Clinical application of RNA sequencing in sarcoma diagnosis
An institutional experience

Jianming Pei, MD, Xiaofeng Zhao, MD, PhD, Arthur S. Patchefsky, MD, Douglas B. Flieder, MD, Jacqueline N. Talarchek, BS, Joseph R. Testa, PhD, FACMG, Shuanzeng Wei, MD, PhD.

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 4 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 glioneuronal 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.[4]

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.[5–18] 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...
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 macrodissected 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 sarcoma) fusions.
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.\textsuperscript{[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.\textsuperscript{[1]} NGS is a fast-growing technology for sequencing both DNA and RNA.\textsuperscript{[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. 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,[25] 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.[12,20,23,29]

Traditionally, sarcomas are managed with a combination of surgery and radiation when disease is localized; and neoadjuvant or adjunct chemotherapy are occasionally involved.[15,24] 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.[15] 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.[26,30,33]

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

Conceptualization: Jianming Pei, Arthur S Patchefsky, Shuanzeng Wei.

Data curation: Jianming Pei, Shuanzeng Wei.

Formal analysis: Jianming Pei, Shuanzeng Wei.

Funding acquisition: Joseph R Testa, Shuanzeng Wei.

Investigation: Jianming Pei, Xiaofeng Zhao, Arthur S Patchefsky, Douglas B Flieder, Jacqueline N Talarache, Shuanzeng Wei.

Methodology: Jianming Pei, Jacqueline N Talarache, Shuanzeng Wei.

Project administration: Shuanzeng Wei.

Resources: Shuanzeng Wei.

Supervision: Arthur S Patchefsky, Douglas B Flieder, Joseph R Testa, Shuanzeng Wei.

Validation: Shuanzeng Wei.

Writing – original draft: Xiaofeng Zhao, Shuanzeng Wei.

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

References

[1] Hoang NT, Acevedo LA, Mann MJ, et al. A review of soft-tissue sarcomas: translation of biological advances into treatment measures. Cancer Manag Res 2018;10:1089–114.

[2] Fletcher CD, Bridge JA, Hogendoorn P, et al. WHO Classification of Tumours of Soft Tissue and Bone. Lyon, France: IARC Press; 2013.

[3] Italiano A, Di Mauro I, Rapp J, et al. Clinical effect of molecular methods in sarcoma diagnosis (GENSARC): a prospective, multicentre, observational study. Lancet Oncol 2016;17:532–8.

[4] Fletcher CD. The evolving classification of soft tissue tumours: an update based on the new 2013 WHO classification. Histopathology 2014;64:2–11.

[5] Mertens F, Tayebwa J. Evolving techniques for gene fusion detection in soft tissue tumours. Histopathology 2014;64:151–62.

[6] Metzker ML. Sequencing technologies: the next generation. Nat Rev Genet 2010;11:31–46.

[7] Pei J, Cooper H, Flieder DB, et al. NEAT1-TFE3 and KAT6A-TFE3 renal cell carcinoma and adenoid cystic carcinoma at our laboratory.[7,24]

[8] Kresse SH, Namlos HM, Lorenz S, et al. Evaluation of commercial DNA and RNA extraction methods for high-throughput sequencing of FFPE samples. PLoS One 2018;13:e0197456.

[9] Williams C, Ponten F, Moberg C, et al. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155:1467–71.

[10] Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999;27:4436–43.

[11] Wei S, Lieberman D, Morrissette JJ, et al. Using “residual” FNA rinse and body fluid specimens for next-generation sequencing: An institutional experience. Cancer Cytopathol 2016;124:224–9.

[12] Jour G, Scarborough JD, Jones RL et al. Molecular profiling of soft tissue sarcomas using next-generation sequencing: a pilot study toward precision therapeutics. Hum Pathol 2014;45:1563–71.

[13] Andersson C, Fagman H, Hansson M, et al. Profiling of potential driver mutations in sarcomas by targeted next generation sequencing. Cancer Genet 2016;209:154–60.

[14] Groszberg R, Roszik J, Conley A, et al. The role of next-generation sequencing in sarcomas: evolution from light microscope to molecular microscope. Curr Oncol Rep 2017;19:78.

[15] Core GM, He J, Choy E. Next-generation sequencing for patients with sarcoma: a single center experience. Oncologist 2018;23:234–42.
[16] Vlenterie M, Hillebrandt-Roeffen MH, Flucke UE, et al. Next generation sequencing in synovial sarcoma reveals novel gene mutations. Oncotarget 2015;6:34680–90.

[17] Walther C, Hofvander J, Nilsson J, et al. Gene fusion detection in formalin-fixed paraffin-embedded benign fibrous histiocytomas using fluorescence in situ hybridization and RNA sequencing. Lab Invest 2015;95:1071–6.

[18] Sweeney RT, Zhang B, Zhu SX, et al. Desktop transcriptome sequencing from archival tissue to identify clinically relevant translocations. Am J Surg Pathol 2013;37:796–803.

[19] Pei J, Al-Saleem T, Uzzo RG, et al. Application of chromosome microarray analysis for the differential diagnosis of low-grade renal cell carcinoma with clear cell and papillary features. Appl Immunohistochem Mol Morphol 2018; doi: 10.1097/PAL.0000000000000704 [Epub ahead of print].

[20] Qadwoum I, Orisme W, Wen J, et al. Genetic alterations in uncommon low-grade neuroepithelial tumors: BRAF, FGFR1, and MYB mutations occur at high frequency and align with morphology. Acta Neuropathol 2016;131:833–45.

[21] Vanden Borre P, Schrock AB, Anderson PM, et al. Pediatric, adolescent, and young adult thyroid carcinoma harbors frequent and diverse targetable genomic alterations, including kinase fusions. Oncologist 2017;22:255–63.

[22] Siggs OM, Beutler B. The BTB-ZF transcription factors. Cell Cycle 2012;11:3358–69.

[23] Ow JR, Ma H, Jean A, et al. Patz1 regulates embryonic stem cell identity. Stem Cells Dev 2014;23:1062–73.

[24] Pei J, Flieder DB, Patchefsky A, et al. Detecting MYB and MYBL1 fusion genes in tracheobronchial adenoid cystic cystic carcinomas by targeted RNA-sequencing. Mod Pathol 2019; doi: 10.1038/s41379-019-0277-x [Epub ahead of print].

[25] Siegfried A, Rousseau A, Maurage CA, et al. EWSR1-PATZ1 gene fusion may define a new glioneuronal tumor entity. Brain Pathol 2019; 29:53–62.

[26] Chougule A, Taylor MS, Nardi V, et al. Spindle and round cell sarcoma with EWSR1-PATZ1 gene fusion: a sarcoma with polyphenotypic differentiation. Am J Surg Pathol 2019;43:220–8.

[27] Leslie T, Perot G, Largeau MR, et al. RNA sequencing validation of the Complexity INdex in SARComas prognostic signature. Eur J Cancer 2016;57:104–11.

[28] Álvarez-Brekenridge C, Miller JJ, Nayar N, et al. Clinical and radiographic response following targeting of BCAN-NTRK1 fusion in glioneuronal tumor. NPJ Precis Oncol 2017;1:5.

[29] Johnson A, Severson E, Gay L, et al. Comprehensive genomic profiling of 282 pediatric low- and high-grade gliomas reveals genomic drivers, tumor mutational burden, and hypermutation signatures. Oncologist 2017;22:1478–90.

[30] Yang K, Liu WO, Xie Y, et al. Co-existence of SYT-SSX1 and SYT-SSX2 fusions in synovial sarcomas. Oncogene 2002;21:4181–90.

[31] Amary MF, Berisha F, Bernardi Fidel C, et al. Detection of SS18-SSX fusion transcripts in formalin-fixed paraffin-embedded neoplasms: analysis of conventional RT-PCR, qRT-PCR and dual color FISH as diagnostic tools for synovial sarcoma. Mod Pathol 2007;20:482–96.

[32] Przybyl J, Sciot R, Rutkowski P, et al. Recurrent and novel SS18-SSX fusion transcripts in synovial sarcoma: description of three new cases. Tumour Biol 2012;33:2245–53.

[33] Ren T, Lu Q, Guo W, et al. The clinical implication of SS18-SSX fusion gene in synovial sarcoma. Br J Cancer 2013;109:2279–85.

[34] Subbiah V, Hess KR, Khawaja MR, et al. Evaluation of novel targeted therapies in aggressive biology sarcoma patients after progression from US FDA approved therapies. Sci Rep 2016;6:35448.

[35] Groisberg R, Hong DS, Hollla V, et al. Clinical genomic profiling to identify actionable alterations for investigational therapies in patients with diverse sarcomas. Oncotarget 2017;8:39254–67.

[36] Brenca M, Maestro R. Massive parallel sequencing in sarcoma pathobiology: state of the art and perspectives. Expert Rev Anticancer Ther 2015;15:1473–88.

[37] Huang M, Wei S. Overview of molecular testing of cytology specimens. Acta Cytologica 2019;1:1–11.