Material and Methods

Mutation screening

FLT3 ITD was examined by amplification of exon 14 and 15 using the primers and annealing temperatures described in Table I, followed by electrophoresis on a 4 % agarose gel containing ethidium-bromide and detection by UV light. FLT3 D835 mutations were detected by amplifying exon 20 (primers and annealing temperatures in Table I) followed by a restriction fragment length polymorphism analyses using *Eco*RV as previously described.[1] Both PCRs were performed in a total volume of 25 µl containing 10 ng of DNA, 6 pmol of each primer, 200 µM of dNTPs, 2.5 mM of $MgCl_2$, 0.75 U HotGoldstar DNA polymerase (Eurogenetec, Seraing, Belgium) and 1× reaction buffer (Eurogenetec). After an initial denaturation step at 95 ^{o}C for 10 min, the samples were incubated for 35 cycles for 1 min at 95 ^{o}C, 1 min at 59-61 ^{o}C (Table I) and 2 min at 72 ^{o}C. The final step consisted of an extension step at 72 ^{o}C for 10 min. When FLT3 ITD and D835 mutations were detected reactions were repeated and confirmed by sequencing the forward and reverse strands in an ABI Prism3700 DNA Analyser (Applied Biosystems) using the same primers used for PCR.

Supplementary Table I – Sequence and annealing temperature of primer pairs used for mutation screening and and $MgCl_2$ concentrations used.

Primer	Forward (5'-3')	Reverse (5'-3')	Annealing Tm (^{o}C)	$MgCl_2$ Conc. (mM)
FLT3 exons 14/15	CAATTTAGGTATGAAAGCC	GTACCTTTCAGCATTTTGAC	59	2.5
FLT3 exon 20	CCGCCAGGAACGTGCTTG	GCAGCCTCACATTGCCCC	61	2.5

FLT3 Expression

cDNA was prepared using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, Breda, The Netherlands). Quantitative real-time PCR (qPCR) mixtures contained (10 μl) of an appropriate cDNA dilution, 1 × SYBR®Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) and 300 nM of forward and reverse primers. Reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using the following conditions: 10 min at 95^{o}C , 45 cycles at 95^{o}C for 15 sec and at 60 ^{o}C for 1 min, followed by a dissociation stage comprised of 15 sec at 95 ^{o}C, 15 sec at 60 ^{o}C and 15 sec at 95 ^{o}C. Each assay was done in duplicate and included a standard curve dilution (raging from 25 ng to 2.5 pg) a negative control, and 250 pg of each sample.

Supplementary Table II – Sequence of primer pairs used for RT-PCR

Primer	Forward (5'-3')	Reverse (5'-3')
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT
FLT3	GATCAGCCATTTTATGCTACAGAAG	GCCGTTTCCTTGAGTCAAAA
CDX2	ATCACCATCCGGAGGAAAG	TGCGGTTCTGAAACCAGATT
PAN3	TTGGTGCCCTCAACATCTCT	TTGATCCCATCGGAACTAGC
FLT1	ATGCCAGCAAGTGGGAGTT	CAAAAGCCCCTCTTCCAAGT
FOXO1A	CATGTGGAAAGCCCAAAGTCA	TACCAAGCCAATGAAGATGCAAT

Reference List

1. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 2001; 97:2434-9.