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Clinical application of RNA sequencing in sarcoma diagnosis

An institutional experience

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Abstract

Accurate diagnoses of sarcoma are sometimes challenging on conventional histomorphology and immunophenotype. Many specific genetic aberrations including chromosomal translocations have been identified in various sarcomas, which can be detected by fluorescence in situ hybridization and polymerase chain reaction analysis. Next-generation sequencing-based RNA sequencing can screen multiple sarcoma-specific chromosome translocations/fusion genes in 1 test, which is especially useful for sarcoma without obvious differentiation. In this report, we utilized RNA sequencing on formalin-fixed paraffin-embedded (FFPE) specimens to investigate the possibility of diagnosing sarcomas by identifying disease-specific fusion genes. Targeted RNA sequencing was performed on 6 sarcoma cases. The expected genetic alterations (clear cell sarcoma/*EWSR1-ATF1*, Ewing sarcoma/*EWSR1-FLI1*, myxoid liposarcoma/*DDIT3-FUS*) in four cases were detected and confirmed by secondary tests. Interestingly, three *SS18* fusion genes (*SS18-SSX2B*, *SS18-SSX2*, and *SS18-SSX4*) were identified in a synovial sarcoma case. A rare fusion gene (*EWSR1-PATZ1*) was identified in a morphologically challenging case; which enabled us to establish the diagnosis of low grade glioneural tumor. In conclusion, RNA sequencing on FFPE specimen is a reliable method in establishing the diagnosis of sarcoma in daily practice.

Abbreviation: PCR = polymerase chain reaction.

Keywords: *EWSR1-PATZ1*, RNA sequencing, sarcoma

1. Introduction

There are more than 100 recognized types of human sarcoma. Obviously, an accurate diagnosis is important for patient management and prognosis. However, precise diagnoses by conventional histomorphology are sometimes challenging.[1–3] Italiano et al found that up to 23% of sarcomas originally diagnosed based on histology and immunophenotype were subsequently modified after molecular genetics testing. It has been suggested that molecular/genetic studies should be mandatory for an accurate diagnosis and classification of sarcoma and appropriate management, even if the histological diagnosis is made by an expert in soft tissue pathology.[3]

Over the past 2 decades, advances in molecular pathology profiling have provided significant insights into sarcoma. Effective treatment based on potentially actionable drug targets requires precise diagnosis and a better understanding of the disease at the molecular genetic level. Many specific genetic aberrations including chromosomal translocations, gene mutations, and gene amplifications have been identified in various sarcomas.[3–5] Some of these molecular genetic abnormalities can be detected by traditional technologies, such as karyotyping, fluorescence in situ hybridization (FISH), and polymerase chain reaction (PCR) analysis. With the development of molecular technologies, next-generation sequencing (NGS) is widely available and cost-effective. NGS can generate hundreds of thousands to hundreds of millions of short DNA or cDNA "reads" in a single run, thus enabling massively parallel sequencing of numerous genetic alterations.[6,7]

Although archived formalin-fixed paraffin-embedded (FFPE) tissues are the most common specimens available in clinical laboratories, DNA and RNA extracted from FFPE samples are often fragmented and damaged due to formalin fixation, which chemically cross-links the nucleic acids with the surrounding proteins and may also modify the nucleotides.[8–11] To date, there have been only a handful of studies reporting on the clinical use of NGS for the diagnosis of sarcomas.[12–18] In this report, we utilized RNA sequencing on FFPE specimens to investigate the possibility of diagnosing sarcomas by identifying disease-specific fusion genes.

2. Materials and methods

2.1. Patients and samples

Six archived cases of sarcoma were retrieved from Department of Pathology at Fox Chase Cancer Center. Five of these cases had confirmative FISH or NGS results (Table 1). A medical record

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Table 1

Clinicopathological information of 6 sarcoma cases.

Case	Age	Sex	Tumor site	Diagnosis	Fusion genes	Confirmative test
1	47	M	Right arm	Clear cell sarcoma	*EWSR1-ATF1*	FISH/*EWSR1+* >50% cells
2	43	M	Left leg	Clear cell sarcoma	*EWSR1-ATF1*	NGS/*EWSR1-ATF1*
3	53	F	Left lateral thigh	Synovial sarcoma	*SS18-SSX2B SS18-SSX2 SS18-SSX4*	FISH/*SS18+* >70% cells
4	34	M	Left chest wall	Ewing sarcoma	*EWSR1-FLI1*	FISH/*EWSR1+* >60 cells
5	45	F	Right popliteal	Myxoid liposarcoma	*DDIT3-FUS*	FISH/*DDIT3+* >80% cells
6	50	F	Cervical spine	Low-grade glial tumor	*EWSR1-PATZ1*	N/D

F = female, M = male, N/D = not done, NGS = FoundationOne NGS panel.

review provided pertinent clinical information. This study was approved by the Institutional Review Board at our institution.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 4-μm sections from FFPE tissue using a Ventana Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ). The following antibodies were used: Neurofilament (monoclonal mouse anti-human antibody, 2F11, Dako; 1:1600), GFAP (rabbit anti-human antibody, DAKO; 1:2500), and CD99 (monoclonal mouse anti-human antibody, 013, Covance; 1:200). The corresponding positive and negative controls were shown to be adequate.

2.3. Next-generation sequencing

A High Pure FFPE RNA Isolation Kit (Roche) was used for RNA extraction according to the manufacturer's protocol. For each tumor, RNA was isolated from FFPE samples using five 10-μm thick tissue sections. RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA). NGS-based targeted RNA-seq analysis was performed using the Illumina TruSight RNA Fusion Panel and a MiniSeq sequencer according to the manufacturer's recommendations (Illumina, San Diego, CA). This targeted RNA fusion panel consists of 507 of the most well-known malignancy-related fusion genes, which covers 7690 exonic regions, with a total of 21,283 probes. The gene list is available at www.illumina.com.

2.4. FISH analysis and confirmative tests

Fluorescence in situ hybridization was performed on FFPE tissue sections using the following dual-color DNA break-apart probes: *EWSR1*, *SS18*, and *DDIT3*. Case 1 was performed at the Hospital of University of Pennsylvania. Cases 3, 4, and 5 were performed at Integrated Oncology (New York). Case 2 was confirmed by a commercial targeted NGS panel (FoundationOne test, Foundation Medicine, Cambridge, MA).

2.5. Chromosomal microarray analysis

Chromosome microarray analysis was performed using Affymetrix OncoScan FFPE Assay kits (Thermo Scientific, Waltham, MA). Based on the H&E slide, tissue sections were macro-dissected to remove obvious necrotic areas, stroma, and adjacent normal tissue. At least 40% tumor cells were achieved. The intensities of probe hybridization were analyzed using Affymetrix software AGCC; and copy number and LOH analysis were performed with the Affymetrix Chromosome Analysis Suite (ChAS), using the default setting.[19]

3. Results

Five cases of sarcoma were processed for NGS, including 1 myxoid liposarcoma (Fig. 1A), 2 clear cell sarcomas (Fig. 1B), 1 synovial sarcoma, and 1 Ewing sarcoma (Fig. 1C). Three patients underwent adjuvant chemo or chemo-radiation therapy (clear cell sarcoma, synovial sarcoma, and Ewing sarcoma). The 2 patients with clear cell sarcoma had recurrence or metastasis, respectively, within 2 years, and both died. Clinicopathological data of the patients and tumors are shown in Table 1.

3.1. Targetable genetic alterations confirmed by NGS

Five sarcoma cases were previously diagnosed based on morphology and immunophenotyping, and in 4 of these cases, by FISH analysis with disease-specific fusion gene probes, including break-apart probes for *SS18* (Synovial sarcoma), *EWSR1* (1 clear cell sarcoma and 1 Ewing sarcoma), and *DDIT3* (myxoid liposarcoma) (Table 1). Another clear cell sarcoma (case 2) was shown by commercial NGS testing (FoundationOne) to have an *EWSR1-ATF1* fusion. All these fusion genes were detected by our RNA sequencing analysis with the RNA Fusion Panel. The 2 cases of clear cell sarcoma each had an *EWSR1-ATF1* fusion gene. The Ewing sarcoma showed a *EWSR1-FLI1*. The myxoid liposarcoma had a *DDIT3-FUS*. Interestingly, the synovial sarcoma showed 3 *SS18* fusion genes, including *SS18-SSX2B, SS18-SSX2*, and *SS18-SSX4*.

3.2. Unexpected targetable genetic alteration identified by NGS

With the success of detecting the fusion gene in the 5 sarcoma cases, we next used NGS to tackle a difficult consultation case. This patient was a 49-year-old female, who presented with a cervical spine tumor, which was an avidly enhancing intradural extramedullary mass extending from C4 to T1. The resection specimen showed both spindle and small round cells (Fig. 1D). Immunohistochemistry results were inconclusive. This case was sent for consultation at an academic institution before being sent to our institution, where it was diagnosed as a low-grade spindle and small round cell neoplasm/sarcoma. We first performed chromosome microarray analysis, which did not reveal any chromosome abnormality. Then RNA sequencing was performed, and a gene fusion was identified: *EWSR1* (Ewing

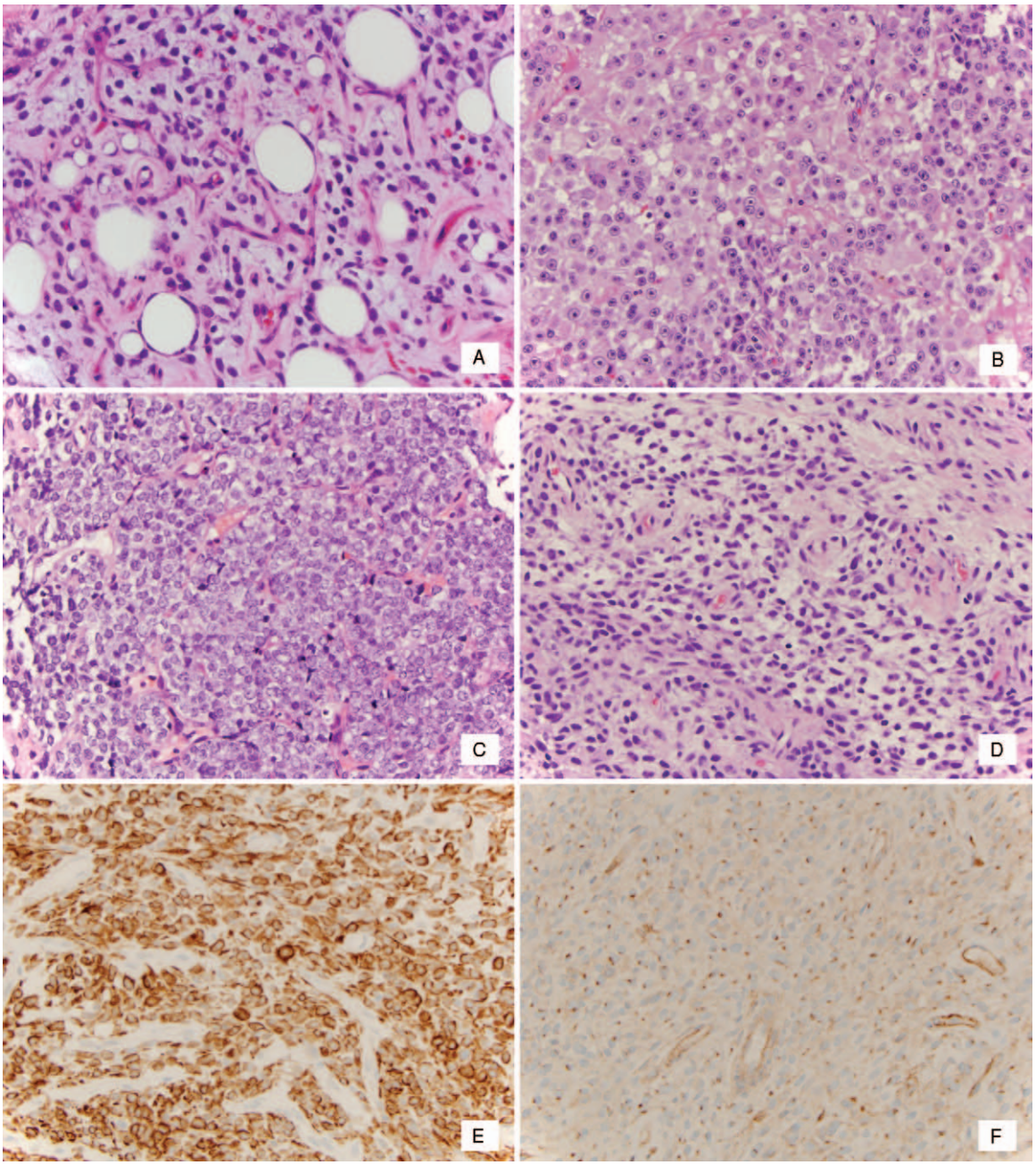

Figure 1. (A) Myxoid liposarcoma (case 5) shows signet-ring lipoblasts in a myxoid stroma with "chicken-wire" capillary vasculature. (B) Clear cell sarcoma shows uniform ovoid cells with prominent nucleoli and eosinophilic cytoplasm with focal clearing (case 1). (C) Ewing sarcoma shows sheets of small round blue cells with indistinct cytoplasmic membranes (case 4). (D) Low-grade glial tumor (case 6) demonstrates monotonous small spindle cells in a background of abundant vasculature. (E) GFAP positivity in glial tumor. (F) CD99 staining in glial tumor demonstrates nonspecific perinuclear dot staining.

Sarcoma Breakpoint Region 1)-*PATZ1* (POZ/BTB and AT Hook Containing Zinc Finger 1). A literature search revealed that *EWSR1-PATZ1* has been reported in rare spindle cell sarcomas and glioneural tumors.[20–23] Additional immunohistochemical analyses were performed, which demonstrated that these tumor cells were strongly and diffusely positive for GFAP and negative for Neurofilament and CD99 (Fig. 1E and F). The overall findings of morphology, phenotype, and the presence of the *EWSR1-PATZ1* fusion gene support the diagnosis of low-grade glial tumor, which may represent either ependymoma or a low-grade glioma. After resection, the patient had relief of symptoms and remains disease-free 1 year later.

4. Discussion

As a group of rare and heterogeneous tumors, sarcomas represent a challenge for precise diagnosis.[1] NGS is a fast-growing technology for sequencing both DNA and RNA.[6] The availability of RNA-based multiplexed gene sequencing panels for interrogating sarcoma-specific chromosome translocations/

fusion genes opens new opportunities to diagnose sarcoma. In this study, we successfully detected 5 sarcoma-defining fusion genes using widely available FFPE specimens, which had been confirmed by FISH or commercial NGS testing. Recently, the same RNA-sequencing panel was also successfully performed in renal cell carcinoma and adenoid cystic carcinoma at our laboratory.[7,24]

The genetic alterations considered hallmarks of sarcoma are not always detectable by FISH analysis, for example, when a chromosomal inversion involves 2 nearby genes or when there is an uncommon translocation. In addition, it is sometimes difficult to choose the specific FISH probe needed for cases lacking morphological or phenotypical evidence of differentiation. The glial tumor presented is an example. Even an expert in soft tissue pathology could not make a definitive diagnosis. The *EWSR1-PATZ1* gene fusion identified by RNA sequencing provided clues for further diagnostic work-up. The diffusely and strongly positive GFAP staining and negative CD99 staining performed after knowing the RNA-sequencing results helped render a diagnosis of low-grade glial tumor. Interestingly, both *EWSR1* and *PATZ1* are located on chromosome arm 22q, and the distance between these 2 gene loci is only 2 Mb. Thus, interpretation of a FISH signal would be difficult and may result in a false-negative test result. The *EWSR1-PATZ1* gene fusion involves the canonical *EWSR1* gene, juxtaposing the entire N-terminal transcriptional activation domain of the *EWSR1* gene and the C-terminal DNA binding domain of *PATZ1*. *PATZ1* is a transcription factor of BTB-ZF (broad-complex, tramtrack, and bric-à-brac zinc finger) gene family,[22] which is an important regulator of pluripotency in embryonal stem cells repressing developmental genes through its BTB domain and essential to maintaining stemness by inhibiting neural differentiation.[23] Fusion of *PATZ1* with *EWSR1* leads to overexpression of this gene, which might be the driving force for tumorigenesis.[25] The *EWSR1-PATZ1* gene fusion was initially implicated only in rare round cell sarcomas,[3,26,27] but has very recently been reported in 4 glioneural tumor.[20,25,28,29] To our knowledge, this case is the fifth glioneural tumor reported with this genetic alteration.[25]

Another interesting example that demonstrates the advantage of RNA-sequencing is the synovial sarcoma in this study; which demonstrates triple SS18 fusion genes: *SS18-SSX2B*, *SS18-SSX2* and *SS18-SSX4*. Multiple fusion pairs involving SS18 have been reported in synovial sarcomas studied;[30] however, which are difficult or impossible to be detected by traditional FISH or PCR technologies.[2,30–33]

Traditionally, sarcomas are managed with a combination of surgery and radiation when disease is localized; and neoadjuvant or adjuvant chemotherapies are occasionally involved.[5,34] By identifying targetable gene alterations in sarcomas, NGS can serve as a useful tool for decisions about inclusion or exclusion of patients for targeted therapy.[35] In addition, with the large number of genes included in targeted sequencing panels, NGS also provides an opportunity to expand our understanding about the genetic alterations of sarcomas beyond what is currently known.[36,37]

5. Conclusions

Targeted RNA sequencing was performed on 6 patients (5 sarcomas and 1 glial tumor). The expected genetic alterations were observed in 5 cases; and the rare fusion gene (*EWSR1-PATZ1*) identified in the sixth patient, enabled us to establish the diagnosis of glioneural tumor in this morphologically challenging case. RNA sequencing is a reliable method in establishing the diagnosis of sarcoma, and holds advantages over conventional molecular detection methods.

Author contributions

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Writing – review & editing: Jianming Pei, Arthur S Patchefsky, Douglas B Flieder, Joseph R Testa, Shuanzeng Wei.

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