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Analysis of *TET2* Exon 3 Mutation in a Small Population of Iranian Acute Myeloid Leukemia Patients

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ABSTRACT

Introduction: Aging is an inevitable biological process and is accompanied by several diseases, such as cardiovascular diseases, cancers, and diabetes, which are among worldwide leading causes of death. One of the prevalent cancers among older adults is acute myeloid leukemia (AML), a clonal and heterogeneous blood cancer. Old AML patients have poor prognosis that underlines the importance of finding new biomarkers and treatment for these patients. Ten-eleven translocation 2 (*TET2*) is one of frequently mutated genes in AML patients and specifically, in elderly patients. Therefore, in this exploratory study we aimed to analyze the existence of mutations in the third exon of *TET2* in a small cohort of Iranian AML patients.

Methods: Whole blood samples were collected from 16 newly diagnosed AML patients older than 40 years, excluding AML-M3 patients. DNA was extracted from blood samples and the third exon of *TET2* was amplified using PCR. Sanger sequencing was performed on the amplified exon 3 samples.

Results: The included patients were predominantly composed of M2 and M1 FAB subtypes. Sanger sequencing showed no sequence variations in the exon 3 of *TET2*, which may be due to several factors, including sample size, and population variations.

Conclusion: Larger cohorts and the implementation of more comprehensive sequencing strategies are required to further elucidate the prognostic value of *TET2* mutations in Iranian AML patients.

Keywords: *TET2*, Mutation, Acute myeloid leukemia (AML)

Introduction

It is predicted that by the year 2050, the Iranian elderly population will reach 21.7% (1). Aging is an inevitable biological process, which can be defined by significant time-dependent functional declines in cellular processes, including DNA repair, proteostasis, and respiration, and also changes in cellular programs, including senescence, stem cell exhaustion, epigenetic alterations, intercellular communication, and deregulated nutrient sensing (2). Moreover, the leading causes of death, such as cardiovascular diseases, cancers, and diabetes, can be considered as age-related complications (3, 4).

Despite advancements in medicine, a majority of cancer types cannot be cured (5). Among them, one of the most prevalent hematological neoplasms in the elderly population is acute myeloid leukemia (AML) (6). AML is a clonal and heterogeneous cancer. High blast counts (20≤) and maturation arrest are the most prominent clinical findings in AML (6). The majority of older adults cannot endure conventional treatments due to multiple factors, including frailty and comorbidities (7). The poor

prognosis of AML among elderly patients highlights the need for investigation of novel biomarkers and therapeutic targets.

Several studies have shown that mutations in *DNMT3A*, *TET2*, *ASXL1*, *NPM1*, *TP53*, and *FLT3-ITD* genes are frequent in AML patients (8). Among them, some somatic mutations are classified as clonal hematopoiesis (CH)-associated mutations. CH is regarded as a premalignant condition in which somatic mutations occur in blood cancer-related genes (9). Due to age-dependent nature of CH mutations, CH is more likely to be found in individuals aged 40 years and older (10). Moreover, ethnicity would be another factor affecting CH occurrence (11). Among CH genes, *DNMT3A*, *TET2*, and *ASXL1*, functioning as epigenetic regulators, are the most mutated genes (10). Although somatic mutations in such mentioned genes have been reported in several studies, their prognostic impact on AML has not been fully elucidated yet.

Ten-eleven translocation 2 (TET2) is a methyl-cytosine dioxygenase (12) and has a key role in the initiation of cytosine demethylation process (13). *TET2* is located on Ch4q24, which has 11 exons (14). Most mutations have been reported to be located in the third exon (14-16). TET2 has an essential role in hematopoiesis (17); due to disruption in its catalytic activity, TET2 mutations can affect normal hematopoiesis (18).

Considering the more common incidence of AML in old adults, and the impact of ethnicity on the occurrence of mutations in CH-associated genes, in this exploratory study, we aimed to investigate the prevalence of mutation/s in the third exon of *TET2* in a small Iranian population of newly-diagnosed AML patients.

Material and Methods

Patient selection and sample collection

This study was conducted under the Ethics Committee of Tarbiat Modares University, Tehran, Iran (IR.MODARES.REC.1403.155). Whole blood samples were collected in K2-EDTA tubes from 16 *de novo* AML patients from Shariati Hospital, Tehran, Iran. Informed consents were signed by patients before sample collection. Patients aged 40 years or older were included in this study and patients diagnosed as M3 subtype were excluded. Patients' clinical data, including AML-type, blast percentage, and complete blood count (CBC), are provided in Table 1.

Table 1: General clinical data of AML patients at diagnosis. SD: standard deviation; FAB: French American British; CBC: complete blood count; WBC: white blood cell; RBC: red blood cell; Hb, hemoglobin; Hct: hematocrit, MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; NA: not applicable.

Variant		Value	Reference range
Gender	male	9	NA
	female	7	
FAB classification	M0	2	NA
	M1	4	
	M2	7	
	M4	2	
	M5	1	
Blast percentage (Average ± SD)		25.63 ± 3.41	NA
CBC (Average ± SD)	WBC ($\times 10^3$ /µL)	35.75 ± 11.59	4.4–11
	RBC ($\times 10^6$ /µL)	Male: 3.58 ± 0.49	Male: 4.5–5.9
		Female: 3.92 ± 0.21	Female: 4.5–5.1
	Hb (g/dL)	Male: 10.37 ± 1.03	Male: 14–17.5
		Female: 10.12 ± 0.56	Female: 12.3–15.3
	Hct (%)	Male: 37.16 ± 1.84	Male: 41.5–50.4
		Female: 38.57 ± 1.39	Female: 35.9–44.6
	MCV (μm^3)	84.96 ± 1.96	80–96
	MCH (pg)	25.29 ± 1.44	27.5–33.2
	MCHC (%)	30.25 ± 0.92	33.4–35.5
	Platelet ($\times 10^3$ /µL)	151.69 ± 11.72	150–450

Table 2: Primer sequences used for the amplification of *TET2* third exon.

Primer	Sequence
Forward	CACACTGTAATGGAAATATCC
Reverse	ATCTAATCCATGTGTCCTAAG

Table 3: The utilized PCR condition for the amplifaction of *TET2* third exon.

Steps		Temperature (Celsius)	Time (seconds)	cycle amount
Initial Denaturation		95	300	1
Amplification	Denaturation	95	30	30
	Annealing	50	40	
	Elongation	72	30	
Final Elongation		72	300	1

DNA extraction

Salting-out method was used for DNA extraction from patient samples (19). Briefly, the blood cells were lysed by cell lysis buffer, containing sucrose, tris, $MgCl_2$, and Triton X-100, two times and thereafter, the nucleus was lysed by nucleus lysis buffer, containing tris, sodium dodecyl sulfate, and EDTA. Consequently, a NaCl solution (6M) was used to isolate and protect the DNA, and chloroform was used for phase separation step. After washing the salt ions by ethanol, the precipitated DNA pellet was dissolved in DNase-free water. The quality of extracted DNA was assessed by spectrophotometry and gel-electrophoresis.

Exon amplification and sequencing

Conventional PCR was used for exon amplification. Eppendorf Mastercycler Gradient (NSC 460, Kell 405) was used for PCR procedure. Taq DNA Polymerase 2x Master Mix RED was used for DNA amplification (Ampliqon, Cat.No.:A180301). The primer sequences used for the amplification of the third exon of *TET2* are presented in Table 2 and the utilized PCR condition is provided in Table 3. Sanger sequencing was performed to screen mutations in the amplified exons (Applied Biosystems 3500). Sequencing data was analyzed by NCBI gene database (https://www.ncbi.nlm.nih.gov/gene) and Gene runner version 6.5.52x64 Beta alignment tool.

Results

Patients laboratory and clinical features

In order to evaluate mutations in *TET2*, a total of 16 patients from both genders were included in this study. As presented in Table 1, the most common subtype was M2 comprising 43.75% of our cases, which was followed by M1 subtype (25%), M4 and M0 (each 12.5%), and M5 (6.25%). The average blast percentage of samples was 25.62%, and the average of CBC indices were as follows: white blood cell (WBC) 35.75×10^3 /µL, red blood cell (RBC) of male samples 3.58×10^6 /µL and female samples 3.92×10^6 /µL, hemoglobin of male samples 10.37 g/dl and female samples 10.12 g/dl, hematocrit of male individuals 37.16%, mean cell hemoglobin (MCH) 25.29 pg, and MCH concentration (MCHC) 30.25% (6). One patient had MCH within normal range. In contrast to female samples, hematocrits of all male samples were out of range. Although the platelet average was in the normal range, the platelet counts of eight individuals were out of normal range.

Mutation analysis by Sanger sequencing

In order to sequence the third exon of *TET2*, DNA was extracted from patients blood samples and the exon was amplified with reasonable quality (Figure 1). Sanger sequencing data revealed complete seqeuence alignment with NCBI gene database (GRCh38.p14 as current reference). As presented in Figure 2, no mutation was found in the third exon of our samples.

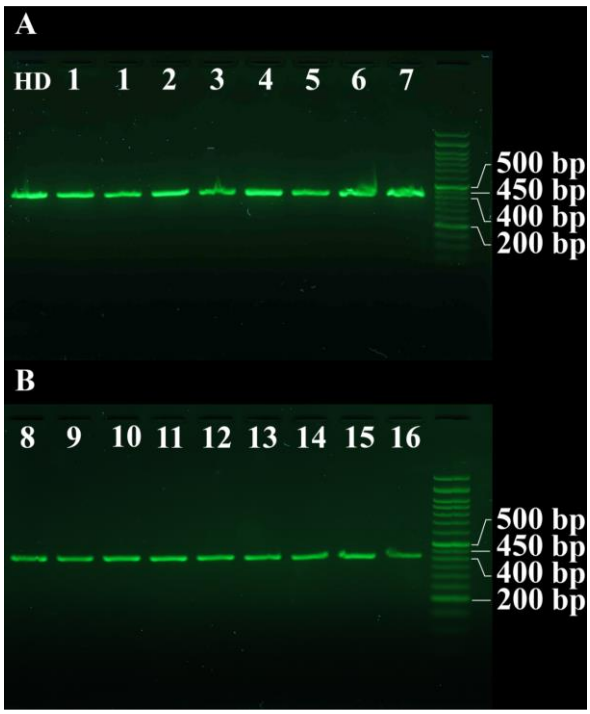

Figure 1: Gel electrophoresis graphs of PCR-amplified *TET2* third exon. (A) The upper gel includes the PCR products of one healthy donor (HD) sample and seven patient samples (patients 1-7). The PCR product of patient 1 is duplicated. (B) The lower gel includes PCR products of patients 8-16. The PCR product length is 419 bp.

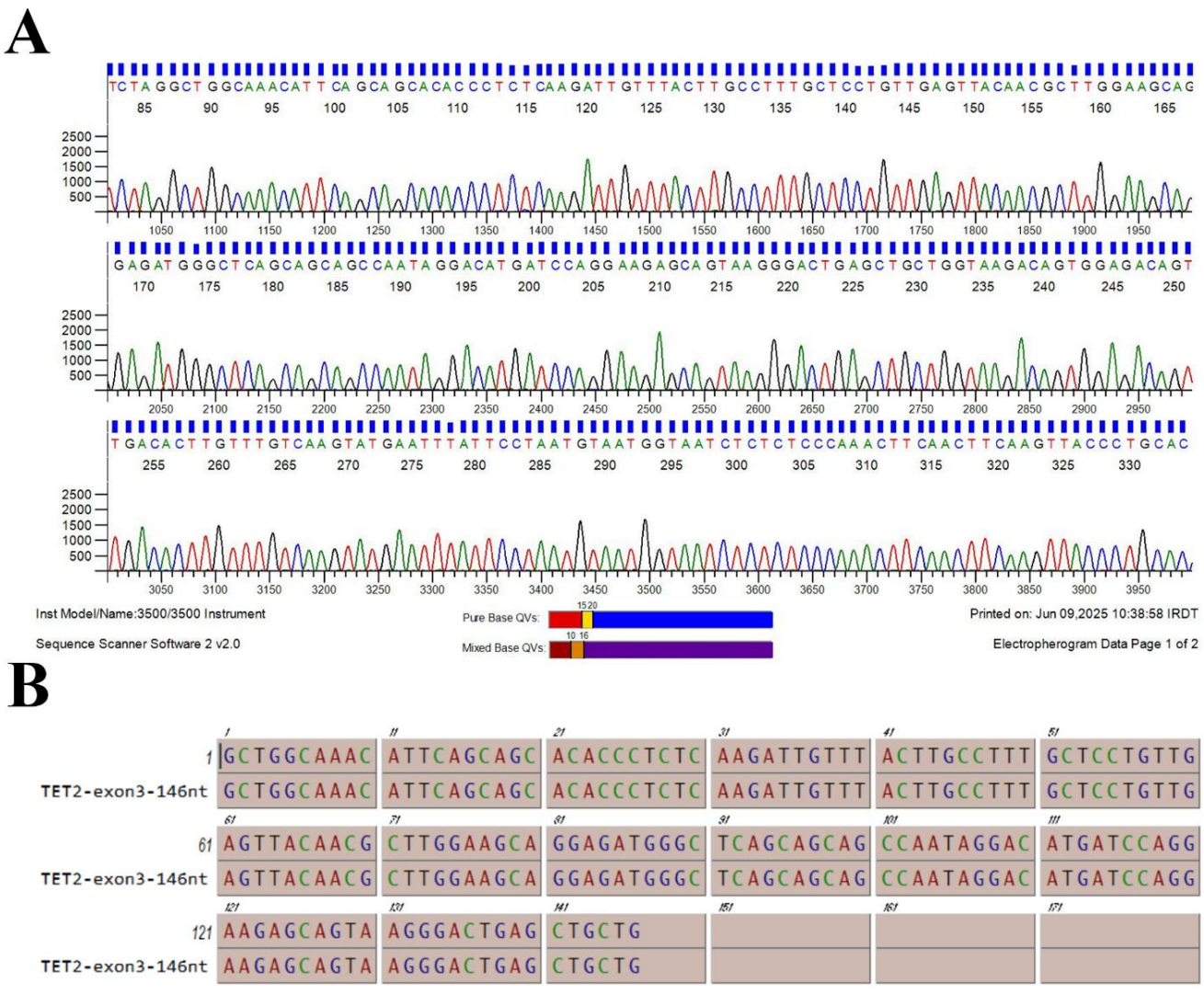

Figure 2: Sanger sequencing result (A) and its alignment result (B) for a patient sample as a representative of all patients.

Discussion

TET2 is a member of TET protein family consisting of three members, TET1, TET2, and TET3 (20). The TET name originates from MLL-TET1 fusion in AML patients with t(10;11) and thus, the term ten eleven translocation was coined for these proteins (21). Epigenetic regulatory function of TET2 plays a key role in hematopoiesis and disruption of its activity is associated with hematological diseases, such as myelodysplastic syndrome (MDS) and AML (22). Numerous studies have been carried out to determine the prognostic value of *TET2* mutation in AML patients, but their results are controversial and further investigation is required to elucidate their prognostic significance in AML patients (23, 24). Moreover, due to exon 3 location upstream of catalytic and functional domains of TET2, it is a common site for occurrence of inactivating mutations (16, 25-28), and thus, makes this exon an attractive target for investigating the impact of mutations on patients clinical outcomes.

In this study, we explored the third exon of *TET2* with the aim of detecting mutation/s as a potential biomarker for AML patients; therefore, M3 subtype patients were excluded from this study because of their satisfactory response to all-trans-retinoic acid (ATRA), which makes M3 subtype a favorable disease with good prognosis (29). We used peripheral blood samples for mutation detection in our study as their collection is simple, non-invasive, fast, and accessible, alongside their accuracy and reliability in the case of biomarker assessment (30).

The third exon of *TET2* was sequenced to assess the mutation occurrence in our patients. Based on previous studies, the mutation occurrence rate in AML patients is 10 – 30 % (23, 31, 32); however, no mutation has been detected in our samples. This contradictory result may be related to some factors. In addition to a relatively small sample size, our study was conducted in a population without any prior genomic and ethnicity analysis, which may be partly attributed to the absence of mutations. It should be considered that geographic region and ethnicity can affect the mutation frequencies among different populations with distinct genetic backgrounds (33). Different aspects of AML (e.g. cytogenetics, economy, and epidemiology) have been studied in Iranian patients (34-36). Among them, cytogenetic studies have gotten more attention recently. For example, Yazdandoust et al. have studied FLT3-ITD mutations and MDR1 gene expression in AML patients, using cDNA for mutation detection (36). Although there are some publications focused on cytogenetics, articles that study epigenetic-related mutations in Iranian population are limited.

Moreover, as explained earlier, since we aimed to reduce clinical heterogeneity, we excluded M3 subtype of AML patients because of their distinct molecular features and favorable clinical outcomes. This exclusion may have affected our observation in this study. Notably, M3 subtype has been excluded from some other similar studies. Moreover, the M2 subtype is the leading subtype in several studies (32, 37-41). Similarly, M2 was the predominant subtype in our study. It has been reported that mutation frequencies may vary across FAB subtypes. For example, Wu et al. have found that more than half of AML patients with somatic *TET2* mutations was grouped under M2 subtype; they hypothesized that this may be associated with TET2 role in differentiation (40). In a study by Shaikh et al., from 130 AML patients that were diagnosed during June 2019 to June 2020 in Sindh, Pakistan, 14 patients (9 males and 5 females) were selected for whole exome sequencing. Contrary to our study, age restriction was not one of their inclusion criteria, and they included adult patients ranging from 23 to 60 years old (average of 40.4 years). Moreover, their samples were mostly obtained from *de novo* AML patients. They reported that the M2 was the prominent AML subtype. Whole blood-derived DNA samples were used, and 17 variants were detected, including four novel variants with potential prognostic values. Genetic variations in exons 3 and 5 of *TET2* were validated by Sanger sequencing. They showed that *TET2* mutations had significant correlations with WBC and blast counts. Although they have found some clinical correlations with detected *TET2* mutations, they stated that the sample size of their cohort was too small to verify statistically significant associations (37).

In a study among 96 AML patients, the incidence of *TET2* mutation was reported to be 13.54% (95% CI, 6.70 – 20.38%) using PCR and direct sequencing of exons 3 to 11 (10). In comparison, our population was considerably

lower in numbers. Considering the small sample size, the absence of mutations may represent the limited statistical power and sample variabilities. Moreover, we used Sanger sequencing to detect mutations; while this method provides high accuracy for targeted regions, its sensitivity may be limited for low-frequency mutations (42), which may be contributed to our observation. One parameter that is overlooked in our mutation detection study is variant allele frequency (VAF), and this technique is incapable to measure this factor exactly and can not detect somatic mutations with low VAF (43). In this regard, increasing the sample size, utilizing whole exome sequencing, implementing next generation sequencing (NGS), and the design of specific primers to detect certain mutations can be beneficial.

This study has several limitations that should be considered. Sample size was relatively small and our sequencing was restricted to the exon 3 of *TET2* gene using Sanger sequencing. Accordingly, incorporating larger studies utilizing comprehensive sequencing approaches, including NGS of the whole *TET2* gene, and the analysis of mutation occurrence at subclonal levels could advance this field.

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Conflict of interest statement

All authors declare no conflict of interest.

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