Case Report

Detection of Alveolar Rhabdomyosarcoma in Pleural Fluid with Immunocytochemistry on Cell Block and Determination of PAX/FKHR Fusion mRNA by Reverse Transcription-Polymerase Chain Reaction

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Background: *Alveolar rhabdomyosarcoma is a primitive malignant round cell neoplasm, which shows skeletal muscle differentiation. Although their histopathologic and immunohistochemical findings are well known, the cytology, immunocytochemistry and molecular study on pleural effusion have not been well documented.*
Objective: *To apply molecular method in the diagnosis and monitoring of alveolar rhabdomyosarcoma.*
Case Report: *The case of a 14-year-old Thai male, who presented with dyspnea and left pleural effusion. Computed tomography of the chest and abdomen showed a huge heterogeneous enhancing mass at the left retroperitoneum. Pleural fluid cytology showed malignant small round blue cells. Immunocytochemical stains on cell block material showed positive reactivity to vimentin, sarcomeric actin, desmin, MyoD1, myogenin, and CD56 in round cell tumor. Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated PAX/FKHR fusion transcript. The patient received chemotherapeutic regimen for advanced-stage rhabdomyosarcoma. Finally, he succumbed to the disease, thirteen months after the diagnosis.*
Conclusion: *Immunocytochemistry on cell block in conjunction with determination of PAX/FKHR fusion mRNA by RT-PCR is a molecular method in the diagnosis and monitoring of alveolar rhabdomyosarcoma in pleural fluid.*

Keywords: *Alveolar rhabdomyosarcoma, Pleural effusion, Cytology, Immunocytochemistry, Reverse transcription-polymerase chain reaction*

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Alveolar rhabdomyosarcoma is a primitive malignant round cell, high-grade mesenchymal neoplasm, which demonstrates evidence of skeletal muscle differentiation. The average age at presentation occurs principally during the first to second decade[1,2]. This tumor shows a male predilection of approximately 1.2:1[2]. The disease predominantly arises in the extremities and to a lesser extent the head and neck, thoracic cavity, abdominal cavity, genitourinary tract, trunk, pelvis, and retroperitoneum. The frequently presenting symptoms of retroperitoneal tumor are primarily abdominal pain, abdominal mass, dyspepsia, vomiting, fever, weight loss, and occasionally dyspnea. The neoplasm possesses a diagnostic challenge in cytologic specimen due to its wide differential diagnoses of small round cell tumors. The present report describes a children with retroperitoneal alveolar rhabdomyosarcoma metastasized to the left pleural cavity, and it is the first reported case of detecting and monitoring *PAX/FKHR* fusion mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Clinical manifestation, cytologic, immunocytochemical, and molecular findings of the pleural fluid concluded the diagnosis of alveolar rhabdomyosarcoma.

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Case Report

A 14-year-old Thai male living in Nakornsawan province Thailand came to Ramathibodi Hospital in April 2008 with the complaints of dyspnea and left pleuritic chest pain for three weeks. The patient had no

history of significant illness in the past. The physical examination revealed decreased breath sound, decreased vocal resonance and dullness on percussion of the left chest. The cervical and inguinal lymph nodes could not be palpated. An evaluation of the abdomen showed a firm non-movable mass at the left upper quadrant. Computed tomography (CT) of the chest and abdomen showed a huge heterogeneous enhancing left retroperitoneal mass measuring 10 x 11 x 10 cm. Multiple intrathoracic and intraabdominal lymph nodes including celiac, aortocaval and pancreatic lymphadenopathies were noted. Left pleural effusion and left pleural thickening were detected. Thoracentesis was performed and revealed clusters of undifferentiated tumor showing small to medium sized cells with hyperchromatic nuclei, inconspicuous nucleoli and scant cytoplasm (Fig. 1). No glandular or pseudorosette formation was present. Immunocytochemistry on cell block of the left pleural fluid was carried out (Table 1) and showed immunopositivity to vimentin, sarcomeric actin, desmin, MyoD1, myogenin, and CD56 in malignant round cells. Tumor cells were negative for cytokeratin (AE1 + AE3), epithelial membrane antigen (EMA), neuron-specific enolase (NSE), leukocyte common antigen (LCA, CD45RB), CD3, CD20 and CD99. The antibody sources, dilutions, and results are shown in Table 1.

Fresh pleural fluid was worked up for RT-PCR on known translocation fusion transcripts of alveolar rhabdomyosarcoma. Total RNA from pleural fluid was extracted using Trizol® (Invitrogen, CA, USA) according to the standard protocol. RNA concentration is determined by DU 730 Life Science UV/Vis Spectro-photometer (Beckman Coulter, CA, USA) and genomic DNA was removed using RQ1 RNase-Free DNase (Promega, WI, USA). 1 µg of total RNA samples were reverse transcribed using ImProm-II™ Reverse Transcription System (Promega, WI, USA) for first-strand cDNA according to the manufacturer's protocol. The cDNA was studied for the *PAX/FKHR* fusion transcripts [t(2;13) and t(1;13)] using the specific primers, *PAX3/PAX7*-1 (5'CCGACAGCAGCTCTGCCTAC3') and *FKHR*-2

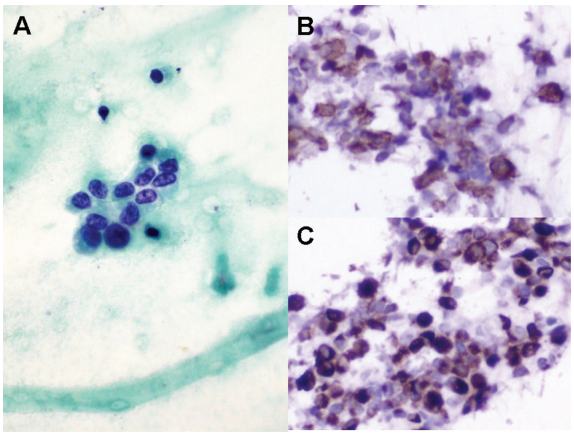

Fig. 1 FNA of the left pleural effusion shows groups of tumorous cells showing small to medium sized, round, hyperchromatic nuclei, inconspicuous nucleoli and scanty cytoplasm (A) Papanicolaou-stained smear, x400. The immunohistopathologic features of malignant round cells show positive to sarcomeric actin (B) and desmin (C), x400

Table 1. Immunohistochemistry-results of primary antibodies used

Antigen	Clone	Dilution	Source	Reactivity
Desmin	D33	1:100	Dako, Glustrup, Denmark	Positive
Myogenin	F5D	1:100	Dako, Glustrup, Denmark	Positive
MyoD1	5.8A	1:50	Dako, Carpinteria, CA, USA	Positive
Sarcomeric actin	Alpha-Sr-1	1:50	Dako, Glustrup, Denmark	Positive
CD56	1B6	1:50	Novo Castra, Newcastle, UK	Positive
Vimentin	Vim3B4	1:200	Dako, Carpinteria, CA, USA	Positive
CD3	Polyclonal	1:80	Dako, Glustrup, Denmark	Negative
CD20	L26	1:400	Dako, Glustrup, Denmark	Negative
CD45RB (LCA)	2B11+PD7/26	1:400	Dako, Glustrup, Denmark	Negative
CD99	12E7	1:50	Dako, Carpinteria, CA, USA	Negative
Cytokeratin (CK)	AE1+AE3	1:200	Dako, Carpinteria, CA, USA	Negative
Epithelial membrane antigen (EMA)	E29	1:100	Dako, Glustrup, Denmark	Negative
Neuron-specific enolase (NSE)	5E2	1:100	Novo Castra, Newcastle, UK	Negative

(5'TGAACTTGCTGTGTAGGGACAG3')[3]. PCR was made up in 25 μL reactions consisting of 1X buffer (Invitrogen, CA, USA), 2 mM of each dNTP (Promega, WI, USA), 0.25 μM of each primer, 1.5 mM of $MgCl_2$ and 1U of Taq DNA polymerase (Invitrogen, CA, USA). PCR amplification condition was one cycle of 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min for final extension. The amplified products were separated by electrophoresis. Briefly 20 μL of the reaction products were mixed with DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (w/v) sucrose in H_2O) and separated on 2% GenePure LE (ISC BioExpress, NC, USA) in TBE buffer (89 mM Tris, 89 mM Boric acid and 2 mM EDTA) at 5 centimeter per volt. The *PAX/FKHR* primers yield a PCR product of 170 base pairs, consistent with the *PAX/FKHR* fusion transcripts of alveolar rhabdomyosarcoma (Fig. 2). The following biopsy of the retroperitoneal mass revealed solid variant of alveolar rhabdomyosarcoma. The immunohistochemical findings were almost identical to the immunocytochemistry of the pleural effusion. The final diagnosis of the pleural fluid was metastatic alveolar rhabdomyosarcoma with *PAX/FKHR* fusion, stage IV. The patient received chemotherapeutic regimen for advanced-stage rhabdomyosarcoma including vincristine, irinotecan, cisplatin, doxorubicin, ifosfamide and etoposide. After 16 weeks of chemotherapy, he underwent pleurodesis to treat left pleural effusion and radiotherapy, total dose of 48 Gray (Gy), at the left chest and the left side of upper abdomen for local control of tumor. Follow-up at five months, pleural fluid cytology and RT-PCR detection of *PAX/FKHR* fusion transcripts revealed no evidence of malignancy. However, after 12 courses of chemotherapy, CT of chest and abdomen revealed newly seen lateral abdominal wall nodules and lateral chest nodules. He subsequently developed pleural and pericardial effusions. Finally, he succumbed to the disease, thirteen months after the diagnosis. No autopsy was performed. This present study was approved by the committee on human research at Ramathibodi Hospital (ID06-51-06).

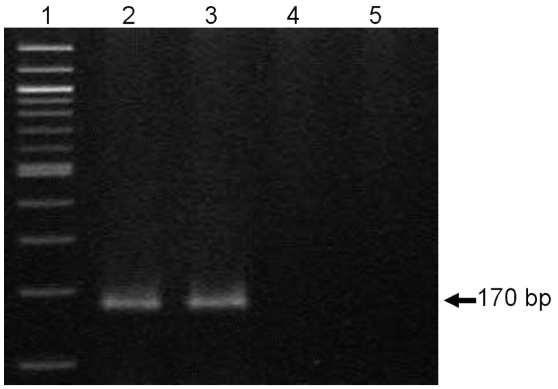

Fig. 2 Detection of the chimera *PAX-FKHR* gene transcript using RT-PCR. Lane 1, 100-bp ladder DNA Ladder (New England BioLabs Inc., MA, USA); lane 2, chimera *PAX/FKHR* gene transcript (reported case); lane 3, chimera *PAX/FKHR* gene transcript (typical alveolar rhabdomyosarcoma section as positive control, 170-bp); lane 4, negative control; lane 5, blank

Discussion

Pleural effusion is uncommon in children and is usually caused by infectious process including tuberculosis. Malignant pleural effusion is rare clinical presentation of metastatic malignant neoplasms in children. Commonly known pediatric malignancies associated with malignant pleural effusion include neuroblastoma and lymphoma. Pleural fluid metastasis from rhabdomyosarcoma is rare[4]. The useful diagnostic tool of metastatic malignant neoplasm to pleural fluid is the pleural fluid cytopathology, which may allow early recognition of tumor. In the previous report, biopsy is usually needed for an accurate immuno-histopathological diagnosis[1,2,5]. However, recently, pleural effusion cytology has been widely performed due to its advantage in early recognition of malignant residual soft tissue tumors by cytology and immunocytochemistry on cell block and determination of the specific fusion transcripts by RT-PCR.

Metastatic small round cell tumor in pleural effusion presents a diagnostic challenge on cytopathology. The microscopic findings of the authors' case demonstrate poorly differentiated round cells showing hyperchromatic nuclei with inconspicuous nucleoli and scanty cytoplasm. The differential diagnoses of malignant small round cell tumor of the pleural fluid include rhabdomyosarcoma, lymphoma, primitive neuroectodermal tumor (PNET)/Ewing sarcoma, small cell carcinoma and neuroblastoma[5]. Negative results of immunocytochemical stains for LCA, CD3, and CD20 may be helpful in excluding lymphoma. The PNET/Ewing sarcoma is cytologically indistinguishable from the other round cell tumor. In the current case, the immunocytochemical stains showed positive result for desmin, sarcomeric actin,

and CD56, which are typically negative in PNET/Ewing sarcoma. Small cell carcinoma demonstrates many cytologic and histologic similarities to alveolar rhabdomyosarcoma. Clinically, small cell carcinoma is associated with a much older patient population and usually originates in the lung. On immunocytochemistry, small cell carcinoma demonstrates immunoreactivity with epithelial markers, including cytokeratin and EMA, but negative for myogenic markers such as desmin and sarcomeric actin. Immunocytochemically, rhabdomyosarcoma is positive for muscle markers, but usually negative for cytokeratin and neural marker including NSE. However, aberrant expression of CD56 neuroendocrine marker, neural cell adhesion molecule, in alveolar rhabdomyosarcoma is reported[6]. Neuroblastoma also shares many cytologic features with alveolar rhabdomyosarcoma, but they occur in young children and typically, have adrenal mass. The expression of sarcomeric actin, desmin, MyoD1, myogenin and CD56 immunohistochemical stain confirmed the diagnosis of rhabdomyosarcoma[6,7]. Finally, the biopsy of the retroperitoneal mass in the presented case was performed to confirm the cytologic diagnosis and to distinguish between alveolar and other types of rhabdomyosarcomas.

Many reports of alveolar rhabdomyosarcoma have identified t (2;13) (q35;q14) and t (1;13) (p36;q14) resulting in a fusion gene between the *PAX3* gene on chromosome 2 as well as *PAX7* gene on chromosome 1 and the *FKHR* gene on chromosome 13[8]. It is postulated that, the resultant chimeric proteins activate downstream transcriptional targets and exert oncogenic effects by altering control of proliferation, apoptosis and differentiation[9]. Moreover, the fusion transcripts can predict clinical outcome, with *PAX3/FKHR* positive alveolar rhabdomyosarcoma behaving in a more malignant fashion than *PAX7/FKHR* positive one[10]. In the presented case, the authors did not examine the nucleotide sequence of these fragments and it was not clear whether these amplified, 170-bp fragments were resulted from *PAX3/FKHR* or *PAX7/FKHR* gene transcription. However, the amplified fragments certainly reflected one of fusion gene transcripts and provided a strong molecular evidence to prove it as alveolar rhabdomyosarcoma.

The treatment of rhabdomyosarcoma requires the application of classic oncologic principles for soft tissue tumors, including sufficient surgical excision, if possible and adjuvant chemoradiotherapy. Aggressive chemotherapy regimen followed by autologous stem cell rescue has been tried with the emphasis on achieving a complete and durable response[11]. Alveolar rhabdomyosarcoma has a highly aggressive clinical course and is usually large, infiltrative, and metastasized at the time of diagnosis. Given that the diagnosis of alveolar rhabdomyosarcoma is difficult cytologically, the immunocytochemistry on cell block and the detection of *PAX/FKHR* fusion gene are useful in the diagnosis and the determination of the appropriate treatment. In conclusion, the RT-PCR detection of *PAX/FKHR* fusion gene could be used to detect microscopic residual disease and subsequent recurrence.

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Potential conflicts of interest

None.

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การพิสูจน์เอกลักษณ์ของ alveolar rhabdomyosarcoma ของน้ำในช่องเยื่อหุ้มปอด โดยวิธี immunocytochemistry และการตรวจพบ PAX/FKHR fusion mRNA โดยวิธี RT-PCR

รัชช์ดล สว่างพาณิชย์, นพดล ลาภเจริญทรัพย์, อาทิตย์ จินาวัฒน์, อัจฉราพร พงษ์ทิพพันธ์, อุษณรัสมิ์ อนุรัฐพันธ์, สุรเดช หงส์อิง

ภูมิหลัง: *มะเร็งกล้ามเนื้อลายชนิด alveolar เป็นเนื้อร้ายของเซลล์รูปร่างกลม ที่แสดงการพัฒนาเป็นกล้ามเนื้อลาย แม้ว่าลักษณะทางพยาธิวิทยาเนื้อเยื่อ และผลทางอิมมูโนวิทยาเนื้อเยื่อของเซลล์มะเร็งกล้ามเนื้อลายนี้จะเป็นที่รู้จักกันเป็นอย่างดีแต่การแสดงออกทางเซลล์วิทยาอิมมูโนเซลล์วิทยา และการศึกษาในระดับโมเลกุลจากสารน้ำในช่องเยื่อหุ้มปอดยังไม่ได้รับศึกษามากนัก*

วัตถุประสงค์: *ใช้วิธีการทางเวชศาสตร์โมเลกุลในการวินิจฉัยและติดตามโรคมะเร็งกล้ามเนื้อลายชนิด alveolar*

รายงานผู้ป่วย: *ผู้ป่วยเด็กชายไทย อายุ 14 ปี มีอาการแสดงด้วยอาการหายใจลำบากตรวจพบว่ามีน้ำในช่องเยื่อหุ้มปอดข้างซ้าย ตรวจทางเอกซเรย์คอมพิวเตอร์พบก้อนขนาดใหญ่ที่บริเวณ retroperitoneum ด้านซ้าย การเจาะดูดที่ช่องเยื่อหุ้มปอดข้างซ้าย พบเซลล์มะเร็งขนาดเล็กกลมสีน้ำเงิน การตรวจทางอิมมูโนเซลล์วิทยาพบว่า เซลล์มีการแสดงออกต่อโปรตีน vimentin, sarcomeric actin, desmin, MyoD1, myogenin และ CD56 ในด้านการศึกษาในระดับโมเลกุลซึ่งตรวจโดยวิธี RT-PCR พบ* PAX/FKHR *fusion transcript ผู้ป่วยได้รับการรักษาด้วยยาเคมีบำบัดสูตรสำหรับมะเร็งกล้ามเนื้อลายระยะรุนแรง แต่ในท้ายที่สุดผู้ป่วยได้เสียชีวิตจากโรค หลังได้รับการวินิจฉัยโรคได้ 13 เดือน*

สรุป: *อิมมูโนเซลล์วิทยา และการศึกษาในระดับโมเลกุล เพื่อตรวจหา* PAX/FKHR *fusion mRNA โดยวิธี RT-PCR เป็นวิธีทางเวชศาสตร์โมเลกุลในการวินิจฉัยและการติดตามมะเร็งกล้ามเนื้อลายชนิด alveolar ในสารน้ำในช่องเยื่อหุ้มปอด*