

Detection of PAX/FKHR fusion by reverse transcription polymerase chain reaction (RT-PCR): an adjunctive molecular diagnosis in pediatric alveolar rhabdomyosarcoma

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Detection of PAX/FKHR fusion by reverse transcription polymerase chain reaction (RT-PCR): an adjunctive molecular diagnosis in pediatric alveolar rhabdomyo-sarcoma
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Abstract:

Background: *Rhabdomyosarcoma (RMS), an uncommon soft tissue sarcoma, is subdivided according to its histopathology into 2 major subtypes; embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS). In certain circumstances, distinguishing between the two subtypes is difficult. Recently, molecular pathology has discovered a potentially new adjunctive diagnostic tool for ARMS; the detection of fusion transcripts PAX/FKHR.*

Objective: *To study PAX3/FKHR and PAX7/FKHR fusion in RMS, and secondarily to look for any correlation between the fusion gene detection and the histopathological findings.*

Methods: *The sample set consisted of 6 ERMS and 5 ARMS. Detection of the fusion gene used reverse transcription polymerase chain reaction (RT-PCR). Positive results were confirmed by nucleotide sequencing.*

Results: *PAX3/FKHR fusion was detected in 3 of 5 ARMS frozen tissue samples. RT-PCR gave a negative result when formalin-fixed paraffin-embedded tissue was used as a template. None of the ERMS gave positive results. Two ARMS that were fusion-gene negative stained negative for desmin, contained no anaplastic histology, presented no rhabdomyoblast differentiation, but both cases had clear evidence of cystic component and electron microscopic evidence suggesting ARMS. The fusion-negative patients seemed to have a better clinical outcome. All ARMS and ERMS showed negative results of PAX7/FKHR.*

Conclusion: *Laboratory detection of PAX3/FKHR fusion gene in ARMS was successfully performed. The test should be useful in differentiating ARMS from ERMS, especially in cases without typical alveolar cystic portion.*

Key words: PAX3/FKHR, PAX7/FKHR, rhabdomyosarcoma, soft tissue sarcoma

บทคัดย่อ:

ความเป็นมา: มะเร็งกล้ามเนื้อลายในเด็กเป็นมะเร็งเนื้อเยื่ออ่อน แบ่งเป็น 2 ชนิด ตามลักษณะทางจุลพยาธิวิทยา ได้แก่ ชนิด embryonal rhabdomyosarcoma (ERMS) และชนิด alveolar rhabdomyosarcoma (ARMS) ในบางรายการแยกแยะระหว่าง ERMS และ ARMS เป็นเรื่องยาก และเมื่อไม่นานมานี้ มีการนำเทคนิคทางอณูชีววิทยามาช่วยในการวินิจฉัยมะเร็งชนิด ARMS โดยตรวจหายีนเชื่อม PAX/FKHR

วัตถุประสงค์: ศึกษา ยีนเชื่อม PAX3/FKHR และ PAX7/FKHR ในมะเร็งเยื่ออ่อนชนิด RMS และประเมินความสัมพันธ์ระหว่างยีนเชื่อมกับลักษณะทางจุลพยาธิวิทยา

วิธีการศึกษา: Ribonucleic acid (RNA) ที่สกัดจากเนื้อเยื่อมะเร็งชนิด ARMS 5 ราย และชนิด ERMS 6 ราย ซึ่งเก็บในอุณหภูมิต่ำ แล้วทำ Reverse Transcription-Polymerase Chain Reaction (RT-PCR) เพื่อศึกษาการปรากฏของยีนเชื่อม (fusion gene) ผลที่เป็นบวกได้รับการยืนยันด้วยการตรวจลำดับเบสโดยตรง

ผลการศึกษา: ผล RT-PCR ยีนเชื่อม PAX3/FKHR ให้ผลบวก 3/5 ราย ในเนื้อเยื่อ ARMS ที่เก็บในอุณหภูมิต่ำ และให้ผลลบในเนื้อเยื่อที่เก็บไว้ในซีลิ่ง สำหรับเนื้อเยื่อ ERMS ทั้งหมดไม่มีผลที่เป็นบวก ใน ARMS 2 ราย ที่ให้

ผลพบว่าเป็นรายที่ให้ผลลบต่อการย้อม desmin ไม่มีลักษณะทางจุลพยาธิวิทยาแบบ anaplastic ไม่พบ rhabdomyoblast differentiation แต่พบลักษณะ cystic component ชัดเจน และมีการตรวจทางจุลทรรศน์ อิเล็กตรอนว่าใช้ ARMS ซึ่งทั้งสองรายมีผลการรักษาที่ดีกว่าผู้ป่วยรายอื่นๆ สำหรับการศึกษาก่อน PAX7/FKHR ให้ผลลบ ทั้ง ARMS และ ERMS

สรุป: การศึกษาประสบความสำเร็จในการตรวจพบยีนเชื่อม PAX3/FKHR ในเนื้อเยื่อ ARMS การตรวจหา ยีนเชื่อมนี้ น่าจะสามารถนำไปประยุกต์ใช้ในการแยก ARMS จาก ERMS โดยเฉพาะอย่างยิ่งในรายซึ่งไม่แสดงลักษณะ alveolar cystic ที่ชัดเจน

คำสำคัญ: มะเร็งกล้ามเนื้อลาย, มะเร็งเนื้อเยื่ออ่อน, PAX3/FKHR, PAX7/FKHR

Introduction

Although rhabdomyosarcoma (RMS) is a rare malignancy in the general population, it is a common soft tissue sarcoma in the pediatric age group.¹⁻² The prognosis of RMS is poor, especially in high risk cases.³ The tumor originates from striated muscle lineage and is divided according to its histopathological pattern into 2 major subtypes: embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS). In most cases, the two subtypes can be distinguished by their clinical characteristics. ARMS usually presents during adolescence in skeletal muscle of the extremities and trunk while ERMS is usually found in a younger age group and has more favorable outcomes.⁴

On a histopathological level, a diagnosis of ARMS is usually made through observation the presence of alveolar-like spaces separated by thick collagenous bands lined by round tumor cells showing variable myogenic features. However, a pathological dilemma sometimes occurs as a poorly differentiated tumor may exhibit ambiguous morphology between the solid variants of ARMS and ERMS.⁵ Furthermore, ARMS must be distinguished from other small round cell neoplasms in children.⁶

Immunohistochemical staining usually helps to demonstrate striated muscle markers, such as desmin, vimentin and muscle-specific actin within tumor cells. However, these proteins may not express in the early myogenic stage. Moreover, the expression patterns in ARMS and ERMS are not different.

Cytogenetic studies have detected repeated genetic changes that seemed to behave like a molecular signature of ARMS, a chromosomal translocation involving chromosome 13 and its partners at chromosome 2 [t(2;13)(q35;q14)] or chromosome 1 [t(1;13)(p36;q14)]. These translocations lead to reciprocal fusion of FKHR (13q14) and the PAX3 (2q35), or PAX7 (1p36), respectively.⁶⁻⁷ PAX3/FKHR and PAX7/FKHR have been detected in 75% and 10% of ARMS,⁸⁻⁹ respectively. Their transcripts were proven to provide transcriptional capacity and perhaps also play an inhibiting role in myogenic differentiation.⁷ Detection of PAX/FKHR fusion in ARMS can not only be used in diagnosis, but recent studies have also demonstrated that tumors with a specific fusion type, PAX7/FKHR, may have a better prognosis.¹⁰⁻¹¹ Moreover, in cases with a known positive fusion gene, the fusion transcript can be used for disease monitoring by means of detecting circulating

tumor cells in peripheral blood after treatment.¹² Concerning its expected advantages, we hope to apply this marker for clinical use in the future. The main objective of this preliminary study was to establish a reliable detection process for the fusion gene in our ARMS cases. The study also put an emphasis on the clinicopathological characteristics of fusion-gene negative ARMS.

Materials and methods

Frozen-tissue samples from 5 patients with a definite diagnosis of ARMS were included in this study. Six cases of ERMS were used as a control. The tissue samples were snap-frozen and stored in liquid nitrogen immediately after completion of the surgical procedures. Storage times of the specimens ranged from 2 months to 3 years. Total Ribonucleic acid (RNA) was extracted from the samples, using an RNAeasy extraction kit (Qiagen Inc.). First-strand complementary Deoxyribonucleic acid (cDNA) was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Qiagen Inc.) and oligo(dT) primers. To detect chimeric transcripts of PAX3/FKHR or PAX7/FKHR, polymerase chain reactions (PCR) were performed with primers specifically designed for cDNA sequences. The primer sequences and PCR conditions followed a previous publication³ with some modifications. In brief, 2 sets of primers were used (5' to 3' direction); PAX3: AGC TAT ACA GAC AGC TTT GT, FKHR-A: CTC TGG ATT GAG CAT CCA CC, and PAX7: GCT TCT CCA GCT ACT CTG AC, FKHR-B: TCC AGT TCC TTC ATT CTG CA. PCR products from 35 rounds of thermal cycling were run

on 2% agarose gel, reacted with ethidium bromide for 3-4 minutes and then displayed under ultraviolet light. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification was used as an internal control.¹³ Positive results were confirmed and analyzed for breakpoint by TA vector cloning (Invitrogen Inc.) and sequencing with M13 primers. To test the effect of different tissue storage conditions, RNA was extracted from formalin - fixed paraffin - embedded (FFPE) tissue in the cases with a positive result and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was attempted.

The reverse transcription PCR results of ARMS were considered together with histopathological parameters, including the presence of cystic (alveolar) morphology, solid morphology, an ERMS-like area, rhabdomyoblast differentiation, multinuclear giant cells and anaplasia, grading of nuclear morphology and Shimada's mitosis karyorrhexis index. Previous immunohistochemistry results were also taken into account.

The use of biological material in this study and access to medical records was approved by the Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University (50/368-006).

Results

Demographic data, tumor sites and surgical staging of ARMS are given in Table 1. Three of the 5 ARMS gave a positive PCR product of PAX3/FKHR (Figure 1A). Moreover, ARMS showed negative results for PAX7/FKHR. Direct nucleotide sequencing of three positive case confirmed the existence of a fusion gene caused by an in-frame translocation

Table 1 Demographic and clinical data of rhabdomyosarcomas

Case	Sex/Age at diagnosis	Site	Histological ambiguity	Electron microscopy	Staging	Treatment	Follow-up duration	Status
ARMS-1	M/8 years	Buttock	yes	malignant small cell sarcoma	T2bNxM0 (II)	SX + CT + XRT	28 months	alive, NOS
ARMS-2	F/14 years	Retropertoneum	yes	rhabdomyosarcoma	T2bNxM0 (II)	CT (Partial response) + SX	26 months	alive, NOS
ARMS-3	M/7 months	Testis	no	NA	T1aN1M0 (III)	SX + CT + XRT	22 months	alive with metastasis
ARMS-4	F/15 years	Breast	yes	NA	T2bN1M0 (III)	SX + CT + XRT	14 months	died of disease
ARMS-5	F/38 years	Thigh	no	NA	T1bNxM1 (IV)	CT (Partial response) + palliative XRT	9 months	alive with metastasis

M: male, F: female, ARMS: alveolar rhabdomyosarcoma, ERMS: embryonal rhabdomyosarcoma, SX: surgery, CT: chemotherapy, XRT: radiation therapy,

NA: not available, NOS: not otherwise specified

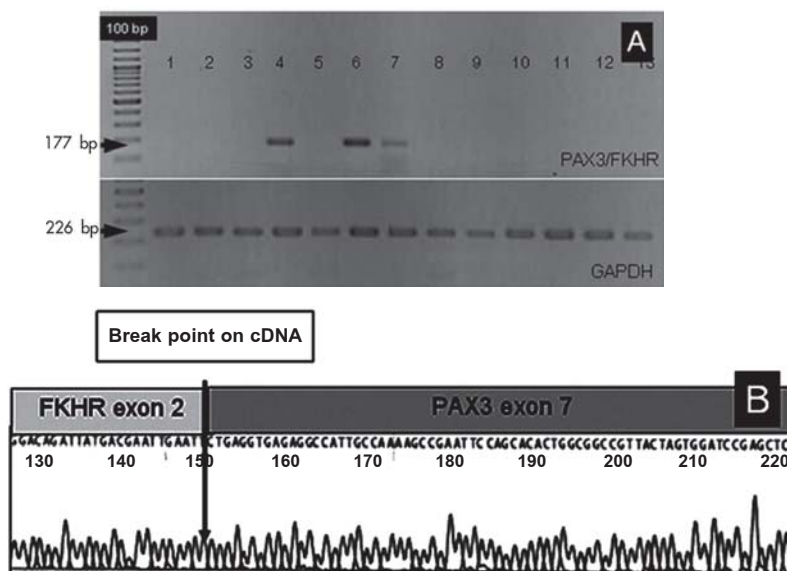


Figure 1 Electropherograms of RT-PCR results A) The upper panel demonstrates bands of positive PAX3/FKHR size 177 bp reverse transcription polymerase chain reaction in three cases of ARMS. (Lane 4, 6, 7) The lower panel shows amplification of GAPDH size 226 bp in all samples examined. (Lane 1 and 5 were negative control using DNA from healthy volunteers and normal tissue of case4 respectively) B) Nucleotide sequencing of three positive case confirmed in-frame fusion between exon 2 of FKHR and exon 7 of PAX3

between PAX3 exon7 and FKHR exon2 (Figure 1B). All FFPE tissue samples gave negative results.

Considering the immunohistochemical profiles, ARMS had varying patterns of histopathology and immunoreactivity. None of the ARMS samples exhibited an ERMS-like area. All cases with a positive PAX3/FKHR fusion transcript had positive desmin staining and presented solid foci together with a cystic part (Figure 2). The three samples also featured anaplastic histology (Table 2).

None of the ERMS samples gave positive results for fusion-gene detection. All revealed positive desmin immunostaining. One of the 6 ERMS

showed a cystic component and four had anaplastic histology.

Discussion

RMS is one of the small round blue cell tumors of childhood that originates from a skeletal myogenic lineage. Like other embryonal tumors, RMS shows varying degrees of differentiation, from primitive cells with subtle lineage-specific features to differentiating rhabdomyoblasts.¹² At the tissue level, patterns of alignment allow most RMS to be subcategorized into ERMS and ARMS. ERMS is diagnosed when a

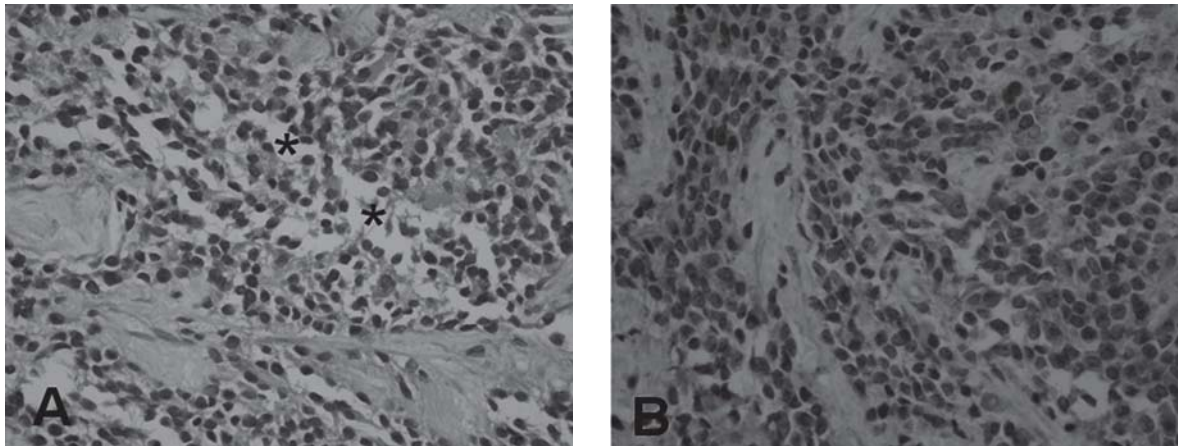


Figure 2 Histopathology of alveolar rhabdomyosarcoma in case ARMS-4. A) Hematoxylin & Eosin stained section shows cystic spaces (asterisked) resembling alveolar spaces lined by small round cells, B) Muscle specific actin staining shows positive immunoreactivity (both 20X magnification).

Table 2 Histopathological details of alveolar rhabdomyosarcomas

Case	Solid component	Cystic component	Rhabdomyoblast differentiation	Multinuclear giant cells	Anaplasia	Nuclear grade	Shimada's mitosis-karyorrhexis index
ARMS-1	yes	yes	no	no	no	1	grade 1
ARMS-2	no	yes	no	no	no	2	grade 1
ARMS-3*	yes	yes	yes	no	yes	2	grade 3
ARMS-4*	yes	yes	yes	yes	yes	2	grade 3
ARMS-5*	yes	yes	no	yes	yes	2	grade 2

* positive PAX3/FKHR

tumor shows a stroma-rich spindle cell appearance and absence of an alveolar pattern: The term was derived from ARMS because of its similarity with the typical histological pictures of ARMS which are composed of densely packed small round cells surrounding the alveolar space-like cystic part⁵ and pulmonary alveoli. However, there is also a solid

variant of ARMS that contains only small round cells of rhabdomyoblast with no cystic component. Although all of the ARMS in this study showed a cystic component on definite pathological findings, three had diagnostic difficulty on the first biopsy when tissue from a Tru-cut biopsy exhibited only a small round cell tumor sheath. Two of these cases (ARMS-1 and

ARMS-2) also gave negative staining for desmin. Besides the histological interpretation difficulty in a situation of limited tissue sampling, some ARMS and ERMS showed overlapping histological features. ARMS without a cystic component have been reported and defined as a solid alveolar variant.¹⁴ On the other hand, one of our ERMS from the orbit of a 1-year-old boy that showed cystic foci, normally marked as ARMS, was an example of overlapping histology. Considering the advantages of distinguishing ARMS from ERMS and the possibility of histological ambiguity, an adjunctive diagnostic tool would have useful role in certain situations.

Molecular pathology has gained more popularity as a diagnostic tool for detecting tumor specific genetic lesions or a 'molecular signature'. In RMS, one study found that 80% of histologically defined ARMS had positive evidence of translocation specific PAX/FKHR fusion.¹⁵ Our study aimed to evaluate the reproducibility of this finding in cases from our own institution. The study successfully used reverse transcription PCR in detection of a fusion transcript of PAX3/FKHR in 3 of 5 samples from cases histologically designated as ARMS and also showed negativity in all ERMS cases.

Two of our 5 ARMS samples gave negative RT-PCR results. The incidence of fusion gene negative ARMS has been reported at around 15-20% in larger series.^{10-12,14} Concerning the oft-found discrepancies between histopathology and molecular diagnosis, one question raised among pathologists has been 'should the classification be changed according to the molecular scheme into PAX/FKHR-fusion related RMS and its negative counterpart?' In technical points, although the

detection of PAX/FKHR is rather straightforward, the results still vary among laboratories. From a clinical point of view, one study with PAX/FKHR negative ARMS showed a prognostic ability intermediate between PAX7/FKHR-positive and PAX3/FKHR positive cases.¹⁰ However, a recent expression array study indicated that fusion-negative tumors might be biologically diverse from the PAX/FKHR-positive ARMS, and have some overlapping of the molecular profile with ERMS.

Technically, not all our PAX/FKHR fusion negatives were true negatives. Our failure to detect fusion-transcripts in some cases might be explained by the fact that expression of a fusion transcript in some ARMS samples was so low that conventional RT-PCR failed to detect them. A recent study found that approximately 20% of seemingly fusion-negative ARMS contained low level expression of PAX/FKHR that could be detected by high-sensitivity RT-PCR or contained a variant fusion between PAX and other rare partners.¹⁵ Our two cases of fusion-gene negative ARMS showed negative immunoreactivity for desmin staining, thus making it unclear if the tumor was ARMS or a variant of ERMS, or something else. However, a closer review of the definitive histopathological examination, there was clear evidenced of cystic alveolar space-like components, which is a pathognomonic pattern of ARMS.

The quality of tissue samples used in RT-PCR was also an important factor in the molecular study crucial as we failed to extract good RNA from all formalin-fixed samples, even though the storage period in some of these samples was less than 72 hours. This technical problem was also not consistent with previous reports that claimed success in detection of ARMS associated fusion gene by

RT-PCR, using FFPE tissue.¹⁶⁻¹⁷ Effectiveness in RNA retrieval from FFPE in our hands may not be adequate; hence frozen storage of surgical specimens is suggested for all rare tumors in which molecular diagnosis may have a role such as soft tissue sarcomas.

Our case number was too low to draw any statistically meaningful conclusions; however, our results were in-line with a recent study that reported a correlation between fusion transcript detection and the presence of cystic components.¹⁸ Moreover, our study also suggested a potential correlation between a positive fusion transcript and more aggressive histology in terms of anaplasia, rhabdomyoblast differentiation and a relatively high mitosis-karhyorhexis index. Considering the clinical outcomes, 2 cases with a negative fusion gene had disease-free survival for at least 2 years, while all 3 cases with PAX/FKHR showed early metastasis or death. This observation was consistent with a report from a large cohort that showed poorer survival in fusion positive cases¹⁰ and the poorer outcome could be an effect of more aggressive biology in fusion-negative tumors.

Conclusion

In summary, we examined frozen tissue samples from RMS cases for PAX/FKHR fusion and found positive PAX3/FKHR in 3 of 5 ARMS. The data suggest that this molecular pathology tool may have use as an adjunctive study in ARMS patients. This preliminary data also suggests a prognosticating role of PAX/FKHR fusion that needs to be verified by a larger scale study.

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