**Abstract**

Biphenotypic sinonasal sarcoma (BSNS) is a rare, low grade spindle cell sarcoma, recently recognized in the WHO classification of head and neck tumors, which is characterized by a dual myogenic and neural differentiation and recurrent gene fusions, often involving PAX3-MAML3, and less commonly PAX3 fusions with other partners such as NCOA1, NCOA2, or WWTR1. Yet, in about 4% of tumors no gene rearrangements are identified. Herein, we describe a *RREB1-MKL2* fusion in a BSNS lesion occurring in a 73-year-old female patient with a right maxillo-ethmoidal angle lesion. The polypoid, moderately cellular tumor with infiltrative submucosal growth was composed of fascicles of relatively bland spindle cells embedded in a loose collagenous matrix. The tumor cells showed moderate amounts of eosinophilic cytoplasm with indistinct borders and uniform, pale, ovoid to slender nuclei. The slowly proliferating neoplastic cells co-expressed smooth muscle actin and S100, and showed focal nuclear positivity for β-catenin, while lacking staining for cytokeratins, desmin, myogenin, caldesmon, glial fibrillary acid protein, and SOX-10. Molecular analysis by targeted RNA-based next-generation sequencing identified an in-frame fusion between exon 8 of *RREB1* and exon 11 of *MKL2*, a genetic event that was reported to be a molecular hallmark of ectomesenchymal chondromyxoid tumor. Gene rearrangements in both genes were independently verified by fluorescence in situ hybridization (FISH). To evaluate its recurrent potential an additional group of 15 fusion negative BSNS were tested for abnormalities in *RREB1* and *MKL2* genes by FISH, but no additional positive cases were identified.

**KEYWORDS**
biphenotypic sinonasal sarcoma, gene fusion, MKL2, RREB1
INTRODUCTION

Biphenotypic sinonasal sarcoma (BSNS), an anatomically restricted low-grade sarcoma with neural and myogenic phenotype, was introduced in the recent WHO classification of head and neck tumors. This tumor entity was first described by Lewis et al in 2012 as low-grade sinonasal sarcoma with neural and myogenic differentiation, and renamed subsequently by the same group as BSNS.

Most BSNS are characterized by recurrent gene rearrangements involving the PAX3 gene, commonly fused to MAML3, a coactivator of the NOTCH signaling pathway, present in approximately 70%. Other rare fusion partners include FOXO1, NCOA1, NCOA2, and WVRT1. In about 4% of BSNS no gene fusions can be detected.

In this study, we describe the case of a female patient with a histologically typical BSNS which showed a RREB1-MKL2 fusion that has not been previously described in this diagnosis. The RREB1-MKL2 fusion represents the genetic hallmark of ectomesenchymal chondromyxoid tumor, a rare benign lesion located in the glossal and extraglottal regions. However, the RREB1-MKL2 fusion has also been reported in a case of oropharyngeal sarcoma with dual neural and myogenic differentiation. Our case raises the hypothesis of a pathogenetic relationship between at least a rare subset of BSNS and the biphenotypic oropharyngeal sarcoma, which may belong to a spectrum of biphenotypic sarcomas of the head and neck region.

METHODS AND RESULTS

INDEX PATIENT

A 73-year-old female presented with moderate nasal airway obstruction, more pronounced on the right side. Endoscopy rhinoscopy revealed a septal deviation to the left and a rounded, yellow mass within the right middle nasal meatus. Tympanic membranes showed some neglectable scars as relics of recurrent otitis media in the childhood. A computed tomography showed a unilateral, rather sharply demarcated mass measuring 3.5 cm within the right maxillo-ethmoidal angle; the origin of the lesion was identified to be the posterior third of the right middle turbinate (Figure 1A,B). Concomitant chronic sinusitis caused a slight opacification of the adjacent dorsal ethmoidal cells. Multifocal positivity was also noted with CD34 (Figure 1H) and EMA, while rare cells showed nuclear ß-catenin expression. H3K27-me3 expression was retained (Figure 1I). Tumor was negative for cytokeratins (AE1/AE3; Figure 1J), desmin (Figure 1K), myogenin, GFAP, and STAT6. A Ki67 proliferation index accounted for less than 5% (Figure 1L).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY OF THE INDEX CASE

Microscopic examination of the initial resection specimen showed a moderately cellular, polypoid mesenchymal tumor with infiltrative submucosal growth and mild hyperplasia of the surface respiratory epithelium (Figure 1C). The tumor was composed of dense fascicles of relatively monotonous spindle cells, embedded in a collagenous stroma (Figure 1D). The tumor cells showed moderate amounts of eosinophilic cytoplasm with indistinct borders and uniform, ovoid to slender nuclei with open chromatin (Figure 1E). The mitotic activity was low with none to one mitosis per 10 high-power fields. Immunohistochemically, the neoplastic cells were diffusely positive for S100 (Figure 1F) and α-smooth muscle-actin (Figure 1G). Multifocal positivity was also noted with CD34 (Figure 1H) and EMA, while rare cells showed nuclear ß-catenin expression. H3K27-me3 expression was retained (Figure 1I). Tumor was negative for cytokeratins (AE1/AE3; Figure 1J), desmin (Figure 1K), myogenin, GFAP, and STAT6. A Ki67 proliferation index accounted for less than 5% (Figure 1L).

RNA SEQUENCING OF THE INDEX CASE

Next generation sequencing on the IonTorrent GeneStudio S5XL/Prime platform revealed an in-frame fusion involving RREB1 exon 8 (NM_001003698.3) and MKL2 exon 11 (NM_014048.3) (Figure 2).

FLUORESCENCE IN SITU HYBRIDIZATION

Fluorescence in situ hybridization (FISH) for RREB1 and MKL2 was performed using standard methods as previously reported. Briefly, custom bacterial artificial chromosome (BAC) clone probes were designed to flank the target genes based on the UCSC genome browser (http://genome.ucsc.edu), and obtained from BACPAC sources of Children’s Hospital of Oakland Research Institute (Oakland, CA: http://bapac.choir.org). DNA from each BAC was isolated and then labeled with fluorochromes by nick translation. The slides were deparaffinized, pretreated, and then hybridized with the denatured probes. Following an overnight incubation, the slides were rinsed, stained with 4',6-diamidino-2-phenylindole, mounted, and examined using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems). A positive score was interpreted when at least 20% of the nuclei showed a break apart signal. Nuclei with incomplete sets of signals were omitted from the score.

In keeping with the RNA sequencing results, the index case showed the presence of both RREB1 and MKL2 gene rearrangements by FISH (Figure 3).

SCREENING ADDITIONAL BSNS CASES FOR RREB1 AND MKL2 GENE ABNORMALITIES

An additional group of 15 cases of BSNS with classic morphology, immunohistochemistry and clinical presentation, lacking known gene
rearrangements in PAX3 or MAML3 genes, were retrieved from the personal consultation files of one of the authors (CRA). Each case was re-evaluated to confirm the diagnosis according to well-defined pathologic criteria. FISH studies were performed on these 15 additional BSNS cases, however, none showed abnormalities in RREB1 or MKL2 genes by FISH. The study was approved by the Institutional Review Board.

3 | DISCUSSION

Sarcomas of the head and neck are rare, accounting for approximately 5% to 10% of sarcomas and 1% to 3% of malignant head and neck tumors in adults. They comprise a heterogeneous group of mostly aggressive mesenchymal malignancies which present a considerable diagnostic and therapeutic challenge.
BSNS is a recently recognized tumor entity of the head and neck belonging to the steadily growing group of translocation-associated sarcomas and, in contrast to most head and neck sarcomas, behaving clinically relatively indolent. They are characterized by slowly progressive growth and typically involve multiple sites in the sinonasal tract, especially the superior aspect of the nasal cavity, and the ethmoid sinus, but may also extend to the orbit or cribiform plate. The recurrence rate is approximately 32%, and up to now, metastatic disease has not been described in BSNS. The only death attributable to this tumor entity was observed in a patient with two recurrences, due to persistent intracranial tumor. The tumor predominantly affects females, with a female-to-male ratio of approximately 1.9:1, and the reported patient age range is 24-87 years with a median in the fifth decade.

BSNS is infiltrative and composed of hypercellular fascicles of monotonous spindle cells, frequently with a herringbone pattern. The tumor cells show indistinct borders with moderate amounts of eosinophilic cytoplasm and uniform, pale, and slender nuclei. Mitotic figures are sparse, and necrosis is absent. Most cases show reactive hyperplasia of surface respiratory or squamous epithelium, and entrapped...
epithelial elements are common. Hemangiopericytoma-like (“staghorn”) vessels are frequently found.\textsuperscript{2,4,15,16} Focal rhabdomyoblastic differentiation may be present.\textsuperscript{2,4,15,16} The tumors exhibit immunoreactivity for smooth muscle actin, S100, and variable staining for nuclear β-catenin; in cases with rhabdomyoblastic differentiation, focal desmin, MyoD1 and, to a lesser degree, myogenin staining is present.\textsuperscript{3,4,6,15-17} SOX10 staining is absent.\textsuperscript{4,17}

The overwhelming majority of BSNS are characterized by gene fusions involving PAX3, most frequently with MAML3 and, in rarer instances, with FOXO1, NCOA1, NCOA2, and WWTR1 as fusion partners.\textsuperscript{3-7} Due to common PAX3-related fusions, BSNS show immunopositivity for PAX3 and co-express PAX8 due to cross-reactivity with other paired box transcription factor family members.\textsuperscript{18}

The PAX3 protein is a member of the PAX family of transcription factors. It is expressed during development of skeletal muscle, central nervous system, and neural crest derivatives, and regulates expression of target genes that impact on proliferation, survival, differentiation, and motility in these lineages.\textsuperscript{19} In alveolar rhabdomyosarcomas, the PAX3-FOXO1 gene fusion, which results from the translocation t(12;13) (q36;q14), generates a potent transcription factor, and also results in high-level expression of the PAX3-FOXO1 fusion protein.\textsuperscript{19}

The sequence of the PAX3-MAML3 fusion in BSNS predicted a PAX3-MAML3 chimeric protein consisting of the highly conserved paired-box DNA-binding domain and the paired-type homeodomain of PAX3 fused to the transactivation domain of MAML3, and it was shown that the PAX3-MAML3 fused BSNS showed altered expression of several genes and signaling networks involved in neural crest, skeletal system and general embryonic development.\textsuperscript{3} These observations suggested that the phenotype of most BSNS might be modulated by PAX3-MAML3 fusion.

RREB1 (RAS-responsive element binding protein 1), located on chromosome 6q24.3, is an alternatively spliced transcription factor implicated in RAS signaling, chromatin modification, and various tumors including, for example, pancreatic and colorectal adenocarcinomas, medullary thyroid carcinomas, and malignant melanomas (reviewed in Ref. 20). Furthermore, it was shown to bind the p53 promoter and transactivate p53 expression on DNA damage in osteosarcoma cells.\textsuperscript{21} Recently, RREB1 was identified as the key molecular integrator of Ras and TGF-β signals to induce epithelial-to-mesenchymal transitions.\textsuperscript{22}

MKL2 (myocardin like 2), also known as MRTFB (myocardin-related transcription factor B), located on chromosome 16p13.12, is a transcription factor that is involved in smooth and skeletal muscle differentiation, and neural development.\textsuperscript{23,24} In addition, MRTFB (MKL2) was shown to be a human colorectal cancer tumor-suppressor gene that functions in part by inhibiting cell invasion and migration.\textsuperscript{25} Together with MKL1 (MRTFA, MAL) and MYOC (myocardin) it belongs to the MRTF (myocardin-related transcription factors) protein family.\textsuperscript{23-25}

The RREB1-MKL2 fusion has not only been detected in 90% of ectomesenchymal chondroid tumors (ECMT), all located in the tongue,\textsuperscript{8} but also in a biphenotypic “oropharyngeal” sarcoma.\textsuperscript{10} Interestingly, Siegfried et al\textsuperscript{10} showed in their case of biphenotypic “oropharyngeal” sarcoma that the RREB1-MKL2 chimeric transcription factor encoded by this fusion gene produced an increase in MKL2 expression, thus mimicking the role of PAX3 in BSNS tumorigenesis. Just recently, a RREB1-MKL2 fusion transcript was described in two cases of mesenchymal tumors involving the mediastinum.\textsuperscript{26} These data suggest a potential role of RREB1 and MKL2 activation in oncogenesis leading to effects in the corresponding downstream pathways.

Given the broad spectrum of differential diagnosis, which ranges from cellular schwannomas, solitary fibrous tumors and sinonasal glomangiopericytomas, to highly aggressive sarcomas such as synovial sarcoma, a molecular genetic verification of BSNS should be attempted, albeit the co-expression of myogenic and neural markers in a cytomorphologically rather bland spindle cell tumor of the sinonasal tract is a strong clue to the diagnosis of a BSNS. The detection of a RREB1-MKL2 fusion transcript in the current case suggests that molecular analysis for rearrangements of the RREB1 and/or the MKL2 genes, which was previously reported to be a genetic feature in ECMT,\textsuperscript{8} or for alternative gene fusions should be performed in presumed BSNS without detectable PAX3 or MAML3 rearrangements. Our data are in line with the notion that fusions, which are highly prevalent or even pathognomonic in a specific histological setting, can occur outside their primary histological context as evidenced, for example, for ALK\textsuperscript{27} or NTRK\textsuperscript{28} gene fusions. In the future, more comprehensive studies are warranted to unravel the relation between the spectrum of tumor types primarily identified by histological features and RREB1-MKL2 fusions.

In summary, we report a case of spindle cell sinonasal sarcoma with an RREB1-MKL2 gene fusion. Our data suggest that this is a BSNS with an RREB1-MKL2 fusion known to be highly prevalent in ectomesenchymal chondromyxoid tumor.

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CONFLICT OF INTEREST

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

DATA AVAILABILITY STATEMENT

Additional data will be made available upon reasonable request.

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