thr-ile									

(*continued on next page*)

Table 1 (*continued*)

No	Gender/ age/	Diagnosis	FAB	Risk group	Cytogenetics-Molecular abnormalities	TET2				CBL				KRAS				FLT3 ITD
						Rs number	Nucleotide change	Localization	Amino acid change	Rs number	Nucleotide change	Localization	Amino acid change	Rs number	Nucleotide change	Localization	Amino acid change	
6	M/7	AML	M4	HR	47, XY, + 22[12] Inv (16;16), Fragile X syndrome	Rs 17253972	1088 C > T	Exon 3	Pro-Leu	–	–	–	–	Novel	1347 T del	Exon 8	Gly- Gly	Negative
						Novel	1842 G del	Exon 3	Gly-Gly									
						Rs 142173406	5162 T > G	Exon 11	Leu-Trp									
						Novel	46 A del	Exon 4	–									
						Rs 150298743	100 C > T	Exon 3	Trp-İle									
						Rs 68431410	652 G > A	Exon 3	Val-Met									
						Novel	5333 A > G	Exon 11	His-Arg									
7	F/9	AML	M2	SR	46, XX	Novel	1842 G del	Exon 3	Gly-Gly	Rs 111836509	310 A > C	Exon 3	Tyr-Stop	Novel	1347 T del	Exon 8	Gly- Gly	Negative
						Novel	46 A del	Exon 4	–									
8	M/8	AML-MDS	M5	HR	46, XX	Rs 12498609	86 G > C	Exon 3	Pro-Arg	–	–	–	–	Novel	1347 T del	Exon 8	Gly- Gly	Positive
						Novel	1842 G del	Exon 3	Gly-Gly					Novel	1111 T > C	Exon 8	*T*ry-His	
						Novel	46 A del	Exon 4	–									

HR high risk; SR standard risk; FAB French–American–British classification.

Fluorescence in situ hybridization (FISH) was performed on interphase nuclei and metaphase chromosomes of bone marrow cells using dual-color/dual-fusion probes for translocations of inv (16;16), *t*(9;22), *t*(4;14), chromosome 19, and dual-color/deletion probe for del 7q, labeled in green and red spectra according to the manufacturer's protocol provided by Cytocell, UK. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI). At least 100 nuclei were analyzed under the Fluorescence microscope, and image capture was performed using Nikon Eclipse 80i equipped with a CCD-camera (CoolCube1), appropriate filters and Isis software (MetaSystems).

2.3. DNA isolation and Next Generation Sequencing

Blood samples were collected with EDTA (Ethylenediaminetetraacetic acid)-containing tubes and DNA was extracted from peripheral blood and bone marrow leukocytes with MagNA Pure automatic DNA isolation instrument (Roche Diagnostics, Manheim, Germany).

We used NGS to study three candidate genes at *TET2, KRAS* and *CBL*. All coding exons of *TET2* (exons 3 and 11) were presented by 27 amplicons. Besides, two primer pairs were amplified known mutational hotspot regions to describe the RING finger domain and linker sequence for *CBL* (exons 8 and 9) and *KRAS* (exons 2 and 3). The analyses were performed as previously described by Kohlmann et al. [18].

Next Generation Sequencing was carried out using 454 GS Junior System Instrument Roche Applied Science. The Data were analyzed using the GS Amplicon Variant Analyzer software version 2.3. (Roche Applied Science, Germany). We used filters which were adjusted to show variants in more than 1% of bidirectional reads per amplicon and per patient to determine the variants.

2.4. Real time PCR

FLT3-ITD and JAK V 617 F mutation were analyzed by Real Time PCR on Light Cycler 480 II instrument (Roche Diagnostics, Gmbh, Mannheim, Germany). Results were analyzed with the High Resolution Melting (HRM) method using genotype profiles. Different plots were created by selecting negative controls as the base-line. Therefore, fluorescence of the all other samples was diagramed relative to this sample. Fluorescence signals were analyzed and significant differences used as indicators of mutations [17,19].

3. Results

In this study, we analyzed mutations affecting the *TET2* coding sequence (exons 3–11), *KRAS* (exons 2–3) and *CBL* (exons 8–9) in our patients with pediatric AML. In total, 20 variants were detected by NGS mutation screening method. The results are shown in Table 1. We detected 16 variants in *TET2*, two variants both *KRAS* and *CBL* genes. Table 1 shows the summary of the association of patients' characteristics and variants of *TET2, KRAS* and *CBL* profiles.

Karyotype analysis was normal in three of the eight (37.5%) patients. Trisomy 14, Trisomy 19, Trisomy 22, monosomy 7, monosomy 14, inv (16;16), *t*(9;22) were found using chromosome banding and FISH analyses. We screened all patients during treatment or relapse. While one patient had 6 *TET2*, 1 *KRAS* gene variants, the other patient had 7 *TET2* variants. Two relapse patients (number 4 and 5) had the same *CBL* variants.

Fig. 1 displays distribution of amplicon reads for patients. The most of TET2 variants were described in the largest exon 3 and 11; 1842 G > -(6/8 patients, 75%) and 1088 C > T(5/8, 62.5%) variants, 5162 T > G(5/8, 62.5%) in exon 3 and exon

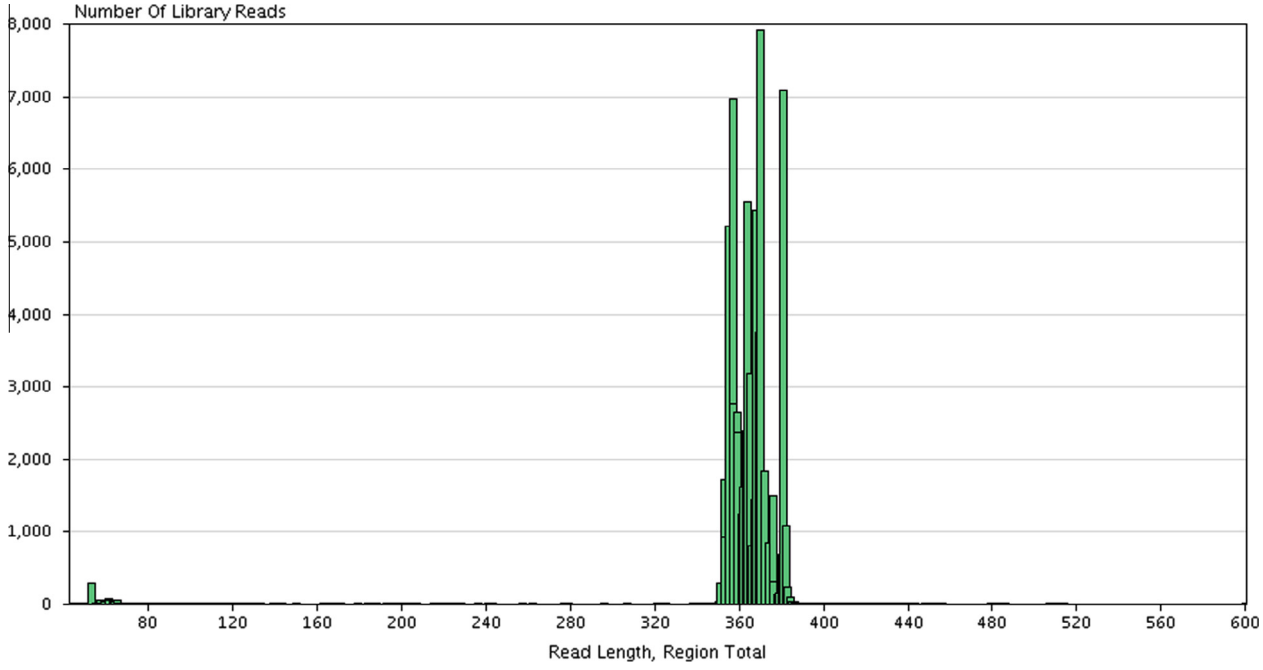

Figure 1 Distribution of generated amplicon sequence reads per patient.

11 of *TET2* gene, respectively. 310 A > C(7/8 87.5%) variation was the highest among the variants in intron 2 of *KRAS* gene. 1347 T > -in exon 8 of *CBL* gene was detected in 6 out 8 patients (75%).

All of AML patients were found negative for JAK V617 F. Three (37.5%) of the eight patients were only FLT3-ITD mutation positive.

4. Discussion

AML is a heterogeneous disorder of hematopoietic stem cells, characterized by multiple genetic events which have an impact on proliferation and differentiation. Some of the genetic and epigenetic alterations play a major role in leukemogenesis; gene mutations, deletions, translocations, and DNA methylation. Recent studies have reported that several genes such as *TET2, KRAS, CBL, FLT3, JAK2* involve in the pathogenesis of AML [1,3,6,10,11,18]. Activated *TET2, RAS* and *CBL* mutations confer proliferative and survival signals.

In this study, we screened the mutations of *TET2, KRAS* and *CBL* genes in childhood AML patients. We used an amplicon based sequencing method to find possible new genetic markers for leukemia diagnosis. *TET2, KRAS* and *CBL* genes were selected based on recent studies on genetic abnormalities in AML and other hematologic malignancies. In all patients, we reported novel mutations at *TET2* and *CBL* genes. 7 of 16 substitutions were missense mutations in the exon and UTR (Untranslated region). These mutations may result in truncated translation of protein.

There are very few studies in childhood AML regarding TET2 mutations. The frequency of TET2 mutations have been reported to be lower in childhood AML when compared with the adult AML [20]. Coenen et al. have performed PCR and sequencing analyses in order to compare childhood AML with the adult AML. They have shown that the frequency of CBL was similar (1–2%). Sano et al. have presented a study using PCR and sequencing analyses and have shown that 29 (18.5%) of the 157 childhood AML patients carried RAS mutations [3]. However, RAS mutations were reported 25–44% in adult AML [8–10].

In this study, we found 16 variants of which 7 of them are novel. The variants were found to be localized from exon 3 to exon 11 in TET2 gene (Fig. 2). Metzeler et al. demonstrated that the patients with favorable risk (according to European Leukemia Net (ELN)) and carrying *TET2* mutations had lower response ratios and higher risk of relapse or death when compared to the patients with *TET2* wild type [6]. *TET2* mutations that have frame shift, nonsense and missense mutations are acquired JAK2 mutations [2]. Our data suggest that TET2 variants are more frequent than CBL and KRAS variants in childhood AML.

Mutant *CBL* affects oncogenic phenotype in different cell lines and induces growth factor independence. Schnittger et al. analyzed 636 patients (MPN/MDS, seconder AML, CMML (Chronic Myelomonocytic Leukemia) and showed several *CBL* mutations localized in LINKER and RING domains in their study group. In addition, the frequency of *CBL* mutations was reported to be higher in patients with mutated JAK2 V617 F when compared to the patients with JAK2 V617 wild type [21]. Reindl et al. have detected exons 8 and 9 deletion transcripts of CBL in three of 279 AML/MDS patients. In PCR screening of AML/MDS patients, one of 116 patients carried a substitution of arginine to glycine at position 420. In addition, PCR screening revealed loss of the transcripts of mutant CBL lacking exons 8 and 9 in remission samples [11].

Several studies have shown that CBL promoted down-regulation of FLT3 activity. Sargın et al. searched the role of c-Cbl in FLT3 regulation and showed that deregulated FLT3 activity has significant roles in AML pathogenesis [22]. Reindl et al. revealed that four of 279 AML/MDS patients were found positive for expression of abnormal CBL transcripts, and hypothesized that CBL mutations might cause the high FLT3 expression and activation of abnormal FLT3 [11]. In a previous study, CBL variants have been detected at the rate of 1–2% [25]. In our study we also confirmed that AML patients had only two CBL variants.

Sano et al. studied 157 pediatric AML patients and reported that *RAS* mutation frequency was higher in AML M4–M5 types than other AML types [3]. Liang et al. searched for mutations in the *RAS* gene in 130 pediatric AML patients. *KRAS* mutation frequency was higher in MLL positive AML patients than MLL negative AML patients; without statistical significance ($P = 0.069$) [23]. Our screening results showed that seven of the eight patients (87.5%) have the same novel KRAS variant (1347 T del), and one patient has two novel variants (1347 T del and 1111 T > C). Relapse patients (number 4 and 5) are showing the same 3 novel TET2 variants, and same KRAS and CBL variants. In addition, they present FLT3-ITD positivity.

We think that 1347 T del (novel KRAS variant) and 1842 G del, 46 A del, 730 A del (3 novel TET2 variants) could be important prognostic markers for childhood AML/relapse patients and should need further study in a larger group of patient.

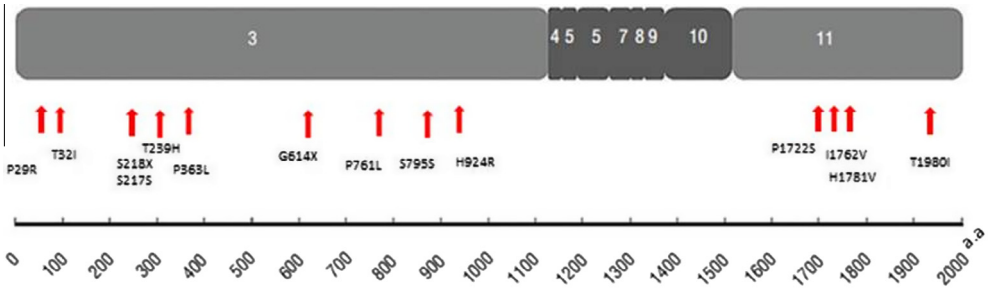

Figure 2 Localization of sequence variations in relation to the TET2 coding sequence.

5. Conclusion

In conclusion, *TET2* mutations are more frequent than *KRAS*, and *CBL* mutations in pediatric AML. In addition, *TET2, KRAS*, and *CBL* may potentially be genetic markers for leukemia diagnosis. However, these results need to be confirmed by further studies on a larger number of patients.

Conflict of interest

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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