Acute promyelocytic leukemia lacking t(15;17): molecular evidence of atypical PML/RAR-α transcriptional variant by gene sequencing

Akutna promijelocitna leukemija bez t(15;17): molekularni dokazi atipične PML/RAR-α transkripcione varijante genskim sekvenciranjem

**Vesna Djordjević*, Nataša Tošić†, Marija Denčić-Fekete*,
Marijana Virijević*‡, Jelica Jovanović*, Ljubomir Jaković*,
Nada Kraguljac-Kurtović*, Andrija Bogdanović*‡, Tatjana Kostić†,
Sonja Pavlović†**

Clinical Center of Serbia, *Clinic of Hematology, Belgrade, Serbia; University of
Belgrade, †Institute for Molecular Genetics and Genetic Engineering, Belgrade, Serbia;
University of Belgrade, ‡Faculty of Medicine, Belgrade, Serbia

Abstract

Introduction. The accurate diagnosis of acute promyelocytic leukemia (APL), not only on the morphological and clinical, but also on the molecular level, is very important for application of targeted therapies. **Case report.** A 62-year-old woman presented with APL. By using conventional cytogenetic analysis as well as applying the fluorescence *in situ* hybridization (FISH) analysis it has not been possible to confirm the presence of t(15;17) in the presented patient. Using reverse transcriptase polymerase chain reaction (RT-PCR) two atypical promyelotic leukemia/retinoic acid receptor alpha (PML/RAR-α) fusion transcripts were identified. Both detected transcripts were isoforms. The larger transcript was in-frame, coding for functional aberrant PML/RAR-α protein, while the shorter transcript was an out-of-frame. **Conclusion.** Our study highlights the need for the application of molecular methodology in daily clinical practice. Precise characteriza-

tion of PML/RAR-α fusion transcript creates a basis for identifying rare individual cases that require special caution when treating such patients. To our knowledge this is only the fifth case of atypical PML/RAR-α transcript containing full PML exon 7a, and among them the only one that was cytogenetically cryptic and FISH negative. All of the herein presented cases had lethal outcome. Therefore, our findings with the additional review of the literature, emphasizes the importance of detailed identification of atypical PML/RAR-α fusions, not only for the purpose of knowing their role in leukemogenesis, but also for the assessment of the impact that they can have on the outcome of the treatment.

Key words:
diagnosis; in situ hibridization, fluorescence; leukemia, promyelocytic, acute; molecular biology; reverse transcriptase polymerase chain reaction.

Apstrakt

Uvod. Precizno dijagnostikovanje akutne promijelocitne leukemije (APL), ne samo na osnovu morfoloških i kliničkih parametara, već i na molekularnom nivou, veoma je važno radi primene adekvatne ciljane terapije. **Prikaz bolesnika.** Prikazali smo bolesnicu, staru 62 godine, sa dijagnozom APL. Primenom standardne citogenetičke analize, kao i primenom fluorescentne *in situ* hibridizacije (FISH), nije bilo potvrđeno prisustvo t(15;17) kod opisane bolesnice. Primenom metode reverzna transkriptaza-lančana reakcija polimeraze (RT-PCR), identifikovana su dva atipična *promyelotic leukemia/retinoic acid receptor alpha* (PML/RAR-α) fuziona transkripta. Oba transkripta su

predstavljala izoforme. Duži transkript je zadržao "okvir čitanja" i kodirao je funkcionalan PML/RAR-α aberantni protein, dok je kraći transkript bio van "okvira čitanja". **Zaključak.** Naša studija ukazuje na potrebu za primenom molekularne metodologije u svakodnevnoj kliničkoj praksi. Precizna karakterizacija PML/RAR-α fuzionih transkipta čini osnovu za identifikovanje retkih bolesnika čije lečenje zahteva dodatni oprez. Prema našim saznanjima, ovo je tek peti slučaj opisanog atipičnog PML/RAR-α transkripta koji u sebi sadrži celokupan PML egzon 7a, a među njima jedini koji se nije mogao detektovati primenom citogenetičke i FISH analize. Svi ovde predstavljeni slučajevi su imali smrtni ishod. Zbog toga, naši rezultati, zajedno sa sličnim slučajevima opisanim u literaturi, naglašavaju

Correspondence to: Vesna Djordjević, Clinical Center of Serbia, Clinic of Hematology, Koste Todorovića 2, 11 000 Belgrade, Serbia.
E-mail: djvesna.kcs@gmail.com

značaj detaljne identifikacije atipičnih PML/RAR-α fuzija, ne samo u svrhu prepoznavanja njihove uloge u procesu leukemogeneze, veći i u smislu procene njihovog uticaja na ishod lečenja.

Ključne reči:
dijagnoza; hibridizacija in situ, fluorescentna; leukemija, promijelocitna, akutna; biologija, molekularna; polimeraza, reakcija stvaranja lanaca, reverzna transkripcija.

Introduction

Acute promyelocytic leukemia (APL) is a well-defined clinical-biological entity of acute myeloid leukemia, characterized by unique morphology of leukemic cells and the specific t(15;17), present in approximately 80% of APL cases. However, this translocation is never found in other subtypes of acute myeloid leukemia (AML), except in rare blasticpromyelocytic leukemic transformation of a chronic myelogenous leukemia [1].

The t(15;17) (q22;q21) fuses the promyelocytic leukemia (PML) gene on chromosome 15 encoding a transcription factor, with the retinoic acid receptor alpha (RAR-α) gene located on chromosome 17, a member of a steroid hormone nuclear receptor family that is important for the regulation of both normal and malignant cellular differentiation and proliferation. The breakpoints in RAR-α gene are always located in intron 2, while different breakpoint cluster regions (bcr) were identified in PML gene. PML breakpoint bcr1 in intron 6 and bcr3 in intron 3, give rise to two different isoforms of PML/RAR-α fusion protein: long, L-isoform (bcr1) is present in about 55% of APL patients, and short, S- isoform (bcr3) is found in about 40% of patients [2, 3]. The rarest is variable, V-isoform, with the breakpoint bcr2 located in PML exon 6 [2–4].

The PML/RAR-α fusion protein has numerous functions in the process of leukemogenesis, but it also mediates response to all-trans retinoid acid (ATRA) therapy treatment [5]. Its oncogenic action, PML/RAR-α fusion protein expresses through disruption of both RAR-α and PML pathways [6, 7]. PML/RAR-α exhibits its dominant negative impact on wild type RAR-α function as a transcriptional factor. It has been suggested that deregulation of the genes that are under RAR-α control lead to a blockage in myeloid differentiation. PML/RAR-α acts as a dominant negative PML mutant. It has the ability to interact with wild type PML causing impaired functioning of PML in programed cell death and genomic stability [8, 9].

It has been known that different PML/RAR-α transcripts can have different prognostic significance. In all 3 most common isoforms, in the in-frame fusion transcripts, the RAR-α part of the PML/RAR-α fusion retains exons 3 to 9, therefore preserving DNA-binding and retinoid acid (RA) ligand binding domain. The RA ligand binding domain is very important for the sensitivity to ATRA treatment. More precisely, it was shown that the expression of PML/RAR-α fusion gene is required for the sensitivity to ATRA treatment [10, 11].

There are several reports describing different response to therapy depending on the type of the PML/RAR-α fusion transcript variants [4, 12–17]. It appears that different parts of PML genes that have been preserved and incorporated into various PML/RAR-α fusion transcripts have a unique impact on the response to therapy.

Here, we report an interesting APL case with cytogenetically cryptic and fluorescence *in situ* hybridization (FISH)-negative PML/RAR-α rearrangement, and a very aggressive course of the disease. We also present the results of RT-PCR and sequencing analysis of the two unusual PML/RAR-α transcripts found in this patient, both containing insertion of exon 7a, and the review of the previously published studies with similar findings.

Case report

A 62-year-old woman was admitted in February 2015 due to widespread muco-cutanous bleeding of two-week duration. After a brisk hematological work-up a diagnosis of intermediate risk APL was made. Namely, her complete blood count was as follows: hemoglobin level 75g/L, WBC count 4.9×10^9/L with 12% blasts and 83% promyelocytes in her leucocyte differential formula, and platelet count of 20×10^9/L. Bone marrow aspirate showed 90% of hypergranular blasts expressing the following markers: myeloperoxidase, CD13 and CD33. However, CD34 and HLA-DR were not expressed on blast cells. Her hemostasis was highly indicative of coagulopathy: fibrinogen 0.9 g/L (reference range: 2–4 g/L); prothrombin time (PT) (Quick) 40% (reference range: 75–120%), activated partial thromboplastin time (APTT) 26.5 s (reference range: 27–35 s); D-dimer 54 mg/L (normal value < 0.5 mg/L).

Cytogenetic analysis showed normal karyotype, 46,XX [20]. Interphase and metaphase FISH studies were performed on bone marrow cytogenetic specimens which were previously used for karyotype analysis. Detection of PML/RAR-α and RAR-α/PML fusion genes were performed using the DF SureFISH® 15q24.1 probe to label PML together with the DF SureFISH® 17q21.2 probe to label RAR-α (Agilent Technologies®, Ceder Creek, TX, USA). FISH analysis detected the presence of normal karyotype in all analyzed cells.

The patient was immediately started with ATRA (45 mg/m²/day). Prednisone (0.5 mg/kg/day) was given to prevent differentiation syndrome. Despite an intensive blood product support the patient expired 4 h after admission due to central nervous system (CNS) bleeding.

For the reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing analyses total RNA was isolated from bone marrow aspirate using Trizol reagent (Invitrogen, USA). Reverse transcription was performed using 1 µg of total RNA, random hexamers and reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) in a total volume of 20 µL, according to the manufacturer's instructions.

For the detection of PML/RAR-α transcript we applied standardized RT-PCR method [18]. Usage of this method enabled us to detect the most common PML/RAR-α transcripts due to the right combination of primers in both first and second (nested) PCR cycles. In parallel with the patient sample the adequate positive, negative and the water control were amplified. Electrophoresis of the PCR analysis was performed on 2% agarose gel stained with ethidium bromide, and visualized on an ultraviolet transilluminator.

The PCR products were cut-out of the gel and purified using QIAquick® Gel Extraction Kit (Qiagen, Germany) and were directly sequenced on ABI 3130 Genetic Analyzer (Applied Biosystems, USA). The primers used for the sequencing (forward and reverse) were the same one used in the RT-PCR analyses.

The classical cytogenetic analyses revealed 46,XX karyotype in all 20 metaphase cells analyzed. Also, the FISH analyses using dual fusion probes for PML and RAR-α genes, indicated signals consistent with normal signal pattern.

In the RT-PCR analyses using primers for the detection of bcr1/bcr2 fusion transcripts (PML-A1 located in exon 5 combined with RAR-α-B primer located in exon 3) yielded two products that were different from typical bcr1 isoform (Figure 1). Two unusually long, but very faint bands were detected in the amplification reaction using primers for bcr3 isoform (PML-A2 primer located in exon 3, in combination with RAR-α-B) (Figure 1). It was evident that it was a case of bcr2 isoform, so we performed sequencing analysis of both bands.

Sequencing analysis of the longer and dominant band showed that it was 690 bp long. Namely, in our sequencing analyses using PML-A1 as a forward primer, i.e. RAR-α-B as a reverse primer, we detected that in addition to complete exon 6 found in typical bcr1 isoform (381 bp long), there was an addition of complete exon 7a (53 bp), a part of the intron 7 (also called exon 7b) in a form of insertion of 229 bp, insertion of 27 bp that is a part of RAR-α gene intron 2, and then RAR-α exon 3 where the RAR-α-B primer is situated (Figure 2). In this fusion transcript the open reading frame (ORF) was maintained.
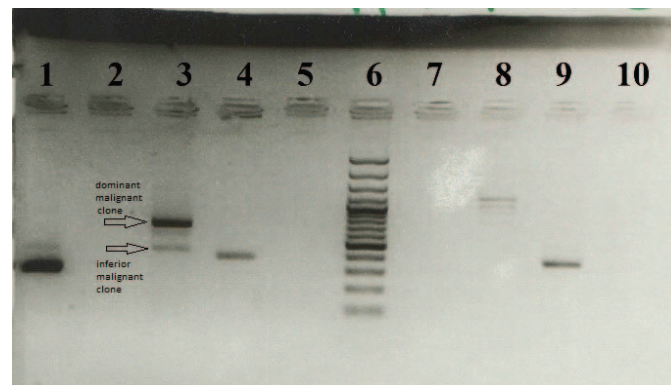

Fig. 1 – Electrophoresis of products of reverse transcriptase polymerase chain reaction (RT-PCR) analysis (1st round). Lane 1 – PCR amplification product for the housekeeping gene (ABL gene); Lanes 2 – 5 represent PCR amplification using promyelocytic leukemia (PML)-A1/retinoic acid receptor (RAR)-α-B primers: Lane 2 – negative control, Lane 3 – patient sample, Lane 4 – positive control (classical bcr1 isoform, 381 bp), Lane 5 – water control; Line 6 – 100 bp DNA marker; Lanes 7 – 10 represent PCR amplification using PML-A2/RAR-α-B primers: Lane 7 – negative control, Lane 8 – patient sample, Lane 9 – positive control (classical bcr3 isoform, 376 bp), Lane 10 – water control.
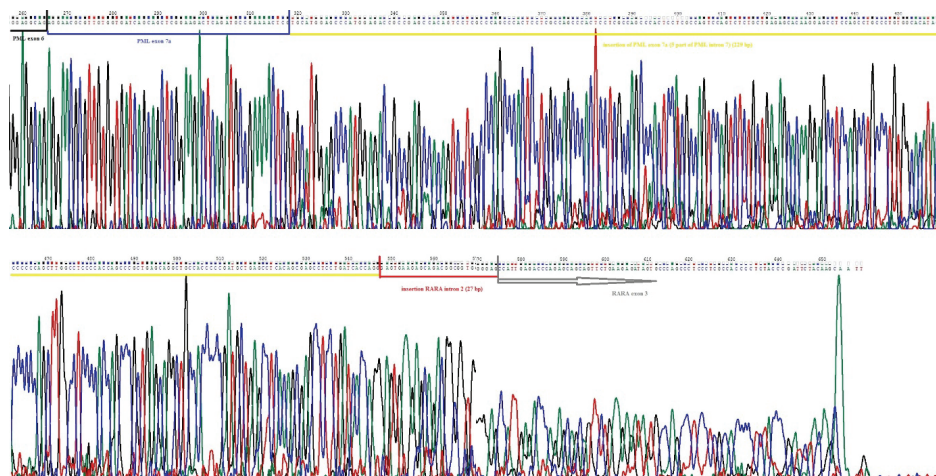

Fig. 2 – Sequencing analyses of the 1st round polymerase chain reaction (PCR) product showing atypical promyelocytic leukemia (PML)/retinoic acid receptor (RAR)-α fusion transcript (reverse complement).
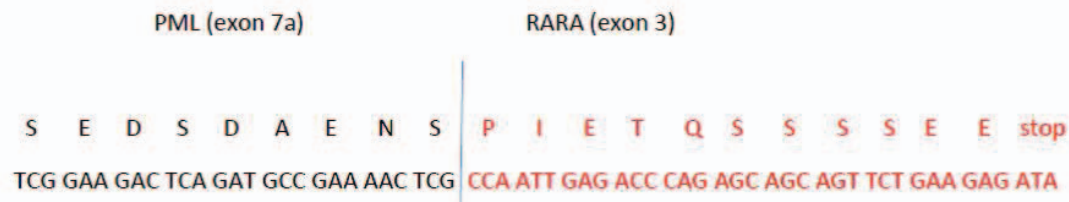
Fig. 3 – cDNA and amino-acid sequence of the promyelocytic leukemia (PML)/retinoic acid receptor (RAR)-α junction between PML exon 7a and RAR-α exon 3 present in our patient, represented as an inferior malignant clone (Figure 1, Lane 3).

The weaker, shorter band, in addition of complete PML exon 6, contained exon 7a (53 bp), that was directly fused to RAR-α exon 3 forming a band of 434 bp. This fusion transcript was not in-frame, since the direct joining of the PML 7a exon to the RAR-α exon 3 generates stop codon in the exon 3 RAR-α gen, precisely at the codon coding for the 12th amino acid of the RAR-α exon 3 (Figure 3).

Discussion

Herein we present a very rare case of the cytogenetically cryptic, FISH-negative, but RT-PCR PML/RAR-α positive APL patient. To our knowledge there has been several cytogenetically cryptic, FISH-negative cases described in the literature [19, 20]. Still, none of these cases refers to such an atypical PML/RAR-α transcript found in our patient.

The reasons for not detecting t(15;17) during routine cytogenetic analyses can be numerous ranging from poor morphology, complex karyotype, but most likely the reason is the presence of submicroscopic insertions leading to cryptic PML/RAR-α fusions [21, 22]. However, the majority of those, cytogenetically cryptic cases, can be detected using FISH (dual-colour or dual fusion probes) analyses. The reports in which the FISH analyses for the PML/RAR-α fusions were negative, while RT-PCR analyses were positive are very rare [19, 21, 23, 24]. These findings can be explained as a consequence of very small insertions, too small for the probe to hybridize to, or the signal that probe produces is too faint to be detected. The difference between all those previously published cases and our case is that in all of them RT-PCR analyses reported the bcr1 or bcr3 isoform. Only one study reported the bcr2 case, but none of them was the case of rare PML/RAR-α transcript with the breakpoint in PML gene after the exon 6 [20].

In our patient we detected the presence of two PML/RAR-α transcripts both with breakpoint after PML exon 7a. The dominant transcript contained entire PML exon 6, exon 7a, 5` part of PML intron 7 (also called PML exon 7b), followed by an insertion of RAR-α intron 2 speeding out through RAR-α exon 3. In addition to this in-frame fusion transcript, we detected a minor PML/RAR-α transcript which differed from the dominant one in that it did not contain neither 5` part of *PML* intron 7, nor the RAR-α intron 2 insertion. Its PML exon 7a was directly fused to RAR-α exon 3, forming an out-of-frame transcript.

In the literature there are only 3 studies describing cases similar to the one here reported (Table 1). Chillon et al. [14] were the first to report two APL patients in which a breakpoint position was outside the coding sequence of exon 7a. In both of the analysed patients the same out-of-frame transcript (PML exon 7a/RAR-α exon 3) as in our patient was detected. One of the patients described had a PML/RAR-α fusion transcript similar to the dominant transcript found in our patient, but without insertion of the part of RAR-α intron 2. This transcript was like in our case in-frame.

In the study by the Barragán et al. [13] in one of the two reported cases, sequencing analyses of the PML/RAR-α fusion transcript showed the presence of the 35 bp insertion originating from RAR-α intron 2, as in our patient. It has been known that the breakpoint in RAR-α gene in t(15;17) is almost always located in the RAR-α intron 2, but there are some cases in which the part of the RAR-α intron 2 is inserted into PML/RAR-α fusion transcript. There are examples of RAR-α intron 2 insertions found in different bcr2 isoforms [25]. Generally speaking, most likely to be inserted are the sequences from RAR-α intron 2 sites that are most prone to breakage. These sites are numerous and spread throughout this 17 kb large intron. Insertion of genomic DNA in the case of bcr2 transcript has been described by others to [4, 7, 13, 14, 16, 26]. There are speculation about the necessity for that part of the DNA sequence in order to ensure an in-frame sequence of the bcr2 transcript [25]. Sometimes the breakpoint in RAR-α gene can be detected inside exon 3, like in the APL patient presented by Park et al. [27]. Namely, one of the PML/RAR-α transcripts detected in the APL patient contained full PML exon 7a, insertion of PML intron and the deletion of first 46 bp from the RAR-α exon 3.

In all of the previously mentioned studies, including our own, a total of 5 patients whose break point in the PML/RAR-α transcript is after exon 7a of PML gene were described. That makes our finding even more significant. The question that remains is: Is the presence of atypical PML/RAR-α transcript causing different response to ATRA therapy? There are reports that patients with different PML/RAR-α fusion transcripts exhibit different sensitivity to ATRA. Significantly worse prognosis of the bcr3 positive patients compared to bcr1 patients have been described [28, 29]. Also, it was shown that patients with bcr2 PML/RAR-α transcript had reduced sensitivity to ATRA [30].

Table 1

Summary of atypical PML/RAR-α fusion transcripts with an insertion of PML exon 7a [13, 14, 27]

Case	Sex	Age, years	Karyo-type	Sequencing analyses of the RT-PCR product (from PML exon 6 to RARα exon 3)	Treatment	Survival	Reference
				3 types of transcript:			
1	M	28	t(15;17)	PML exon6, exon 7a, 5`part of exon 7b / RARα exon 3 (in-frame) PML exon6, exon 7a /RARα exon 3 (out-of-frame) PML exon 6 deletion, exon 7a / RARα exon 3 (out-of-frame)	No	Dead at day 2 (intracerebral hemorrhage)	[14]
2	M	28	t(15;17)	PML exon6, exon 7a / RARα exon 3 (out-of-frame)	ATRA + chemotherapy	Dead at day 13 (multiorgan failure)	[14]
3	M	50	NA*	PML exon6, exon 7a /RARα exon 3 (out-of-frame)	LPA-99 protocol	Dead at day 16 (multiorgan failure)	[13]
4	M	38	t(15;17)	PML exon6, exon 7a, insertion of intron 7/ RARα exon 3 deletion (46bp) (in-frame)	ATRA + anthacycline	Dead at day 14 (pulmonary hemorrhage)	[27]
				2 types of transcript:			
5	F	62	Cryptic, FISH-negative	PML exon6, exon 7a, 5`part of exon 7b / RARα intron 2 insertion (27bp), RARα exon 3 (in-frame) PML exon6, exon 7a / RARα exon 3 (out-of-frame)	ATRA + pred-nisone	Dead at day 1 (intracerebral hemorrhage)	Present case

*NA – not available; RT-PCR – reverse transcriptase polymerase chain reaction; PML – promyelocytic leukemia; ATRA – all-trans retinoid acid; RAR – retinoid acid receptor; FISH – fluorescence *in situ* hybridization.

This was later confirmed by *in vitro* studies [16]. In our study APL patent died too soon for the conclusion about the sensitivity to ATRA to be made. The same outcome was detected in patients reported by other studies [13, 14, 27]. Sadly, the number of reported patients (five) is too small to draw any kind of conclusion regarding prognostic relevance of reported atypical PML/RAR-α transcript.

Conclusion

Our results are an important addition to the limited number of reports describing the existence of atypical PML/RAR-α fusion transcripts in APL patients. Namely, this is only the fifth case of the APL patient with PML exon 7a incorporated in the PML/RAR-α transcript, associated with aggressive course of the disease. In order to clarify the biological significance and prognostic value of atypical PML/RAR-α fu-

sion transcripts like the one reported in our study, it is important to address to every such case and apply an extended analysis including detailed characterization and sequencing of the fusion transcript.

Ethics

This study adhered to the tenets of the Declaration of Helsinki. We obtained written informed consent from the patient, and also the study was approved by the Ethical Committee of the Clinic of Hematology, Clinical Center of Serbia.

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