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t(3;17)(p25;q21) APL Represents a Cryptic Insertion of PML-RARA into the 3p25 Locus

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Abstract

We previously reported a case of a 72 year old man with acute promyelocytic leukemia with karyotype 47XYt(3;17)(p25;q21), +8. Fluorescent in-situ hybridization failed to show rearrangement of the PML locus but did demonstrate relocalization of the retinoic acid receptor alpha (RARA) to chromosome 3. We performed a modified panhandle PCR analysis to investigate the unknown 5' partner. Our analysis indicates that the fusion partner is PML. This karyotype therefore results in a cryptic PML-RARA fusion inserted into the 3p25 locus. Our case highlights the need for molecular analysis of seemingly novel karyotypic abnormalities.

Keywords

chromosome translocation; Acute promyelocytic leukemia; PML-RARA

Introduction

Acute promyelocytic leukemia (APL) is characterized by a balanced translocation involving the retinoic acid receptor alpha (RARA) locus at 17q21-23 (Melnick and Licht 1999). The most common translocation partner is the PML locus at 15q21, and indeed t(15;17)(q21;q21) occurs in over 95% of APL. Expression of the resultant PML-RARA fusion protein induces the characteristic myeloid maturation arrest and leukemic phenotype. Indeed, expression of PML-RARA in murine bone marrow cells induces leukemic transformation similar to human APL (Brown, et al. 1997; He, et al. 1997). Treatment with all-trans retinoic acid (ATRA), the ligand for RARA, induces differentiation of the blasts, through an incompletely understood mechanism that likely involves both activation and degradation of the PML-RARA protein (Kogan 2009).

APL patients with variant RARA translocations are rare (Redner 2002). The first of these to be reported, t(11;17)(q23;q21) fuses the PLZF transcriptional repressor to the same C-terminal sequences of RARA as are expressed in PML-RARA. The second most common translocation, t(5;17)(q35;q21) fuses nucleophosmin (NPM) to RARA. t(11;17)(q13;q21) fuses NUMA to RARA. One case has been described of a STAT5b-RARA fusion in a patient with der 17, and one case has been described of a fusion of RARA with the

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regulatory subunit of the cyclic adenosine monophosphate dependent protein kinase PRKAR1A on 17q24 (Catalano, et al. 2007). Altogether these infrequent variants represent less than 2% of APL (Grimwade, et al. 2000; Grimwade, et al. 1997). Although rare, such variant translocations represent “experiments of nature” with similar phenotypes, though different genotypes, and provide valuable model systems with which to test the molecular basis of APL.

We previously reported a case of a 72 year old man with leukocytosis with t(3;17)(p25;q21) that involved the RARA locus (Redner, et al. 2006). Bone marrow analysis revealed an abnormal population of chloracetate esterase positive myeloblasts marking for CD117, CD13/CD33, partial CD15, CD33, and myeloperoxidase, but lacking CD34 and HLA-DR. Cytogenetic analysis demonstrated 47XYt(3;17)(p25;q21), +8. Fluorescent in-situ hybridization (FISH) using the Vysis LSI PML-RARA dual color single fusion probe showed no evidence for PML-RARA fusion. Hybridization with the LSI RARA dual color break apart DNA probe indicated rearrangement of the RARA locus on chromosome 17, partnered with chromosome 3, as determined through rehybridization with the Visas CEP3 DNA probe (Redner, et al. 2006). RNA was of insufficient quality for RT-PCR or sequencing.

In this report we describe use of a modified panhandle PCR to identify the breakpoint in the genomic DNA isolated from the bone marrow specimen. Sequencing of the amplified DNA indicates that t(3;17)(p25;q21) represents a cryptic rearrangement of PML-RARA.

Methods

Southern Blot Analysis

The use of stored bone marrow was approved by the University of Pittsburgh Committee for Oversight of Research Involving the Dead. The mononuclear cell fraction was enriched over a ficoll gradient before being pelleted and frozen at -80°C until processing. Genomic DNA was extracted from the marrow sample using the QIAamp DNA Blood kit (Qiagen). To obtain adequate amounts of DNA for further analysis whole genome amplification was performed using REPLI-g kit (Qiagen).

The RARA breakpoint in APL consistently lies within intron 2 of RARA. The configuration of RARA intron 2 was analyzed by Southern blotting. Twenty µg of genomic DNA was digested with restriction enzyme KpnI (NEB) and fractionated by electrophoresis on a 1% agarose gel. The DNA was depurinated, denatured, neutralized and transferred onto a nylon membrane (Roche). The blot was successively hybridized with three 32P labeled RARA probes (Rediprime II DNA labeling system (Amersham)) corresponding to sequence 5864-6405 (Probe 1), 11826-12330 (Probe 2), and 13730-14278 (Probe 3) of the 16919 bp long RARA intron 2 (NCBI Reference Sequence NC_000017.10).

Panhandle PCR

To make the panhandle, genomic DNA was digested with KpnI and the cleaved DNA was dephosphorylated using calf intestinal alkaline phosphatase (NEB). A single stranded 5' phosphorylated adaptor oligonucleotide (complementary to the sense strand from 12126-12160) was then ligated to the DNA. The complimentary strand of the adaptor was filled-in using the Klenow fragment of DNA polymerase I (NEB).

A 50 µl reaction mixture containing 250 ng of adaptor ligated DNA, 200 µM dNTP's, and 5 µl of 10× PCR reaction buffer was heated to 94°C, followed by a 10% ramp to 72°C to facilitate intrastrand annealing of the complimentary strand of the ligated oligo to RARA sequence at the 5' end. The handle was then extended by continuing the reaction at 72°C for

30 sec. At the end of the incubation, 200 nM panhandle primers RARA PH-1 (5' gga ttc tca cca gag ca 3') and RARA PH-2 (5' att ctt ctc acc aga gca gc 3') were added to the reaction mix and the breakpoint region was amplified using the following conditions: denaturation 94°C 1min; 20 cycles at 94°C, 10 sec; 60°C, 15sec(decrement 0.5°C/cycle); 68°C, 2min; 25 cycles at 94°C, 10 s; 50°C, 15 sec; 68°C, 2 min. Nested PCR was conducted using 1 µl aliquot of the primary PCR product and primer set RARA PH 3 (5' tcc act cct ggg gat gaa ag 3') and RARA PH-4 (5' ctc cac tcc tgg gga tga aag 3'). PCR products were subcloned into a TOPO vector (Invitrogen), and the sequence analyzed by the University of Pittsburgh's Genomics and Proteomics Core Laboratories on an Applied Biosystems (ABI) 3730xl DNA Analyzer.

Single Specific Primer PCR (SSP PCR)

5 µg of genomic DNA was digested with KpnI and EcoRI. The restricted DNA was then ligated into a plasmid vector pBlueScript (Stratagene) digested with the same restriction enzymes. To amplify the genomic region containing the breakpoint, a two step PCR analysis was performed. The first round of PCR was performed with the vector specific M13 reverse primer and RARA gene specific primer (5' ccc caa gtg aag tcag ggt a 3'). A 1-µl aliquot of the first round of PCR product was used for the second round of PCR using vector-specific primer T3 and a nested RARA specific primer (5' att ctt ctc acc aga gca gc 3'). The PCR product was cloned into a TOPO TA cloning vector (Invitrogen) for sequence analysis.

Results

Attempts to harvest RNA from the limited patient material of adequate quality to perform RT-PCR or RACE analysis were unsuccessful. We therefore turned to an analysis of the genomic DNA to further characterize the translocation. RARA intron 2 is approximately 17 kb, with three Kpn 1 sites (Figure 1A; NCBI Reference Sequence NC_000017.10). Southern blot analysis revealed a unique band corresponding to 5 KB rather than the expected 7 KB band corresponding to the 3' Kpn 1 fragment (Figure 1B). Further analysis with Eco R1 indicated that the breakpoint was located within the Kpn 1 – EcoR1 fragment immediately 5' to Probe 2.

In order to identify the unknown sequence that was fused to the second intron of RARA, we pursued two complementary strategies of panhandle PCR and SSP-PCR. Prior use of panhandle PCR has focused on identification of unknown sequences 3' to the known sequence (Megonigal, et al. 1997; Robinson and Felix 2009). In our case, the unknown sequence was 5' of the known sequence (RARA). In order to adapt panhandle PCR to this situation, we developed a strategy to amplify the complementary (non-coding) strand. Prior publications made use of restriction digests with enzymes that would generate a 5' overhang (Megonigal, et al. 1997; Robinson and Felix 2009); we therefore further adapted the strategy to address DNA restricted with Kpn 1, which generates 3' overhangs. The schematic is presented in Figure 2. Briefly, an adapter sequence partially complementary to the Kpn 1 restricted overhang was ligated to the Kpn 1 digested DNA.

The complementary strand of the adapter sequence was filled-in, forming a sequence complementary to 12126-12160 in RARA, to allow formation of a stem-loop structure. The stem-loop structure was amplified using nested primers, and the amplified sequence of approximately 1 KB was subcloned for sequencing.

An alternative strategy was simultaneously pursued using SSP-PCR (Shyamala and Ames 1989). Southern blotting indicated that the breakpoint lay within the approximately 1 KB Kpn1-Eco R1 fragment identified in the map of Figure 1. Genomic DNA was restricted with Kpn1 and Eco R1, and the digested DNA subcloned into pBlueScript. PCR was performed

using nested primers, with 5' primers corresponding to the sequence of the cloning vector, and 3' primers complementary to the known sequence in intron 2 of RARA. Amplified sequences of the appropriate size for the rearranged RARA (smaller than wild-type RARA sequence) were subcloned and sequenced.

Both strategies identified PML sequence as the unknown (see Figure 3). The breakpoint corresponds to the previously described bcr3 breakpoint within intron 3 of PML (Pandolfi, et al. 1992).

Discussion

Molecular analysis and direct sequencing of the genomic DNA revealed that t(3;17) (p25;q21) represents a cryptic translocation of PML and RARA. The RARA breakpoint is in RARA intron 2, as is typical of t(15;17) (Melnick and Licht 1999; Redner 2002). The breakpoint in PML is in intron 3, consistent with production of the shorter bcr3 transcript and protein (Pandolfi, et al. 1992). Expression of PML-RARA protein would be consistent with the blast phenotype (Melnick and Licht 1999), and the previously described sensitivity to the differentiating effects of ATRA (Redner, et al. 2006). We have not extended our sequence analysis to identify the locus on chromosome 3 in which the cryptic PML-RARA translocation was inserted.

Numerous cryptic translocations resulting in expression of a PML-RARA fusion transcript have been described, representing up to 5% of APL. (Grimwade, et al. 2000; Grimwade, et al. 1997) They respond to ATRA treatment, as in classical t(15;17) APL, and convey an equivalent prognosis. Insertion of PML-RARA into the 3p25 locus has not been previously reported.

It is surprising that FISH analysis failed to detect rearrangement of the PML locus. The rearrangement in our case presumably involved a limited sequence of DNA that was insufficient to hybridize with the Vysis PML-RARA dual color probe, thus allowing the PML rearrangement to go undetected. Indeed, Choghule et al. have previously reported a cryptic PML-RARA rearrangement in a patient who exhibited normal karyotype by both G-banding and FISH analysis (Choghule, et al. 2009). Our case illustrates a limitation with the large genomic probes routinely used for FISH analysis, and highlights the need for molecular analysis of novel karyotypic abnormalities in leukemia.

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Figure 1.

Panel A. Schematic map of intron 2 of RARA. The approximate location of the breakpoint is indicated by the boldface vertical arrow. Panel B. Southern blot of control U937 genomic amplified DNA (Lane 1) and the patient's genomic amplified DNA (Lane 2). DNA was digested with Kpn 1. The blot was probed with Probe 3, (indicated in Panel A). The unique approximately 5 kb band representing the fusion DNA is indicated by the arrow.

Figure 2. Schematic of the strategy that was employed for panhandle PCR. The lower panel indicates the formation of the panhandle stem-loop structure.

*GCCACCCAGAGGGGACACATTTTACTAATTTACAAGACTCTGTGGATTGACCTAGCGATGACTCATGGGGAACACG
TGAAAGTTGAAAACTGCAAAGCTGAGAATCAGACCCAGTATCATTTTGGCGCCAGAGGGACCAAGCACTTCAGGA
GGTTTTCTTAAGC***CAGCTGCTCTGCTCGGATGGCGCCGCCGGCTGANTGACGGGGGCGGCGCGCAGNACTTCCCA
GCTCGGACCTCTTGCCCTTCGAGGGGAAAGATGTACGAGAGTGAAAAGTGGGGGTCCACCCCTAATCCCTTCCTA
GNGGTGGATTTTATA**

Figure 3.

The fusion sequence. The genomic PML sequence at the 5' end is represented in italics; the 3' genomic RARA sequence is represented in bold.