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Investigation of FMS-Like Tyrosine Kinase 3 Mutation Frequency in Myelodysplastic Syndrome

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: FMS-Like Tyrosine Kinase Class 3 (FLT3) mutations harbor poor prognosis, high relapse, and decreased overall survival in acute myeloblastic leukemia (AML). This mutation is also known to be demonstrated in myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia and acute lymphoblastic leukemia. This study included 94 MDS-diagnosed patients and we tried to investigate FLT3 mutation frequency (as tyrosine kinase domain-TKD and internal tandem duplication-ITD).
Materials and Methods: Polymerase chain reaction (PCR), restriction fragment length polymorphism, and agarose-gel electrophoresis methods were used to analyze the mutation. The blood samples were collected in K3-EDTA tubes, and total DNA was isolated using genomic DNA

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isolation kits (GeneMark, Cat No: DP023P). For the detection of FLT3-ITD mutation, PCR was performed to amplify a 330- base pair fragment of exons 11 and 12 of *FLT3* using FAM (Carboxyfluorescein)-labeled ITD-11F and HEX (Hexachloro-Fluorescein)-labeled ITD-12R primers in a thermal cycler (Eppendorf). Similarly, to detect D835 mutation, a 115- bp region of exon 17 of the *FLT3* gene region was amplified using primers.

Results: One patient was found FLT3-ITD positive (1.1%). The patient was 64-year-old and diagnosed with MDS-excess blast type 2 according to the World Health Organisation 2016 myeloid neoplasm classification. He transformed to AML within 19 months and subsequently died after 1 month. No patient with tyrosine kinase domain mutation was detected.

Conclusion: FLT3 mutation is considered a significant parameter to define prognosis in AML. The routine workup of FLT3 screening and the potential of targeting FLT3 inhibition for high-risk MDS may be taken into consideration in the future.

Keywords: Cytopenia; FMS-like tyrosine kinase mutation; myelodysplastic syndrome; prognosis.

1. INTRODUCTION

Myelodysplastic syndrome (MDS) is a heterogeneous clonal stem cell disorder that is characterized by cytopenia and abnormal cellular proliferation of bone marrow hematopoietic cells. MDS is associated with serious clinical problems, including morbidity due to cytopenia as well as a potential transformation to AML [1]. FMS-Like Tyrosine Kinase 3 is a class 3 receptor tyrosine kinase family member. It plays a role in the proliferation and differentiation of hematopoietic stem/progenitor cells. FLT3 is the most frequently mutated gene in AML (30%), and patients who harbor this mutation have higher relapse rates and lower overall survival (OS) [2]. FLT-3 mutations are also observed in MDS patients. Currently, there is a heterogeneity of data on the frequency of FLT-3 mutation in MDS and it varies between 0% and 7% [3-10]. Therefore, in this study, we aimed to quantify FLT-3 mutation frequency in MDS and to determine its relationship with prognostic scoring systems.

2. MATERIALS AND METHODS

2.1 Study Design

The study was designed to be single-center, multidisciplinary, analytic, and cross-sectional. Ninety-four patients who were diagnosed with MDS according to the World Health Organization (WHO) 2016 myeloid neoplasms classification in Aydın Adnan Menderes University Hematology Department between August 2018 and August 2019 were included in the study [11].

2.2 Selection Criteria

Patients above 18 years of age who had been diagnosed with MDS were included in the study. Individuals were excluded if they were less than 18 years of age and had MDS-AML transformation. Informed consent was obtained from all patients.

2.3 Specimen Collection and Laboratory Method

The investigation of FLT3 mutation status was performed at diagnosis and it was assayed for one time. The blood samples were collected in K3-EDTA tubes, and total DNA was isolated using genomic DNA isolation kits (GeneMark, Cat No: DP023P). For the detection of FLT3-ITD mutation, polymerase chain reaction (PCR) was performed to amplify a 330- base pair (bp) fragment of exons 11 and 12 of *FLT3* using FAM-labeled ITD-11F: 5′′- GCA ATT TAG GTA TGA AAG CCA GC-3′′ and HEX-labeled ITD-12R: 5′′-CTT TCA GCA TTT TGA CGG CAA CC-3′′ primers in a thermal cycler (Eppendorf) [12]. Wild-type PCR products were expected to consist of 330 bp in the absence of ITD mutation while ITD mutant PCR products were to be larger than 330 bp. We preferred to use double-labeled forward and reverse primers to increase both the sensitivity and specificity of the ITD PCR. Otherwise, peaks caused by nonspecific PCR products would be created, leading to false results if a single fluorescent labeled primer was used. We decreased the likelihood of getting a false-positive result by choosing double-labeled fluorescent primers. PCR was then performed in a final volume of 25 µL containing 12.5 µL PCR ready master mix, 0.4µL primers (-20 µM each), 1 µL (100 ng) template DNA, and 10.7 µL sterile dH_2O. The amplification program started with an initial denaturation at 95 ℃ for 1 min; 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 56 ℃ for 1 min, extension at 72 ℃ for 2 min and a final extension at 72 ℃ for 5 min. Subsequently,

amplified fragments were loaded onto a 1% agarose gel and visualized under UV light. Finally, PCR products were sent to Macrogen Inc. (Seoul, South Korea) for fragment analysis to obtain allele sizes of FLT3 amplicons. The allele sizes of FLT3-ITD mutation of each patient were determined by the Peak Scanner program (Peak Scanner software 2.0, Thermo Scientific). Individuals with fragments of 330 bp were identified as individuals with wild-type gene regions; individuals with fragments of longer than 330 bp were identified as mutated individuals. Based on this, individuals with both 330 bp and higher than 330 bp were considered heterozygous individuals.

Similarly, to detect D835 (aspartic acid) mutation, a 115- bp region of exon 17 of the *FLT3* gene region was amplified using primers D835-17F: 5′′-CCG CCA GGA ACG TGC TTG-3′′ and D835-17R: 5′′-GCA GCC TCA CAT TGC CCC-3′′ in a thermal cycler [13]. PCR was performed in a final volume of 25 µL containing 12.5µL PCR master mix, 0.4µL primer (20 µM each), 1 µL (100 ng) template DNA, and 10.7 µL sterile dH_2O. The amplification program consisted of an initial denaturation at 95 ℃ for 5 min, 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 60 ℃ for 45 s, an initial extension at 72 ℃ for 45 s and finally followed by another extension at 72 ℃ for 7 min. Subsequently, amplified fragments were loaded onto a 1% agarose gel and visualized under UV light. A restriction fragment length polymorphism (RFLP) analysis was carried out by using the *EcoR* V enzyme, which recognizes GAT'-ATC sequences. The reaction mixture for RFLP consisted of 1 µL 10 × RFLP buffer, 1 µL *EcoR* V enzyme, 5 µL PCR product, and 11 µL sterile distilled water. The mixture was incubated at 37 °C for 4 h in a thermal cycler to perform the RFLP reaction. The reaction was stopped by incubating at 65 °C for 20 min. Products were loaded onto a 3% agarose gel and run at 50 mA for 90 min. In the absence of the D835 mutation, band digestion occurred, creating fragments (68 bp and 47 bp). However, the base change leading to the D385 mutation destroys the enzyme recognition site and therefore yields an undigested band profile (115 bp). Finally, undigested and three randomly selected PCR products were sent to Macrogen Inc. (Seoul, South Korea) to obtain amplified FLT3-D835 sequences.

2.4 Statistical Analysis

Statistical analyses were performed using the SPSS computer software 21.0 (IBM). The variables were expressed as mean ± standard deviation in quantitative data, and using frequency (count-percentage) for categorical data. The Mann-Whitney U test was used to compare the differences not normally distributed ordinarily differences between groups. A p-value below 0.05 was the cutoff for statistical significance.

3. RESULTS

Ninety-four previously/recently diagnosed MDS patients were included in the study. Forty-nine (52.1%) patients were females and 45 (47. 9%) were males. The median age of the patients was 73 ± 10 years. There was no statistically significant difference in age distribution between the sexes. The follow-up duration was 41. 88 ± 25 months; 67 (71.3%) patients were alive while 27 (28.7%) had died. The cumulative survival rate for the first year was 94.6 ± 2.3% and then decreased to 65.6 ± 6.3% by the fifth year. Forty patients(42.5%) had single lineage dysplasia(SLD), 22 patients (23.4%) had multi-lineage dysplasia(MLD), 1 patient(1.06%) had MDS-Ring sideroblasts (RS) with SLD, 2 patients (2.1%) had MDS-RS-MLD, 3 patients (3.2%) had MDS with deletion 5q abnormality, 14 patients (14.9%) had MDS-EB-1 and 12 patients (12.8%) had MDS-EB-2 according to the WHO 2016 myeloid neoplasm classification. Twenty-two patients were excluded from the prognostic scoring because no karyotype analysis could be performed due to technical reasons. The characteristics of the patients are shown in Table 1. Fragment analysis revealed a 330 bp (wild type) product derived from juxtamembrane region of FLT3 in 93 of 94 patients with no evidence of ITD abnormalities (98.9%). A heterozygous ITD mutation was detected in only 1 patient (1.1%). In this patient, the fragments were 330 and 387 bp in length. RFLP analysis showed that none of the 94 patients had the FLT-tyrosine kinase domain (TKD) D835 mutation. It also showed that the 115-bp PCR products obtained from all patients were cut into two fragments of 68 bp and 47 bp by *EcoR* V due to the absence of mutation. Identification of the ITD mutation and image of the fragment analysis are shown in Figs 1 and 2, respectively. The FLT3-mutated patient had 10% blasts in the initial bone marrow examination (MDS-EB-2) but prognostic indices could not be calculated due to insufficient results from the karyotype analysis. The follow-up duration for the FLT3-mutated patient was 20 months and was diagnosed with secondary AML 19 months after the initial MDS diagnosis. The

follow-up duration after AML diagnosis was 1 month and the patient then died from sepsis.

4. DISCUSSION

FLT3 is a transmembrane ligand-activated receptor kinase that is expressed in hematopoietic stem/progenitor cells. It is a key regulator of the development of myeloid and lymphoid precursors. FLT3-ITD mutation, which is present at a frequency of 25% in AML, is a driver mutation that causes high leukemic burden and is associated with poor prognosis. Compared to FLT3-ITD, the FLT3-TKD mutation is infrequent (7-10%) and its biologic significance is less clear [2]. FLT3 mutations can also be seen in MDS and chronic myelomonocytic leukemia (CMML). Trials in which the frequency and prognostic significance of FLT3 mutations are examined have generally been focused on AML and they are limited in number for MDS [3-10]. Previously, 7 patients (7%) positive for FLT3 ITD mutation were reported by Horiike et al; in a study involving 92 MDS, CMML, and AML patients. This study included 49 MDS patients (except CMML); and no FLT3 mutations were detected in the refractory anemia (RA) and refractory anemia-ring sideroblastic(RARS) subgroups while 1 of 12 patients in the refractory anemia and excess blasts in transformation (RAEB-T) subgroup had an FLT3 ITD mutation(allocation to the subgroup was based on the French-American-British (FAB) classification, see reference 3).

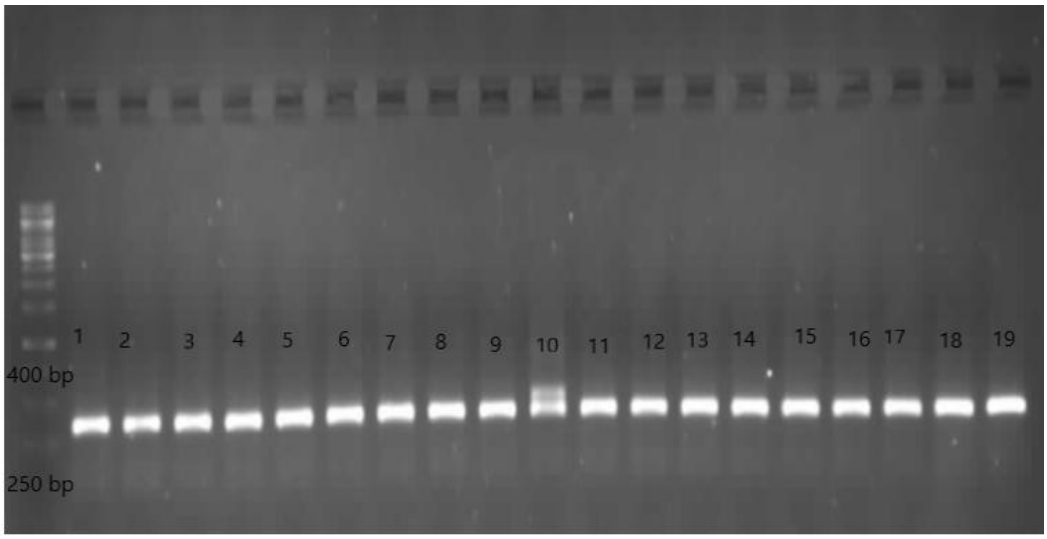

Fig. 1. The image of ITD mutation on agarose gel

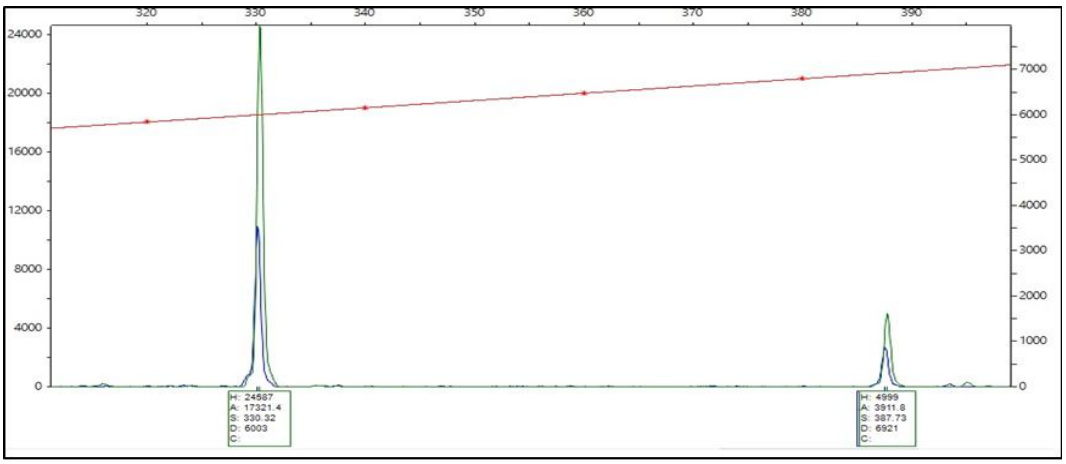

Fig. 2. Fragment analysis result of heterozygous FLT - ITD positive patient having 330 and 387 bp bands [Blue: FAM (Carboxyfluorescein fluorescent dye), Green: HEX (Hexachloro-Fluorescein fluorescent dye

Table 1. The characteristics of the patients

Parameters	n (%)	Median (±SD)
Age	(-)	73 ± 10
Gender		
Male	45 (47.9)	(-)
Female	49 (52.1)	
Hemoglobin(g/dL)	94 (100)	9.8 ± 1.8
Hematocrit (%)	94 (100)	30.3 ± 5.6
Leukocyte(10^3/µL)	94 (100)	5625 ± 4427
Platelet(10^3/µL)	94 (100)	178500 ± 164441
LDH(U/L)	94 (100)	198 ± 179
BM blast (%)		
0%	50 (53.29	(-)
1-4%	18 (19.1)	
5-9%	14 (14.8)	
10-19%	12 (12.7)	
MDS subtype	40 (42.5)	(-)
SLD	22 (23.4)	
MLD	1 (1.06)	
MDS-RS-SLD	2 (2.1)	
MDS-RS-MLD	3 (3.2)	
MDS with del5q abnormality	14 (14.9)	
MDS-EB-1	12 (12.8)	
MDS-EB-2		
Cytogenetic abnormalities		(-)
Normal	46 (48.9)	
Y chromosome loss	3 (3.1)	
Trisomy 8	1 (1)	
Del 11q	1 (1)	
Del 7q	0	
Del 20q	0	
3≤ complex abnormality	2 (2.1)	
Other	19 (20.2)	
Missing	22 (23.4)	
Treatment		
Observation or BSC including Tx	28 (29.7)	(-)
Growth factors (ESA, TPO-RA)	22 (23.4)	
Lenalidomide	11 (11.7)	
Hypomethylating agents	30 (31.9)	
Targeted agents (e.g., IDH inhibitors)	0	
SCT	3 (3.1)	

BM: Bone marrow, BSC: Best supportive care, Del: Deletion, ESA: erythropoiesis-stimulating agent, IDH: isocitrate dehydrogenase, LDH: Lactate dehydrogenase, MDS: Myelodysplastic syndrome, SCT: stem cell transplantation, TPO-RA: thrombopoietin receptor agonist, Tx: Transfusion, SD: Standard deviation

Shih et al. reported a 6% frequency of FLT3-ITD mutations in newly diagnosed MDS patients (including CMML). In this trial, FLT-ITD mutation was demonstrated in 82 MDS patients who later progressed to AML. One patient among 33 RAEB and 3 patients among 27 RAEB-T patients were FLT3-ITD positive. No FLT3 mutations were detected in 11 patients in the RA and RARS category. One of 11 CMML patients also had FLT-ITD mutation. No statistically significant differences for age, sex, blood count, circulating blast ratio, FAB classification subgroup, cytogenetic or International Prognostic Scoring System (IPSS) results were detected. A higher bone marrow-blast ratio has previously been associated with the presence of FLT3-ITD mutation [4].

Another trial by Bacher et al. involving 359 MDS patients (excluding CMML) revealed that 8 patients (2.2%) had FLT3-ITD positivity. All the positive patients were in the RAEB (bone marrow blasts, 5-19%) subgroup. FLT3-TKD mutation was found in 1 patient (0.4%) in the RA/RARS category [5].

Twelve patients (4.3%) had FLT3 mutations (including ITD and TKD) among 1232 MDS patients who were diagnosed between 1997-2010 in the largest trial performed by the MD

Table 2. FLT 3 mutation frequency in MDS patients based on the literature

	Total MDS patient number (excluding CMML)	FLT3-ITD positivity (%) at diagnosis	FLT3-TKD positive patient number at diagnosis	RA/RARS or SLD/MLD/del5q/other low risk category patient number/FLT3 mutated patient number	MDS-EB (RAEB) category patient number/ FLT3 mutated patient number	MDS-EB-T (RAEB-T) patient number/ FLT3 mutated patient number
Horikee et al.[3]	49	1 (2)	0	17/0	20/0	12/1
Shih et al.[4]	71	4 (5.6)	1 (1.4)	11/0	33/1	27/4
Bacher et al.[5]	359	8 (2.2)	1 (0.2)	28/1	293/8	n.a.
Daver et al.[6]	1232	9 (0.7)	3 (0.2)	560/1	651/11	n.a.
Xu et al[7]	304	7 (2.3)	n.a.	116/0	183/7	34/8 ¶
Yu et al.[8]	93	0	n.a.	66/0	27/0	n.a.
Badar et al.[9]	152	5 (3.2)	n.a	n.a	n.a	n.a
Bezerra et al.[10]	84	1 (1.1)	n.a	63/0	25/1	n.a
Our Study	94	1(1)	0	68/0	26/1	0

¶ Five patients developed the mutation in the course; CMML: Chronic myelomonocytic leukemia, del: deletion, FLT 3: FMS-Like Tyrosine Kinase Class 3, MDS: Myelodysplastic syndrome, MDS-EB: MDS with excess blasts, MDS-EB-T: MDS-EB in transformation, MLD: multilineage dysplasia, n.a: Not Applicable, RA/RARS: Refractory anemia/ refractory anemia with ring sideroblasts, RAEB: refractory anemia with excess blasts, RAEB-T: RAEB in transformation, SLD: single lineage dysplasia

Anderson Cancer Center. Nine of them (75%) were FLT3-ITD positive while 3 of them (25%) had FLT3-TKD mutations. The positive patients were generally from the RAEB category and tended to be younger (60 vs. 68). One refractory anemia patient out of 12 had an FLT3 mutation [6]. It is suggested that FLT3 mutations are generally associated with decreased OS and event-free survival, but the MD Anderson trial, with an expanded patient number showed that the FLT3 mutations conferred no significant survival alteration when compared with FLT3 wild-type.

Another study by Xu et al. demonstrated 7 patients (2.3%) with FLT3-ITD mutation in the cohort consisting of 304 de-novo MDS diagnosed patients. AML transformation rate of FLT3-ITD positive patients was 42.9%. A total of 34 patients (11.1%) were transformed into AML and three and five of them had FLT3-ITD mutation initially and gained later in the course, respectively [7]. Interestingly, another study by Yu et al. detected no FLT3 mutation in a group of 93 de-novo MDS-diagnosed patients [8].

Badar et al. also investigated the frequency of FLT3 mutation. It was detected that the rate of positivity for the mutation (ITD or TKD, not specified) was 3.2% (five in 152 patients) among MDS-diagnosed patients and 3.4% (five in 145 patients) among AML patients transformed from MDS [9].

Recently, Bezerra et al. detected one patient (1.1%) with FLT3-ITD mutation in a study consisting of 88 MDS-diagnosed patients including CMML. The patient was included in the MDS-EB-1 subtype. Also, this mutation was investigated in 35 patients with AML who were transformed from MDS, and the mutation rate increased to 14% in this group [10].

Although there is debate about the impact of FLT3 mutation on OS, all the trials provide evidence that FLT3-ITD mutations can have a significant role in MDS-AML transformation [3-10]. A summary of FLT3 ITD/TKD positive frequencies in patients in these trials is shown in Table-2. FLT3 mutation was present at a frequency of 1.1% in our study, which is consistent with values from other trials. The outcomes may be affected by lower patient numbers and/or the inclusion of MDS patients with prior treatment history. Consistent with the other trials' conclusions, our patient with FLT3-ITD mutation had a poor prognosis; the MDS transformed to AML in a relatively short time (19 months) and the patient died in one month thereafter.

The limitations of our study are that the study has been carried out using blood samples instead of bone marrow samples and screening for FLT3 mutations was performed by PCR instead of the next generation sequencing (NGS), as a more sensitive method. The NGS technology also allows the detection of mutational alterations along the disease course from diagnosis to progression/relapse [14]. Another restriction in the study is the absence of allele burden measurement due to technical limitations. While it is suggested that a high allele burden for FLT3-ITD- positive AML worsens prognosis, this is less clear in the case of MDS [15]. The incidence of FLT3-ITD mutation in AML may be lower in Eastern Asians when compared to Caucasians, however, there is no data to suggest that this may impact the FLT3 mutation frequency in Turkey [16]. An important aspect of our study is that, unlike many other studies focusing on the ITD mutation, it also investigated TKD mutations in MDS [7,8,10]

5. CONCLUSION

FLT-3-ITD mutation screening has been adopted in routine AML work-up and positive screening result signifies poor prognosis, high relapse rate, and short overall survival. This mutation is also found in MDS, CMML, and ALL. In AML, first-generation FLT3 inhibitors such as Midastaurin, Sunitinib, Lestaurtinib, Sorafenib, Ponatinib, and Tandutinib and second-generation inhibitors such as Crenolanib, Gilteritinib, and Quizartinib are either approved or under investigation in clinical trials [16]. The FDA and European Medicines Agency have approved Midastaurin in combination with chemotherapy for FLT3-mutated adult AML [17]. FLT-3 mutation investigation is not in routine use in MDS work-up and to the best of our knowledge, there have been no studies demonstrating the benefit of altering treatment based on FLT3 mutational status. We suggest that routine FLT3 screening for high-risk MDS should be considered in the future. Whether the FLT3-ITD mutation shortens overall survival remains unclear, there seems to be a consensus that the mutation increases the risk of MDS transformation to AML. The presence of the mutation potentially worsens prognosis in cases where secondary leukemia is already present. More extensive trials are necessary to accurately determine FLT3

mutational frequency, and the significance of TKD mutations in MDS as well as the impact that mutations should have on decisions related to the treatment regimen.

CONSENT AND ETHICS APPROVAL

All patients provided their written informed consent to receive each regimen, and treatment was administered according to the principles of the Declaration of Helsinki. Approval from the ethical committee (Adnan Menderes University, Aydın, Turkey) had previously been obtained (2018/1455)

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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