Detection of EWSR1 fusions in CCOC by targeted RNA-seq

Camron M. Rivera, BS,a,b William C Faquin, MD, PhD,c,d Julia Thierauf, MD, PhD,c,d Amir H. Afrogheh, BChD, MSc, MChD,e Alexandre Jaquinet, MD,f A. John Iafrate, MD, PhD,c,d Miguel N. Rivera, MD,c,d and Maria J. Troulis, DDS, MSca

Objective. To describe the application of a targeted RNA sequencing assay to detect fusion transcripts in formalin-fixed paraffin-embedded (FFPE), non-decalcified samples of clear cell odontogenic carcinoma (CCOC) and related tumors, and to add to knowledge of the genetic drivers of CCOC.

Study Design. Five FFPE tissues, including intraosseous CCOC (n = 3), clear cell carcinoma of the salivary gland (CCC, n = 1), and Ewing sarcoma (ES, n = 1), were analyzed by targeted RNA-seq to detect fusions.

Results. The 3 intraosseous CCOC samples harbored EWSR1 translocations: EWSR1-ATF1 (n = 2) and EWSR1-CREM (n = 1); the CCC sample contained an EWSR1-ATF1 fusion; and the ES sample contained an EWSR1-FLI1 fusion detected by RNA-seq.

Conclusions. These results demonstrate that targeted RNA-seq is a valuable tool to detect fusions in FFPE samples of rare tumors such as CCOC and CCC. The results also confirm the observations that CCOC is driven by fusions between EWSR1 and CREB family transcription factors, including ATF1 and CREM. To our knowledge, this is the second report of CCOC with an EWSR1-CREM translocation. (Oral Surg Oral Med Oral Pathol Oral Radiol 2022;000:e1–e5)

Targeted RNA-seq is a method for the detection of fusion transcripts that has been shown to have robust performance in paraffin-embedded samples, broad coverage of common fusions, and the ability to detect novel fusion partners.1-4

Clear cell odontogenic carcinoma (CCOC) is a rare jaw tumor, with 107 cases documented between 1985 to 2019 worldwide.5 The tumor was first described in 1985 by Hansen et al.6 as a benign odontogenic tumor with a tendency for local recurrence. The behavior, which is seen in many benign jaw tumors, was deemed to be locally aggressive. Recognizing the malignant behavior with potential for metastasis, the WHO reclassified this tumor in 2005 as a “malignant” CCOC.7

The classification of CCOC is relevant for guiding clinical treatment. Currently, accurate diagnosis is based on immunohistochemical classification. Three histologic variants of CCOC have been described: biphasic, monophasic, and ameloblastic variants.5,7-10 The biphasic variant is the most common and presents as islands and strands containing 2 populations of cells: clear cells with well-defined cytoplasmic borders and centrally placed nuclei, and polygonal cells with eosinophilic cytoplasm and eccentrically placed hyperchromatic nuclei. Both cell types are embedded in a dense fibrous/hyalinized stroma. The monophasic variant is the least common and is composed almost entirely of clear cells. The ameloblastic variant, in addition to clear cells, exhibits columnar ameloblast-like cells at the periphery of the tumor islands.

Aside from immunoreactivity for various keratins and epithelial membrane antigen (EMA), the immunohistochemical profile of CCOC is nonspecific.5 Additional studies, including molecular analysis, are needed to define the relationship of the histologic variants to one another as well as clinical behavior. In general, the diagnosis of CCOC is one of exclusion because these cancers lack overt histologic and immunohistochemical features of other tumors, including squamous cell carcinoma, adenocarcinoma, myoepithelial carcinoma, and mucoepidermoid carcinoma. Currently, molecular

Statement of Clinical Relevance

In this manuscript, we report the gene fusion transcripts detected via targeted RNA-seq and establish the technique as a powerful methodology for detection of fusion transcripts in formalin-fixed paraffin-embedded samples of rare tumors.
characterization of the tumor in combination with histology and/or immunohistochemistry is the most reliable approach to accurately diagnosing CCOC.

To date, several studies have described the genetics of CCOC. Seventeen CCOC specimens have been tested for Ewing sarcoma breakpoint region 1 (EWSR1) translocations using either fluorescence in situ hybridization (FISH) or real-time polymerase chain reaction (RT-PCR) and FISH analysis. Genetic analysis showed that EWSR1 was partnered with cyclic AMP-dependent transcription factor (ATF1) in 7 samples, cAMP response element-binding protein (CREB) in 1 sample, and recently, cAMP responsive element modulator (CREM) in 1 sample. The remainder of the samples had an unknown rearrangement location and/or partner. Five specimens were negative for an EWSR1 translocation. The significance of this finding is unknown but may reflect limitations of the sequencing technologies.

Translocations creating an EWSR1-ATF1 or -CREB are frequently associated with CCOC, angiomatoid fibrous histiocytoma, conventional clear cell sarcoma, clear cell sarcoma-like tumor of the gastrointestinal tract, hyalinizing clear cell carcinoma of the salivary gland, and primary pulmonary myxoid sarcoma. EWSR1-CREM translocations are commonly found in mesenchymal tumors, including intracraniomyxoid mesenchymal tumor, pulmonary mesenchymal neoplasms, and others.

The goal of this study is to show that targeted RNA-seq is a feasible method to detect fusion transcripts in FFPE CCOC samples.

MATERIALS AND METHODS

Five non-decalcified, FFPE tissues, including intraosseous COCC (n = 3), clear cell carcinoma of the salivary gland (CCC, n = 1), and Ewing sarcoma (n = 1), were analyzed by a targeted RNA-seq assay at Massachusetts General Hospital (Boston, MA; Solid Fusion Assay v2) to detect fusions. The CCC and Ewing sarcoma samples served as controls because their fusion partners are well documented. The clinical cases had been reviewed by their respective local institutional tumor boards and deemed to be primary tumors. Once received by our group, each tumor was reviewed to confirm the diagnosis. The head and neck cases (CCOC [n = 3], CCC [n = 1]) did not receive preoperative or postoperative chemotherapy and underwent wide resection with clear margins. Only the patient with CCC received postoperative radiation. To our knowledge, none of the head and neck cases have recurred, 4 to 6 years postoperatively.

Total nucleic acid was extracted from ten 5-μm FFPE tumor sections using the Formapure RNA Isolation Kit (Agencourt AMPure; Beckman Coulter Life Sciences, Indianapolis, IN). Double-stranded cDNA was synthesized and ligated with a half-functional adapter. Anchored multiplex PCR reactions were performed using custom ArcherDx (Boulder, CO) primers. Libraries were sequenced using an Illumina NextSeq. Sequences were aligned, and split reads were analyzed using a custom bioinformatics pipeline.

RESULTS

The 3 intraosseous CCOC samples harbored EWSR1 translocations. Two of these were EWSR1-ATF1 fusions, and 1 was an EWSR1-CREM fusion. An EWSR1-ATF1 fusion was also detected in the clear cell carcinoma of the salivary gland. The Ewing sarcoma sample contained an EWSR1-FLI1 translocation (Figure 1).

Analysis of RNA-seq data further showed that the CCOC fusion junctions involved exons 12 and 3 of EWSR1 and ATF1, respectively, or exons 10 and 7 of EWSR1 and CREM, respectively (Figure 1). This result is similar to fusions of EWSR1 to CREB family members described in other tumor types, such as clear cell sarcoma.

DISCUSSION

Our results, which are consistent with those of other investigators, show that CCOC appears to be driven by translocations that produce fusions between EWSR1 and CREB family transcription factors, including ATF1 and CREM. To our knowledge, this is the second report of intraosseous CCOC with an EWSR1-CREM translocation—the first being reported by Breik et al. in February 2021. These fusions (EWSR1-ATF1 and EWSR1-CREM) are also detected in CCC. If defined by the gene translocation and partner (as is done here) (Figure 1), hematoxylin and eosin (Figure 2), immunohistochemical profile (Figure 2), and presence of clear cells and behavior, then CCOC may be considered the bony counterpart to CCC.

CCOC is a rare jaw tumor that remains poorly understood and represents a diagnostic and therapeutic challenge. Genetically, fusions between EWSR1 and CREB family transcription factors have been suggested as a hallmark of this disease, but the numbers of cases analyzed by molecular diagnostic methods remains limited (n = 20). We have now developed and tested a robust pipeline for the genetic characterization for these tumors by next generation sequencing technologies using targeted RNA-seq.

FISH and quantitative RT-PCR methods have been predominantly used for fusion detection, including for COCC. These methods typically only test for the presence of a single fusion gene and are time-consuming and costly paths to diagnosis. Additionally, they are unable to identify novel fusion gene partners or resolve complex structural rearrangements. For example, documented negative results in the literature may
be the consequence of nontested or novel fusion genes and isoforms.2

Of the 17 COCC specimens that have been tested (and results published in the literature) for \textit{EWSR1} translocation using these techniques, 6 remain with an unknown rearrangement location and/or fusion partner. Five specimens were negative for an \textit{EWSR1} translocation.11-15 This result may be due to the limitations of FISH and RT-PCR.

RNA-seq can address many of these limitations by providing genome-wide surveillance of fusion genes with nucleotide-level resolution of fusion junctions. However, due to the sheer size of the transcriptome, RNA-seq suffers from poor sensitivity for detecting fusion genes that are less highly expressed.2,3

Targeted RNA-seq is a highly sensitive method for selecting and sequencing specific transcripts of interest. It offers both quantitative and qualitative information. Targeted RNA-seq can be achieved via either enrichment or amplicon-based approaches, both of which enable gene expression analysis in a focused set of genes of interest. For example, targeted RNA-seq can be used for the identification of oncogenic driver mutations from low-quality RNA. Enrichment assays also provide the ability to detect both known and novel gene fusion partners in many sample types, including FFPE tissue. In this study, targeted RNA-seq was applied as developed by Zheng et al.3 and detected the \textit{EWSR1-CREM} fusion.

In 2014, Zheng et al.3 described a rapid target enrichment method for next-generation sequencing, termed anchored multiplex PCR (AMP), that is compatible with low nucleic acid input from FFPE specimens. AMP is an effective technique in detecting gene rearrangements when the fusion partners are unknown (as in rare tumors, such as CCOC). Zheng et al.3 performed validation of the gene rearrangement panel using 319 FFPE samples and showed 100% sensitivity (95% confidence limit: 96.5%-100%) and 100% specificity (95% confidence limit: 99.3%-100%) compared with reference assays.

---

**Fig. 1.** Schematic overview of 4 \textit{EWSR1} fusion positive cases with breakpoint location and fusion partner. All cases fuse the N-terminal transcriptional activation domain (TAD, light blue boxes) of \textit{EWSR1} to a partner with a DNA binding domain (BLZ, basic leucine zipper domain, light red). The red line indicates the fusion breakpoint. Numbers in parenthesis indicate protein positions. The green box indicates a methionine site in ATF1 Exon 2. *\textit{EWSR1} exon numbering corresponds to transcript NM_138986 except case 3, which uses NM_1163287 and is therefore annotated as Exon 9b.*
Furthermore, they demonstrated that AMP is a scalable and efficient next-generation sequencing target enrichment method for research and clinical applications. Our findings further support the results obtained by Zheng et al.3

Our results show that targeted RNA-seq is an effective methodology for the detection of fusions in FFPE samples of the rare tumors CCOC and CCC. This information is important in disease entities in which availability of tissue is limited due to the rarity of the tumor. Procurement of fresh tissue for analysis and study is exceedingly difficult for rare tumors.

In addition to the more common EWSR1-ATF1 fusion, another EWSR1-CREM fusion was detected. A similar result was recently reported by Breik et al. (February 2021) also using a next-generation sequencing methodology.1-3 This finding highlights the fact that broad coverage fusion partners and the possibility of detecting novel fusions by RNA-seq are important for the analysis of rare tumors such as CCOC, in which the frequency of fusion partners is not fully established.

Finally, in this study, a sample of clear cell carcinoma of the salivary gland was evaluated using targeted RNA-seq and was found to harbor a EWSR1-ATF1 fusion. This finding further supports the hypothesis that hyalinizing clear cell carcinoma is the soft tissue counterpart of the intraosseous CCOC.5,14

We are in the process of collaborating with colleagues worldwide to analyze paraffin-embedded specimens of CCOC for detection of fusions using this RNA-seq technology. This information will add to the knowledge of this rare tumor. A better understanding of CCOC will allow for the guidance of more appropriate treatments.

**CONCLUSIONS**

Our results show that targeted RNA-seq is an effective methodology for the detection of EWSR1 fusions in FFPE CCOC samples. Furthermore, we have demonstrated that EWSR1-ATF1 and EWSR1-CREM fusions commonly occur in CCOC.

**ACKNOWLEDGMENTS**

We acknowledge Dr. Fernando P.S. Guastaldi for his help in the initial writing of the IRBs.

**FUNDING**

This study was funded in part by the Bertarelli Foundation (Gstaad, Switzerland), the Bertarelli Fund for Advancing Innovative Research Tumors (MJT, MNR, CMR), the Bertarelli Rare Cancers Fund (MNR, AJI, MJT), and the Walter C. Guralnick Fund (Haseotes-Bentas Foundation, Boston, MA, USA). MNR is supported by the Thomas F. and Diana L. Ryan MGH Research Scholar Award.
REFERENCES