Implications of Molecular Advances for Diagnostic Pediatric Oncological Pathology

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Abstract: An increasing number of pediatric malignant soft tissue tumors are associated with diagnostic and characteristic molecular genetic alterations, which may be detectable routinely on histological samples using established laboratory techniques such as PCR and FISH. This change in approach to laboratory diagnosis has led to changes in methods of clinical investigation, with increasing use of small image guided needle biopsies, which require modification of laboratory handling to optimize use of such limited tissue samples. Future developments in technological aspects of molecular investigation, such a expression profiling and proteomic approaches are likely to lead to even greater reliance of tissue samples, not only for provision of diagnosis, but for determination of prognostic and therapeutic information.

Keywords: Needle biopsy, molecular, PCR, FISH, tumor, malignancy.

INTRODUCTION

There have been significant changes in laboratory medicine over recent decades with regard to the approach and investigation of diagnostic tissue samples. This has been particularly the case for pediatric tumor specimens, since many pediatric neoplasms represent distinct biological entities, most of which are types of embryonal sarcomas, as opposed to the much more common carcinomas encountered in adult practice. Furthermore, a large number of these tumors are associated with specific molecular genetic alterations, which in the majority of cases are important mechanisms in the underlying pathogenesis of the disease. In addition, many pediatric neoplasms recapitulate varying stages of embryological tissue appearances, and therefore may have minimal differentiating morphological characteristics on small biopsies; a common phenotype being that of the category of 'small round blue cell tumors'. The recognition of these changes in the laboratory approach to tumor specimens has however, resulted in alterations in the strategy of clinical investigation of these diseases in relation to the approach to tissue biopsy, and is likely to change significantly in future years. In general, the histomorphological diagnosis of many pediatric tumors has gradually become superseded by the findings of immunophenotyping based on immunohistochemical staining of tissue sections, and in the last decade, a range of molecular diagnostic investigations have become of primary importance. This article will discuss the implications of these changes on biopsy techniques and tissue handling for pediatric pathologists, discuss some specific molecular investigations and provide examples of pediatric tumors in which the diagnostic approach has changed based on the improved understanding of the underlying cellular and molecular mechanisms of disease.

TISSUE SAMPLING APPROACH TO PEDIATRIC TUMORS

The exact approach to obtaining tissue for diagnosis in pediatric tumors depends on a range of factors, including the anatomical site, patient age and specific protocols followed according to individual institution policies. Nevertheless, whilst open (surgical) biopsies can continue to provide diagnostic material, with improvements in diagnostic capability from increasingly small tissue samples, the role of initial minimally invasive needle core biopsy diagnosis has become of greater importance. Image-guided 'tru-cut' type needle core biopsies provide much more limited tissue compared to the traditional open approach, but are associated with reduced morbidity and, with the application of newer laboratory techniques, can provide adequate diagnostic material in most cases. In a review of 13 studies examining the adequacy of needle core biopsies for tissue diagnosis of pediatric tumors it was demonstrated that around 95% of cases provided adequate tissue for diagnosis and in a similar proportion, a definite diagnosis could be made from the material [1]. Potential theoretical disadvantages of this approach are that the material obtained may be unrepresentative of a large tumor and that there may be insufficient material to perform formal morphological studies or molecular analysis. The issue of representativeness is only of importance for those tumors which are either heterogeneous or in whom no diagnostic immunohistochemical or molecular markers are available; in practice, this is a relatively small issue for the majority of pediatric tumors. However, it should be recognised that needle core biopsies must be interpreted with caution in the settings of highly heterogeneous tumors, for example Wilms tumor, and particular care must be taken in the interpretation of biopsies

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from organs which may be only focally involved by disease. For example, focal nodal involvement by Hodgkins disease may be missed by a needle biopsy compared to an open lymph node biopsy. It is likely that, in future, the issue of representativeness will become of less importance with the introduction of newer imaging methods of targeting specific biopsy sites. Promising techniques to identify areas of viable tumor, as opposed to areas of necrosis or posttherapy change, include the assessment of apparent diffusion coefficient (ADC) for assessment of tumor cellularity using magnetic resonance imaging [2], and the use of a PET (positron emission tomography), to identify foci of viable tumor most likely to provide a diagnostic biopsy [3, 4]. The argument that needle biopsy is maybe too small for formal morphological studies is also theoretically correct but in practice is of little importance for the increasing number of pediatric tumors in whom diagnostic immunohistochemical and/or molecular investigations are available. Furthermore, with the increasing number of molecular investigations being performed, the diagnostic and prognostic role of previously described morphological features is becoming of relatively less importance [5]. Finally, it should be recognized that many of diagnostic molecular investigations can now be performed with minimal tissue, such that needle core biopsies can provide adequate material for the full range of diagnostic investigations in most cases. In our centre, adequate material for molecular studies is obtained in >95% of needle core biopsies. Fluorescence *in situ* hybridization (FISH) requires only tumor imprints on glass slides, a negligible amount of material, whilst even RT-PCR (reverse transcriptase polymerase chain reaction) requires only a tiny fragment of tumor.

BIOPSY HANDLING OF PEDIATRIC TUMOR SAMPLES

It will be apparent that appropriate laboratory handling of these small specimens, whether needle biopsies or traditional open biopsies, is of prime importance. Specimens should ideally be received fresh in the laboratory immediately following the biopsy procedure, at which time the sample can be divided into aliquots for FISH and molecular studies such as RT-PCR, (either snap-frozen material or placed in RNA-protection medium such as 'RNA-later' (Ambion, Applied Biosystems), cytogenetic studies, fixed in glutaraldehyde for electron microscopy if required, and the remainder processed for routine fixation, paraffin embedding and sectioning for morphological examination and immunohistochemical staining. For these reasons, pediatric tumors should optimally be handled in specialist centres with laboratories dedicated to processing these types of specimen.

PRACTICAL ISSUES WITH MOLECULAR DIAGNOSTIC TECHNIQUES

Many pediatric soft tissue tumors are associated with specific gene fusion transcripts which are detectable using molecular techniques [6] (Table **1** and Fig. **1**). A potentially wide range of laboratory investigations may be applied, but in practice, whilst many of gene translocations were originally identified using classical cytogenetic techniques, this has been superseded by other methods, the two most commonly used being RT-PCR and FISH. PCR essentially involves mixing oligonucleotide primers with template DNA in a reaction using a thermal cycler and DNA polymerase, resulting in massive and highly specific amplification of the DNA fragment targeted based on the primer sets used. The specific PCR products are then be detected and identified using techniques such as gel electrophoresis, restriction fragment length polymorphisms (RFLPs), other hybridisation techniques, such as Southern blotting, or sequence analysis, with all reactions performed in the presence of appropriate positive and negative controls. The advantages of the PCR method are that the technique is sensitive and specific, and can be performed rapidly. In addition, only tiny amounts of starting nucleic acid are required and the technique does not require fully intact nucleic acids; it can therefore be applied to both fresh and paraffin-embedded tissue, (although modifications are required for optimization for use with formalin-fixed paraffin embedded (FFPE) material). However, since the technique is so sensitive it is also easily contaminated and good laboratory technique with appropriate controls must be ensured for correct interpretation. Furthermore, the main disadvantage is that known target primers for the region of interest must be used, such that the presence of a negative result, with appropriate laboratory safeguards, may exclude the presence of a specific translocation but does not then provide positive criteria regarding another diagnosis. In addition, due to the potential marked variability of the breakpoint position for a given translocation, all of which result in a common spliced fusion transcript, assessment of tumor RNA is required in most cases rather than DNA. Since RNA in tissue is much more sensitive to degradation, tissue must be optimized for RT-PCR, by being processed as soon as possible following the biopsy or by undergoing immediate snap-freezing or being placed in RNA protection medium. RT-PCR may still be feasible in FFPE material, although detectable fragment sizes will be shorter and the success rate for a diagnostic test is lower than for fresh or snap-frozen material. Previous studies have reported that adequate RNA can be obtained in up to 95% of routine diagnostic FFPE blocks [7].

FISH involves denaturation and hybridisation of target DNA with fluorescent labelled probes of a known and specific sequences, followed by a detection step. FISH probes can be used for detection of translocations using either breakpoint flanking probes, which will be moved apart if translocation has occurred, or fusion probes, which will be brought together with a specific translocation. FISH is also useful as a rapid and reliable technique for detection of amplification of regions of DNA, for example MYCN amplification in neuroblastoma, and also using specific probes for detection of deletions or gains, such as 1p loss or 17q gain in neuroblastoma [8, 9]. FISH is performed on imprints and fresh and FFPE tissue, with modifications. The main disadvantage of FISH is related to technical issues with both performance and appropriate interpretation. For these reasons, translocation breakapart probes appear most reliable [10]. Also, since FISH using breakapart probes flanking, for example, the Ewing's sarcoma (EWS) gene, can potentially detect all EWS-gene-related translocations, the range of translocations detectable is greater than with a specific PCR, for which the exact translocation partner site must also be

Table 1. Documenting the More Common Characteristic Molecular Genetic Alterations Associated with Paediatric Tumours and Applicable to Diagnostic Practice. (Modified from Yu & Parham 2009[33])

Tumour	Translocation	Molecular Feature
PNET/Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) + others	EWS-FLI-1 EWS-ERG +others
Infantile fibrosarcoma	t(12;15)(p13;q25)	ETV6-NTRK3
Alveolar rhabdomyosarcoma	t(1;13)(p36;q14) t(2;13)(q25;q14)	PAX7-FKHR (FOXO1) PAX3-FKHR (FOXO1)
Desmoplastic small round cell tumour	t(11;22)(p13;q12)	EWS-WT1
Alveolar soft part sarcoma	t(X;17)(p11;q25)	ASPL-TFE3
Synovial sarcoma	t(X;18)(p11.2;q11.2)	SYT-SSX1/2/4
Dermatofibrosarcoma protuberans	t(17;22)(q21;q13)	COL1A1-PDGFB
Clear cell sarcoma of soft tissue	t(12;22)(q13;q12)	EWS-ATF1
Myxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	FUS-CHOP EWS-CHOP
Low grade fibromyxoid sarcoma	t(7;16)(q33;p11) t(11;16)(p11;p11)	FUS-CREB3L2 FUS-CREB3L1

known in advance. However, since breakapart FISH does not detect the translocation partner, whilst an EWS translocation is reliably detected, the exact fusion product may not be known. For these reasons, in practice, FISH and PCR are used as complimentary techniques and their application is exemplified by the planned mainstream incorporation of these techniques into new clinical trials such as the European soft tissue sarcoma group study, which will require molecular diagnostic confirmation of alveolar rhabdomyosarcoma (ARMS; [11]).

ISSUES WITH INTERPRETATION OF MOLECULAR FINDINGS

Increasing experience of these techniques has demonstrated that given molecular findings may not be entirely specific for the initially described tumor type, leading some to question the role of molecular diagnostics. For example, alveolar soft part sarcoma (ASPS) and Xp1-related renal cell carcinoma are both associated with similar TFE3 gene fusion products but are clinically and morphologically entirely different entities [12-15]. Similarly, it is recognised that the t(12;15) ETV6-NTRK3 translocation is associated with cellular mesoblastic nephroma, infantile fibrosarcoma and also secretory breast carcinoma [16-19]. However, in all of these settings, the morphological and clinical characteristics are quite different and the findings are unlikely to mislead the appropriate differential diagnosis.

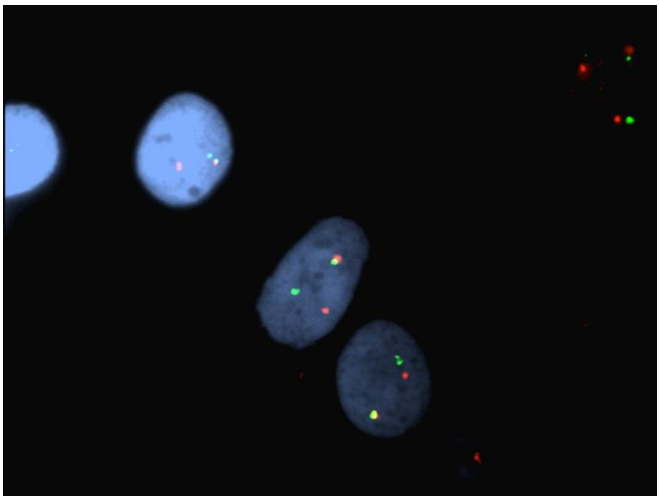

Fig. (1). FISH with a dual colour breakapart probe in a case of PNET demonstrating disruption of the EWS gene with split red and green signals.

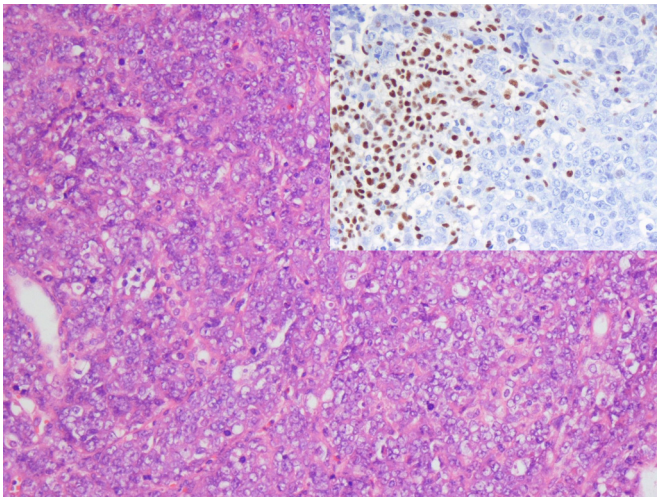

Fig. (2). Photomicrograph of a malignant rhabdoid tumour demonstrating diagnostic absent nuclear expression of INI1 on immunostaining (inset).

Conversely, there is evidence that molecular features of a tumor may be more important than the morphologic phenotype for determination of prognosis and response to therapy as well as providing the diagnosis. For example, congenital and infantile tumors with an ETV6-NTRK3 gene fusion present, are identified as a group which are highly responsive to chemotherapy, even those cases in which the morphological features are not classical for infantile fibrosarcoma [20]. Other examples, include the diagnostic presence of the SSX-SSY translocation in cases of monophasic synovial sarcoma, in which morphological and immunophenotypic findings may be nondiagnostic [21]. Further examples include the expanding spectrum of primitive neuroectodermal tumors (PNET) following the recognition of specific EWS-related translocations, including epithelioid and spindle cell subtypes [22-25]. Furthermore, it has been demonstrated in laboratory models that induced expression of PAX3-FKHR can transform the behaviour of embryonal rhabdomyosarcoma to a more aggressive clinical

type consistent with ARMS, without causing a shift in morphology to that of classical alveolar phenotype [26].

However, perhaps most importantly for future developments of molecular tumor diagnostics, it is progressively reported that molecular findings may provide additional prognostic and/or therapeutic information, in addition to the diagnosis. In a multivariate analysis of overall survival of patients with PNET, the specific EWS-fusion transcript type is associated with differences in clinical outcome, type 1 transcripts having a better overall survival [27, 28]. Similarly, in patients with fusion-positive ARMS, presence of the PAX3-FKHR transcript is associated with significantly worse prognosis in those with metastatic disease compared to those with PAX7-FKHR [29].

CURRENT STATE AND FUTURE DIRECTIONS

In addition to the increased application of molecular studies for determination of diagnosis and prognosis in primary tumor samples, as described above, future applications of molecular techniques such as PCR are likely to include detection of minimal residual disease (MRD), particularly in bone marrow specimens. It has been estimated that PCR can detect one tumor cell in 10^5 normal cells and in one study using PCR for MRD detection in patients with ARMS, reported that PCR evidence of MRD was associated with a significantly worse prognosis and preceded clinical relapse [30]. Finally, the area which is likely to have the largest overall long-term impact in the practice of diagnostic pediatric pathology is the widespread introduction of testing based on gene expression profiling and microarray techniques [31]. The application of this methodology may identify specific gene signature patterns which could provide both diagnostic and therapeutic information for an individual tumor, in addition to the determination of further prognostic data being available from the initial diagnostic biopsy. For example, characteristic molecular signatures predicting subsequent metastatic disease, present in primary solid tumors, have been identified in a range of adult cancer [32].

A hypothetical example demonstrating how molecular techniques have changed, and will continue to influence, the diagnostic pathology approach to pediatric tumor specimens would be a child presenting with a soft tissue mass undergoing image guided needle core biopsy. Based on morphological features, positive nuclear staining for myogenin and RT-PCR demonstrating the presence of PAX3-FKHR transcript, the diagnosis of ARMS can be made with certainty in this limited amount of tissue. Knowledge of the precise transcript present influences prognosis, and in the near future it is highly likely that with the introduction of high-throughput profiling-based analysis, the likelihood of subsequent metastatic disease and even response to specific chemotherapeutic agents may be predicted.

Examples of specific pediatric tumors in which molecular testing is either an integral part of diagnostic investigation, or in which molecular findings have led to development of immunohistochemical based diagnostic techniques include the following:

Neuroblastoma, in which prognostic stratification, and hence treatment strategy, is based heavily on biological features of the primary tumor such as MYCN amplification, 1p deletion, 17q gain, and an expanding list of other biological markers. Such assessment of tumor biology is now an integral part of their initial investigation.

Malignant Rhabdoid tumor, in which the diagnosis historically was extremely difficult, being a diagnosis of exclusion. The recognition of INI1 abnormalities in these tumors led to the development of highly reliable and rapid INI1 immunohistochemical staining, which now provides a rapid and specific diagnosis even on small tissue samples (Fig. **2**).

Rhabdomyosarcoma, in which it has been recognised for many years that distinction between alveolar and embryonal subtypes is of major importance for stratifying prognosis and treatment, but in which classical alveolar architecture may not always be present. Definite diagnosis of ARMS is now reliably achieved using detection of specific PAX-FKHR fusion transcripts.

PNET, in which the morphological phenotype may be that of a non-specific embryonal small round blue cell tumor but in which the combination of characteristic immunohistochemical staining for membranous CD99 and presence of EWS gene fusion transcripts now allows specific diagnosis, with specific transcript product detection providing further prognostic information.

CONCLUSION

An increasing number of pediatric malignant soft tissue tumors are recognized as being associated with diagnostic and characteristic molecular genetic alterations, which may be detectable routinely on histological samples using established laboratory techniques such as PCR and FISH. This change in approach to laboratory diagnosis has led to changes in methods of clinical investigation, with increasing use of image guided needle biopsies for primary diagnosis, which require modification of laboratory handling to optimize use of such limited tissue samples. Future developments in technological aspects of molecular investigation are likely to lead to even greater reliance of tissue samples, not only for provision of diagnosis, but for determination of prognostic and therapeutic information.

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