Research Article

Mutations of FLT3 Gene in Pakistani Acute Myeloid Leukemia Patients

Sehrish Nadyme[1], Nadia Sajid[2], Saqib Mehmood[3]

[1]Demonstrator, Biochemistry Department, King Edward Medical University, Lahore; [2]Consultant Hematologist, Inmol Hospital, Lahore; [3]Assistant Professor, Human Genetics and Molecular Biology Department, University of Health Sciences, Lahore

Abstract

Background: Acute myeloid leukemia, AML, is a hematological malignancy, with diverse genetic abnormalities. Mutations in FLT3 gene are the most common aberrations found in AML. The two important FLT3 mutations are ITD and D835 mutation. Both of these mutations are of prognostic significance in AML and are target of currently developing new drugs which are part of strategies to improve outcome of AML patients.

Objectives: The objective of this study was to determine the frequency of ITD and D835 mutations of FLT3 gene in AML patients in Pakistani population.

Methodology: 47 untreated and diagnosed male and female AML patients of all age groups were included in the study. After DNA extraction, exons 14 and 15 (for ITD) and exon 20 (for D835) of FLT3 gene were amplified by PCR. For ITD mutation detection, PCR products were analyzed by amplified fragment length polymorphism analysis, while D835 mutations were detected by restriction fragment length polymorphism by using EcoRV restriction enzyme.

Results: Frequencies of both FLT3/ITD and FLT3/D835 mutations were found to be 6.4%. ITD mutation shows a significant association with old age and high WBC count.

Conclusion: The frequency of FLT3/ITD mutations, found in the present study, is lower than that reported in other studies, while that of D835 mutation is within the range observed by most researchers. This lower ITD frequency than usual while normal D835 frequency showed the possibility that, instead of ITD mutation, other mutations of the FLT3 gene (like D835) may be more common in the local population.

Received | 29-05-2018: **Accepted** | 13-04-2019

Corresponding Author | Dr. Saqib Mehmood, Assistant Professor, Human Genetics and Molecular Biology Department, University of Health Sciences, Lahore, **Email:** sqb_medgen@yahoo.com

Keywords | fms-Like Tyrosine Kinase 3, Leukemia, Myeloid, Acute, ITD, D835

Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic stem cell disorder which is characterized by acute over-poliferation of stem cells of myeloid lineage along with failure in their differentiation into functional leukocytes and in some instances erythrocytes. This results in accumulation of myeloblasts, along with hematopoietic insufficiency. AML results from the genetic and epigenetic changes that disturbed the key cell processes[1]. Among these genetic changes, mutations in FMS like tyrosine kinase 3 (FLT3) gene were one of the most common aberrations found in AML.[2]

FLT3 gene is located on chromosome 13q12 and it codes for a cell surface tyrosine kinase receptor, called FLT3 receptor. Mutations in FLT3 gene results

in dimerization of these receptors in a ligand-independent manner, leading to autonomous growth in the mutant cells and therefore, cause leukemia. Out of these FLT3 mutations, two most commonly occurring ones were ITD (internal tandem duplication) and D835 (tyrosine kinase domain TKD) mutations.[3-5]

In recent years, FLT3/ITD mutation had been found as the most common mutation in AML.[3,4] In the FLT3/ITD mutation, a fragment of the juxtamembrane (JM) domain-coding sequence in exons 14 and 15 of the FLT3 gene is duplicated in direct head-to-tail orientation.[6] The duplicated region is variable in both size and location in different individuals and is always in frame. The overall frequency reported in adult patients, by different studies, varied with a range between 13.2% and 32% [3]. It is associated with poor prognosis.[4,5,7]

The second most frequent FLT3 mutation found in AML patients is a point mutation in exon 20 of FLT3 gene. This region of FLT3 gene coded for aspartic acid (D) at 835th position in second tyrosine kinase domain of FLT3 receptor.[8] The frequency of D835 mutation varied with a range of 5% to 10%. Although, at present, the true prognostic significance of this mutation is controversial, however, the meta-analysis suggested that its presence had adverse effects on the outcome.[4]

The clinical outcome of acute myeloid leukemia is quite worse, despite intensive chemotherapy. That's why, new strategies to improve outcome of AML patients are very important. FLT3 activating mutations are a promising target for therapeutic interventions. Over the last decade, more than a dozen FLT3 tyrosine kinase inhibitors have been studied and are in different stages of development.[9] Clinical trials of few, especially of sorafenib, Crenolanib and quizartinib, had shown measurable clinical responses.[4,5]

As it is reasonable to say that future of AML therapy will include FLT3 inhibitors, so it is necessary to know the frequency of FLT3 mutations, in a specific population. This would provide knowledge which would help recommend routine testing of FLT3 mutations in AML patients and plan treatment strategies according to the findings. Due to insufficient data available regarding FLT3 mutations in Pakistan, their frequency in our local AML patients is very important. The objective of this study was to determine the frequency of two most common types of FLT3 mutations (ITD and D835) in the AML patients from local population and to find their relation with demographic and clinical variables likes age, gender, hematological parameters and FAB subtype.

Methods

A total of 47 untreated patients of acute myeloid leukemia, diagnosed on the basis of bone marrow morphology and cytochemistry, were selected from different tertiary care hospitals of Lahore, from 2011 till 2013. Male and female patients of all age groups were included. While patients who had started receiving and/or had received complete treatment for AML or patients who had AML secondary to chronic myeloid leukemia (CML) or myelodysplastic syndrome (MDS) were excluded from the study. Consecutive sampling was done.

After ethical approval from IRB of UHS, written and informed consent was obtained from the patients. Then complete clinical history and laboratory investigation data, including Hb levels, WBC count, platelet count, blast count and FAB subtype of AML, was collected on a proforma.

Using aseptic measures, 3 ml peripheral venous blood samples and 1 ml bone marrow aspirates were collected in E.D.T.A vacutainers.

Genomic DNAs were extracted from all collected blood and bone marrow aspirate samples by using phenol chloroform method.[10] The final volume of DNA extraction was 50µl.

To detect ITD mutation, exons 14 and 15 of FLT3 gene were amplified by PCR (BIO-RAD, Hercules, California, USA) by using primers 14-F (5'-(6-FAM) GCA ATT AAG GTA GGG AAG GGA GC-3') and 15-R (5'-CTT TGA CCA TTT AGT GGC AAC CC-3').[11] The primers were of 23bp each. The forward primer was labeled with FAM (Fluorescein amidite). By using 1 µl solutions of these primers each, along with 12.5 µl PCR master mix (by Fermentas, Massachusetts, USA), 1 µl previously extracted sample DNA and 9.5 µl nuclease free water, a total of 25µl reaction mixture was prepared in a 0.2ml PCR tube.

Denaturing, annealing, and extension steps were performed for 1 minute each at 95 °C, 60 °C and 72 °C respectively, for 35 cycles. There was an initial 5 min denaturation step at 95 °C and a final extension step at 72 °C for 10 min. Fluorescently labeled PCR products were then processed for amplified fragment length analysis and loaded onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). A peak of 329 bps was considered of wild-type (non-mutated) allele, while peak of more than 329 bps was considered of mutated allele.

To detect D835 mutation, exon 20 of FLT3 gene was amplified by PCR (BIO-RAD, Hercules, California, USA) by using primers 20F (5'-CATCACCGG TACCTCCTACTG-3') and 20R (5'-TAACGAC ACAACACAAAATAGCCGT-3'). Primers were of 21bp and 25bp respectively. By using 1 µl solutions of these primers each, along with 12.5 µl PCR master mix (by Fermentas, Massachusetts, USA), 1 µl previously extracted sample DNA and 9.5 µl nuclease free water, a total of 25µl reaction mixture was prepared in a 0.2ml PCR tube. Denaturing, annealing, and extension steps were performed for 30 seconds each at 95 °C, 58 °C and 72 °C respectively, for 35 cycles. There was an initial 5 min denaturation step at 95 °C and a final extension step at 72 °C for 10 min. Then PCR products were digested by 1 µl of 5 U EcoRV restriction enzyme (by Fermentas, Massachusetts, USA) at 37 °C for 1 hour. After 1 hour, the digested products were mixed with loading dye (0.25% bromophenol blue) and loaded into the wells, along with control ladder, on 2% agarose gels stained with ethidium bromide. Electrophoresis was performed at 100 volts for 45 minutes in 1X TBE buffer, on gel electrophoresis apparatus (by BIO-RAD, Hercules, California, USA). Digested products of 188 bps and 90 bps fragments considered negative, while undigested product of 278 bps considered positive for D835 mutation.[12]

Statistical analysis was done by using SPSS 18.0. Mean and standard deviations of normally distributed quantitative variables (that is age, Hb levels and blast count), median and IQR of skewed quantitative variables (that is WBC count and platelet count) and frequencies and percentages of qualitative variables (that is ITD and D835 mutation, gender and FAB subtype) were calculated. To determine the relation of ITD and D835 mutations with normally distributed quantitative variables student t test was applied, and with skewed quantitative variables Mann Whitney U test was applied and p-value was calculated. A p-value of less than 0.05 was considered significant. While for relation of ITD and D835 mutations with qualitative variables, percentages in AML patients with and without mutations were compared.

Results

A total of 47 diagnosed patients of AML were included in the study. There were 28 (60%) male and 19 (40%) female patients. Their ages ranged from 5 years to 68 years, with the mean age of 31(± 16.7) years. Regarding FAB classification, the highest frequency of patients belong to M2 subtype, that is 15 patients (31.9%), followed by M3 subtype, which had 14 patients (29.7%).

For FLT3/ITD mutation, graph for amplified fragment length polymorphism analysis showed a single peak of normal allele of 329 bps in 44 (93.6%) patients, while double peaks, one of normal allele and other of mutated allele of more than 329 bps were detected in 3 (6.4%) patients (Figure 1). These 3 patients were considered positive for FLT3/ ITD mutation and all of these patients were heterozygous for this mutation.

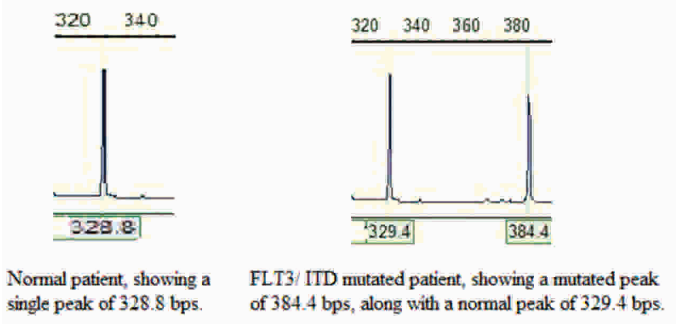
Out of theses ITD mutated patients, 2 (66.6%) were males and one (33.6%) was female. All the three patients were of M3 subtype. In this study, ITD mutation was found in older patients with higher WBC count and blast counts which was statistically significant (t-test showed p-value $\leq$0.05). However there is no significant differences in hemoglobin (Hb) levels and platelet counts (t-test showed p-value > 0.05) (Table 1).

For FLT3/ D835 mutation detection, the gel imaging showed that DNA product of 44 (93.6%) patients were digested into two fragments of sizes 188 bps and 90 bps. This showed presence of normal allele in these patients. Amplified DNA product of the rest 3 (6.4%) patients showed both digested fragments of 188 and 90 base pairs and undigested fragment of 278 base pairs. This showed the presence of mutated allele in these patients in heterozygous form. Thus frequency of D835 mutation in the present study was found to be 6.4%. All patients were males and one each of the three patients had M1, M2 and M4 FAB

Table 1: *Relation of ITD mutation with quantitative variables.*

Parameter		ITD Mutation Absent n=44, Present n=3	Total (n=47)	t-test p-value	Males (n=28)	Females (n=19)
Age (years)		Absent	29.3±15.93		29.1±16.02	29.8±16.27
Mean ± S.D		Present	55±8.88	0.021	53.5±12	58±N/A
Hb (gm/dl)		Absent	7.9±2.1		8.15±2.15	7.56±2.28
Mean ± S.D		Present	7.8±2.5	0.95	8.55±3.18	6.3±N/A
WBC (x 10^3/µl)		Absent	22.3(80-17.4)		52.1(86.2-17.7)	19.65(29.7-5.3)
Median (IQR)		Present	131.0 (N/A)	0.013	110.5(N/A)	135.6 (N/A)
Platelet (x 10^3/µl)		Absent	45.5(75.2-27)		40.5(64.5-28)	53.5(97.7-26.2)
Median (IQR)		Present	60.0 (N/A)	0.790	77.5(N/A)	60.0(N/A)
Blast count (%)		Absent	63.5±22.03		59.77±22.36	69.05±20.96
Mean ± S.D		Present	83.3±10.59	0.058	82.5±14.85	85.0±N/A
FAB type	M1	Absent	5		4	1
Frequency		Present	0		0	0
	M2	Absent	15		8	7
		Present	0		0	0
	M3	Absent	11		6	5
		Present	3		2	1
	M4	Absent	8	N/A	6	2
		Present	0		0	0
	M5	Absent	3		1	2
		Present	0		0	0
	M6	Absent	2		1	1
		Present	0		0	0
	M7	Absent	0		0	0
		Present	0		0	0

subtype. This mutation showed no significance association with age and hematological parameters (t-test showed p-value >0.05) (Table 2).



Normal patient, showing a single peak of 328.8 bps.

FLT3/ ITD mutated patient, showing a mutated peak of 384.4 bps, along with a normal peak of 329.4 bps.

Figure 1: *Genotype Curves of a Non Mutated Patient and an ITD Mutated Patient*

Discussion

The present study was conducted to find out the frequency of FLT3/ ITD mutation and FLT3/ D835 mutation in local AML patients, both of which was found to be 6.4%.

A total of 47 diagnosed AML patients were included in the study. Out of 47 patients, 28 that are about 60% were males, while 19 that are about 40% were females. This is in accordance with U.S Leukemia and Lymphoma Society, 2018 report, which stated that AML is more prevalent in males.[14]

Worldwide, the frequency of FLT3/ITD mutation, reported by different researchers varied in a range between 13.2% and 32%.[3] Compared to these statistics, the frequency of ITD mutation in the present study was quite low. Two other studies conducted in Pakistan, reported 13.3% (by Ishfaq M et al [15]) and 17% (by Ali A et al[3]) ITD frequency in local AML patients. Though these figures were also low, they were within the global range. There could be a number of reasons for this difference. A small sample size and different selection criteria in the present study can be a major reason. Majority of patients, in this study, were of younger age group with a mean age of 31 (± 16.7) years. While frequency of

Table 2: *Relation of D835 mutation with quantitative variables.*

Parameter		D835 Mutation Absent n=44, Present n=3	Total (n=47)	t-test p-value	Males (n=28)	Females (n=19)
Age (years)		Absent	31.1±17.01		31.08±17.32	31.28±17.09
Mean ± S.D		Present	29.0±15.09	0.83	29.0±15.09	0
Hb (gm/dl)		Absent	7.8±2.20		8.07±2.2	7.49±2.23
Mean ± S.D		Present	9.1±1.96	0.377	9.1±1.96	0
WBC (x10^3/µl)		Absent	25.1(86.7-17.5)		60.0(89.9-17.9)	19.8(32-6.1)
Median (IQR)		Present	60.0 (N/A)	0.854	60.0 (N/A)	0
Platelet (x10^3/µl)		Absent	45.5(75.2-27)		37.0(69-27)	57.0(92-27)
Median (IQR)		Present	50.0 (N/A)	0.886	50.0 (N/A)	0
Blast count (%)		Absent	65.0±22.44		61.4±23.43	69.89±20.69
Mean ± S.D		Present	61.3±15.94	0.733	61.3±15.94	0
FAB type	**M1**	Absent	4		3	1
Frequency		Present	1		1	0
	M2	Absent	14		7	7
		Present	1		1	0
	M3	Absent	14		8	6
		Present	0		0	0
	M4	Absent	7	N/A	5	2
		Present	1		1	0
	M5	Absent	3		1	2
		Present	0		0	0
	M6	Absent	2		1	1
		Present	0		0	0
	M7	Absent	0		0	0
		Present	0		0	0

FLT3/ITD mutation was higher in elderly patients.[15] Also cytogenetic aberrations were not excluded, while FLT3/ITD mutation was mostly present in cytogenetically normal patients.

However, results of some other studies correlated with the findings of present study. A study conducted in Iraq has reported only 1.88% frequency of ITD mutation.[16] This frequency is even lower than that found is our study. Another research in Brazil had found 7.4% ITD mutation frequency in their population[17]. So, one of the reasons of low frequency of ITD mutation in the present study could be that this mutation is less prevalent in local population than in western countries. The fact which supported this argument is that there were some other disease genes whose frequencies in Pakistani population were found lower than that of other populations.[18-20]

Patients, who found to have FLT3/ITD mutation, in this study, belong to M3 FAB subtype, are elderly and had a higher white blood cell (WBC) count and blast count as compared to ITD negative patients. This is in accordance with the findings of other researchers.[15]

As for FLT3/ D835 mutation the frequency reported was in a range of 5% to 10% worldwide.[13] The only other study conducted on Pakistani population, had found a frequency of 7% mutation rate in AML patients.[21] Unlike results of ITD mutation, the frequency of D835 mutation in the present study fell in this range and was comparable to findings from other researches.

In the present study, FLT3/ D835 mutation patients did not show any significant trend regarding age group, hematological parameters and FAB subtype. As about gender, in this study, all patients having D835 mutation were male. However, as the data was small, there was a strong probability that this observation was due to chance alone.

Considering FLT3/ITD and FLT3/D835 mutations simultaneously, it had been observed that both of these mutations occurred independently of each other. However, an unusual finding of this study was the presence of equal number of ITD and D835 mutations in AML patients. This result was only comparable to few researches, which had found a slightly higher frequency of D835 mutation than ITD mutation in their populations.[17]

This study gives an insight about genetic makeup of Pakistani AML patients. On the basis of this, it can be suggested that, in future, AML patients should be tested for the presence and type of FLT3 mutations. Positive patients could get benefit from FLT3 inhibitor drugs most suitable for the specific type of mutation they had. These inhibitors include type I inhibitors like crenolanib, and gilteritinib, which inhibit FLT3 signaling in AML cells with either ITD or TKD mutations and type II inhibitors like quizartinib, and ponatinib which inhibit FLT3 signalling in cells with ITD mutation, but not with TKD mutations[22]. This, hopefully, could cause a remarkable change in outcome of AML patients.

In the present study, there were limitations which may have affected the results. Like, karyotyping was not performed on the recruited patients and the sample size of this study was small, which may contribute to erroneous results. Results obtained in this study need to be further evaluated by sequencing of the target FLT3 gene.

Conclusion

It is concluded from the present study that, in local population, frequencies of both FLT3/ITD mutation and FLT3/D835 mutation was 6.4%. This lower ITD frequency than usual while normal D835 frequency showed the possibility that, instead of ITD mutation, other mutations of FLT3 gene (like D835) are more common in local population.

Association of ITD mutation with older age group, high WBC count and high blast count signifies that patients with these factors have greater chance of having ITD mutation and should be tested for it on priority basis. Also, all of these factors are associated with poor prognosis. Thus ITD mutation can also be considered as poor prognostic factor. On the other hand, D835 mutation was not found to be associated with any prognostic or clinical variable. Thus, D835 mutation may not be an indicator of prognosis and all patients have equal chance of having this mutation regardless of their clinical picture.

As there is limited data available, regarding frequencies of FLT3 mutations in AML patients in Pakistani population, it is important that further studies are conducted to verify these results and to determine other mutations as well.

Ethical Approval: Given
Conflict of Interest: None
Funding Source: None

References

1. Khwaja A, Bjorkholm M, Gale RE, Levine RL, Jordan CT, Ehninger G, et al. Acute myeloid leukaemia. Nat Rev Dis Primers [Internet]. 2016 Mar [cited 2019 Mar 28];2:16010. Available from: https://www.nature.com/articles/nrdp201610
2. Van Deusen B, Bessette M, Johnson L, Berlin A, Banos M, Griffin L, et al. Comprehensive Detection of Driver Mutations in Acute Myeloid Leukemia Including Internal Tandem Duplications with Anchored Multiplex PCR and Next-Generation Sequencing. Blood. 2016; 128(22): 5251.
3. Ali A, Siddique MK, Shakoori AR. Frequency of FLT3/ITD Mutations in Pakistani Acute Myeloid Leukemia Patients. Pak J Zool. 2013; 45(2): 495-501.
4. Galanis A, Ma H, Rajkhowa T, Ramachandran A, Small D, Cortes J, et al. Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. Blood. 2014; 123:94-100.
5. Kazi JU, Chougule RA, Li T, Su X, Moharram SA, Rupar K., et al. Tyrosine 842 in the activation loop is required for full transformation by the oncogenic mutant FLT3-ITD. Cell Mol Life Sci. 2017; 74: 2679 –2688.
6. Allahyari A, Sadeghi M, Ayatollahi H, Yazdi HN, Tavakol M. Frequency of FLT3 (ITD, D835) Gene Mutations in Acute Myelogenous Leukemia: a Report from Northeastern Iran. Asian Pac J Cancer Prev. 2016; 17 (9): 4319-4322.
7. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. N Engl J Med. 2017; 377:454-464.
8. Galanis A, Ma H, Rajkhowa T, Ramachandran A, Small D, Cortes J, et al. Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. Blood. 2014 Jan 2; 123(1):

94-100.

9. Leick MB, Levis MJ. The Future of Targeting FLT3 Activation in AML. Curr Hematol Malig Rep. 2017;12(3):153-167.
10. Renshaw MA, Olds BP, Jerde CL, McVeigh MM, Lodge DM. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol–chloroform–isoamyl alcohol DNA extraction. Mol Ecol Resour. 2015 Jan; 15(1): 168-76.
11. Qiu QC, Wang C, Bao XB, Yang J, Shen HJ, Ding ZX, et al. The impact of FLT3 mutations on treatment response and survival in Chinese de novo AML patients. Hematology. 2017; 23(3):131–138.
12. Zaker F, Mohammadzadeh M, Mohammadi M. Detection of KIT and FLT3 Mutations in AML with Different Subtypes. Arch Iran Med. 2010; 13 (1): 21 – 25.
13. Elyamany G, Awad M, Fadalla K, Albalawi M, Al Shahrani M, Al Abdulaaly A. Frequency and Prognostic Relevance of FLT3 Mutations in Saudi Acute Myeloid Leukemia Patients. Adv Hematol. 2014; 2014: 141-360.
14. The Leukemia and Lymphoma Society. Updated data on blood cancers. Available from: https://www.lls.org/sites/default/files/file_assets/PS80_Facts2017-2018.pdf [Accessed 16 February 2019]
15. Ishfaq M, Malik A, Faiz M, Sheikh I, Asif M, Khan MN, et al. Molecular characterization of FLT3 mutations in acute leukemia patients in Pakistan. Asian Pac J Cancer Prev. 2012; 13(9): 4581-5.
16. Abdulateef S, Almothaffar A, Al-khafaji KR. Molecular Study of FLT3-ITD Mutation in Iraqi Adult Acute Myeloid Leukemia Patients; Its Correlation with Clinicopathological Parameters. Pathology and Laboratory Medicine. 2017; 1(1):79-82.
17. Burnatt G, Licínio MA, Gaspar PC, Ferreira AS, Reis ML, Moraes AC, et al . Analysis of the presence of FLT3 gene mutation and association with prognostic factors in adult and pediatric acute leukemia patients. Braz J Pharm Sci. 2017; 53(2):53.
18. Shahid SU, Hasnain S. Identification of genetic basis of obesity and mechanistic link of genes and lipids in Pakistani population. Biosci Rep. 2018; 38(4):38.
19. Sajjad Z, Khan SA, Malik HS, Altaf C, Khan N, Khurshid A. Frequency of prothrombin gene mutation in venous thromboembolism in northern Pakistan. Pak Armed Forces Med J. 2018; 68 (1): 04-07.
20. Khan A, Ayyub M, Ahmed S, Altaf C, Malik HS. Frequency of three different gene mutations (TelAm l1, E2a-Pbx1 and Mll-Af4) in acute lymphoblastic leukaemia. Hematol Transfus Int J. 2016; 2(4): 82–85.
21. Ali A, Siddique MK, Shakoori AR. Detection of FLT3/TKD and IDH1 Mutations in Pakistani Acute Myeloid Leukemia Patients by Denaturing HPLC. J Cell Biochem. 2017; 118: 1174–1181.
22. Larrosa-Garcia M, Baer MR. FLT3 Inhibitors in Acute Myeloid Leukemia: Current Status and Future Directions. Mol Cancer Ther. 2017; 16(6):991-1001.