Molecular methods shown to push cases forward: Case studies in hematopathology

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October 2020—B-ALL with aberrant expression of myeloid markers should be investigated further for specific gene abnormalities, including *ZNF384* rearrangements, and microarray analysis may play an important role.

That was the crux of a case presented at last year's AMP annual meeting by Shweta Bhavsar, MBBS, MD, molecular genetic pathology fellow, University of Pittsburgh Medical Center and School of Medicine.

A second presentation in the same session, by Jeffrey SoRelle, MD, of the University of Texas Southwestern, was of an aggressive case of MDS in which molecular analysis alone led to earlier treatment for the patient.

Dr. Bhavsar

Drs. Bhavsar and SoRelle spoke with CAP TODAY recently.

In the case of B-lymphoblastic leukemia, the four-year-old female patient, who had a significant medical history of asthma, presented with familiar B-ALL symptoms: pallor, fatigue, weight loss, and petechiae, predominantly on her face, Dr. Bhavsar says. "She was not doing well."

The CBC and peripheral blood exam results revealed leukocytosis (26.3×10^9/L) with approximately 74 percent circulating blasts, and anemia (Hb 8.2 g/dL). "Morphologically the blasts were typical lymphoblasts with no specific findings," Dr. Bhavsar says, describing them as small to intermediate in size, with a high nucleus-to-cytoplasmic ratio, scant amount of cytoplasm, fine chromatin, and prominent nucleoli.

The bone marrow aspirate results, which showed 92 percent blasts and reduced trilineage hematopoiesis, further supported the diagnosis of B-ALL, she says. "The bone marrow biopsy, unfortunately, was inadequate in this case because it showed predominantly cartilage."

Flow cytometry revealed that while the blasts, at 86 percent of total events, were positive for CD45 (dim), CD34, HLA-DR, TdT, and the B-cell markers CD19 and CD22, they were negative for CD10, CD20, CD117, cytoplasmic CD3, and myeloperoxidase. "Usually most of the B-ALLs are CD10 positive, but in this case they were CD10 negative, and they showed some myeloid marker expression—CD13 (partial dim) and CD33," Dr. Bhavsar says. These immunophenotypic findings have been described in association with *ZNF384* translocations and some other genetic types, she adds.

On routine karyotypic analysis, five cells showed an inv2 (q11.2q31), which was of unknown significance, as "there is no known specific association of inversion 2 in B-ALLs," Dr. Bhavsar says. No abnormality was seen on chromosome 12 (*ZNF384* is located on Chr 12).

FISH studies—performed for the most common oncogenic alterations in the B-ALL including trisomies 4 and 10, *AFF3, BCR/ABL1, KMT2A, ETV6/RUNX1,* and *CRLF2* gene rearrangements—were all negative.

The turning point in the case, Dr. Bhavsar says, was the result of the microarray analysis performed and interpreted by Svetlana Yatsenko, MD, associate professor, Department of Pathology, and director of the Pittsburgh

cytogenetics laboratory. (See “Deletion/breakpoint in this case.”)

“While balanced translocations cannot be picked up on microarray studies, even when the translocation appears to be balanced, there may be loss of some genetic material at the breakpoints,” Dr. Bhavsar explains. “It is important to review these small deletions in clinically significant genes and look for translocations involving those genes.” This case is an example of such a phenomenon, she says. The microarray results demonstrated a small (62 kb) deletion in the 5′ region of the *ZNF384* gene, which is strongly indicative of a translocation involving this gene. Nidhi Aggarwal, MD, hematopathologist and director of UPMC’s molecular hemato-oncology service, Division of Molecular and Genomic Pathology, signed out the morphology on the case. When she reviewed the results of ancillary studies for final classification, she recognized this and further ordered the confirmatory FISH testing. She found recent descriptions of the *ZNF384* rearrangement in the literature (Lilljebjorn H, et al. *Blood*. 2017;130[12]:1395–1401).

“In addition,” Dr. Bhavsar says, “there was a secondary alteration seen in about 20 percent of the cells in chromosome 10, which showed a deletion of the *BLNK* gene (2.356 Mb), but this was separate from the *ZNF384* rearrangement.”

“A lot of people were looking into these B-ALLs, which were initially not categorized in the WHO risk stratification categories,” Dr. Bhavsar says, noting that 20 to 30 percent of B-ALLs do not fall into the established genetic subtypes. “People have been exploring those to see other genetic alterations that could be disease-defining and help to prognosticate and treat patients.”

ZNF384 fusions are one of those new oncogenic subtypes. They have a distinct gene expression pattern that is enriched in hematopoietic stem cell features, “which would explain the aberrant expression of myeloid markers,” she says. *ZNF384* fusions in B-ALL have an incidence of about one to six percent in pediatric cases and five to 15 percent in adult cases.

Microarray analysis is a core protocol at UPMC for pediatric B-ALL cases. “Obviously, the main use for microarray is to look for copy number alteration and loss of heterozygosity to recognize hypodiploidy presenting as pseudohyperdiploidy in B-ALL, which can help in prognosis,” Dr. Bhavsar says. “If it’s hyperdiploidy, then these patients do really well, versus hypodiploidy—those patients do badly. So that is one of the key things we look for.”

But it can also be a clue to look for these rearrangements if one identifies the small deletions in relevant genes, as in this case, she says. “Otherwise you would have to do specific fusion analysis or RNA sequencing.”

Dr. Aggarwal agrees that microarray analysis is a good screen for copy number and copy neutral changes in any of the genes. For balanced rearrangements, though, it is not a good screen, she says. “As long as the genetic material is present where it is present—whether the rearrangement is present or not—it will not show up on microarray.”

Studies have shown that whenever there are rearrangements—“even rearrangements that we truly think are balanced—at the breakpoint, they actually lose some material. So we think they are balanced but they are not. And that is how we have picked up a few rearrangements in our institute, as demonstrated in this case.”

“That is the important part: Whenever you find these small alterations in microarray, usually the chromosomal analysis does not show anything specific.” If she had not found the *ZNF384* rearrangement, Dr. Aggarwal says she would have sent the specimen out for additional testing.

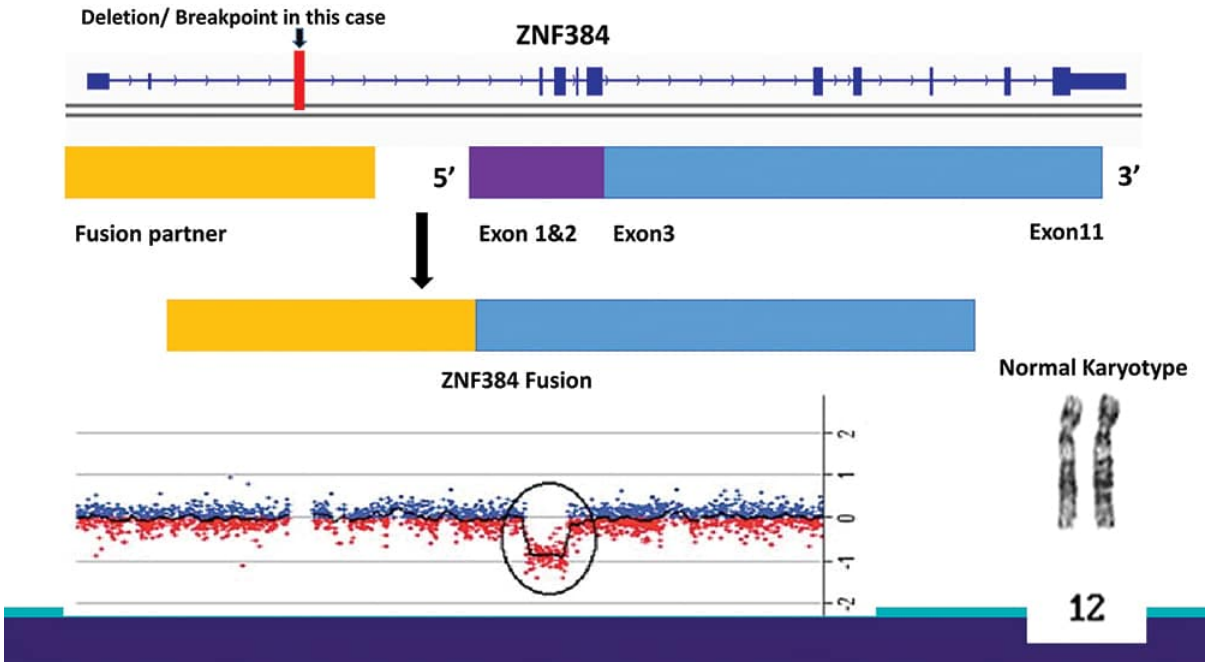
“I was lucky that when I looked at it there was *ZNF384* rearrangement, and it was very well described in the literature by that time, although still very new,” she says.

These *ZNF384* rearranged acute leukemias are actually a spectrum, Dr. Aggarwal says. While they are described with the B-lymphoblastic leukemias, “they are also described with B/myeloid mixed phenotype acute leukemias or mixed lineage acute leukemias,” she says. “Some people believe that maybe these should just be called as acute leukemia with *ZNF384* rather than trying to classify it.”

ZNF384, which has also been known as *CIZ* and *NMP4*, encodes a zinc finger transcription factor that is involved in the regulation of matrix metalloproteinases, Dr. Bhavsar says. The exact mechanism of action of *ZNF384* fusion proteins is still unknown (Alexander TB, et al. *Nature*. 2018;562[7727]:373–379).

"One of the unique things about the translocation is the partner gene sequence attaches at the 5′ end of almost the entire gene," she says. "The entire coding sequence of *ZNF384* is present in *ZNF384* fusions."

So far, nine fusion partners have been identified for *ZNF384: ARID1B, BMP2K, CREBBP, EP300, EWSR1, SMARCA2, SYNRG, TAF15,* and *TCF3*. "The most common one is *TCF3*, and there has been a study that talks about how these patients tend to have a poorer prognosis," Dr. Bhavsar says. "They have a poorer response to steroids and tend to present with higher white blood counts" (Lilljebjorn H, et al. *Blood*. 2017;130[12]:1395–1401).



Associations with epigenetic regulator genes, such as *CREBBP* or *EP300*, have been believed to play a cooperative role in *ZNF384* fusions, Dr. Bhavsar says. In in vitro studies, *ZNF384* fusions with *EP300* and *CREBBP* have been reported to show increased sensitivity of leukemia cells to histone deacetylase inhibitors. Mutations in the RAS signaling pathway genes (*NRAS, KRAS, PIK3CD, PTPN11*) are also common (Qian M, et al. *Genome Res*. 2017;27[2]:185–195).

"So we were able to confirm the *ZNF384* rearrangement by FISH and identify its translocation partner to be *EP300*," Dr. Bhavsar says of Dr. Yatsenko's work in the Pittsburgh cytogenetics laboratory. Depending on the partner, "the prognosis of these *ZNF384* rearranged cases might vary."

Most of the time, Dr. Aggarwal says, *ZNF384* "is a complete gene. It is not missing anything, and the partner gene has the C terminus that is missing."

Some cases with *ZNF384* rearrangement could also have higher expression of *FLT3*, and there is a secondary question of whether that could be useful for treatment. "That is what was unique about this gene rearrangement," Dr. Aggarwal says. "It was not just in B-lymphoblastic but also in a spectrum with mixed lineage, so it can present with mixed lineage."

Dr. Bhavsar points out one report of a myeloid lineage switch that occurred after a patient had therapy for B-lymphoblastic lymphoma. "Then it went on to present like an AML," she says. "So where these can be B/myeloid lineage, they can also have this switch where you should monitor both diseases. It might not only be B-

lymphoblastic. It could be of mixed lineage."

Even though there is not much data about *ZNF384* rearrangements, identifying them can help clinicians "because, one, they now have a genetic signature to it," Dr. Aggarwal says. "So in terms of the clinical features and outcomes of these patients, of course this is a relatively newly described translocation, so there is not much data about it. But as in this case, where the patient was put on a standard protocol and did not achieve remission and hence was put on a very high-risk arm," with enough cases it might be possible to know whether to put the patient on a high-risk arm at the outset.

The patient responded well to treatment in the very high-risk arm and remained in remission as of August 2020.

Dr. Aggarwal

Knowing the genetic alteration of the patient can guide the clinician in looking for residual disease, and "if the patient doesn't do very well, then maybe they can explore if *FLT3* mutation is present," Dr. Aggarwal says.

It's important for the clinician to follow up, and that was part of the reason for presenting the case at the AMP meeting, she says. "A number of gene arrangements are known to be missed on normal karyotypes."

FISH is useful for confirming a rearrangement, she adds, but "the drawback of FISH is that you have to know what you're looking for. So if you're doing other modalities other than just karyotype and FISH, it is important to look at the results of those modalities in greater detail and be aware that these might be subtle ways of picking up translocations in a technique that otherwise is known to not pick up translocations."

In summing up, Dr. Bhavsar says B-ALL cases with aberrant expression of myeloid markers CD13 and/or CD33 with weak or absent CD10 should be investigated for specific gene abnormalities, including *ZNF384* rearrangements, and a multiple-modality approach is needed to identify different genetic alterations, with microarray analysis playing an important role.

Without microarray analysis and knowledge of the *ZNF384* rearrangement, "we probably wouldn't have picked this up," she says. "People are doing specific fusion analysis to look for these newly described variants of B-ALL including rearrangements of *ZNF384*. We were not doing FISH studies for *ZNF384* as a routine."

Molecular analysis of normal hematolymphoid specimens led to identification of high-risk mutations and earlier treatment for MDS in the case presented by Dr. SoRelle, assistant instructor, Department of Pathology, University of Texas Southwestern.

Dr. SoRelle's colleague in the department, Flavia Rosado, MD, had reviewed the case of a 64-year-old man who presented with several symptoms over a one-year period. "He had low platelets, he was edematous all over his body, and he had a very large spleen," Dr. SoRelle says. "So there was concern for some kind of lymphoma. They wanted to get a bone marrow biopsy to do surveillance to see if there was an explanation for what was going on."

The bone marrow biopsy revealed no abnormalities. "It was a little hypercellular at 70 percent, but there was no real dysplasia or evidence of cancer, no abnormal morphology of any of the megakaryocytes, the red blood cell precursors, or the white blood cell precursors," Dr. SoRelle says.

Cytogenetics was significant for 16 out of 20 cells being positive for 20q- and three out of 20 cells positive for 5q-, he says, but no aberrant populations were found by flow cytometry. "It was one of those cases where it seemed like not much was jumping in the face as an abnormality."

The patient's case was sent to the group of Dr. SoRelle and Jeff Gagan, MD, PhD, for next-generation sequencing because of the severity and duration of the symptoms and the high grade of suspicion in the context of a negative bone marrow biopsy, he says.

"In spite of the mostly normal bone marrow and flow cytometry analysis, there were several large, high-frequency molecular abnormalities," he says. There was an *IDH2* classical mutation present in about 46 percent variant allele frequency. There was also an *SRSF2* mutation (p.P95T, 51 percent) at the common hotspot location, as well as CBL (p.R499*, 47 percent), which is involved in a tyrosine kinase ubiquitination, and a *KRAS* mutation (p.K117N, 12 percent).

The subclonal population of *KRAS* was in a "pretty rare hotspot site," Dr. SoRelle says, "but it is more common in the hematolymphoid cells when there is a mutation in *KRAS*." The 20q deletion was also visible, but they did not see a chromosome 5 aberration because it was likely below the limit of detection.

In a subset of these genes, these mutations confer increased proliferation and susceptibility to progress to MDS and could continue to progress to acute myeloid leukemia, he says. *IDH2*, for example, is a "red flag for being a high-risk allele."

In this case, "the first few mutations were all 45 to 50 percent frequency, so very high. We can detect quite low, and see low underlying mutations, but these were all very high. If they're at 50 percent and they're heterozygous, that means it's essentially the whole bone marrow that has the mutation present."

Dr. SoRelle

Some of the mutations found in the molecular analysis pointed to a clonal hematopoiesis of indeterminate potential (CHIP). "But it was especially the *IDH2* mutation that made us look closer," Dr. SoRelle says. "We know that when we have these *IDH1* or *2* or *TP53* mutations, there's been a good amount of data that look at the odds ratio [28.5/47.2] of progressing to AML from MDS. And these were 30 times higher, whereas the other mutations in CHIP, such as *TET2* and *DNMT3A*, were only about two to three times higher than usual" (Abelson S, et al. *Nature*. 2018;559[7714]:400–404).

Progression to AML is a prognostic indication based on the mutational profile, Dr. SoRelle says. The high-risk mutations of *IDH1* and *2* and *TP53* accelerate the onset of AML by four or five years. Spliceosome accelerates onset of AML by three years, and *DNMT3A* by two to three years. *TET2* does not have a significant acceleration prognosis factor, unless there is a biallelic mutation present. "It's interesting to see that if RDW is increased [>14], it's a prognostic risk factor for accelerated onset of AML, and our patient had an increased RDW of about 15.7" (Desai P. *Nat Med*. 2018:24[7]:1015–1023).

The abnormal molecular results "made us push a little harder and recommend that they classify this case as MDS" rather than take a watch-and-wait approach for three months, Dr. SoRelle says.

The patient was started on azacitidine, and when he returned for his three-month checkup, "we saw significant changes to the bone marrow. There was megakaryocytic and erythroid hyperplasia and megakaryocytic dysplasia, and even reticulin fibrosis."

The presence of these abnormalities reflected the aggressive nature of the patient's disease, Dr. SoRelle says, and there likely would have been a worse outcome had the patient not been diagnosed with MDS as a result of the molecular analysis. The next step in the patient's treatment plan was stabilization in preparation for a bone

marrow transplant.

“This is a very important way to show that morphologically normal hematolymphoid specimens in patients who have symptoms can be aided by using ancillary molecular panel testing,” Dr. SoRelle says. “We also know that precursor CHIP mutations in a morphologically normal sample should be taken seriously. And high-risk mutations warrant pushing clinicians forward in diagnosing MDS. This is more of a clinical role in showing them the significance of high-risk mutations.”

“What’s interesting,” he says, “is that molecular analysis alone was what pushed this case forward in getting a diagnosis and got the patient onto an earlier treatment than otherwise would have been the case.”

Amy Carpenter Aquino is CAP TODAY senior editor.