Acute promyelocytic leukemia with *PML/RARA* (bcr1, bcr2 and bcr3) transcripts in a pediatric patient

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Abstract. Patients with acute promyelocytic leukemia (APL) exhibit the t(15;17)(q24.1;q21.2) translocation that produces the promyelocytic leukemia (*PML*)/retinoic acid receptor α (*RARA*) fusion gene. Different *PML* breakpoints yield three alternative molecular transcripts, bcr1, bcr2 and bcr3. The present study reports the simultaneous presence of three *PML/RARA* transcripts in a pediatric female patient diagnosed with APL, according to the clinical characteristics, immunophenotype and karyotype of the patient. The simultaneous presence of the *PML/RARA* transcripts were detected using reverse transcription-quantitative PCR (RT-qPCR). This was confirmed with HemaVision-28N Multiplex RT-qPCR, HemaVision-28Q qualitative RT-qPCR and the AmpliSeq RNA Myeloid Panel. To the best of our knowledge, the pediatric patient described in the present study is the first case found to exhibit all three PML/RARA transcripts (bcr1, bcr2 and bcr3). Additionally, a microarray analysis was performed to determine the expression profile, potential predictive biomarkers and the implications of this uncommon finding. According to the information obtained from molecular monitoring, the results reported in the present study were associated with a good patient prognosis. In addition, upregulated genes that are rare in acute myeloid leukemia were identified, and these genes may be promising diagnostic biomarkers for further study. For example, CCL-1 is present in leukemic stem cells, causing treatment failure and relapse, and α- and β-defensins have been reported exclusively in chronic myeloid leukemia. However, the results of the present study confirmed that they may also be present in APL. Thus, these findings suggested a possible signaling pathway that involves the PML/RARA oncoprotein in APL.

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Abbreviations: APL, acute promyelocytic leukemia; PML, promyelocytic leukemia; RARA, retinoic acid receptor α

Key words: pediatric, acute promyelocytic leukemia, bcr1, bcr2, bcr3

Introduction

Acute promyelocytic leukemia (APL) represents ~10% of all childhood acute myeloid leukemia (AML) cases (1,2). Cytomorphologically, APL is classified as hypergranular (or typical M3) or as hypogranular (or variant M3V) (3). APL presents a balanced reciprocal translocation t(15;17)(q24.1;q21.2), involving the promyelocytic leukemia (*PML*) gene on chromosome 15 and the retinoic acid receptor α (*RARA*) gene on chromosome 17 (4,5). There are three distinct *PML* breakpoint cluster regions, namely bcr1, bcr2 and bcr3, that generate a *PML/RARA* fusion gene of varying mRNA lengths. The bcr1 transcript results from a break in intron 6 of *PML* with exon 3 of *RARA*; transcript bcr2 results from a break in exon 6 of *PML* with exon 3 of *RARA*; transcript bcr3 results from a break in intron 3 of *PML* with exon 3 of *RARA* (6). The frequency of the different breakpoints is dependent principally on ethnicity and geographical location as the bcr1 transcript is more frequent among individuals of Latin origin (3). The aforementioned transcripts have been detected using reverse transcription-quantitative PCR (RT-qPCR) (7,8). The *PML/RARA* fusion gene generates a PML/RARA oncoprotein that blocks myeloid precursor differentiation, leading to the accumulation of abnormal promyelocytes in the bone marrow (9,10). The PML domain of the PML/RARA oncoprotein can affect the senescence

pathway, facilitating the acquisition of mutations that drive the development of leukemia (9-11). Investigations involving children and adults have provided information on the typical and atypical transcripts of *PML/RARA*. Typical transcripts are the most common (identified in 90-95% of cases) and have three breakpoints in intron 6, exon 6 and intron 3 of the *PML* gene and only one in intron 2 of the *RARA gene*. Atypical transcripts have breakpoints in intron 4, exon 6 or exon 7 of the *PML* gene, and in intron 3 of the *RARA gene*. However, the biological function of atypical transcripts in the initiation and evolution of APL have yet to be defined (11). The present study aimed to describe a novel pediatric case that presents atypical bcr1, bcr2 and bcr3 transcripts, as well as the clinical and molecular aspects present in this APL case. To the best of our knowledge, the present study is the first to identify the three aforementioned atypical *PML/RARA* transcripts.

Materials and methods

Case report. A 12-year-old female patient was admitted in March 2018 to the Civil Hospital of Guadalajara (Guadalajara, Mexico) with gingival bleeding, hyperplasia, petechiae, ecchymosis, paleness and traces of bleeding in the oral cavity, with no lymph node enlargement, hepatomegaly or splenomegaly. Laboratory analyses revealed the following: Hemoglobin levels, 10.8 g/dl (normal range, 12-16 g/dl); leucocytes, 2,670/μl (normal range, 5,000-10,000/μl); platelets, 8,000/μl (normal range, 150,000-400,000/μl); prothrombin time, 13.1 sec (normal range, 9-13 sec); activated partial thromboplastin time, 25.7 sec (normal range, 26-40 sec); and D-Dimer levels, >1,500 ng/ml (normal range, 340-729 ng/ml). Bone marrow aspiration revealed that 98% of nucleated cells were replaced by myeloblasts, that hypergranular promyelocytes were densely packed, bright-pink, reddish-blue or dark-purple granules, and that there were numerous Auer rods. Immunophenotyping revealed a population of 91% of promyeloblasts, which was $CD13^+$, human leukocyte antigen (HLA)-DR^-, $CD38^+$, $CD117^+$ and $CD45^+$. All the aforementioned data were compatible with Acute Myelomonocytic Leukemia or French-American-British (FAB) M3 classification (12). The patient was staged at intermediate risk according to the PETHEMA APL 2012 protocol proposed by Spanish Society of hematology and hemotherapy (13). The patient achieved remission on day 50 after receiving consolidation therapy with three chemotherapy cycles, which was maintained for 2 years according to the protocol. The patient completed treatment 4 years ago (2018-2022). The patient has a good prognosis and continues to be followed up.

Ethical considerations. The present study was submitted and accepted by The Research Committee and The Research Ethics Committee of The Civil Hospital of Guadalajara (approval no. 00116). Bone marrow aspirates from the patient and reference (wild-type control) were obtained prior to treatment. Written informed consent was obtained from the parents and the institutional review boards approved the use of excess diagnostic material for research purposes. These studies were conducted in accordance with the Declaration of Helsinki.

Karyotyping and fluorescence in situ hybridization (FISH) analysis. A bone marrow sample of the patient was obtained and G-banding karyotyping was performed. FISH analysis was then performed for the detection of the translocation, t(15;17)(q24.1;q21.2). Cells were dropped onto glass slides to perform the FISH assays, which were conducted following the manufacturer's recommendations. Images were captured using an AXIO ImagerMI (Zeiss AG) microscope, and the images were analyzed using ISIS software (MetaSystems). A total of 200 interphase cells were reviewed in each slide. The PML and RARA genes were analyzed using a Vysis LSI *PML/RARA* Dual Color probe, Dual Fusion Translocation Probe (cat. no. 05J70-001; Abbott Molecular, Inc.). For the dual-color probe, cells with 1 orange, 1 green and 2 fusion signals were considered positive for the PML/RARA fusion.

RNA isolation and RT-PCR. RNA was isolated from lymphocytes of the bone marrow using the method of TRIzol™ (cat. no. 15596026; Thermo Fisher Scientific, Inc.) proposed in the study by Rio *et al* (14). The Applied Biosystems™ High-Capacity cDNA RT kit (cat. no. 4368813; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for cDNA synthesis. The reaction included 4 μl 10x RT buffer, 4 μl 10x RT random primers, 1.8 μl 25x dNTP Mix (100 mM), 50 U/μl MultiScribe® Reverse Transcriptase and 1 μg RNA, and was conducted using an Applied Biosystems ProFlex PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 10 min at 25˚C, 2 h at 37˚C and 5 min at 85˚C.

Detection of PML-RARA rearrangements. cDNA was analyzed using the HemaVision-28N Multiplex RT-qPCR kit in the Applied Biosystems ProFlex PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the Qualitative HemaVision-28Q RT-qPCR kit (cat. no. HV01-28Q; DNA Diagnostic A/S) in the Rotor-Gene® Q system (cat. no. 1070452EN; Qiagen, Inc.). These methodologies can identify 28 chromosomal translocations and >145 gene breakpoints associated with leukemia. The procedures were performed according to the manufacturer's protocols. Duplicate analysis was considered in the methodology using quality and negative controls from the kit.

An adapted RT-qPCR protocol from the study by Gabert *et al* (15) was used to identify the PML/RARA fusion gene. The primers used were as follows: i) ENF903 (bcr1 forward, 5'-TCTTCCTGCCCAACAGCAA-3'; 19 bp); ii) ENF906 (bcr2 forward, 5'-ACCTGGATGGACCGCCTAG-3'; 19 bp); iii) ENF905 (bcr3 forward, 5'-CCGATGGCTTCGACGAGTT-3'; 19 bp); iv) ENR962 (bcr1-3 reverse, 5'-GCTTGTAGATGCGGGGTAGAG-3', 21 bp); and v) ENP942 (probe, 5' FAM-AGTGCCCAGCCCTCCCTCGC-BHQ-1 3', 20 bp). The RT-qPCR was conducted using the following reagents: 12.5 μl TaqMan Gene Expression Master Mix (cat. no. 4369016; Applied Biosystems; Thermo Fisher Scientific, Inc.), 1.2 μl forward and reverse oligonucleotides, 0.5 μl probe, 8.6 μl nuclease-free water and 1 μl cDNA. The samples were placed in a 96-well plate and analyzed using the 7900 HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SDS version 2.4 program (Thermo Fisher Scientific, Inc.). The thermocycling

conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, 95˚C for 15 sec, and 60˚C for 1 min (40 cycles). To confirm the expression of *PML/RARA* transcripts, the NB4 human cell line [derived from the leukemic cells of a relapsed acute promyelocytic leukemia (M3) patient and carrying the t(15;17) translocation; CVCL_0005; Cellosaurus, https://www.cellosaurus.org/CVCL_0005] was donated by St. Jude Children's Research Hospital (Memphis, USA), and validated samples positive for bcr1, bcr2 and bcr3 variants were used as positive controls. The NB4 cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11875093) with 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. A4766801), 2 mmol/l L-glutamine (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 25030081), 1X antibiotic (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 15240062) and incubated in a humidified incubator at 37˚C with 5% CO_2. The RNA isolation and RT-qPCR protocol for the NB4 cells was the same as aforementioned. Non-template controls (nuclease-free water) were also included in each experiment. The quantification of relative expression of the transcripts was calculated using the $2^{-\Delta\Delta Cq}$ method, with β-glucuronidase was as the housekeeping gene.

Genomic analysis. cDNA obtained using RT was analyzed to detect simultaneous PML/RARA transcripts and myeloid leukemia-associated genes using the Illumina® AmpliSeq RNA Myeloid Panel (cat. no. 20024478; Illumina, Inc.). The procedure was performed by Illumina Inc., and each RT reaction required 10-100 ng total RNA.

Expression microarray. A wild-type sample (from a healthy 10-year-old patient) and the APL case were used for the expression microarray. RNA reconstituted in UltraPure™ DEPC-Treated Water (cat. no. 750023; Invitrogen; Thermo Fisher Scientific, Inc.) was quantified and examined for RNA quality using a NanoDrop 2000™ spectrophotometer (cat. no. ND-2000; Thermo Fisher Scientific, Inc.). The A260/A280 and A260/A230 ratios between 1.8 and 2.2 were used to determine the RNA quality. RNA integrity was evaluated using a 15% agarose gel stained with Gel Red (cat. no. 41003; Biotium, Inc.), visualized using a FirstLight® Uniform UV Illuminator (model, LM-20; single intensity; 302/365 nm UV; filter size, 20x20-cm; 230 V; cat. no. 95-0449-02).

To detect transcripts, the human Clariom™ D Assay (cat. no. 902922; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used. The Applied GeneChip System 3000Dx v.2 included the GeneChip® Hybridization Oven 645 with GeneChip® Fluidics Station 450 Dx v.2 and workstation. Array images were acquired using a GeneChip® Scanner 3000Dx v.2 with AutoLoaderDx and Affymetrix Molecular Diagnostic Software (Affymetrix; Thermo Fisher Scientific, Inc.).

Data analysis and functional classification of differentially expressed genes. All data were captured using Applied Biosystems Transcriptome Analysis Console (TAC) software (version 4.0.2; Thermo Fisher Scientific, Inc.) and microarray data were deposited in the Gene Expression Omnibus (GEO) database following the Minimum Information about a Microarray Experiment (MIAME) and Minimum Information about a Next-generation Sequencing Experiment (MINSEQE) guidelines (https://www.ncbi.nlm.nih.gov/geo/). Finally, to identify candidate differentially expressed genes, the microarray data were analyzed using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (version 6.8; https://david-d.ncifcrf.gov/). A fold change ±1.5 and $P<0.05$ were considered to indicate a statistically significant difference in expression. However, only those genes with a fold change > ±10.0 (the highest selection) were selected.

Results

Conventional cytogenetics. Karyotyping analysis of the patient revealed 46,XX,t(15;17)(q24;q21)[20] and the FISH result was nuc ish(*PML,RARA*)x3(*PML,RARA*)x2[148/200] (*PML,RARA*)x2[52/200], with [n/n] representing the number of cells counted with the alteration out of the total (Fig. S1).

HemaVision RT-qPCR test. All three transcripts were identified using the HemaVision-28N Multiplex RT-PCR kit, with a size of 353 bp for bcr1 (*PML*ex6-*RARA*ex3), 97-350 bp for bcr2 (*PML*δex6-*RARA*ex3) and 325 bp for bcr3 (*PML*ex3-*RARA*ex3) (Fig. S2). Furthermore, the atypical transcripts were corroborated using the Qualitative HemaVision-28Q RT-qPCR assay. The Cq values were as follows: i) 29.83 (bcr1, *PMLex6a-RARAex3*); ii) 30.59 (bcr2, *PMLex5-RARAex3*); and iii) 30.12 (bcr3, *PMLex3-RARAex3*). It should be noted that the breakpoints reported are dependent on the primer design in each kit.

RT-qPCR data. The measurable residual disease was calculated at diagnosis through the relative expression quantification of the simultaneous transcripts using the Livak method (16). The results were 100% for bcr1 (Cq, 28.931), 38.5% for bcr2 (Cq, 30.08) and 0.83% for bcr3 (Cq, 35.86). After the first month of treatment, expression of the three *PML/RARA* transcripts decreased to 0%.

Genomic analysis of simultaneous transcripts in the patient with APL

Genomic analysis with RNA sequencing. Genomic analysis using the AmpliSeq RNA Myeloid Panel Illumina® revealed two simultaneous transcripts, bcr1 and bcr2 (Fig. 1).

Data analysis of differentially expressed genes. First, the microarray data were deposited in the GEO database (accession no. GSE205372; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205372), following the MIAME and MINSEQE guidelines. Then, the overall pattern of gene expression in the microarray was assessed. Using TAC Software version 4.0.2, a cut-off fold change value of ±10.00 was set to generate a reduced gene list since only 1 case was analyzed. As a result, 613 differentially expressed genes were identified (139 upregulated and 473 downregulated genes) compared with the control (wild-type). The top upregulated and downregulated genes are listed in Table SI.

Classification of differentially expressed genes in the APL case. Using the data obtained of the 613 differentially expressed genes, two different analyses were performed: i) First, a search was performed for groups of genes arranged by functional similarity and related to APL from the list using

Table I. Functional annotation clusters obtained by Database for Annotation, Visualization and Integrated Discovery analysis.

Functional annotations	No. of genes	Enrichment score[a]	Benjamini value[b]
Lectin C-type	20	10.08	$4.5x10^{-9}$
SH2 domain	15	5.36	$4.5x10^{-4}$
MHC class II, α/β chain, N-terminal	7	4.05	$2.9x10^{-3}$
Immunoglobulin C1-set	12	4.96	$8.3x10^{-4}$
AIG1	5	3.88	$7.8x10^{-3}$
Graft-vs. -host disease	9	3.79	$8.0x10^{-4}$
Peptidase SI	12	2.80	$5.5x10^{-2}$
Pleckstrin homology domain	20	2.68	$4.0x10^{-2}$
Mammalian defensins	4	2.62	$2.9x10^{-3}$
Btk motif	4	2.58	$7.8x10^{-2}$

[a]The enrichment score is a modified form of the P-value of the exact Fisher test. [b]The Benjamini value is the adjusted P-value resulting from the Benjamini and Hochberg method. MHC, major histocompatibility complex; SH2, Src-homology 2.

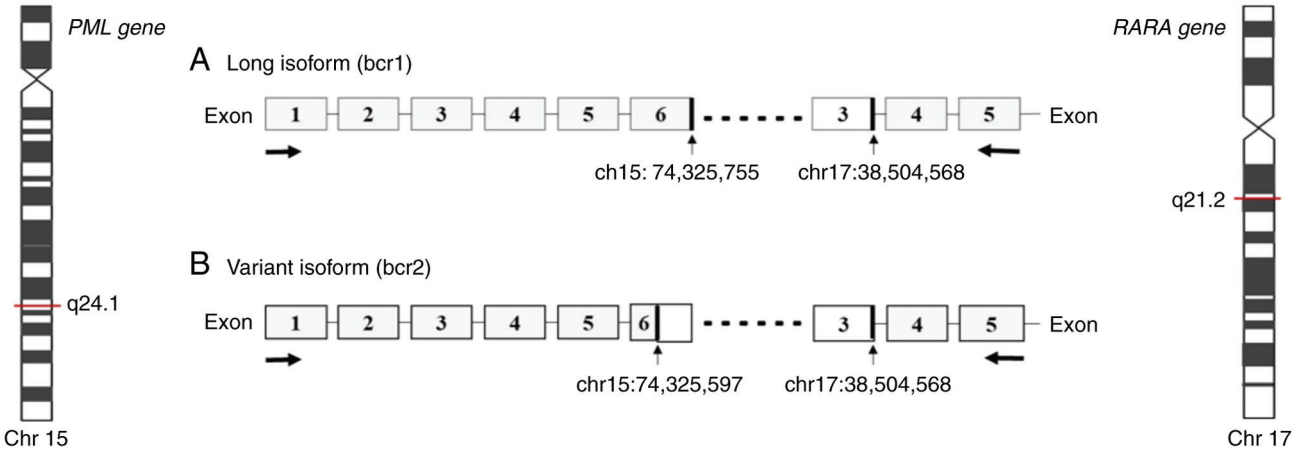

Figure 1. *PML/RARA* transcripts were detected using AmpliSeqRNA in the present study. (A) Long isoform (bcr1) was detected, describes the breakpoints in the exons of PML and RARA genes, and B) Variant isoform (bcr2) was detected, describes the breakpoints in the exons of PML and RARA genes. These are molecular subtypes of *PML/RARA* in patients with acute promyelocytic leukemia (41). *PML*, promyelocytic leukemia; *RARA*, retinoic acid receptor α.

the Functional Annotation Clustering setting in DAVID. An enrichment score >2 was deemed a significant enrichment with the highest stringency. The analysis generated several functional clusters of significantly upregulated or down-regulated genes (Table I), such as: i) Immune system-related clusters: Immunoglobulin C1-set molecules involved in the immune system, major histocompatibility complex (MHC) class II and the loss of HLA-DR antigen expression; ii) C-type lectin cluster; iii) Src-homology 2 domain (SH2) cluster; and iv) mammalian defensins cluster. Additional clusters unrelated to APL were also found, including graft-vs. -host disease (the patient was previously transfused), Btk motif, AIG1, peptidase SI and Pleckstrin homology domain. The enrichment score is a modified form of the P-value of the exact Fisher test, and the Benjamini value is the adjusted P-value resulting from the Benjamini and Hochberg method.

ii) Second, from the list generated and presented in Table SI, only 21 genes with a fold change >20 and 24 genes <-50 were selected that can be associated with clinical and molecular characteristics of APL for future research. The selected genes are part of different signaling pathways, such as for the cell cycle, proliferation, differentiation and adhesion, in addition to MHC and cytokine genes.

Literature review. The present study suggested a possible signaling pathway involving the PML/RARA oncoprotein that leads to cell proliferation or the evasion of apoptosis, based on the microarray analysis (613 up or downregulated genes) and a literature search. The identified genes were selected as 'key words' in the literature search in association with characteristic clinical and molecular APL. The following databases were searched: PubMed (https://pubmed.ncbi.nlm.nih.gov/), Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/), Gene-Cards (https://www.genecards.org/), UniProt (https://www.uniprot.org/) and Ensembl (https://www.ensembl.org/index.html).

Discussion

APL is a subtype of AML with a unique molecular appearance distinguished cytogenetically by balanced

reciprocal translocation t(15;17) and *PML/RARA* gene fusion. Furthermore, it is often associated with a complex coagulopathy known as disseminated intravascular coagulation (DIC), resulting in a high hemorrhage rate and thrombosis. Patients with APL present more frequently with severe DIC due to the increased expression of tissue factors and annexin II, which activates fibrinolysis. During the induction period, hemorrhaging is responsible for the majority of deaths (17,18).

Case studies with *PML/RARA* transcripts are common among adults and uncommon among children (10,19). Generally, one transcript is usually detected in children from common or typical breakpoints and rarely from atypical breakpoints. The bcr1 or bcr3 transcripts are the most frequent (90-95%) and bcr2 is infrequent (11,19-22). Previously, a case bearing two transcripts, bcr1 and bcr2, was detected using the HemaVision RT-qPCR kit, in a pediatric patient with APL detected by immunophenotyping, but with normal results from karyotyping and FISH; however, there was insufficient evidence to define a prognostic factor (23,24). To the best of our knowledge, there have been no cases reporting the presence of the three different transcripts (bcr1, bcr2 and bcr3). In the present study, alternative methods, such as genomic analysis, were applied to identify the breakpoints between the *PML* and *RARA* genes and only bcr1 and bcr2 transcripts were detected. Due to the large size of the transcriptome, the RNA sequencing methodology cannot detect fusion genes that are expressed at low levels in the leukemic clones, which may have affected the detection of bcr3 (25).

The effect of the *PML/RARA* molecular transcripts on APL continues to be controversial as there have been no conclusive results. The reason for the generation of the three bcr1, bcr2 and bcr3 transcripts remains unknown, although it may involve molecular heterogeneity or unidentified secondary alterations. Adaptive advantages provided by such events may contribute to a mutated phenotype during the development of APL (26). To the best of our knowledge, no studies to date mention differences in the oncoprotein expressed depending on the transcript present; thus, it would be of interest to obtain further knowledge of the molecular function of oncoproteins in association with the prognosis of these patients. It can be hypothesized that different PML breakpoints that lead to protein variants could affect the prognosis or therapeutic response; however, this issue remains unsolved (27).

Previous studies have reported that pediatric patients have a >25% frequency of the hypogranular morphological subtype, and a higher frequency of the bcr2 and bcr3 transcripts, compared with adult patients (3,17). In addition, a higher incidence of the bcr1 transcript has been observed in the Latin American population, and the bcr3 transcript is associated with a worse prognosis, the M3v subtype, hyperleukocytosis and a higher frequency of mutations in the Fms-like tyrosine kinase 3 (FLT3) gene. According to the clinical follow-up of the patient, it is suggested that the presence of the three transcripts infers a good prognosis. As the expression of bcr1 and bcr2 inhibits the effects of bcr3, we speculate that there could be environmental influences determining the breakpoint in the *PML* gene, or there is an additional secondary alteration, as mutations in the *FLT3* gene may alter cells to infer an adaptive advantage; however, the present case did not present with *FLT3* mutations, or other clinical risk characteristics (17,28).

In the present study, to predict the possible functional interactions of the PML/RARA oncoprotein, an expression microarray analysis was performed. Only gene expression profiles of genes consistently associated with APL were selected, identifying the following clusters of genes: i) Immunoglobulin C1-set molecules involved in the immune system, MHC class II and the loss of HLA-DR antigen expression; ii) C-type lectin; iii) SH2; and iv) mammalian defensins. HLA-DR is a molecule of antigen-presenting cells. The principal function of HLA-DR is to initiate and promote the immune response, and its expression is present in the early stages of the APL disease (29). HLA-DR- and low CD34 expression characterizes malignant promyelocytes. A study by Dunn *et al* (30) examining the mechanisms of immune evasion indicated an association with the downregulation of HLA-DR antigen expression in tumor cells. The mechanism of immune evasion is an immunoediting process that has been described in transplanted patients with AML (30). The transplanted immune cells exert selective pressure against AML cells that can be recognized immunologically. Tumor clones evolve in response to selective pressure mediated by the immune system and finally escape, leading to resistant clones and relapse. These epigenetic alterations suggest that therapeutic strategies to re-sensitize AML cells to the graft-vs. -leukemia effect may be feasible (29,30). Studies have shown that the expression levels of either CD56, CD34 or FLT3-internal tandem duplication (ITD) markers are associated with a poor patient prognosis (31-33). However, the prognosis of patients with APL expressing CD2, CD4, HLA-DR and FLT3-ITD mutation remains controversial. To the best of our knowledge, pediatric cases have not yet been reported.

The C-type lectin cluster was also found in the RNA analysis of the present study. C-type lectin functions as a recognition molecule in the immune system and has a variety of roles in the defense against pathogens, immune regulation and prevention of autoimmunity (34). Human C-type lectin-like molecule-1 (CLL-1; CLEC12A) is a transmembrane glycoprotein that plays a role in immune regulation as an inhibitory receptor. CLL-1 is present in granulocytes, monocytes and certain types of myeloid progenitors in the bone marrow. Furthermore, CLL-1 is detected in 77.5-92% of AML blasts at diagnosis and is also present in leukemic stem cells, causing treatment failure and leukemia relapse (34). However, the association between the expression of CLL-1 and other classical AML markers remains unclear, and the predictive value of CLL-1 expression in patients with AML has rarely been reported.

SH2 was another representative cluster found in the present study. SH2 domain-containing phosphatase 2 (SHP2; PTPN11) is a positive regulator of receptor tyrosine kinase-driven signaling in response to growth factors and cytokines, including signaling through the Ras/RAF/extracellular signal-regulated kinase (ERK), and the JAK/STAT pathways. Hyperactive SHP2 is associated with tumorigenesis, tumor maintenance, metastasis and therapeutic resistance (35). Numerous somatic gain-of-function mutations that similarly cause the constitutive activation of SHP2 are found in leukemia (35).

In the present study, other clusters observed in the functional analysis were mammalian defensins, which are endogenous

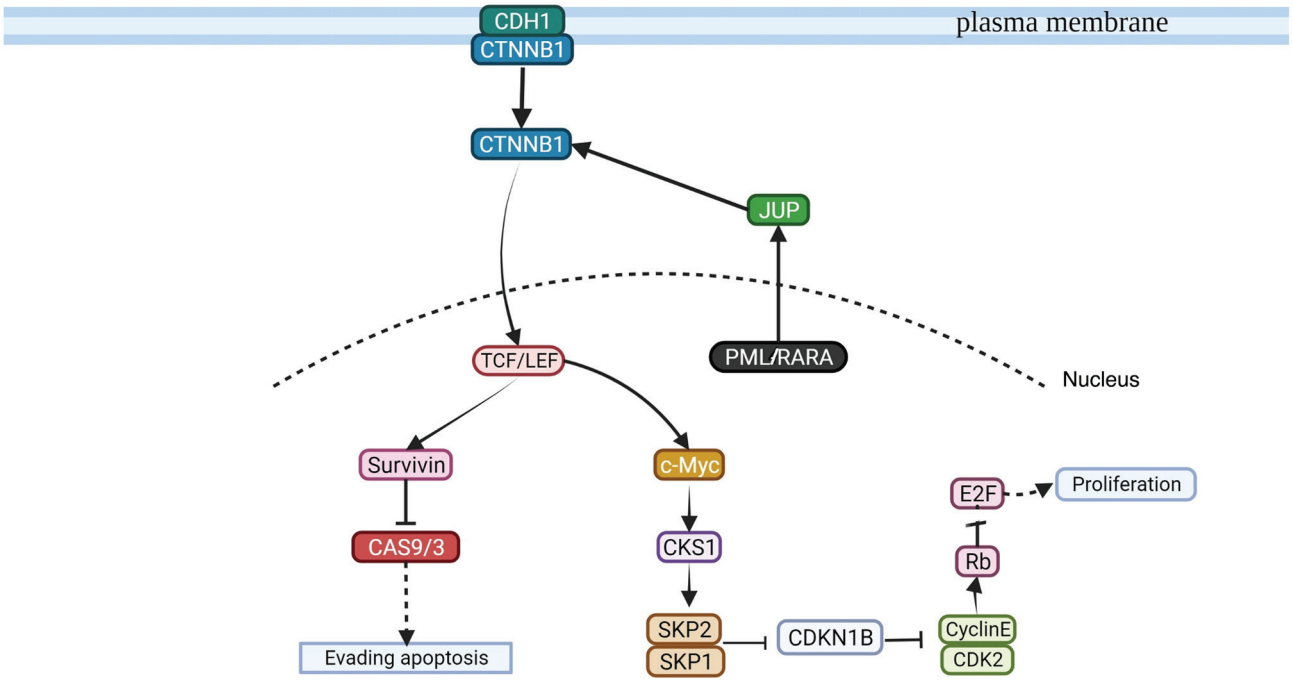

Figure 2. Suggested PML/RARA-related signaling pathway that leads to cell proliferation and the evasion of apoptosis. The PML/RARA oncoprotein is active when interacting with JUP, and in-turn interacts with CTNNB1. CTNNB1 is active in the cellular membrane or in its free form in the cytosol. CTNNB1 is internalized into the nucleus by TCF/LEF, leading to cell proliferation or evasion of apoptosis. For cell proliferation, the TCF/LEF protein activates c-Myc which then interacts with CKS1. CKS1 then activates SKP1/2, which in-turn inhibits the function of CDKN1B and CDK2/cyclin E. Active Rb inhibits E2F, which promotes the constant proliferation of immature promyelocytes. For the evasion of apoptosis, TCF/LEF activates survivin to inhibit the function of CAS9/3. PML, promyelocytic leukemia; RARA, retinoic acid receptor α; JUP, junction plakoglobin; CDH1, cadherin 1; CTNNB1, catenin β 1; TCF/LEF, T-cell factor/lymphoid enhancer factor; survivin, surviving, baculoviral IAP repeat-containing protein 5; CAS, caspase; c-Myc, Myc proto-oncogene protein; CKS1, cyclin-dependent kinase regulatory subunit 1; SKP, S-phase kinase-associated protein; CDKN1B, cyclin-dependent kinase inhibitor 1B; CDK2, cyclin-dependent kinase 2; Cyclin E, cyclin-dependent kinase E; Rb, retinoblastoma-associated protein; E2F, transcription factor E2F1. Created with BioRender.com (2020).

peptides produced by certain leukocytes and epithelial cells. In humans, α-defensins are packaged in azurophilic granules of neutrophils or secreted by intestinal Paneth cells. In addition, β-defensins are constitutively expressed in various mucosa and epithelial cells, where they are upregulated in response to infectious and inflammatory stimuli (36). Humans produce six different α-defensins, including four peptides (HNP-1 to HNP-4) in neutrophils and two peptides (HD5 and HD6) in Paneth cells of the small bowel. Several tumor types, including lung, esophageal and skin cancer, exhibit a deregulated expression and secretion of α- and β-defensins (36). The reasons for this deregulated expression and the role of defensins in oncogenesis remain poorly understood.

To the best of our knowledge, to date there have been no studies that indicate an association of defensins with AML. Nonetheless, α- and β-defensins have been previously reported to be associated with chronic myeloid leukemia (37-39). However, the present study did not establish an association between *PML/RARA* transcripts and the functionally identified clusters using gene expression microarray analysis as the identified clusters do not participate in the PML/RARA oncoprotein pathway. It is considered that CCL-1 may emerge as a promising diagnostic biomarker (34). In addition, the HLA-DR class II implication in promyelocytes was negative. Furthermore, the present study suggested a possible signaling pathway involving the PML/RARA oncoprotein that leads to cell proliferation or the evasion of apoptosis, based on the microarray analysis and literature search (Fig. 2).

In conclusion, to the best of our knowledge, the present study is the first to report a pediatric patient with AML with three simultaneous transcripts. The three transcripts may be protective as the patient exhibited a positive response to treatment. As it has been 4 years since the end of the treatment, the patient is considered to be cured. Moreover, the *PML/RARA* transcripts (bcr1, bcr2 and bcr3) coincide with the good patient prognosis observed in the present case. Further accumulation of data on similar cases may provide relevant clinical evidence for pediatric APL. The functional clusters identified in the patient of the present study may be related to APL biology and suggest promising biomarkers, such as CCL-1, and α- and β-defensins. The PML/RARA oncoprotein signaling pathway suggested in the present study may be associated with the leukemogenic events involved in APL.

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Availability of data and materials

The gene expression datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (40) repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205372). This was in-line with the MIAME and MINSEQE guidelines. All other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

JSH contributed to the design of the study, performed technical procedures, drafted the article and interpreted genomic data. LMM acquired data and performed qPCR procedures. ICQ performed technical microarray procedures. AMM and GSS performed cytogenetics technical procedures. SABJ and RMCO interpreted the clinical data. UFSB contributed to the interpretation and analysis of results. DOS contributed to the interpretation and analysis of microarray results. FASZ coordinated the clinical management. RCR interpreted clinical results and reviewed the manuscript. ACR designed the study and reviewed the submitted version. LBM designed the study and coordinated the final approval of the submitted version. ACR and LBM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted following the principles of The Declaration of Helsinki. The present study was submitted and accepted by The Research Committee and The Research Ethics Committee of The Civil Hospital of Guadalajara (approval no. 00116). Written informed consent and assent were obtained for participation in this study.

Patient consent for publication

Written informed consent was obtained from the patient and their parents to publish the present study.

Competing interests

The authors declare that they have no competing interests.

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