Detection of *FLT3/TKD* and *IDH1* Mutations in Pakistani Acute Myeloid Leukemia Patients by Denaturing HPLC

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Abstract : Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the marrow and an arrest in their maturation. Various genetic mutations are associated with AML. FMS-like tyrosine kinase 3 (*FLT3*), a member of the class III receptor tyrosine kinase family, plays an important role in stem cell survival, and the development of dendritic and natural killer cells. *FLT3/TKD* mutations are generally missense mutations or in-frame alterations of residues D835 and I836 within the activation loop of the FLT3 protein. D835 mutations have been reported to occur in ≈ 7% of AML patients. Mutations have also been reported in exon 4 of isocitrate dehydrogenase 1 (IDH1) in ≈9% of AML patients. Mutations in FLT3/TKD and IDH1 genes were studied in AML patients from Pakistan and correlated with the laboratory findings. FLT3/TKD mutations were found in 7%, while IDH1 mutations were found in 10% Pakistani AML patients. Neither of these mutations was significantly correlated with age and sex, although the incidence of these mutations was higher in female patients. These mutations were found to be positively associated with each other. IDH1 mutations were positively associated with FAB type M1 and negatively associated with FAB type M2. In conclusion, the overall incidence of all these mutations in Pakistani patients was within the globally reported ranges.

Key words: AML, acute myeloid leukemia, FLT3/TKD mutations, IDH1 mutations, WBC Count.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the marrow and an arrest in their maturation, frequently resulting in hematopoietic insufficiency (granulocytopenia, thrombocytopenia, or anemia), with or without leukocytosis.

FMS-like tyrosine kinase 3 (FLT3), also known as stem cell tyrosine kinase-1 (STK1) or fetal liver tyrosine kinase-2 (FLK-2), is a member of class III tyrosine kinase (TK) receptors. It includes five immunoglobulin-like loops in the extracellular region, a transmembrane region, an intracellular juxta-membrane (JM) domain, two tyrosine kinase (TK) domains interrupted by a kinase insert and a C-terminal tail (Agnes *et al.,* 1994). The gene is located at chromosome 13q12 and consists of 24 exons (Rosnet *et al.,* 1991; Abu-Duhier *et al.,* 2001a).

The expression of FLT3 is found mainly in hematopoietic stem cells in the bone marrow, lymph nodes and thymus (Rosnet *et al*., 1993), but is also seen in other tissues including cerebellum, placenta, gonads, and brain (Maroc *et al*., 1993). After the ligand binds, the receptor dimerizes and autophosphorylates, which activates downstream signalling pathways, resulting in the proliferation of pluripotent stem cells (Lyman, 1995). Other cytokines such as Kit ligand also influence this interaction. In fact, FLT3 ligand or KIT ligand alone induce very little proliferation of *in vitro* human progenitor cells. But, when used together, they have a synergistic effect on stem cell proliferation (Hannum *et al*., 1994).

The *FLT3* gene is expressed in 93% of AML, 100% of B cell ALL and 87% of T-cell ALL patients (Drexler, 1996). In AML, over expression of FLT3 mRNA has been reported in many studies, suggesting that this high level of expression may have some critical role in increased proliferation and survival of the leukemic cells (Carow *et al*., 1996). Two types of mutations have been reported in the *FLT3* gene in AML patients *viz.,* internal tandem duplications (*FLT3/ITD*) and mutations in the second tyrosine kinase domain (*FLT3/TKD*). *FLT3/ITDs* result from the duplication and tandem insertion of a portion of the juxtamembrane (JM) region. The duplicated region is always present between exons 14 and 15 of the *FLT3* gene (Nakao *et al.,* 1996). The *FLT3/ITDs* are always in frame and produce functional FLT3 protein. *FLT3/ITDs* are one of the most frequent mutations reported in AML, found in 13 to 32% of adult AML patients (Gale,

2003), and are associated with worse prognosis. *FLT3/TKD* mutations in exon 20 include at least six different substitutions within the D835 codon, predominantly tyrosine and histidine, less frequently valine, glutamate and asparagine. The deletion of I836 and insertion of nucleotides have also been detected, but the sequence always remains in frame. The incidence of these mutations varies between 5% and 7% in adult AML patients (1, 2). The impact *of FLT3/TKD* mutations on prognosis is unclear.

Isocitrate dehydrogenase (IDH) is an enzyme that catalyzes the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate and carbon dioxide in the citric acid cycle. Three IDHs have been reported: one NAD(+)-dependent IDH, which is cytosolic, and two NADP(+)-dependent IDHs, one of which is mitochondrial and the other cytosolic. Each NADP(+)-dependent isozyme is a homodimer. The *IDH1* gene is on chromosome 2 at 2q33.3 and consists of 30.12kb. It encodes one of the NADP(+)-dependent isocitrate dehydrogenases, which is found in the cytoplasm and peroxisomes. It plays a significant role in cytoplasmic NADPH production.

Point mutations in the exon 4 (R132) of the *IDH1* gene were first reported in 12% cases of glioma (3). Similar *IDH1* mutations were also found in more than 70% of the WHO grade I and III astrocytomas and in glioblastomas. These mutations have also been identified in 9% of primary AML patients, and are more associated with normal karyotype AML (16%) (Mardis *et al.,* 2009). In a study on *de novo* AML adult patients, 10% patients with normal karyotype were found to have *IDH1* R132 mutations. These mutations were found to be associated with isolated trisomy 8, *NPM1* mutations and FAB subtype M1 (Chou *et al.,* 2010).

In the present study, *FLT3/TKD* and *IDH1* mutations were studied in 70 AML patients from Pakistan and correlated with hematological findings.

MATERIALS AND METHODS

Subjects

Samples from 70 Pakistani AML patients were included in this study. The AML patients were selected according to standard hematological and clinical parameters with the help of consultant hematologists at different hospitals in Lahore, Pakistan from January 2006 to February 2009. Clinical and laboratory findings were obtained on a prescribed proforma from the hospital. DNA was extracted from all the samples using the Guanidine Thiocyanate and ethanol precipitation method (Malferrari *et al*., 2002). DNA concentrations were measured on a NanoDrop ND-1000 spectrophotometer and each DNA sample was diluted to a final working concentration of 30ng/µl with TE buffer for further use in PCR.

PCR amplification

For the screening of *FLT3/TKD* mutations, PCR was used to amplify a 278 base pair fragment covering *FLT3* exon 20. For *IDH1* mutations screening, a 369 base pair fragment covering *IDH1* exon 4 was amplified. For both *FLT3/TKD* and *IDH1*, the PCR mixture contained 1X Optimase buffer, 1.5mM $MgSO_4$, 0.2mM dNTPs, 0.5mM each primer (17F2 and 17R3 for *FLT3/TKD*, 4F and 4R for *IDH1*, Table 1), 0.5 units Optimase polymerase (Transgenomic Limited, Glasgow, UK) and 30ng DNA in a total reaction volume of 20 µl. Cycling conditions for PCR were: Initial denaturation at 95°C for 5 minutes and then 35 cycles each of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. A known wild type and mutant-positive sample were also amplified along with patient samples. Five micro liters of each PCR product were run on a 2% agarose gel in 1X TBE buffer to confirm the presence of PCR product. To enhance the formation of heteroduplexes before running on DHPLC, the PCR products were denatured at 95°C for 4 min and then allowed to cool down slowly to room temperature at a rate of 1°C/min.

DHPLC running conditions

Flow rate of mobile phase was 0.9 mL/min. The gradient of buffer A and B contained 55% buffer B at the beginning with an increase of 2% per minute to 63% at the end. The duration of the gradient was 4 minutes. The temperature of the column oven was 59.0°C for *FLT3/TKD* and 57.0°C and 59.0°C for *IDH1*. Volume of PCR product per injection was 5µl. The ultraviolet detector was set at 260 nm. Heterozygous profiles were identified visually on the basis of the appearance of additional earlier peaks of eluting DNA on the chromatograms. Homozygous samples showed only one peak. The relative mutant level was visually assessed using the height of the mutant peak compared with that of wild type peak of the same sample run.

Confirmation of FLT3/TKD mutations by EcoRV digestion and sequencing

The samples which showed an additional peak on the DHPLC chromatogram were PCR amplified and digested with *Eco*RV. The PCR mixture contained 1X Bioline buffer, 1.0mM $MgCl_2$, 0.2mM dNTPs, 0.5mM each primer (17F2 and 17R3), 0.5 units Taq polymerase (Bioline, London, UK) and 30ng DNA. The total reaction volume was 20 µl. Cycling conditions for PCR were: 35 cycles each of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. A known wild type and mutant-positive sample were also amplified along with unknown samples. The PCR products were run on a 2% agarose gel. Five micro liters of each PCR product were digested with *Eco*RV (New England BioLabs, Hitchin, UK). The digestion mixture contained 20 units *Eco*RV, 1X NEB buffer 3 and 1µg BSA. The final volume was adjusted to 10µl and placed at 37°C for 4-5 hours. The digested products were run on a 2% agarose gel. The wild type fragments were digested into two fragments of 187bp and 91bp in size while the mutant products remained uncut at 278bp. The PCR products generated using Bioline reagents were cleaned up using QIAquick PCR purification kit as per manufacturer's protocol and were sent to an in-house sequencing service.

Confirmation of IDH1 p.R132 mutations by restriction digestion

For R132H mutations, amplicons of 200bp were generated using Bioline PCR with primers IDH1ex4/F2 and IDH1/R132H/MM(R) (Table 1). The PCR mixture contained 1X Bioline buffer, 1.0mM $MgCl_2$, 0.2mM dNTPs, 0.5mM each primer, 0.5 units Taq polymerase (Bioline, London, UK) and 30ng DNA. The total reaction volume was 20 µl. Cycling conditions for PCR were: 35 cycles each of 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were digested with *Bsp*HI (New England BioLabs, Hitchin, UK) using manufacturer's recommendations. The digested products were run on a 2% agarose gel. Wild-type (WT) amplicons were uncut (200bp) and R132H-positive amplicons were reduced to 168bp.

For R132C, R132S and R132L mutations, amplicons of 199bp were generated using Bioline PCR with primers IDH1ex4/F2 and IDH1/R132C/MM(R) (Table 1). The PCR mixture contained 1X Bioline buffer, 1.0mM $MgCl_2$, 0.2mM dNTPs, 0.5mM each primer, 0.5 units Taq polymerase (Bioline, London, UK) and 30ng DNA. The total reaction volume was 20 µl. Cycling conditions for PCR were: 35 cycles each of 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were digested with *Taq*I (New England BioLabs, Hitchin, UK) using manufacturer's recommendations. The digested products were run on a 2% agarose gel. WT amplicons were reduced to 168bp but amplicons carrying an R132C, R132S or R132S mutation remained uncut (199bp). All the *IDH1* mutations were also confirmed by sequencing.

RESULTS

AML patients

Out of these 70 patients, 38 were male and 32 were female. Median values and range of age, WBC count, platelet count and %blasts for males and females along with FAB types are shown in Table II.

There was no difference in the median values for WBC count (*P* = .38), platelet count (*P* = .26) and % blasts (*P* = .13) of the male and female patients. Median age of male patients was significantly higher than that of female patients (*P* = .03). There was no patient with either M0 or M7 FAB type. FAB type M2 had the maximum number of patients followed by M1 and M4. Relatively smaller number of patients was seen in M3, M5 and M6 (Table II).

Frequency of mutations

FLT3/TKD mutations were detected in 5 patients (7%) while *IDH1* mutations were detected in 7 patients (10%). The incidence of *FLT3/TKD* mutation was almost equal among male (8%) and female (6%) patients (P = .95) while that of *IDH1* mutations was higher in male (13%) than female (6%) patients but this was not statistically significant (P = .34, Table III). The overall incidence of both the mutations was slightly higher in male (21%) than in the female (12.5%) patients but this was not significant (P = .42).

FLT3/TKD mutations were found to be equally distributed among all age groups (*P* =.66). A significantly higher incidence of the *IDH1* mutation was seen in elderly patients (>50 years of age, *P* = .02). Neither of the mutations was significantly associated with sex, any specific FAB type, WBC count, platelet count or blast percentage.

FLT3/TKD mutations

In the 5 *FLT3/TKD* mutant-positive cases, D835Y was the most common *FLT3/TKD* mutation, found in 4 samples as a sole mutation, accounting for 80% of the mutant-positive patients and 6% of the total cohort. The fifth patient had three different mutations with different levels; D835H being high, R834R intermediate and D835Y being low (Figs. 1 and 3). The restriction digestion results confirmed the DHPLC results (Figs. 1 and 2).

IDH1 mutations

In the 7 *IDH1* mutant-positive cases, 3 different types of mutations were detected. R132C and R132H were detected at the same frequency (3 in 7, 43%). R132S mutation was seen in one case. The DHPLC results (Fig. 4) were confirmed by restriction digestion (Fig. 5) and sequencing (Fig. 6).

DISCUSSION

In this study, the mutations in *FLT3/TKD* and *IDH1* (exon 4) genes were studied in AML patients of Pakistan. The association of these mutations with each other and with clinical characteristics of the patients was also studied.

The male to female ratio in Pakistani AML patients was 1.2:1.0 (56% male, 45% female) which was in accordance with US National Cancer Institute statistics about Asian populations.

FLT3/TKD mutations

Developing appropriate molecular biology techniques to detect different molecular abnormalities in human malignancies is a major challenge. Even in AML alone, there are many different reported genetic mutations. Information derived from the detection of some of these

mutations is important for clinical management of the disease. Low level mutations can be very difficult to detect using conventional mutation detection techniques. It is necessary therefore to use sensitive and high throughput techniques that are capable of detecting these mutations.

Using DHPLC, *FLT3/TKD* mutations were detected in 7% of the Pakistani cohort. There are a number of advantages in using DHPLC over the standard *EcoRV* restriction enzyme digestion method that has been used in the majority of previous studies. Firstly, it is more sensitive. In the current study, there was one case which was *Eco*RV negative but DHPLC positive. The *Eco*RV-digested PCR product was therefore used as the template in a fresh *FLT3/TKD* PCR. When the product was digested with *Eco*RV, a mutant band was prominent on the gel. This suggests that the mutation level in that patient was very low. In addition, interpretation of *Eco*RV digestion greatly depends on the efficiency of the restriction digest. In some cases, it was extremely difficult to differentiate between an undigested low level mutant and the incomplete digestion of FLT3/WT. Using DHPLC however, such low level mutants were detected efficiently. This observation highlights the sensitivity of the DHPLC technique for the detection of mutations that are present in a small proportion of the amplified alleles. Another major limitation of restriction digestion screening is that it detects mutations only within a very specific region of DNA, usually 4-6 base pairs. Although the most frequent *FLT3/TKD* mutations occur within codons 835 and 836 and disrupt the *EcoRV* digestion site, a number of mutations occur outside this region. The DHPLC technique, on the other hand, detects mutations across a wider region of DNA. Furthermore, the chromatogram profile produced by different mutations was found to be highly specific and reproducible, in most cases enabling direct mutation identification from the WAVE pattern without the need for sequencing (Figure 1 and 4). Considering the clinical management of AML patients with *FLT3/TKD* mutations, there are clear advantages to the use of DHPLC.

In the current cohort, a D835 mutation was seen in 4 cases as a sole mutation. Interestingly, one of the mutant positive patients had three different mutations; D835H, D835Y and R834R. Presence of more than one mutation has already been reported (Yamamoto *et al.*, 2001). The *Eco*RV digested PCR product was used as a template for re-amplification of the PCR product for sequencing in that case.

FLT3/TKD mutations were not associated with age or sex. This was in accordance with other studies (Yamamoto *et al*., 2001; Mead *et al*., 2007). The mutations were not found to be associated with any of the FAB types. Some studies have shown a positive correlation of the mutations with FAB type M5 (Thiede *et al*., 2002; Bacher *et al*. 2008) and a negative correlation with FAB types M2 and M6 (8) while others have shown no association with any of the FAB types (Mead *et al*., 2007; Whitman *et al*., 2008).

IDH1 mutations

Acquired mutations in the *IDH1* gene have been identified in 8% (Mardis *et al*., 2009) and 5.5% (Chou *et al*., 2010) of newly diagnosed AML cases. In the current study, *IDH1* mutations were seen in 10% (Pakistani samples) of AML patients. R132C and R132H compete for being the most frequent IDH1 mutations in AML (Mardis *et al*., 2009; Abbas *et al*., 2010). In present study, R132C and R132H were detected at the same frequency (3 in 7, 43%). The mutations were found to be positively associated with higher age (P = 0.02, Table 3). No association of the mutations with sex or FAB types was seen.

The overall frequency of the *FLT3/TKD* and *IDH1* mutations in this study was higher than most of the other studies. The most likely reason for this is the use of DHPLC, which is a more sensitive technique than other conventional mutation detection techniques.

Coincidence of FLT3/TKD and IDH1 mutations with other described mutations

Using DHPLC, we have previously reported the frequency of *NPM1* mutations and *FLT3/ITD* mutations in the Pakistani AML patients (Ali *et al*., 2013a,b). Table IV shows the coincidence of *FLT3/TKD* mutations with the previously described *FLT3/ITD* and *NPM1* mutations. The percentage of *FLT3/TKD* mutations in *FLT3/ITD* and *NPM1* mutant-positive patients was not much different from that of the wild type patients. No *FLT3/TKD* mutation was seen in IDH1 mutant-positive patients. No significant association was seen between *FLT3/TKD* mutation and any of the other three mutations.

Table V shows the coincidence of *IDH1* mutations with previously reported *FLT3/ITD* and *NPM1* mutations in the acute myeloid leukemia patients of Pakistan**.** Although no *IDH1* mutation was seen in *FLT3/ITD* mutant-positive patients, this was not statistically significant (*P* = .11). Same was the case with *FLT3/TKD* mutant-positive patients (*P* = .29). Incidence of the *IDH1* mutations in mutant-positive patients was almost equal to that of the wild type patients (*P* = .83). So, IDH1 mutations were not found to be significantly associated with any of the three mutations.

CONCLUSIONS

Mutations in FLT3/TKD and IDH1 genes were studied in AML patients from Pakistan and correlated with the laboratory findings. FLT3/TKD mutations were found in 7%, while IDH1 mutations were found in 10% Pakistani AML patients. Neither of these HPLC mutations was significantly correlated with age and sex, although the incidence of these mutations was higher in female patients. These mutations were found t be positively associated with each other. IDH1 mutations were positively associated with FAB type M1 and negatively associated with FAB type M2. In conclusion, the overall incidence of these mutations in Pakistani patients was within the globally reported ranges.

Statement of conflict of interest

Authors have declared no conflict of interest

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FIGURE LEGENDS

Fig. 1. Wave (DHPLC) pattern of samples with (a) wild type *FLT3/TKD*, (b) a D835Y mutation and (c) D835H, R834R and D835Y mutations

Fig. 2. A representative gel demonstrating *FLT3/TKD* mutations using *Eco*RV restriction enzyme digestion. Lane 1 contains undigested PCR product. Lanes 2 and 3 contain *Eco*RV-digested mutant-negative and mutant-positive controls respectively. Lanes 4 and 8 show complete digestion of the PCR product, indicative of absence of the mutation in these samples. Lanes 5, 6 and 7 show the presence of mutant and wild type bands, indicative of mutant-positive samples.

Fig.3. Sequencing chromatogram showing *FLT3/TKD* mutations. (a) D835Y (G→T) mutation and (b) three different mutations in one patient, namely D835H (G→C) being high, R834R (A→G) intermediate and D835Y (G→T) being low. The arrows indicate the mutations.

Fig. 4. Wave (DHPLC) patterns of samples with different *IDH1* mutations.

Fig. 5. Representative gels showing mutant-specific restriction enzyme digestion of *IDH1* mutations.

(a) *BspH*I restriction digestion for R132H mutations: Lane 1, wild type amplicons are uncut (200bp); Lanes 2-6 contain R132H mutant-positive amplicons which are reduced to 168bp after restriction digestion.

(b) *Taq*I restriction digestion for R132C, S or L mutations: Lane 1, undigested PCR product; Lanes 2 and 4, wild type amplicons are reduced to 168 bp after restriction digestion; Lanes 3, 5 and 6, amplicons carrying R132C, R132S and R132L remain uncut (199bp)

Fig. 6. Sequencing chromatograms showing different *IDH1* mutations. The arrows indicate the mutations.

Table I. Oligonucleotide primers used for mutation screening

Gene/Exon	Primer Sequence	WT Product Size (bp)
FLT3/20	17F2: 5′-CATCACCGGTACCTCCTACTG-3′	278
	17R3: 5′-TAACGACACAACACAAAATAGCCGT-3′	
IDH1/4	4F: 5′-CATTTGTCTGAAAAACTTTGCTTC-3′	369
	4R: 5′-ACATGCAAAATCACATTATTGCCA-3′	
IDH1/4	4F2: 5′-ATCACTCCTGATGAGAAGAGGGTTGAG-3′	200
	R132H/MM(R): 5′-ACATGACTTACTTGATCCCCATAAGCATCA-3′	
	R132C/MM(R): 5′-CATGACTTACTTGATCCCCATAAGCATGTC-3′	199

Table II. Patient characteristics (Median and range) of all patients, male and female patients.

	Total (n=70)	Male (n=38)	Female (n=32)
Age (Years)			
Median	30	32	24
Range	9-68	16-68	9-50
WBC count (x10^9 per litre)			
Median	26	24	32
Range	0.8-196	0.8-141	1.2-196
Platelet count (x10^9 per litre)			
Median	64	53	76
Range	7-322	7-187	12-322
% Blasts			
Median	42	48	41.5
Range	15-98	15-98	18-82
FAB Types			
M0	0	0	0
M1	18	11	7
M2	24	13	11
M3	4	2	2
M4	9	5	4
M5	7	2	5
M6	3	2	1
M7	0	0	0
Unknown	5	3	2

Table III. Demographic characteristics according to the *FLT3/TKD* and *IDH1* mutations

	Total	FLT3/TKD				IDH1			
		WT	MT (%)	*P*	% MT	WT	MT (%)	*P*	% MT
Sex									
Male	38	35	3 (60)	.79	8	33	5 (71)	.34	13
Female	32	30	2 (40)		6	30	2 (29)		6
Age (Years)									
<25	30	27 (41)	3 (60)	.66	10%	28 (44)	2 (29)	.02	7
26-50	36	34 (52)	2 (40)		6%	33 (52)	3 (43)		8
>50	4	4 (6)	0 (0)		0%	2 (3)	2 (29)		50
FAB Types									
M1	18	17 (26)	1 (20)	.76	6%	15 (24)	3 (43)	.27	17%
M2	24	22 (34)	2 (40)	.78	8%	22 (35)	2 (29)	.74	8%
M3	4	4 (6)	0 (0)	.43	0%	4 (6)	0 (0)	.35	0%
M4	9	8 (12)	1 (20)	.62	11%	9 (14)	0 (0)	.15	0%
M5	7	7 (11)	0 (0)	.29	0%	6 (10)	1 (14)	.69	14%
M6	3	3 (5)	0 (0)	.50	0%	3 (5)	0 (0)	.42	0%
Unknown	5	4 (6)	1 (20)		20%	4 (6)	1 (14)		20%
WBC count x10^9/L									
<10	24	23 (35)	1 (20)	.87	4%	24 (38)	0 (0)	.22	0%
10-50	19	17 (26)	2 (40)		11%	17 (27)	2 (29)		11%
51-100	13	12 (18)	1 (20)		8%	10 (16)	3 (43)		23%
>100	9	8 (12)	1 (20)		11%	8 (13)	1 (14)		11%
Unknown	5	5 (8)	0 (0)		0%	4 (6)	1 (14)		20%
Platelet count x10^9/L									
01-50	25	23 (35)	2 (40)	.25	8%	24 (38)	1 (14)	.37	4%

51-100	21	18 (28)	3 (60)		14%	19 (30)	2 (29)		10%
>100	18	18 (28)	0 (0)		0%	15 (24)	3 (43)		17%
Unknown	6	6 (9)	0 (0)		0%	5 (8)	1 (14)		17%
% Blasts									
01-50	39	36 (55)	3 (60)	.81	8%	37 (59)	2 (29)	.29	5%
51-100	26	24 (37)	2 (40)		8%	22 (35)	4 (57)		15%
Unknown	5	5 (8)	0 (0)		0%	4 (6)	1 (14)		20%

Table IV. Coincidence of *FLT3/TKD* mutations with previously reported *FLT3/ITD* and *NPM1* mutations in acute myeloid leukemia patients of Pakistan.

	FLT3/TKD WT (% in total WT)	FLT3/ TKD mutant (% in total mutants)	*p*	% FLT3/TKD mutant
Total (100)	93	7	----	7
FLT3/ITD Wild type	77 (83)	6 (86)	.84	7
FLT3/ITD Mutant	16 (17)	1 (14)		6
NPM1 Wild type	73 (78)	5 (71)	.66	6
NPM1 Mutant	20 (22)	2 (29)		10
IDH1 Wild type	58 (89)	5 (100)	.29	8
IDH1 Mutant	7 (11)	0 (0)		0

Table V. Coincidence of *IDH1* mutations with previously reported *FLT3/ITD* and *NPM1* mutations in acute myeloid leukemia patients of Pakistan.

	IDH1 WT (%)	IDH1 mutant (%)	*p*	% mutant
Total (70)	63	7		10
FLT3/ITD Wild type	52 (83)	7 (100)	.11	12
FLT3/ITD Mutant	11 (17)	0 (0)		0
FLT3/TKD Wild type	58 (92)	7 (100)	.29	11
FLT3/TKD Mutant	5 (8)	0 (0)		0
NPM1 Wild type	52 (83)	6 (86)	.83	10
NPM1 Mutant	11 (17)	1 (14)		8

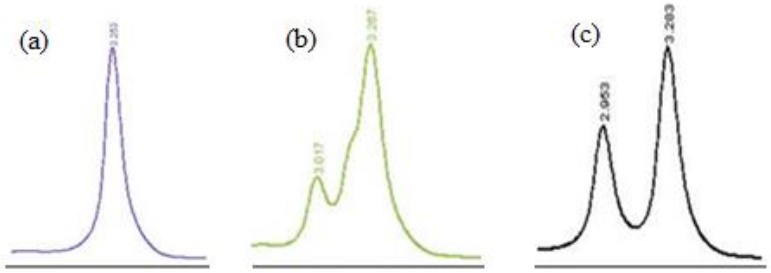

Fig. 1. Wave (DHPLC) pattern of samples with (a) wild type *FLT3/TKD*, (b) a D835Y mutation and (c) D835H, R834R and D835Y mutations

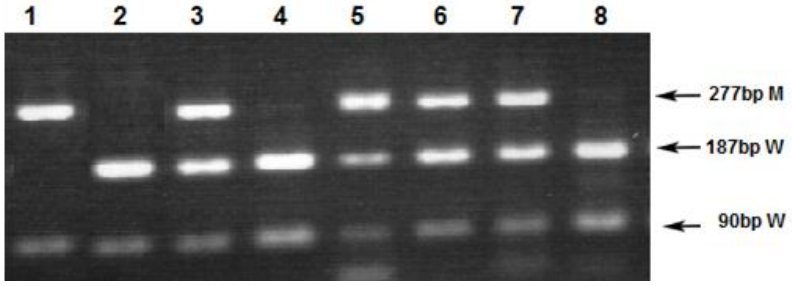

Fig. 2. **A representative gel demonstrating *FLT3/TKD* mutations using *Eco*RV restriction enzyme digestion. Lane 1 contains undigested PCR product. Lanes 2 and 3 contain *Eco*RV-digested mutant-negative and mutant-positive controls respectively. Lanes 4 and 8 show complete digestion of the PCR product, indicative of absence of the mutation in these samples. Lanes 5, 6 and 7 show the presence of mutant and wild type bands, indicative of mutant-positive samples.**

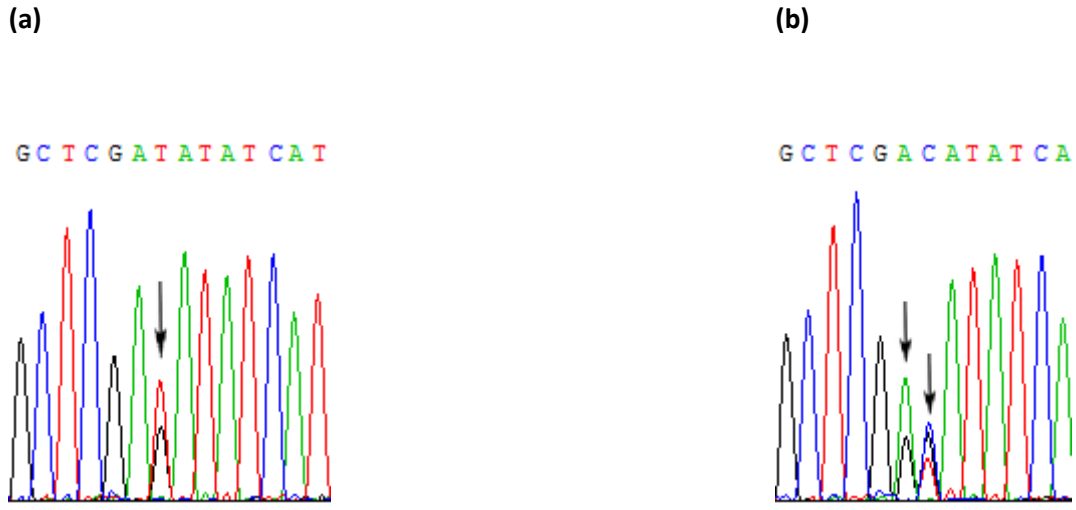

Fig.3. **Sequencing chromatogram showing *FLT3/TKD* mutations. (a) D835Y (G→T) mutation and (b) three different mutations in one patient, namely D835H (G→C) being high, R834R (A→G) intermediate and D835Y (G→T) being low. The arrows indicate the mutations.**

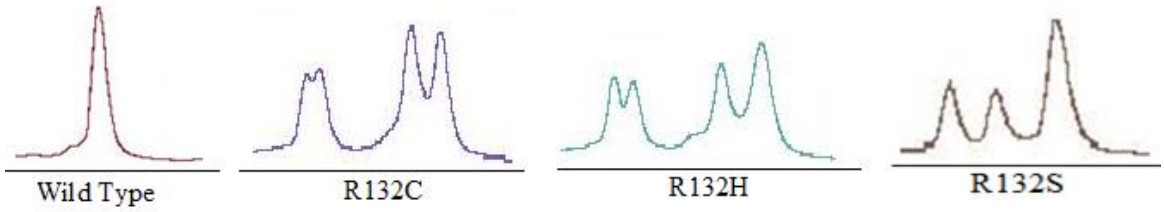

Fig. 4. Wave (DHPLC) patterns of samples with different *IDH1* mutations.

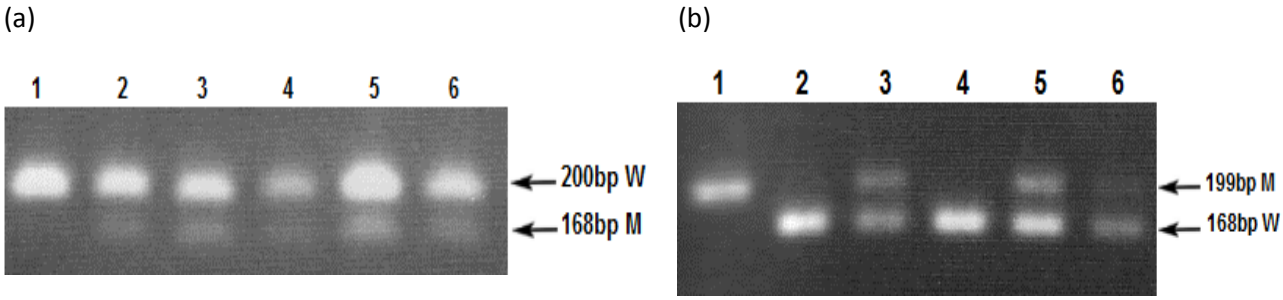

Fig. 5. Representative gels showing mutant-specific restriction enzyme digestion of *IDH1* mutations.

(a) *BspH*I restriction digestion for R132H mutations: Lane 1, wild type amplicons are uncut (200bp); Lanes 2-6 contain R132H mutant-positive amplicons which are reduced to 168bp after restriction digestion.

(b) *Taq*I restriction digestion for R132C, S or L mutations: Lane 1, undigested PCR product; Lanes 2 and 4, wild type amplicons are reduced to 168 bp after restriction digestion; Lanes 3, 5 and 6, amplicons carrying R132C, R132S and R132L remain uncut (199bp).

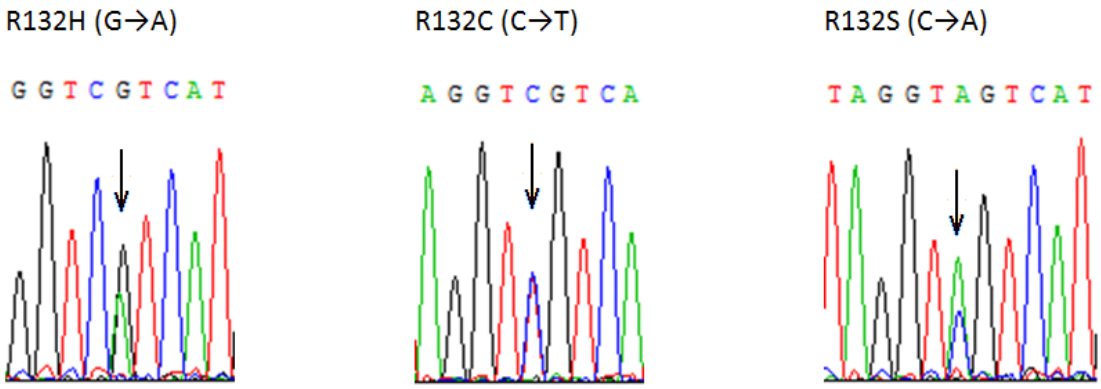

Fig. 6. Sequencing chromatograms showing different *IDH1* mutations. The arrows indicate the mutations.