An alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis

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Introduction

Diverse transcripts are generated by chromosomal translocation 8;21 (1-6). We reported that one previously unidentified and alternatively spliced transcript, AML1-ETO including ETO exon 9a (AE9a) (Figure 1, ref. 1), is leukemogenic in mice and is widely detected in t(8;21) AML patients (7). Here, we describe the methods to identify and quantitate the specific A/E9a transcript in t(8;21) patients samples relative to the AML1-ETO transcript encoding the well known full-length 752 amino acid AML1-ETO protein (AE).

Reagents

Reagents:

BM or blood cells from t(8;21) and non-t(8;21) leukemia patients obtained with proper consent

RNA isolation reagents: RNeasy mini kit (Qiagen).

ThermoScriptTM RT-PCR system (Invitrogen).

FailSafeTM PCR system (Epicentre Biotechnologies)

Fluorescent Probes (IDT), 2 µM stock

Forward Primer (IDT), 10 µM stock

Reverse Primer (IDT), 10 µM stock

IQ Reaction Supermix (BioRad)

Nuclease Free dH_20

Real-Time PCR plate (USA Scientific Template 1 PCR plate 1402-9596)

Template RT Optical Film (USA Scientific, 2978-2100)

Filter Pipet Tips for PCR

Primers:

First round PCR for AML1-ETO:

AML1 (RUNX1) exon 4/5 boundary sense primer: 5'-GAGGGAAAAGCTTCACTCTG -3'

ETO (RUNX1T1, MTG8) exon 9 antisense primer: 5'-CTGTGAAGGAATTCCCGATG-3'.

Second round real time PCR for AE quantitation:

ETO exon 8 sense primer: 5’-AGAGTCCCGTCAACCCAGAC-3’

ETO exon 9 antisense primer: 5’-ATCTCCTCTGGCACGTATCC-3’

probe HEX-CAGTTGCACTAGACGCGCATC-IABLACK (located across the border of exon 8 and exon 9).

Second round real time PCR for AE9a quantitation:

ETO exon 9a sense primer: 5’-TGAGCATTGCTGTCCTGGGTCATA-3’

ETO exon 9 antisense primer: 5’-TTGGATACTAGATACTGCAAGGGCCG-3’ETO

Exon 9a probe: FAM-TGAGGTCACATTGCTTCTCCAAAGGC-TAM

Software:

For primer and probe design: we used PrimerQuest software available at idtdna.com and Beacon designer 2.0 (Biosoft International)

Icycler iQ software (BioRad)

Equipment

Real-Time Thermocycler (BioRad iCycler). Thermocycler (Whatman Biometra)

Procedure

RNA preparation and RT-PCR:

1. Purify total RNA using the RNeasy mini kit (Qiagen) from the patient samples (numbers of cells are variable) according to the manufacturer’s protocol. DNase treatment can be performed to eliminate the DNA contamination.
2. cDNA synthesis: Perform the reverse transcription reaction in a 20 µl reaction volume assembled in a thin-walled 0.2 ml PCR tube containing: 1 µg of total RNA

1 µl Oligo (dT)20 primer (50 µM)

2 µl 10 mM dNTP

add DEPC-treated dH_2O to 12 µl

incubate at 65 °C for 5 min for denature and place on ice.

Add following mix to each tube

4 µl 5x cDNA synthesis buffer

1 µl 0.1m DTT

1 µl Rnase OUT

1 µl DEPC-treated dH_2O

1 µl ThermoScript RT

Transfer the tubes to the PCR machine and incubate at 55 °C for 1 hour and terminate the reaction at 85 °C for 5 minutes.

3. To distinguish AML1-ETO fusion transcripts containing ETO exon 9 or exon 9a from endogenous ETO transcripts, perform PCR to only amplify the fusion transcripts: With 1 µl of cDNA template and AML1 exon 4/5 boundary sense primer and ETO exon 9 antisense primer, perform PCR amplifications with the FailSafeTM PCR system (Epicentre Biotechnologies) for 40 cycles in a volume of 50 µl as follows: denaturating 45 s at 94 °C, annealing 45 s at 58 °C, and extension 90 s at 72 °C.

Relative quantitation of the AE9a and the AE transcripts:

1. Dilute PCR products from step 3 1:30 in nuclease free dH_20. For relative quantitation of AE9a or AE transcripts, real-time PCR should be performed with fluorescent probes specific for either exon 9 or exon 9a of ETO. To assemble 20 µl reactions prepare the following mastermix: pipet 10 µl of 2x iQ Supermix with sense and antisense primers specific for either exon 9 or exon 9a (see reagents). In a separate mastermix, add 1 µl 30x diluted PCR products from step 3 above as template and H_20. Add the fluorescent probe last since it is light sensitive.
2. Seal the plate with optical film and place in a 96-well thermocycler. Program the thermocycler as follows: 95 ºC 2', 95 ºC 1' and 55 ºC 25 cycles, 4 ºC hold.
3. Check for product amplification on a 1% agarose gel and perform quantification with the BioRad iQ software.
4. To confirm and correct for the relative efficiency of probe and primer pairs for exon 9 and exon 9a transcripts, perform a standard curve using serial dilutions of MigR1-A/E or MigR1-A/E9aP DNA as the template.

Timing

Three days

References

1. Miyoshi, H. *et al.* t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. *Proc. Natl. Acad. Sci. U. S. A* **88**, 10431-10434 (1991).
2. Nisson, P. E., Watkins, P. C. & Sacchi, N. Transcriptionally active chimeric gene derived from the fusion of the AML1 gene and a novel gene on chromosome 8 in t(8;21) leukemic cells. *Cancer Genet Cytogenet.* **63**, 81-88 (1992).
3. Erickson, P. *et al.* Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. *Blood* **80**, 1825-1831 (1992).
4. Kozu, T. *et al.* Junctions of the AML1/MTG8(ETO) fusion are constant in t(8;21) acute myeloid leukemia detected by reverse transcription polymerase chain reaction. *Blood* **82**, 1270-1276 (1993).
5. Tighe, J. E. & Calabi, F. Alternative, out-of-frame runt/MTG8 transcripts are encoded by the derivative (8) chromosome in the t(8;21) of acute myeloid leukemia M2. *Blood* **84**, 2115-2121 (1994).
6. Era, T. *et al.* Expression of AML1 and ETO Transcripts in hematopoietic cells. *Leukemia* **9 Suppl 1**, S26-S28 (1995).
7. Yan, M. *et al.* An alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. *Nat. Med.* Published online: 30 July 2006; doi:10.1038/nm1443 (2006).

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