Case Study: An APL Case without a Detectable *RARA* Gene Rearrangement

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Abstract

Acute Promyelocytic Leukemia (APL or APML) is a subtype of Acute Myeloid Leukemia (AML). APL is well characterized by its genetic abnormality, a reciprocal translocation between chromosomes 15 and 17 (i.e., t(15;17)(q22;q12)). This translocation results in the expression of the fusion gene, *PML-RARA* which eventually activates the translation of an oncoprotein blocking differentiation of myeloid cells. Just like the symptoms of AML, APL patients usually experience anemia, pancytopenia, fever, and bleeding. Although the median survival of untreated APL is less than 30 days, the introduction of ATRA (all trans retinoic acid) therapy combined with chemotherapy, results in complete remission (CR) rates of approximately 80 to 95% in as little as 38 to 44 days. In addition, the longer term "cure rate" is as high as 80%, making APL the most curable subtype of AML.

The most common tests that accurately detect the t(15;17) include cytogenetics, FISH, and RT-PCR on both bone marrow and peripheral blood. The main reason for the high accuracy of these tests is that 92% of APL cases have an exact breakpoint in *RARA* (on chromosome 17) with only three breakpoint cluster regions on *PML* (chromosome 15). A *RARA* translocation to any of the three breakpoints on *PML* would not affect the diagnosis or the effectiveness of ATRA therapy. Therefore, the ability to detect the (15;17) translocation makes the three tests listed above the preferred method of testing.

However, there is a subset of APL patients in the 8-9% range with variant translocations, and which may require different treatment strategies. In general, these remaining APL cases can be separated into three groups: 1) a small insertion of *RARA* into *PML* (4%, cryptic by cytogenetics); 2) complex translocations involved in chromosome 15 and 17 (2%); and 3) one of the seven alternative *RARA* fusion partners from chromosomes 4, 5, 11, 16, 17 and X (less than 2%).

We highlight a case where our patient, a 22 year-old female, presented to her physician's office with symptoms. A bone marrow evaluation was ordered as part of her work-up, which came back suspicious for APL; however, both FISH and cytogenetics were reportedly normal. The physician suspected that the patient's symptoms were related to APL and sent a part of the bone marrow sample for repeat testing to our laboratory. Based on a limited remaining sample that was already 5 days old, the physician prioritized the repeat testing in the following order: *PML-RARA* dual fusion probe by FISH, *RARA* break-apart probe by FISH, cytogenetics, and RT-PCR for the *PML-RARA* transcript. Due to the limited amount of bone marrow received, only the FISH and cytogenetic tests were able to be performed. Cytogenetics appeared normal as did the *RARA* break-apart probe set by FISH. However, the *PML-RARA* FISH test revealed an atypical, abnormal fusion signal pattern of 2R1G1F in just over 84% of the interphase nuclei scored. Further investigation and close examination with metaphase FISH (M-FISH) using this dual color, dual fusion *PML-RARA* probe set revealed a small red signal on top of one of the green signals, forming a fusion. This M-FISH finding confirmed a small insertion of *PML* from the long arm of chromosome 15 into the long arm of chromosome 17, juxtaposing the *PML* and *RARA* genes.

There have been reports in the literature that have suggested different probe designs can affect the interpretation of atypical *PML-RARA* gene rearrangements. To follow-up on this, our lab tested the dual color, dual fusion t(15;17) probe sets from 4 different companies including DAKO, MetaSystems, Abbott Molecular, and CytoCell. Three of the four probe sets resulted in an abnormal, atypical FISH signal pattern, interestingly with different intensities of the fused red signal. However, one probe set revealed a normal FISH result. Such a finding confirms that although 92% of APL patients have exactly the same break point, some atypical APL cases may not be detectable based on probe set design. *PML-RARA* (or any other probe set) designs are not standardized and are viewed as proprietary by many companies. This lack of standardization results in probes of different sizes, colors, intensities, and hybridization patterns, all of which have the potential to lead to a false negative result. Using this case as an example, we hope to raise awareness of a potential pitfall in *PML-RARA* gene rearrangement studies utilizing FISH especially for those 8% of atypical APL cases.

Background

- Acute Promyelocytic Leukemia (APL or APML), a subtype of Acute Myeloid Leukemia (AML), represents close to 10% of all AML. APL is identified cytogenetically by a reciprocal translocation between chromosomes 15 and 17 (i.e., t(15;17)(q22;q12)).
- Hypergranular (kidney-shaped) promyelocytes, auer rods, and faggot cells are often observed in morphology studies.[1]
- The detection of CD13 and CD33 by flow cytometry, as well as absence or minimum presence of CD34 and HLA-DR in immunophenotype, are often hints of APL.[1]
- The most common tests that accurately detect the t(15;17) include cytogenetics, FISH, and RT-PCR on both bone marrow and peripheral blood.[4,5] The main reason for the high accuracy of these tests is that 92% of APL cases have an exact breakpoint in *RARA* with only three breakpoint cluster regions (*Bcr*) on *PML*, intron 6 (*Bcr1*), exon 6 (*Bcr2*), and intron 3 (*Bcr3*).[3]

Case Study

A 22-year-old female presented to her physician's office with symptoms suggestive of APL. A bone marrow evaluation was ordered as part of her work-up and the result came back suspicious for APL. However, results from both FISH and cytogenetics testing were reported negative. Due to these perplexing results, the physician sent a portion of the bone marrow sample to our laboratory for repeat testing. The physician prioritized testing in the following order: *PML-RARA* dual fusion FISH, *RARA* break-apart FISH, cytogenetics, and RT-PCR for the *PML-RARA* transcript. Only the FISH and cytogenetic tests could be performed due to the limited amount of bone marrow received.

Result

Cytogenetics appeared normal as did the *RARA* break apart FISH. However, the *PML/RARA* dual fusion FISH test revealed an atypical abnormal fusion signal pattern (2R1G1F) in just over 84% of the interphase nuclei analyzed. Further investigation and close examination with metaphase FISH (M-FISH) using this dual color, dual fusion *PML/RARA* probe set revealed a small red signal on top of one of the green signals forming a fusion. This M-FISH finding confirmed a small insertion of *PML* from the long arm of chromosome 15 into the long arm of chromosome 17 juxtaposing the *PML* and *RARA* genes.

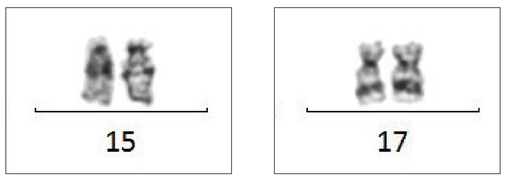

Figure 1: Normal sets of chromosome 15 and 17 were observed in both 24- and 48- hour cultures.

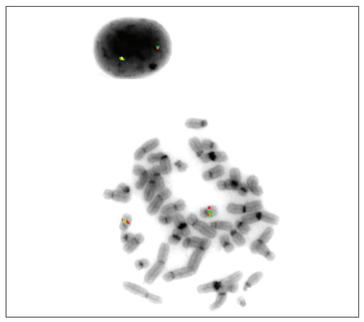

Figure 2: Normal result, 2F, was observed with the *RARA* break-apart probe (DAKO probe set).

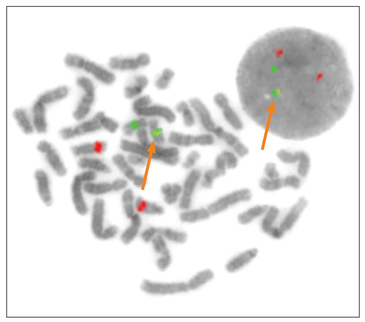

Figure 3: An atypical fusion signal pattern (2R1G1F), identified under close examination with the dual fusion *PML-RARA* probe (MetaSystems probe set).

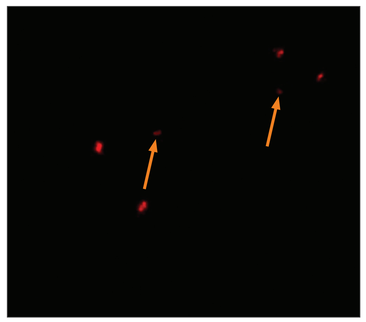

Figure 4: A third small red signal can be better recognized in these two cells using a single-pass red filter.

Discussion

Campbell, from Victorian Cancer Cytogenetics Service in Australia, has shown that the size of the dual color, dual fusion *PML-RARA* probe can affect the diagnostic result. From 2002 to 2013, 10 cases previously reported to be negative for *PML-RARA* fusion by FISH showed positive results by molecular studies. Further analysis and only under careful examination, three of the ten cases, were indeed positive with a 2R1G1F pattern.[2] The remaining seven cases were reported negative utilizing the *PML-RARA* dual fusion probe from Abbott Molecular. However, an atypical positive result (1R2G1F) could be clearly detected with the *PML-RARA* translocation probe from CytoCell. Campbell's study concluded the small third fused green signal becomes hidden in Abbott Molecular probe set when there are two larger and brighter signals sitting next to the fused signal. Due to the smaller size of CytoCell's *RARA* probe, the fused green signal stands out more, relative to Abbott Molecular's.[2]

To follow-up on our patient, our laboratory tested the dual fusion *PML-RARA* probe sets from 4 companies: Abbott Molecular, CytoCell, DAKO, and MetaSystems. An abnormal, atypical FISH signal pattern (2R1G1F) was detected using probe sets from Abbott Molecular, DAKO, and Metasystems, with remarkable difference in intensity of the fused red signal. However, the probe set from CytoCell revealed normal results (2R2G). A representative image of the negative result from CytoCell can be seen in Figure 5.

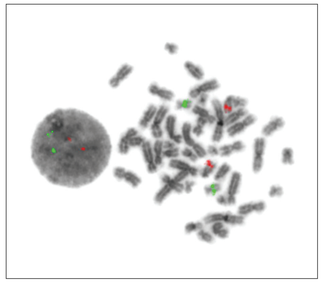

Figure 5: Normal result, 2R2G, could be found with the dual fusion t(15;17) probe(CytoCell probe set).

Figure 6: No third red or fused red can be seen with single-pass red filter.

Experiments were performed to investigate if the difference in probe size and design from different vendors resulted in discrepancies of results and intensity of the fused red signal when applied to our case study. A summary of the probe size and the intensities of the fused red are listed in Table 1.

	Abbott Molecular	CytoCell	DAKO	Metasystems
***PML* Probe Size**	517KB	325KB	749kb	422KB
Fused Red Intensity	++	N/A	+	+++

Table 1: *PML* probe size per vendor and observed intensity of fused red signal

As shown by Table 1, the *PML* probes from Abbott Molecular and DAKO have broader coverage than that of MetaSystems. However, MetaSystems' red fused signal intensity was the strongest. On the other hand, the CytoCell probe set has the smallest *PML* probe coverage and was the only probe to not detect the fused red. This finding suggests that the size alone of the *PML* probe does not exclusively affect the detection and intensity of the fused red signal in this case. However, the probe position is the key factor determining the intensity of the fused red signal observed in this case study. Figure 7 illustrates location of the *PML* gene in relation to the *PML* probes from CytoCell and Abbott Molecular based on published probe maps.

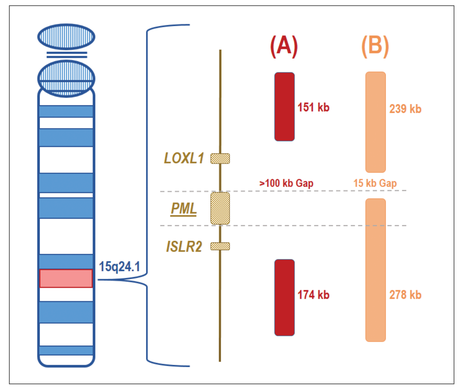

Figure 7: Estimated *PML* probe location of (A) CytoCell and (B) Abbott Molecular relative to the PML gene located on 15q24.1.

Even though the probe maps for these two companies are just representative images to illustrate the relationship of the actual location of *PML* gene and the designed location for the *PML* probe, it can be easily seen that *LOX1* and *ISLR2* is included in the *PML* probe from Abbott Molecular, but not in that of CytoCell. Furthermore, CytoCell has a gap larger than 100kb within the *PML* probes, while Abbottt Molecular's *PML* probe gap is around 15kb. It is thus concluded that the designed location of the probe is the main reason behind the discrepancy in results.

In addition, BAC (Bacterial Artificial Chromosome) clones and DNA printing are only two of the most common ways to design and develop FISH probes. Without an FDA-standardized guideline that specifically dictates the probe design, location, and the ratio of the fluorescence material, different companies may have different ways to produce their own probe sets. This lack of unified guidelines results in the existence of various sizes, colors, intensities, and hybridization patterns for the various commercially available probe sets. Eventually this variation may lead to inconsistent inter-laboratory results.

The case presented exemplifies how the different intensities of the fused red could potentially cause technologists to interpret false negative results as well as inaccurate abnormal cell percentages. Three experienced technologists were asked to participate in a double-blinded study to analyze the case study and normal samples as controls. Ten slides probed with a mix of D-FISH, enumeration, and break-apart probe sets were provided. The technologists were told the purpose of this study was to identify the target chromosome. The participants were also asked to count 100 cells and to record the patterns observed. The abnormal sample results from each of the three technologists are summarized in Table 2.

		Abbott Molecular	CytoCell	DAKO	Metasystems
Technologist A	2R2G 2R1G1F	[100/100] [0/100]	[100/100] [0/100]	[100/100] [0/100]	[100/100] [0/100]
Technologist B	2R2G 2R1G1F	[47/100] [53/100]	[100/100] [0/100]	[98/100] [0/100]	[28/100] [72/100]
Technologist C	2R2G 2R1G1F	[33/100] [59/100]	[92/100] [0/100]	[28/100] [65/100]	[4/100] [94/100]

Table 2: Result of the double-blinded study for the positive sample

The atypical abnormal pattern is unusual and difficult to detect. Technologist A did not detect the atypical abnormal patterns using the probes designed by all four companies. Technologist B could identify the abnormal pattern from two out of the four companies, but failed to identify the abnormal pattern from DAKO's *PML-RARA* probe, which was the dimmest detectable fused red out of the four companies. Technologist C was the only technologist in this study who correctly detected all the patterns with relatively uneven percentages of the abnormal cells. This finding also emphasizes the importance of peer review and quality control as a necessary process to minimize false or discrepant diagnosis.

Conclusion

The literature and this case study confirm that although 92% of APL patients have the same break point, some atypical APL cases may not be detectable due to variation of probe design. *PML-RARA* (or any other probe set) designs are proprietary and unique to the manufacturing company. This lack of standardization results in probes of different sizes, genomic location, colors, intensities, and therefore different hybridization patterns, which have the potential to lead to a false negative result.

Although the introduction of ATRA therapy combined with chemotherapy results in complete remission (CR) rates of approximately 80 to 95% and the long-term cure rate is 80%, it is important to know that the median survival of untreated APL is less than 30 days.[5] The false negative result can easily lead to misdiagnosis as well as delayed treatment. The same situation could apply to molecular studies, such as RT-PCR, since results are highly dependent on primer design. Not only could those results affect the decisions of the doctors and the treatments of the patients, but a laboratory could also be susceptible to litigation due to misdiagnosis. Using this case as an example, we hope to raise awareness of a potential pitfall in *PML-RARA* gene rearrangement studies when utilizing FISH probes from a single vendor, especially for those 8-9% of atypical APL cases.

Reference

1. Amare PK, Baisane C, Nair R, et al. Characterization of cryptic rearrangements, deletion, complex variants of PML, RARA in acute promyelocytic leukemia. *Indian J Hum Genet*. 2011;17(2):54-8.
2. Campbell LJ, Oei P, Brookwell R, et al. FISH detection of PML-RARA fusion in ins(15;17) acute promyelocytic leukaemia depends on probe size. *Biomed Res Int*. 2013;2013:164501.
3. Grimwade D, Lo coco F. Acute promyelocytic leukemia: a model for the role of molecular diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia. *Leukemia*. 2002;16(10):1959-73.
4. Jad J. Wakim and Carlos A. Tirado (2012). Acute Promyelocytic Leukemia Lacking the Classic Translocation t(15;17), Myeloid Leukemia - Clinical Diagnosis and Treatment, Dr Steffen Koschmieder (Ed.), ISBN: 978-953-307-886-1
5. Redner RL. Variations on a theme: the alternate translocations in APL. *Leukemia*. 2002;16(10):1927-32