Clinica Chimica Acta 453 (2016) 38–41

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Rapid diagnosis of acute promyelocytic leukemia with the *PML-RARA* fusion gene using a combination of droplet-reverse transcription-polymerase chain reaction and instant-quality fluorescence in situ hybridization

Shohei Shigeto [a], Kazuyuki Matsuda [a,*], Akemi Yamaguchi [b], Akane Sueki [a], Masayuki Uehara [b], Mitsutoshi Sugano [a], Takeshi Uehara [a], Takayuki Honda [a]

[a] Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan
[b] Core Technology Development Center, Seiko Epson Corporation, Suwa, Japan

ARTICLE INFO

Article history:
Received 22 October 2015
Accepted 1 December 2015
Available online 2 December 2015

Keywords:
FISH
PML-RARA
Droplet-RT-PCR
APL
IQ-FISH

ABSTRACT

Background: Acute promyelocytic leukemia (APL) with the *PML-RARA* fusion gene can be effectively cured using molecular-targeted therapies, which require both detection and quantification of the *PML-RARA* fusion gene. Here, we developed a rapid assay for identifying and measuring the *PML-RARA* fusion gene in patients with APL using droplet-reverse transcription-polymerase chain reaction (droplet-RT-PCR) and instant quality-fluorescence in situ hybridization (IQ-FISH).

Methods: RNA for droplet-RT-PCR and fixed-cell suspensions for IQ-FISH were prepared from five patients with APL and three controls. We evaluated the amplification efficiency and reaction time with droplet-RT-PCR and signal clarity and hybridization time with IQ-FISH.

Results: The reaction using droplet-RT-PCR was completed in 26 min. The *PML-RARA* fusion gene was detected in all samples from the five patients. IQ-FISH yielded clear signals after 1 h of hybridization. There were no significant differences in signal clarity or positive signal ratios between IQ-FISH and conventional FISH.

Conclusions: Simultaneous droplet-RT-PCR and IQ-FISH, in addition to morphological examination of blood smears, can be used to diagnose patients as having APL within 4 h based on molecular/cytogenetic results. Rapid diagnosis can allow effective therapies to be started promptly.

1. Introduction

Acute promyelocytic leukemia (APL) constitutes 5–8% of all cases of adult acute myelogenous leukemia (AML) [1]. The presence of the *PML-RARA* fusion gene, which results from a balanced reciprocal translocation, i.e., t(15;17)(q24;q21), is the molecular hallmark of APL. APL is a highly aggressive disease presenting hemorrhagic complications from a characteristic coagulopathy [2]. All-*trans* retinoic acid (ATRA) with chemotherapy or ATRA with arsenic trioxide (AsO_3) is effective and yields higher cure rates with complete remission than previous therapies. ATRA and AsO_3 have specific reactivity for different parts of the PML-RARA fusion protein [3], which serves as the molecular basis for highly effective molecular targeted therapies [1,4–6].

Rapid detection and quantitative assessment of the *PML-RARA* fusion gene, carried out using reverse-transcription polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH), influence the prognoses of the patients with APL. Quantitative RT-PCR (RT-qPCR) is more sensitive than FISH for detection of the fusion gene; however, the expression level of fusion gene by RT-qPCR does not indicate the ratio of cells having the fusion gene to the total number of APL cells. On the other hand, FISH analysis can show the ratio of cells having the fusion gene by counting abnormal signals as needed during the treatment course.

Unfortunately, conventional analysis by RT-PCR and FISH requires one to two days. Therefore, given the high efficacy of ATRA and AsO_3 therapies for patients with APL having the *PML-RARA* fusion gene, it is important to diagnose APL as soon as possible in order to improve patient outcomes and quality of life.

In this study, we developed a systematic rapid identification/quantification assay for the *PML-RARA* fusion gene using droplet-RT-PCR and instant quality (IQ) FISH (IQ-FISH).

Abbreviations: APL, acute promyelocytic leukemia; AML, acute myelogenous leukemia; ATRA, all-*trans* retinoic acid; AsO_3, arsenic trioxide; droplet-RT-PCR, droplet-reverse transcription-polymerase chain reaction; FISH, fluorescence in situ hybridization; IQ, instant quality; RT-qPCR, quantitative RT-PCR; PB, peripheral blood; BM, bone marrow.

* Corresponding author at: Department of Laboratory Medicine, Shinshu University Hospital, 3-1-1, Asahi, Matsumoto 390-8621, Japan.
E-mail address: kmatsuda@shinshu-u.ac.jp (K. Matsuda).

http://dx.doi.org/10.1016/j.cca.2015.12.001
0009-8981/

Table 1
Clinical and laboratory data from five patients with APL.

Patient no.	Sex	Age (years)	Sample type	FISH signal for *PML/RARA* (%)		Type of fusion gene	Karyotype
				Conventional FISH	IQ FISH		
1	Male	38	PB	86.8	85.0	(+) Long type	No growth
2	Female	74	BM	85.8	86.8	(+) Long type	46,XX,t(15;17)(q24;q21)[3]/46,XX[15]
3	Male	71	BM	81.0	79.0	(+) Long type	46,XY,add(11)(q11.2),t(15;17)(q24;q21)[3]/46,XY[11]
4	Male	63	BM	91.4	90.0	(+) Long type	46,XY,t(15;17)(q24;q21)[18]/46,XY[2]
5	Male	48	BM	95.0	96.4	(+) Short type	46,XY,t(15;17)(q24;q21)[7]/46,XY[5]

PB, peripheral blood; BM, bone marrow; IQ FISH, instant quality fluorescence in situ hybridization; Long type, the long form of the *PML-RARA* mRNA transcript (breakpoint in intron 6 of *PML*); Short type, the short form of the *PML-RARA* mRNA transcript (breakpoint in intron 3 of *PML*). Patients 1 and 2 were also used in our previous study [7].

2. Materials and methods

2.1. Materials

Peripheral blood (PB) and bone marrow (BM) samples from five patients with APL and three controls were used in this study (Table 1). Conventional semi-nested RT-PCR and FISH confirmed that all samples from the patients had the *PML-RARA* fusion gene, whereas all samples from the controls did not contain the fusion gene. Two patients (nos. 1 and 2) were included in our previous study [7]. This study was approved by the Institutional Review Board of Shinshu University (No. 351).

2.2. RNA extraction

Total RNA was extracted from PB or BM samples using a QIAamp RNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.3. One-step droplet-RT-PCR

The primers and TaqMan probes for the *PML-RARA* fusion gene were designed as previously reported [8]. The one-step RT-PCR reaction mixture contained RNA, SuperScript III/Platinum Taq Mix (Life Technologies, Grand Island, NY), reaction buffer composed of Tris–HCl pH 9.0, KCl and $MgCl_2$, 0.8 μmol/L of each primer, and 0.2 μmol/L of the TaqMan probe in a final volume of 10 μL. One microliter of each reaction mixtures was used for the droplet-PCR assay. The reaction conditions used in the present study were as follows: 50 °C for 300 s for RT, 95 °C for 10 s for inactivation of the reverse transcriptase, and 50 cycles at 95 °C for 5 s and 62 °C for 25 s. In this study, we set an arbitrary standard for evaluating the amplification; samples were considered positive when the fluorescent level of amplification was more than 2.0.

2.4. Conventional FISH

Conventional FISH was performed using Vysis LSI Hybridization buffer and Vysis LSI Dual-color, Dual-Fusion Probe (Abbott Molecular/Vysis. Des Plaines, IL) as previously described [9]. We scored 500 nuclei from PB or BM samples to determine the number of yellow fusion signals using a fluorescence microscope (Axio Imager Z2) equipped with ISIS system software (MetaSystemes Hard & Software, Althlussheim, Germany).

2.5. IQ-FISH

IQ-FISH analysis was performed using an IQ-FISH Fast Hybridization Buffer, Sure FISH *PML-RARA* Dual fusion probe (Agilent Technologies, West Cedar Creek, TX) and a Dako Cytology FISH Accessory Kit (Dako, Glostrup, Denmark) containing pretreatment buffer, wash buffer and stringent wash buffer. The sample slides were chemically aged by treatment with pretreatment buffer at 95 °C for 10 min and then cooled at room temperature for 10 min. The slides were dehydrated through an ethanol series (70%, 85%, and 100%), and the probe mixture was then applied. The slides were sealed with coverslips, denatured at 66 °C for 10 min, and incubated at 45 °C for 1 h. Thereafter, the slides were washed with wash buffer and stringent wash buffer according to the manufacturer's instructions. The nuclei were stained and mounted with DAPI II counterstain (Abbott Molecular/Vysis. Des Plaines, IL) and VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). The signals obtained by IQ FISH were analyzed as described for conventional FISH.

3. Results

3.1. Droplet-RT-PCR for detection of the PML-RARA fusion gene

The *PML-RARA* fusion gene was detected by droplet-RT-PCR in all samples from the five patients with APL within 26 min (Fig. 1). No amplification was observed from control samples. Long-type and short-type fusion genes were amplified from four and one samples, respectively. Short-type of *PML-RARA* (patient no. 5) was amplified faster than the long-type of *PML-RARA* (patient nos. 1–4).

3.2. IQ-FISH and conventional FISH

The IQ-FISH and conventional FISH protocols were completed within 4 h in which probe hybridization time was 1 h, and 2 days in which probe hybridization time was 18 h, respectively. IQ-FISH yielded yellow fusion signals from all samples from patients with APL, showing the same strong, clear signals as obtained by conventional FISH (Fig. 2). There were no significant differences in positive signal percentage obtained by IQ-FISH and conventional FISH (Table 1).

4. Discussion

In this study, we developed a rapid assay system for detecting and quantifying the *PML-RARA* fusion gene by using droplet-RT-PCR and IQ-FISH simultaneously. This molecular/cytogenetic assay system, combined with morphological examination, was completed within 4 h (Fig. 3); morphological examination required 1 h, droplet-RT-PCR required 1 h (with an RT-PCR time of 26 min), and IQ-FISH required 4 h (with a probe hybridization time of 1 h). Droplet-RT-PCR and IQ-FISH compensate the morphological examination and would allow patients to start molecular-targeted therapies with ATRA and AsO_3 more quickly than conventional analyses.

RT-PCR is a robust method that can be used to confirm the presence of the *PML-RARA* fusion gene. APL is initially diagnosed by morphologic examination of blood smears; however, morphological examination alone cannot provide molecular information required for effective therapy. Thus, detection of the *PML-RARA* fusion gene is important for selecting an effective molecular-targeted therapy, and RT-PCR can be used for this purpose, even in the case of cryptic *PML-RARA* rearrangement without t(15; 17) on G-banding [10].

RT-PCR can detect the *PML-RARA* fusion gene faster than the other molecular/cytogenetic methods such as G-banding and FISH; however, conventional RT-PCR or RT-nested PCR requires about 2–6 h. Additionally, APL can be morphologically diagnosed within 1 h by examining PB and/or BM smears subjected to May-Grunwald–Giemsa, peroxidase, or