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Commentary

Molecular Diagnosis of Ewing Family Tumors

Too Many Fusions. . . ?

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In the field of sarcoma molecular diagnosis, the "test" most frequently requested is for the gene fusions generated by the chromosomal translocations in Ewing family tumors (EFTs). Of note, this test is perhaps the most complicated of all of the molecular assays in the sarcoma diagnostic toolbox. Starting with the frequent 11;22 translocation involving the *EWS* gene on chromosome 22 and the *FLI1* gene on chromosome 11, the chromosome breakpoints are spread among four introns in the *EWS* gene and six introns in the *FLI1* gene to give a large number of possible EWS-FLI1 fusion products.[1] The size of the fusion transcripts can vary over a 700-bp range, thereby necessitating cautious investigation of a large range of product sizes in diagnostic reverse transcription-polymerase chain reaction (RT-PCR) assays. To increase the complexity further, a relatively frequent variant 21;22 chromosomal translocation generates a fusion of *EWS* to *ERG*, which encodes an ETS domain-containing transcription factor highly related to FLI1 with a comparable distribution of chromosomal breakpoints.[1] Next, as described in the article by Wang and associates in this issue of *The Journal of Molecular Diagnostics*,[2] the complexity continues to rise as there are three additional translocations [t(2;22), t(7;22), and t(17;22)] that juxtapose *EWS* to genes, encoding three additional members of the ETS transcription factor family (*FEV*, *ETV1*, and *E1AF*, respectively) in small numbers of EFT cases. In addition to these rare variant fusions, there is also a second set of rare variants involving the *FUS* gene, which encodes an RNA-binding protein highly related to EWS. This second set includes a 16;21 translocation in which *FUS* is juxtaposed to *ERG*,[3] which was found in a small group of cases and, as described by Ng and colleagues[4] also in this issue of the *JMD*, a *FUS-FEV* fusion resulting from a novel 2;16 translocation found in a single case. Based on these collective findings, a definitive investigation of the gene fusions associated with EFT is, to put it simply, a daunting task.

If this list of possible gene fusions in EFT was not long enough, the situation becomes even more bewildering with the identification of a group of EFT-like tumors with novel gene fusions.[2] Each of these tumors has a small round cell microscopic pattern but does not show the diffuse CD99 immunohistochemical staining characteristic of the classic EFT. Furthermore, instead of a fusion of *EWS* or *FUS* to one of the ETS family genes, two types of gene fusions have been reported in these EFT-like tumors. In one subset, *EWS* is joined to a gene encoding a non-ETS transcription factor, which is not typical of any of the fusions involving *EWS* or *FUS* in other established sarcoma categories. In three reported cases, *EWS* is juxtaposed with genes encoding transcription factors that have not previously been implicated in EFT pathogenesis: *SP3* (described by Wang et al in this issue[2]), *ZNF278*, and *POU5F1*. In the second subset of cases, a completely novel gene fusion, *CIC-DUX4*, which does not involve genes related to *EWS* or the ETS family, was described in two cases of EFT-like tumors. Although rare, these EFT-like tumors add to the differential diagnosis of undifferentiated small round cell tumors. Furthermore, additional cases of these two subsets will likely be identified now that the existence of these tumors is recognized.

Laboratory Challenges

When faced with so many potential fusions that can be assayed, there are many inevitable questions of how many and which fusions should be assayed as part of the

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molecular diagnostic test for EFT. The first issue to be addressed is how this multitude of EFT-associated fusions impacts on the choice of a testing methodology. As noted in the articles by Wang et al[2] and Ng et al,[4] all but one of the five rare gene fusions reported in the two articles in this issue were initially recognized by standard cytogenetic analyses, and thus the rare fusion was apparent from the outset. A clear attribute of standard chromosomal analysis is that this technology can detect countless different genetic alterations without prior selection of one or a few specific alterations for testing. However, cytogenetic analysis requires fresh tissue and cell culture, has a significant failure rate in sarcomas, and is labor-intensive. In addition, the characteristic translocations are not recognizable in some cases because of additional chromosomal changes that obscure the translocation.

For many laboratories, RT-PCR is the technology of choice for detecting gene fusions associated with EFT. RT-PCR procedures can be performed on fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) tissues and are amenable to being performed in batches. For fresh or frozen tissues, the isolated RNA is sufficiently intact so that RT-PCR assays can be used that will detect the full range of fusion transcript sizes. Most published studies used either individual assays for *EWS-FLI1* and *EWS-ERG* or a single assay with a set of "consensus" primers that will detect either *EWS-FLI1* or *EWS-ERG*.[5,6] In large published studies, which often used nested PCR reactions, 91 to 96% of histologically defined EFT cases were positive for either the *EWS-FLI1* or *EWS-ERG* fusion.[5,7] Based on these results, 4 to 9% of cases are estimated to potentially contain rare variant gene fusions that may be detected by use of additional RT-PCR assays. As described in the article by Wang et al,[2] a set of primers has been developed to detect *EWS-FLI1*, *EWS-ERG*, or *EWS-ETV1* in one reaction, or alternatively, primers have been developed to detect *EWS-ETV1* and *EWS-E1AF* in one reaction.[8] Therefore, for fresh or frozen material, three reactions may suffice to detect all five fusions involving *EWS* and ETS transcription factor family genes. Additional reactions would be necessary to detect the fusions involving *FUS* and ETS family genes.

These considerations change when the tumor is received as an FFPE specimen in which the RNA is likely to be less intact because of hydrolytic events and crosslinking. In this case, RT-PCR assays are needed that amplify a small segment, and thus the full range of fusion product sizes cannot be targeted by a single reaction. Instead, an assay must be specifically designed for the most common fusion products such as the type 1 (*EWS* exon 7-FLI1 exon 6) and type 2 (*EWS* exon 7-*FLI1* exon 5) *EWS-FLI1* fusions, and other assays must be considered to assess fusions larger or smaller than the type 1 and type 2 categories. As the goal of many laboratories is to develop one platform for both fresh/frozen and FFPE samples, these considerations will even affect the use of assays on fresh/frozen specimens in the coming years. Therefore, RT-PCR assays may not be able to test readily for all EFT-associated gene fusions in a clinical setting, and basic decisions must then be made to test for the most frequent fusions and accept the potential false-negative results.

An alternative molecular diagnostic approach is the fluorescent *in situ* hybridization (FISH) assay, which can be applied to fresh, frozen, or FFPE specimens. Although FISH assays can detect a gene fusion with differentially labeled probes from two chromosomal loci, such as *EWS* and *FLI1*,[9] a more useful assay detects the splitting of a single locus, such as *EWS*.[10,11] Differentially labeled "break-apart" probes flanking the two sides of the *EWS* breakpoint region determine when a rearrangement event involving *EWS* has occurred. In this way, an EFT case will score "positive" regardless of the participating ETS family gene or the size of the fusion product. Furthermore, the splitting assay has fewer false positives than the fusion assay, in which there can be a superimposed appearance of signals in different planes when viewed in two dimensions. Two large FISH studies of FFPE cases confirmed the utility of these assays and detected *EWS* rearrangements in 83 and 91% of histologically defined EFT cases.[10,11] If desired, as shown in the study by Ng and colleagues,[4] a comparable splitting assay can be used to evaluate involvement of the *FUS* locus in the small subset of EFT cases with negative results in the *EWS* splitting assay.

A potential problem with the FISH *EWS* splitting assay, however, is that it cannot provide definitive proof of an EFT-associated gene fusion because the assay only clearly indicates that *EWS* is rearranged, and this gene is involved in multiple other sarcoma-associated gene fusion categories. In addition, the data reviewed by Wang et al[2] emphasize that a subset of EFT-like tumors also have fusions involving *EWS* and a non-ETS family gene, and although the number of these cases is small, the involvement of a different transcription factor suggests that the biology of these EFT-like tumors will be different from the classic EFT. Therefore, a follow-up assay with either a FISH fusion assay or RT-PCR assays is useful to determine definitively the composition of the gene fusion. Furthermore, if the size of the fusion is prognostically useful, as suggested by several retrospective studies,[12] these data are currently best obtained via RT-PCR-based assays.

If the FISH probe detects all fusions involving the *EWS* gene, an explanation is needed for why the reported FISH-positive rates for the *EWS* break-apart probe are not higher than the RT-PCR-positive rates for *EWS-FLI1* and *EWS-ERG* assays.[5,7,10,11] One possible explanation is that the two FISH studies were performed on FFPE tissues, and there may have been technical issues affecting the performance of these assays on less than optimal tissue samples. However, 9 to 17% of these cases were scored as negative on FISH and not unsatisfactory, indicating that intact *EWS* loci were present in these cells. Of note, in several of the cases called negative by FISH, the corresponding RT-PCR assay was positive.[10,11] In these studies, the RT-PCR assays used either a nested procedure or up to 40 cycles of amplification. In one of the large RT-PCR studies of EFT cases, the single and nested RT-PCR procedures were compared.[5] After a single round of RT-PCR, 54 of 64 cases (84%) were positive

for the *EWS-FLI1* or *EWS-ERG* fusion, and after a nested procedure, an additional four cases were positive for a total of 58 of 64 positive cases (91%). The question arises as to what is the nature of these low-expression fusion-positive EFT cases that require this additional level of sensitivity.

Although there is no clear answer in the EFT literature on low expression fusion-positive cases, there is information available from studies of alveolar rhabdomyosarcoma (ARMS). In particular, high-sensitivity RT-PCR assays identified low-level expression of *PAX3-FKHR* and *PAX7-FKHR* gene fusions in a small subset of ARMS cases.[13] Examination of these ARMS cases by FISH with an *FKHR* break-apart probe found only intact *FKHR* loci (no evidence of a gene fusion), indicating that the low-level expression is most likely the result of rare fusion-positive cells in the tumor. In other studies, low-expressing cases were identified by quantitative RT-PCR assays and shown by microarray studies to have genome-wide expression profiles that were strikingly different from the rest of the fusion-positive ARMS cases and more similar to the fusion-negative embryonal rhabdomyosarcoma cases.[14] Therefore, low expression of the gene fusion is of questionable biological significance in ARMS, and caution must be exercised when using high-sensitivity assays to amplify fusion transcripts from sarcomas.

Clinical Challenges

The final issues to be addressed are the clinical applications of this test for EFT-associated gene fusions. There are several situations in which this molecular test is requested. In some instances, a pathologist orders this test for a genuinely challenging case that presents as an undifferentiated sarcoma without the classic histological and immunohistochemistry pattern of an EFT. In other instances, this test is ordered in the setting of a case that presents with a more classic histological and immunohistochemical pattern of an EFT. For a pathologist experienced in pediatric sarcomas, this test will help confirm the diagnosis, and for pathologists less experienced with these lesions, this test thereby helps to establish the diagnosis.

An important consideration is the practical consequences of a negative or positive molecular test for these EFT-associated gene fusions. In the usual situation, a sample is analyzed for the *EWS-FLI1* and *EWS-ERG* gene fusions by RT-PCR or the *EWS*-associated fusions by FISH. If the result is positive, the diagnosis is confirmed, and the ordering pathologist is at least temporarily satisfied with the capabilities of molecular pathology. However, if the test is negative, different reasoning must ensue. If the initial histological pattern and immunohistochemistry are not strongly consistent with a histopathological diagnosis of EFT, a negative result casts doubt on a diagnosis of EFT, and alternative diagnoses should be considered. In contrast, if the histological pattern and immunohistochemistry are consistent with a diagnosis of EFT, then the available data suggest that this case may be part of the EFT subset with rare gene fusions. The question then arises as to whether to pursue further fusion testing. Although the overall goal is to get the most specific diagnosis for every patient, that goal must be balanced with the realization that resources are finite.

If further testing for rare variants is pursued, an interesting problem is the interpretation of a case with the histopathological appearance of an EFT and negative FISH results for both the *EWS* and *FUS* loci. A scientific study has not yet been conducted to determine the size of this subset of *EWS*- and *FUS*-negative "EFT" cases, and although it is likely to be small, there will no doubt be cases with this description. Here, the question arises as to what defines a case as EFT, histopathology or molecular genetics. Similar to the situation in other sarcomas, the diagnosis of EFT is ultimately based on the histopathological features, but the absence of a detectable fusion may have significant biological ramifications that need to be explored in future studies.

An understanding of the impact of these diagnostic decisions on treatment is an important consideration in using these molecular assays. There are no known specific guidelines for treating an undifferentiated sarcoma of the bone, and generally, such a lesion would receive the same treatment as an EFT. In contrast, for a soft tissue lesion, the distinction between EFT and undifferentiated sarcoma may be more significant. The Children's Oncology Group has different protocols, with very different treatments for non-rhabdomyosarcoma soft tissue sarcomas and EFT (including cases arising in soft tissue sites). As part of the non-rhabdomyosarcoma soft tissue sarcoma protocol (*http://www.clinicaltrials.gov/ct/show/NCT00346164?order = 1*; accessed July 13, 2007), undifferentiated sarcomas that are unresectable, metastatic, or more than 5 cm in diameter are treated with two-drug chemotherapy; resectable tumors less than 5 cm are not treated with chemotherapy at all. In contrast, all EFT cases are treated with five-drug chemotherapy (*http://www.clinicaltrials.gov/ct/show/NCT00334867?order = 9*; accessed July 13, 2007). In this case, the histology and immunohistochemistry data become significant factors in the determination of a final diagnosis and treatment, and the decision to perform additional molecular testing may thus weigh more heavily.

Finally, the possibility of prognostic differences associated with rare gene fusions should be examined. For the fusions that involve novel gene families and are found in EFT-like tumors, the biology of these novel transcription factors and the corresponding tumors is an entirely open question, and significant differences relative to *EWS-FLI1*-positive EFT are expected. However, the fact that there are only one or two cases with each novel fusion will limit definition of detailed clinical differences. For the variant fusions of EWS or *FUS* to ETS family members, the resulting transcription factors will more closely resemble the prototypical *EWS-FLI1* fusion, and thus fewer biological and clinical differences are expected. For example, there were no clinical differences found between *EWS-ERG*-positive and *EWS-FLI1*-positive EFTs.[15] Although the rarer fusions have greater structural differences relative to *EWS-FLI1*, the small number of cases in any cat-

egory will again complicate analysis. Combination of all of the rare variants into a single subset may be one way to deal with this numbers problem. As noted in the article by Wang et al,[2] this subset is enriched in extraosseous cases, and thus differences in outcome between osseous and extraosseous EFT will be a relevant issue. This question has been addressed to date in only small studies, and the conclusions differ with some studies indicating a comparable outcome for the two EFT subsets and other studies indicating that extraosseous EFT has a poorer outcome.[16,17]

Conclusions

In summary, since the *EWS-FLI1* fusion was first described in 1992,[18] EFT and now EFT-like tumors continue to amaze molecular pathologists by the complexity of rearrangements that occur in this set of cancers. Clearly, this cornucopia of molecular alterations provides fascinating glimpses into the complex biology of these tumors and the fundamental steps in etiology. In addition, these data have provided remarkable opportunities for improving the ways that these tumors are diagnosed. However, the growing complexity has indicated that the diagnostic rules will not be absolute and decisions need to be made how best to assay for a subset of these gene fusions in molecular diagnostic situations. Furthermore, these decisions will not be static but will change over time as additional data regarding these fusions are discovered, as well as newer technology becomes available for detecting these fusions. In this way, the molecular pathology community will learn and grow as the field evolves and progresses.

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