Myogenin and MyoD1 expression in paediatric rhabdomyosarcomas

N J Sebire, M Malone

The diagnosis of paediatric solid tumours is often based on small tissue needle biopsies in which many different entities demonstrate a “small round cell tumour” phenotype and in which there may be insufficient tissue to allow the interpretation of diagnostic architectural features, which may be present in larger specimens. Therefore, the extensive use of a panel of immunohistochemical markers is part of the routine handling and investigation of such biopsies to reach a definite diagnosis. However, in some cases the morphological and routine immunohistochemical findings may be insufficient for a precise diagnosis or they may be difficult to interpret in the given clinical context. Although many paediatric tumours exhibit characteristic chromosomal translocations with resultant specific fusion transcripts, these require molecular methods for their detection, usually on fresh tissue samples, which may not always be available. As more immunohistochemical markers become available, more precise diagnosis on such small biopsies may be possible. This review examines the use of the immunohistochemical markers, MyoD1 and myogenin, in the diagnosis of paediatric rhabdomyosarcoma, including its subtypes.

Rhabdomyosarcoma (RMS) is the most common paediatric solid tumour.¹ The diagnosis is almost always on the basis of a small tissue biopsy supplemented by immunohistochemical confirmation for the definitive diagnosis. Although many historical classification schemes have been reported,² the most widely used is the modified World Health Organisation classification,³ which describes two main morphological subtypes, embryonal RMS (ERMS) and alveolar RMS (ARMS). The third major type, pleomorphic or anaplastic rhabdomyosarcoma, is essentially a tumour that affects adults; in children, ERMS cases may demonstrate pronounced cellular pleomorphism but their classification remains ERMS. The diagnosis of RMS and its subtypes is important because ARMS is reported to have a worse prognosis, with a greater frequency of disseminated metastases.⁴ Overall, multimodal treatment has increased survival in RMS from 25% in 1970 to more than 70% in recent studies, with particular improvements in those patients with residual tumour after the initial treatment.⁵

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The diagnosis of most paediatric solid tumours requires an extensive panel of immunohistochemical markers because many entities exhibit a non-characteristic “small round cell tumour” phenotype. The main differential diagnoses in this age group are lymphoma, neuroblastoma, primitive neuroectodermal tumour, and RMS, most of which can be readily distinguished on the basis of their simple immunohistochemical profile. In some cases, however, the material submitted and/or the immunohistochemical profile may not be diagnostic, and further investigations are required. This review examines the use of the immunohistochemical markers MyoD1 and myogenin in the diagnosis of paediatric RMS.

**SUBTYPES OF PAEDIATRIC RHABDOMYOSARCOMA**

RMS may be classified as embryonal, including the pathologically distinct botryoid subtype, spindle cell, and alveolar, including the solid variant.¹ Most adult cases are of the pleomorphic type, occurring in skeletal muscles, whereas in the paediatric age group, RMS mainly occurs in the head and neck region (especially periorbital), the urogenital tract, the biliary tract, and the trunk or limb. The classification of RMS was initially based purely on the morphological and cytological appearances of the tumour. ERMS refers to a tumour composed of primitive mesenchymal cells with varying stages of morphological skeletal muscle differentiation (strap cells, striated cytoplasm, etc) in a loose, myxoid, or cellular collagenous stroma. There may be nuclear pleomorphism but nucleoli are inapparent. The botryoid subtype simply refers to an ERMS in a subepithelial location, which exhibits a layer of densely cellular neoplastic cells just beneath the epithelium (cambium layer). ARMS classically demonstrates an architectural pattern with thin fibrous septae lined by tumour cells, some of

Abbreviations: ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; RMS, rhabdomyosarcoma; RT-PCR, reverse transcriptase polymerase chain reaction.
which are centrally dissociated or discohesive. However, it has become apparent that this architectural pattern may not always be present (solid variant ARMS) and the morphological
diagnosis of ARMS therefore relies on the cytological char-
acteristics of round tumour cells with hyperchromatic nuclei,
coarse chromatin, and prominent nucleoli. Cytological fea-
tures of skeletal muscle differentiation may be absent,
particularly in small biopsy specimens.

The distinction of ARMS from ERMS is important because
ARMS carries a worse prognosis and requires a modified
therapeutic regimen. Definitive diagnosis of ARMS may
require molecular and/or cytogenetic investigation because
most cases exhibit characteristic translocations enabling sub-
type diagnosis even on the basis of small tissue samples. Two
characteristic gene fusion products have been described in
ARMS: the fusion of PAX3 to FKHR 10 and PAX7 to FKHR, 11
corresponding to the t(2;13) 10 and t(1;13) translocations, respectively. 12–21

MOLECULAR DIAGNOSIS OF ALVEOLAR RHABDOMYOSARCOMA

Because most cases of ARMS express the PAX3–FKHR or
PAX7–FKHR gene fusions, resulting from the t(2;13) or
t(1;13) translocations, respectively, molecular methods have
been increasingly used in their diagnosis, both on fresh and
paraffin wax embedded tissue. In a study of 171 childhood
cases of RMS, including 78 cases of ARMS, the reverse
transcriptase polymerase chain reaction (RT-PCR) was able to
identify either the PAX3–FKHR or the PAX7–FKHR fusion
transcripts in 55% and 22% of patients with ARMS,
respectively. Importantly, no case of ERMS expressed either
transcript. 22 In a further study of 91 cases of primary RMS, the
PAX3–FKHR or PAX7–FKHR translocations were present in
more than 80% of ARMS cases, with PAX3–FKHR expression
appearing to be an adverse prognostic factor. 23 Several other
studies have confirmed the high specificity for the diagnosis
of ARMS because no cases of ERMS exhibit these fusion
products. 24–26 Furthermore, molecular and morphological
review of RMS cases suggests that, in some cases, the
detection of translocations may clarify the histopathological
diagnosis in cases where morphological features are
equivocal. 27 Although such cytogenetic abnormalities are
often detected, in some RMS cases the PAX3–FKHR or
PAX7–FKHR fusion gene products can be detected by molecu-
lar methods, but the translocation cannot be identified
cytogenetically, 28,29 and rarely other variant translocations may
be present. Nevertheless, in some ARMS cases these gene
fusion products cannot be detected, for example congenital
ARMS. 30

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Patients with ERMS do have characteristic translocations,
hyperl diploid or hypertetraploid karyotypes have been
reported, 31 and many ERMS cases show breakpoints at the
1p11–q11 region or loss of heterozygosity at 11p15. 32 Although
fusion products are not present, ERMS cells have been
reported to express increased amounts of wild-type PAX3 or
PAX7 compared with non-RMS myoblasts. 33

IMPLICATIONS FOR PROGNOSIS

In patients with localised ARMS, the presence or absence of
the PAX3–FKHR or PAX7–FKHR fusion products does not
appear to be associated with differences in outcome. However,
in patients with metastatic ARMS, survival is significantly
worse for those expressing the PAX3–FKHR translocation. 22 23

MOLECULAR MECHANISM OF ARMS FUSION
PRODUCT FUNCTION

The t(2;13)(q35;q14) and t(1;13)(p36;q14) translocations
rearrange PAX3 and PAX7, which are members of the paired
box transcription factor family. These are juxtaposed with
FKHR, a member of the fork head transcription factor family.
The fusion genes thus produced encode chimaeric proteins
containing the PAX3/PAX7 DNA binding and the FKHR
transcriptional activation domains, allowing transcriptional acti-
vation with a higher potency than the wild-type PAX
proteins. 10 11 In addition to their altered functional behaviour,
such fusion products are also overexpressed in ARMS because
of the amplification of PAX7–FKHR and an increase in the
transcription rate of PAX3–FKHR. 34,35 This aberrant gene
expression presumably contributes to the malignant behav-
our of ARMS by affecting cellular growth, apoptosis, and
differentiation. 36–38 The PAX3–FKHR and PAX7–FKHR translo-
cations differ with regard to the presence of reciprocal
translocation products and amplification, further suggesting
differences between the mechanisms of these translocation
events. 39

RELATION BETWEEN PAX AND MORPHOLOGY

Although PAX3–FKHR is only found in ARMS, RMS cell lines
transfected with the PAX3–FKHR translocation and grown as
tumour xenografts in immunodeficient mice show faster
growth, more invasion, and have a more pleomorphic appear-
ance, although the characteristic alveolar architecture is not
apparent. 39 Furthermore, the proportion of tumour cells stain-
ing with Ki67 or in terminal deoxynucleotidyl transferase
mediated dUTP nick end labelling based assays is greater in
tumours expressing PAX3–FKHR. 40 Clearly, such fusion gene
products have important effects on biological behaviour, but
may be insufficient in isolation to transform cells to an ARMS
phenotype.

MYOGENIC REGULATORY PROTEINS: MYOD1 AND
MYOGENIN

The myogenic nuclear regulatory proteins are a group of DNA
binding proteins, which act as transcription factors and
stimulate myogenesis. Transfection into multipotential meso-
dermal cells stimulates myogenic differentiation, 41–45 and a
variety of differentiated cell types can be converted to skeletal
muscle after transfection with MyoD1. 46 MyoD induces differ-
tiation by activating muscle specific genes and is important
in the switch from cellular proliferation to differentiation. Loss
of this normal control could theoretically lead to the
formation of RMS tumours, which have lost control of cell
proliferation. 47 Northern blot analyses demonstrate expression
of both MyoD1 and myogenin in RMS cell lines, which act as
lineage markers and differentiation markers, respectively. 48
Fetal myoblasts express both MyoD and myogenin in culture,
whereas adult myoblasts are negative. 49 That these genes are
important for normal skeletal muscle differentiation can be
demonstrated by reports that myogenin knockout mice show
severe skeletal muscle defects, differing in different regions,
despite expressing normal amounts of MyoD, with committed
cells unable to form muscle sheets without the presence of
myogenin. Therefore, myogenin and MyoD appear to have dif-
ferent roles in myogenesis rather than there simply being dif-
ferences in expression. 50 51

“MyoD induces differentiation by activating muscle
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Traditionally, immunohistochemistry to detect myoid dif-
ferentiation has been based around expression of the intermed-
iate filament desmin, the contractile protein actin, or
the oxygen transport molecule, myoglobin. In one early study, histological examination of 65 RMS samples reported that cross striations were seen on light microscopy in about 25–30% of cases; however, immunohistochemical staining for myoglobin was present in only 30% of ERMS and 70% of ARMS cases. Overall, 64% of ERMS and 80% of ARMS cases showed either positive immunostaining or ultrastructural features of skeletal muscle differentiation. These traditional immunomarkers require considerable differentiation along the myogenic pathway before cellular expression occurs. In contrast, the analysis of the expression of the myogenic nuclear regulatory proteins, MyoD1 and myogenin, should allow the identification of primitive tumours that are relatively undifferentiated. However, because they are markers of skeletal muscle differentiation rather than RMS, these molecules may also be expressed in many other tumours demonstrating skeletal muscle differentiation, such as rhabdomyosarcomas. Nuclear expression of MyoD1 and myogenin in most cases of RMS is localised to the nuclei (cytoplasmic staining may occur). MyoD1 immunostaining is positive in almost all RMS cases, uses an epitope near the C-terminus of the MyoD1 protein. Myogenin is stronger than for MyoD1 in cases with differentiated tumour cells, but was less prominent in cases in which myogenin staining was present in occasional desmoid, infantile myofibromatosis, and infantile fibrosarcoma specimens, but this may have represented entrapment, regenerating non-neoplastic skeletal muscle.

Figure 1 Photomicrographs showing embryonal rhabdomyosarcoma (A,B) and alveolar rhabdomyosarcoma (C,D) immunostained with antibodies to MyoD1 (A,C) and myogenin (B,D). Note that both tumours demonstrate nuclear positivity but staining is much more widespread and intense with myogenin in rhabdomyosarcomas of the alveolar subtype (original magnification, ×400).

Note that both tumours demonstrate nuclear positivity but staining is much more widespread and intense with myogenin in rhabdomyosarcomas of the alveolar subtype (original magnification, ×400).

Antigen (epitope) retrieval techniques are required for both antibodies and only true nuclear expression should be considered positive.

**IMMUNOCYTOCHEMICAL DIFFERENTIATION OF EMBRYONAL FROM ALVEOLAR SUBTYPES**

Although essentially all RMS samples show some degree of immunostaining with antibodies to MyoD and myogenin, the expression patterns differ between ERMS and ARMS. In one study examining 68 RMS cases, there was extensive nuclear myogenin staining in most cases of ARMS (expression in > 75% of tumour cells), whereas in ERMS, although all cases showed some tumour cells with positive nuclear expression, immunopositivity was less uniform, and in many cases < 25% of tumour cells were positive. Similarly, Cessna and colleagues examined 32 ARMS cases and reported that there was strong nuclear staining for myogenin, which was most pronounced in the tumour cells lining fibrous septae,
highlighting the alveolar architecture. Again, ERMS showed consistent positivity but with a much more variable and focal staining pattern. In another series, all nine ARMS cases stained strongly positively for myogenin, whereas in the 15 ERMS, staining was weak, patchy, and a larger proportion of tumour cells were negative for myogenin (fig 1).64

Solid variant alveolar rhabdomyosarcoma

In the only small study examining 15 cases of ERMS, classic alveolar ARMS, and solid variant ARMS, sections were immunostained for both myogenin and MyoD1 and semiquantitative scoring of each section was carried out for percentage tumour cells stained and staining intensity. In all cases, the tumour cells stained with both antibodies, but in the ARMS groups staining for myogenin was much stronger and most (>90%) of the tumour cells stained. However, there was no difference in staining extent or intensity between the classic and solid ARMS variants, suggesting that myogenin immunohistochemistry is useful even in patients with atypical morphological findings on needle biopsy.65

RELATION BETWEEN IMMUNOSTAINING AND MOLECULAR ALTERATIONS

Few studies have directly examined the inter-relation between the presence of the fusion gene products and immunostaining patterns. However, in one small series, in six of seven ARMS cases with strong nuclear immunostaining for myogenin the presence of PAX3–FKHR or PAX7–FKHR was demonstrated by RT-PCR. Furthermore, there was one tumour, which was initially diagnosed as ERMS on morphological grounds, but which stained strongly for myogenin and was retrospectively found to be positive for the PAX3–FKHR transcript. In this study, western blotting for myogenin was also carried out and there was good correlation between the extent of immunohistochemical staining and western blot findings. ARMS cases were found to express three times more myogenin than ERMS. It is therefore possible that ERMS results from an early block in myogenesis, before the expression of myogenin, whereas ARMS originates from cells later in the myogenic pathway.66 Further evidence for the inter-relation is provided by a study in which PAX3–FKHR was introduced into cell lines and gene expression changes analysed by means of cDNA microarrays. Expression of the PAX3–FKHR product stimulated myogenic differentiation, including the induction of MyoD and myogenin expression.67

“It is possible that embryonal rhabdomyosarcoma results from an early block in myogenesis, before the expression of myogenin, whereas alveolar rhabdomyosarcoma originates from cells later in the myogenic pathway”

RT-PCR FOR THE DETECTION OF METASTATIC DISEASE

The detection of minimal residual disease or micrometastases in RMS may be difficult. PCR based detection of MyoD1 may be of value in both ARMS and ERMS, in which characteristic fusion products are absent. MyoD1 mRNA may be detected in tissue specimens using RT-PCR. In one series of 35 cases of RMS, the MyoD1 transcript was detected in almost all RMS cases, whereas no expression was found in non-RMS samples.68 However, in a similar study, the detection of MyoD1 mRNA was not specific for RMS, being amplified in some other childhood tumours.69 The detection rate of metastatic disease is significantly higher with RT-PCR than by morphological means. RT-PCR is positive in all patients with morphological evidence of metastatic disease and also in some in whom metastases were identified by RT-PCR alone. Therefore, such methodology may be particularly useful for the detection of minimal bone marrow involvement in children with RMS, although the clinical relevance of such micrometastases remains uncertain.64

SUMMARY

The immunohistochemical staining of paediatric rhabdomyosarcomas with antibodies to MyoD and myogenin provides sensitive and specific diagnostic information. Almost all cases demonstrate nuclear expression of both products, but myogenin immunostaining is usually more clinically useful because it is more consistent and is associated with less nonspecific staining. Furthermore, widespread and intense immunostaining for myogenin in RMS is significantly associated with tumours of the alveolar subtype, both alveolar and solid variants. Further studies are required to investigate the precise relation between the immunohistochemical expression of myogenin, the presence of PAX3–FKHR or PAX7–FKHR gene fusion products, and prognosis.

Take home messages

- Rhabdomyosarcoma is the most common malignant soft tissue tumour of childhood
- Rhabdomyosarcoma may be categorised into embryonal (including botryoid), alveolar (including solid variant), and spindle cell subtypes according to morphology and immunohistochemical findings
- Prognosis and treatment are different for the alveolar subtype, which shows characteristic chromosomal translocations and gene fusion products involving chromosome 13 (2:13 PAX3–FKHR and 1:13 PAX7–FKHR)
- On small biopsy specimens, morphology may be difficult to determine and immunohistochemistry plays an important role
- Essentially all paediatric rhabdomyosarcomas show nuclear expression of myogenin and MyoD1 (myogenic nuclear regulatory proteins), whereas almost no other paediatric tumours demonstrate positive immunostaining
- Rhabdomyosarcomas of the alveolar subtype, alveolar or solid variants, demonstrate widespread and strong myogenin expression compared with those of the embryonal subtype

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