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RESEARCH ARTICLE

A morphologic and molecular reappraisal of myoepithelial tumors of soft tissue, bone, and viscera with *EWSR1* and *FUS* gene rearrangements

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Abstract

Myoepithelial tumors (MET) represent a clinicopathologically heterogeneous group of tumors, ranging from benign to highly aggressive lesions. Although MET arising in soft tissue, bone, or viscera share morphologic and immunophenotypic overlap with their salivary gland and cutaneous counterparts, there is still controversy regarding their genetic relationship. Half of MET of soft tissue and bone harbor *EWSR1* or *FUS* related fusions, while MET arising in the salivary gland and skin often show *PLAG1* and *HMGA2* gene rearrangements. Regardless of the site of origin, the gold standard in diagnosing a MET relies on demonstrating its “myoepithelial immunophenotype” of positivity for EMA/CK and S100 protein or GFAP. However, the morphologic spectrum of MET in soft tissue and bone is quite broad and the above immunoprofile is nonspecific, being shared by other pathogenetically unrelated neoplasms. Moreover, rare MET lack a diagnostic immunoprofile but shows instead the characteristic gene fusions. In this study, we analyzed a large cohort of 66 MET with *EWSR1* and *FUS* gene rearrangements spanning various clinical presentations, to better define their morphologic spectrum and establish relevant pathologic-molecular correlations. Genetic analysis was carried out by FISH for *EWSR1/FUS* rearrangements and potential partners, and/or by targeted RNA sequencing. Then, 82% showed *EWSR1* rearrangement, while 18% had *FUS* abnormalities. *EWSR1-POU5F1* occurred with predilection in malignant MET in children and young adults and these tumors had nested epithelioid morphology and clear cytoplasm. In contrast, *EWSR1/FUS-PBX1/3* fusions were associated with benign and sclerotic spindle cell morphology. Tumors with *EWSR1-KLF17* showed chordoma-like morphology. Our results demonstrate striking morphologic-molecular correlations in MET of bone, soft tissue and viscera, which might have implications in their clinical behavior.

KEYWORDS

EWSR1, *FUS*, myoepithelial tumors, *PBX1*, *PBX3*, *POU5F1*

1 | INTRODUCTION

The spectrum of myoepithelial tumors (MET) represents a family of lesions with variable terminology, which based on anatomic location and the presence of ductal structures are designated as pleomorphic adenoma in the salivary gland and benign mixed tumor in the skin or soft tissue.^{1,2} Tumors lacking ducts or an overt biphasic epithelial-myoepithelial phenotype are often designated as myoepithelioma or myoepithelial tumor at various sites, being composed of pure populations of epithelioid or spindle cells embedded in variable stromal components. Moreover, the MET arising in soft tissue and bone lack a corresponding cell of origin, compared to the lesions from salivary gland or skin, which appear to relate to the normal myoepithelial cells surrounding tubulo-acinar glandular structures.³ Furthermore, significant differences exist between assessing the risk of malignancy of MET at various anatomic sites; salivary gland malignant MET are defined based on the extent of capsular invasion,^{4,5} while malignancy in soft tissue MET relates to nuclear pleomorphism and mitotic activity.¹ To complicate things further, the emerging genetic signatures of MET from different sites have suggested a dichotomy of molecular events, with *PLAG1* and *HMGA2* gene abnormalities being prevalent in salivary gland and skin,^{6,7} while in bone, soft tissue and other viscera recurrent *EWSR1* and *FUS* related gene fusions are detected in half of MET.⁸ Despite this conflicting evidence toward a unified family of MET, the current diagnostic criteria have been rather homogeneous across different clinical presentations and molecular phenotypes, relying on demonstrating the so-called “myoepithelial immunophenotype”. This immunoprofile consists of co-expression of cytokeratins (CK) or epithelial membrane antigen (EMA) in combination with S100 protein, calponin or glial fibrillary acidic protein (GFAP). If in the salivary gland location these diagnostic criteria appear reliable, in soft tissue and bone MET the above immunoprofile is far from specific, encompassing other look-alike mesenchymal neoplasms.^{2,9} Furthermore, the pathogenetic relationship between the fusion-positive and fusion-negative cohorts of MET sharing similar histology and immunoprofile has not been yet elucidated. It is possible that some of the molecularly negative cases might represent alternative diagnoses, which may be inappropriately lumped together based on histologic or immunohistochemical grounds.

In this study, we evaluate the clinicopathologic features of a large cohort of MET selected based on their positive gene fusion signature, spanning a broad spectrum of morphologies, and clinical presentations. We further sought to investigate whether fusion type correlates with histologic features, myoepithelial marker expression, and histologic grade.

2 | MATERIALS AND METHODS

We retrieved 66 MET with *EWSR1* and *FUS* gene rearrangements from the consultation files of the authors (CRA, CDF, and BD), 30 of which were included in earlier publications.^{8,10,11} The molecular results were obtained by FISH in 50 cases. In 13 cases, the molecular diagnosis

was established by one of the following platforms of targeted sequencing, including nine cases using targeted RNA sequencing, two cases by Archer, and two cases being tested by Foundation One. In three cases, the diagnosis of a MET was confirmed by conventional karyotyping results. When material was available, FISH was also performed to confirm the RNA sequencing or karyotype results.

Clinical data, including age, gender, and anatomic site, as well as IHC results, were retrieved from pathology reports. Hematoxylin and eosin-stained slides from resection specimens were re-reviewed by two of us (A.S., C.R.A.). For each tumor, special emphasis was put on the cytomorphology of myoepithelial cells (epithelioid, plasmacytoid, spindle, and small cell), histologic growth pattern (solid sheets, nests, reticular pattern, and fascicular growth) and the presence of stromal changes (myxoid, myxohyaline, and sclerotic).^{3,12} Criteria for malignancy included moderate to severe nuclear atypia (nuclear enlargement and hyperchromasia), easily discerned prominent nucleoli, and mitotic activity, typically defined as ≥ 5 MF/10 HPFs.^{1,12} In most cases immunohistochemical stains for CK, EMA, S100, and GFAP were available for review, however, more than half of the cases had a much wider spectrum of immunostains performed, including markers for smooth muscle differentiation such as calponin, SMA, and so forth. As the four immunostains mentioned above are the most sensitive markers defining myoepithelial phenotype in soft tissue and bone anatomic sites, and most cases in the current series had these results available, we only included these markers. Although most cases fulfilled the previous pre-requisite criteria of a positive “myoepithelial immunoprofile”, the few cases that fell short of this requirement were not excluded, as our main inclusion criteria was the confirmed molecular abnormalities in *EWSR1* and *FUS* genes. However, cutaneous myoepithelial tumors with *EWSR1-PBX3* fusions diagnostic of syncytial myoepithelioma and benign cutaneous myoepithelial tumors/benign mixed tumors with *PLAG1/HMGA2* gene rearrangements were not included in this investigation.

2.1 | Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was used on FFPE sections for the examination of *EWSR1*, *FUS*, *POU5F1*, *PBX1*, *PBX3*, *ZNF444*, *KLF15*, or *KLF17* gene rearrangements.^{8,10} Tumors were first tested for *EWSR1* gene rearrangements, and if negative were subsequently tested for *FUS* gene abnormalities. MET positive for *EWSR1* and *FUS* rearrangements were further tested to interrogate a potential fusion partner, including *POU5F1*, *PBX1*, *PBX3*, *ZNF444*, *KLF15*, and *KLF17*. If no partner was detected, further FISH testing was performed in most cases to exclude alternative diagnoses, including abnormalities in *FLI1*, *ERG*, *NR4A3*, *ATF1*, *CREB1*, and *CREM* genes. Custom probes made by bacterial artificial chromosomes (BAC) clones flanking the genes of interest according to UCSC genome browser (<http://genome.ucsc.edu>) and obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (Oakland, CA; <http://bacpac.chori.org>). DNA from each BAC was isolated according to the manufacturer's instructions. The BAC clones were labeled with

fluorochromes (fluorescent-labeled dUTPs, Enzo Life Sciences, New York, NY) by nick translation and validated on normal metaphase chromosomes. The 4 μm -thick FFPE slides were deparaffinized, pretreated, and hybridized with denatured probes. After overnight incubation, the slides were washed, stained with 4',6-diamidino-2-phenylindole, mounted with an antifade solution, and then examined on a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany) controlled by Isis 5 software (Metasystems).

2.2 | Targeted RNA sequencing

In eight cases analyzed by targeted RNA sequencing, RNA was extracted from FFPE tissue using Amsbio's ExpressArt FFPE Clear RNA Ready kit (Amsbio LLC, Cambridge, MA). The fragment length was assessed with an RNA 6000 chip on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA-seq libraries were prepared using 20 to 100 ng total RNA with the TruSight RNA Fusion Panel (Illumina, San Diego, CA). Targeted RNA sequencing was performed on an Illumina MiSeq platform. Reads were independently aligned with STAR (version 2.3) against the human reference genome (hg19) and analyzed by STAR-Fusion.

2.3 | Anchored multiplex RNA sequencing (Archer Dx)

The detailed procedure for the two cases studied by Anchored multiplex RNA sequencing assay has been previously described.¹³ In short, unidirectional gene-specific primers were designed to target specific exons in 62 genes known to be involved in oncogenic fusions in solid tumors. In brief, RNA was extracted from formalin-fixed paraffin-embedded (FFPE) specimens, followed by cDNA synthesis and library preparation. Anchored Multiplex polymerase chain reaction amplicons were sequenced on Illumina MiSeq, and the data were analyzed using the Archer software.

3 | RESULTS

The current series included 66 MET which were positive for gene rearrangements or gene fusions often seen in MET. There were 38 females and 28 males, with a wide age range at diagnosis (1-64 years; median age 27 years). About one-third of patients ($n = 21$) were younger than the age of 18, while nine patients were older than 50 years of age. Cases had a wide anatomic distribution, with 48 tumors arising in diverse soft tissue locations, of which 30 were extremity-based, seven occurred in the trunk, and five located in the head and neck. Twelve tumors presented as primary bone tumors, of which six originated in long bones, two in pelvic bones, two in the mandible, and one each in the vertebral body and navicular bone. Six tumors were located in visceral organs, including four in the lung, and one each in the kidney and urinary bladder.

By FISH, targeted RNA sequencing, or cytogenetics, *EWSR1* rearrangement was found in the large majority of MET, occurring in 54 (82%) cases, while *FUS* rearrangement was detected in 12 (18%) cases. The *EWSR1* fusion partners included *POU5F1* ($n = 15$, 28%), *PBX3* ($n = 10$, 19%), *PBX1* ($n = 6$, 11%), *ZNF444* ($n = 3$, 6%), *KLF15* ($n = 2$, 4%), and *KLF17* ($n = 1$, 2%). In 17 (31%) cases, no *EWSR1* partner was identified. Among the 12 MET cases with *FUS* gene rearrangements, only two partners were identified, including the most common *KLF17* ($n = 8$, 67%) gene and less frequently *POU5F1* ($n = 2$, 17%; Figure 1). No fusion partner was detected in two MET with *FUS* rearrangements.

Microscopically, the MET lesions included in this study showed a heterogenous morphologic spectrum, often composed of epithelioid to ovoid cells embedded in a variable amount of myxoid or collagen-rich stroma (Figure 2). At low power, the tumors often displayed a multinodular growth pattern, with mostly well-defined, non-encapsulated borders. Rare cases showed a more infiltrative growth pattern within subcutis or muscle. The degree of cellularity varied significantly between cases and between different areas within one lesion, which inversely correlated with the amount of stromal component. Thus, some cases resembled primitive undifferentiated round cell tumors, while others were deceptively bland, hypocellular and markedly fibrotic. Although most tumors had a predominant epithelioid phenotype, arranged in cords, nests, and sheets, at least focal areas of cells with ovoid or short spindle cells with pale eosinophilic cytoplasm organized in vague fascicles were also noted. At higher power, the epithelioid cells showed moderate amounts of pale to densely eosinophilic cytoplasm and round, often eccentric uniform nuclei, with fine chromatin and only a mild to moderate degree of pleomorphism. Infrequent patterns included foci of more basaloid epithelioid cells with scant cytoplasm (15%) and plasmacytoid (10%) morphology. A consistent feature seen in most MET was the presence of

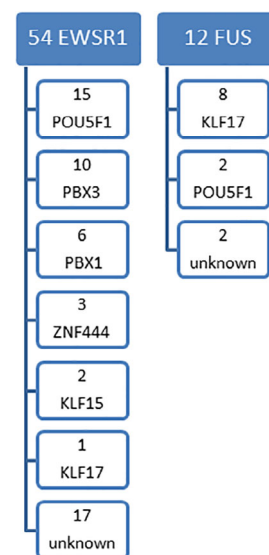


FIGURE 1 Distribution of gene fusions in the present cohort of 66 MET of soft tissue, bone, and visceral organs [Color figure can be viewed at wileyonlinelibrary.com]

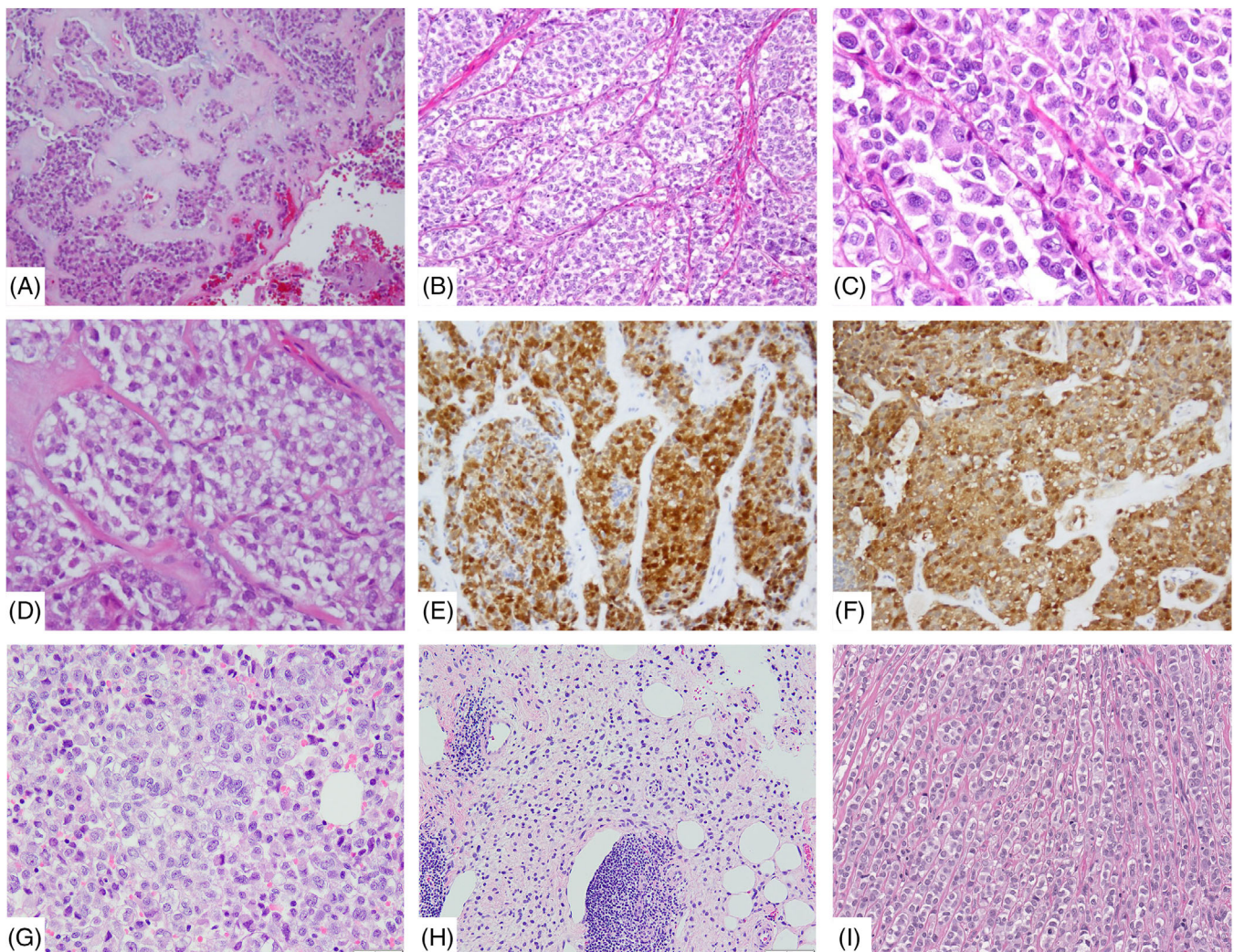


FIGURE 2 Morphologic spectrum of MET with *POU5F1* gene rearrangements. (A-H) MET with *EWSR1-POU5F1* showing at low power a lobulated growth within a prominent myxochondroid stroma (A), often a well-defined nested pattern (B-D), being composed of epithelioid cells with overtly malignant features and lightly eosinophilic cytoplasm (C) or clear cytoplasm (D). These classic examples often show diffuse positivity for cytokeratin (E) and S100 protein (F). Rare examples showed unusual features such as more solid growth of epithelioid cells with scant clear cell cytoplasm (G) or an infiltrative growth pattern within subcutaneous fat, with ill-defined cell borders (H). Rare *FUS-POU5F1* positive cases showed epithelioid morphology with light eosinophilic to clear cytoplasm organized in nests or linear arrangements (I) [Color figure can be viewed at wileyonlinelibrary.com]

a prominent but variable extracellular matrix, alternating from densely sclerotic, hyalinized, fibromyxoid, myxochondroid, or purely myxoid. None of the lesions displayed ducts or glandular structures. Notably, several of these histologic patterns were associated with certain gene fusions; these phenotype-genotype correlations are summarized below. In this series, the number of malignant MET ($n = 30$) was only slightly smaller than the MET with benign histology ($n = 36$), undoubtedly reflecting referral bias of diagnostically challenging malignant examples. Typical features of malignancy were moderate to severe cytonuclear atypia and prominent nucleoli, whereas mitoses were often $\geq 5/10$ HPFs. Necrosis was only rarely observed, almost exclusively in tumors with undifferentiated morphology. The majority of tumors ($n = 51$; 81%) expressed S100 or GFAP in combination with EMA or cytokeratin. Six (10%) MET expressed either only epithelial

markers or only S100, whereas six MET lacked all four myoepithelial markers. In three cases, the IHC data were not available.

3.1 | *EWSR1-POU5F1* fusions are associated with malignant MET with nested epithelioid growth with clear cell morphology

There were 15 MET with *EWSR1-POU5F1* fusions and two harboring *FUS-POU5F1* fusions (Table S1). These 17 tumors occurred in 10 females and 7 male patients, with an age range of 3 to 49 years (median age 26 years). Tumors with *EWSR1-POU5F1* had the following anatomic distribution: 11 were located in soft tissue (all in the extremities), two in the bone (mandible and pelvic bone), and in one

case each located in the kidney and vulva. Notably, 11/15 (73%) MET with *EWSR1-POU5F1* fusions had overtly malignant features, which occurred preferentially in children and young adults, nine patients being younger than the age of 30. Morphologically, these 11 malignant MET with *EWSR1-POU5F1* fusions consisted of nests and sheets of epithelioid cells, often with clear cell cytoplasm, showing moderate pleomorphism, prominent nucleoli, and increased mitotic activity, but no necrosis (Figure 2). By IHC, the malignant MET consistently expressed S100 with either EMA or CK, except for one case showing only cytokeratin expression. Four *EWSR1-POU5F1* positive MET were benign, displaying classic histology, with epithelioid cells arranged in a reticular pattern within a myxoid stroma, alternating with a spindle cell component admixed with a variable fibromyxoid stroma. Three of the four benign *EWSR1-POU5F1*-positive MET lacked markers of myoepithelial differentiation (S100, EMA, and CK). The two *FUS-POU5F1*-positive MET cases (arising in the trunk of a 34-year-old female and in the hand of a 31-year-old male, respectively) displayed benign classic histology, with epithelioid and ovoid spindle cells in a collagen-rich stroma.

3.2 | MET with *EWSR1-PBX1/3* fusions are often benign and associated with bland spindle cell and sclerotic morphology

There were 10 MET with *EWSR1-PBX3* and 6 with *EWSR1-PBX1* fusions. These 16 patients had an age range at diagnosis of 2-75 years (median 29 years). Eight tumors arose in extremity soft tissue locations, seven presented as primary bone tumors and one occurred in the lung. In fact, *EWSR1-PBX1/3* fusions were the most common gene fusions in primary skeletal MET, occurring in 7 out of 12 (58%) cases. Most tumors with *EWSR1-PBX1/3* (13/16, 81%) consisted of benign-appearing spindle cells arranged in fascicles, often embedded in hyaline sclerotic stroma (Figure 3). Only 3/16 (19%) of MET cases with *EWSR1-PBX1/3* showed malignant cytologic features. By IHC, 13 tumors showed positivity for EMA and S100 (Figure 3) but lacked cytokeratin expression. Two cases showed only S100 protein positivity, while one case, occurring in the bone, was negative for all markers possibly related to decalcification.

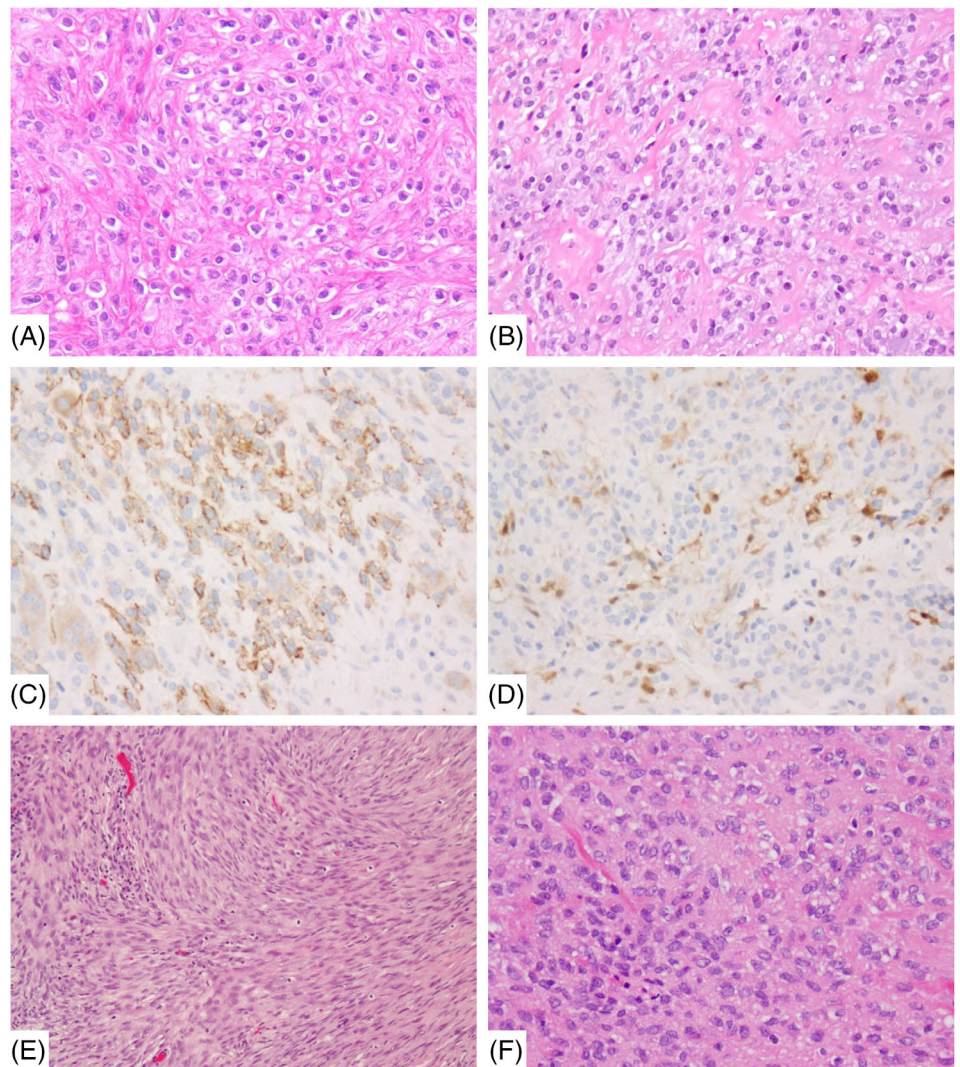


FIGURE 3 Pathologic features of MET harboring *EWSR1-PBX1/3* fusions. Tumors with *EWSR1-PBX1* fusions often show a bland epithelioid to ovoid phenotype, with scant clear cytoplasm, embedded in a delicate fibrous collagenous stroma (A,B). Tumors are frequently positive for EMA (C) and S100 protein (D). MET with *EWSR1-PBX3* fusions displays a more ovoid to spindle cell appearance, with benign histologic features (E,F) [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | *FUS-KLF17* fusions are associated with chordoma-like morphology (so-called parachordoma)

Eight MET harbored *FUS-KLF17* fusions. Patients had an age range at diagnosis of 8–58 years (median 33 years). Five tumors were located in soft tissue, two in the lung or pleura, one in the periosteum of bone, and one intradurally involving the lumbar spine. Microscopically, the predominant pattern seen in 6/8 tumors consisted of radiating cords of epithelioid cells with eosinophilic or clear cytoplasm. The tumors were often embedded in ample myxoid or myxohyaline stroma, reminiscent of chordoma pattern¹⁴ (so-called parachordoma; Figure 4). Three of the cases (37%) were associated with malignant histologic features. By IHC, all tumors were consistently positive for cytokeratins (or EMA) and S100.

3.4 | MET with rare gene fusions (*EWSR1-ZNF444*, *EWSR1-KLF15*, and *EWSR1-KLF17*)

Three MET harbored *EWSR1-ZNF444* fusions. One of the cases which was previously reported,⁸ occurred in a 64-year-old female as a 13 cm malignant pulmonary MET with classic morphologic features and coexpression of cytokeratin and S100. The patient subsequently developed multiple local and distant recurrences over a 10-year follow-up period. Two additional new cases with this fusion occurred as a temporal head mass in a 2-year-old female and as a thigh mass in a 44-year-old female. Both cases had overtly malignant features, with an undifferentiated small blue round cell phenotype, and revealed a nonspecific immunophenotype (negative for S100, EMA, and cytokeratin). Notably, the malignant MET in the 2-year-old resembled a desmoplastic small blue round cell tumor, consisting of small nests of undifferentiated small blue round cells embedded in a loosely arranged spindled stromal cells (Figure 4). The tumor from the 44-year-old from the thigh was initially diagnosed as a Ewing sarcoma with *EWSR1* gene rearrangement, until the Archer results showed a *EWSR1-ZNF444* fusion.

Two MET had *EWSR1-KLF15* fusions. The first case concerned a visceral MET arising in the urinary bladder of a 12-year-old female, which showed highly variable but classic histology and immunoreactivity for S100, EMA, and cytokeratin. The second case was a malignant soft tissue MET arising in the thigh of a 7-year-old male. This tumor had an uncommon biphasic appearance, being composed of reticular cords of epithelioid cells and nests of malignant undifferentiated round cells (Figure 4). By IHC, tumor was positive for S100 and cytokeratin, while negative for EMA.

The single MET with an *EWSR1-KLF17* fusion previously reported represented a benign MET, occurring in the foot of a 20-year-old male.¹¹ Morphologically, it was composed of radiating cords and clusters of epithelioid cells arranged in myxoid and hyalinized stroma. The tumor cells expressed cytokeratin, EMA, and S100.

4 | DISCUSSION

The molecular abnormalities of bone and soft tissue MET have been recently elucidated as harboring *EWSR1/FUS*-related gene fusions in at least half of the cases, involving *EWSR1* and *FUS* with various partner genes encoding for transcription factors, including *POU5F1*, *PBX1*, *PBX3*, *ZNF444*, *KLF15*, and *KLF17*.^{8,10,11,15} Presumably, other novel gene fusions not yet defined might be responsible for the other half of MET lacking *EWSR1/FUS* gene abnormalities. In contrast, MET arising in the skin and salivary gland frequently show distinct *PLAG1* and *HMGA2* related gene fusions.^{6,7} Controversy still remains regarding the pathogenetic link between fusion-positive vs fusion-negative MET, as well as the relationship between MET characterized by either *EWSR1/FUS* or *PLAG1/HMGA2* type fusions. To avert these challenges, the current study focused on MET with *EWSR1* and *FUS* gene rearrangements arising in soft tissue, bone, and viscera, which were characterized in most but not all cases by a classic morphologic appearance and displayed a “myoepithelial” immunophenotype.

In keeping with prior published data, the current study cohort showed a predilection for young adults (median age 27 years) and extremity soft tissue locations (72%), whereas skeletal MET (18%) had a highly variable anatomic distribution in long bones, pelvic bones, and mandible. Although our results reveal a wide morphologic spectrum of architectural and cytologic heterogeneity, common phenotypes emerged, including reticular or trabecular growth patterns with prominent myxoid stroma, or areas of more nested or solid growth and hyalinized stroma.^{1,8,12} Tumor cells ranged from epithelioid, ovoid to short spindled, typically containing uniform nuclei, and eosinophilic to clear cytoplasm. Another common histotype was that of a deceptively bland spindle cell neoplasm associated with a prominent fibrotic stromal component. A small subset of malignant myoepithelial tumors displayed undifferentiated round cell features showing histologic overlap with small blue round cell tumors. By IHC, the majority (81%) of fusion-positive MET expressed cytokeratins (CK) and/or EMA in combination with S100 protein or GFAP, in keeping with the well-established myoepithelial immunoprofile. Among the 12 cases (20%) that did not meet the IHC criteria (either displaying positivity for only one marker or negativity for all), there were four tumors with classic, predominant epithelioid morphology and *EWSR1/FUS-POU5F1* fusions, three tumors with classic predominant spindle cell morphology and *EWSR1-PBX1/3* fusions, and five tumors with undifferentiated round cell morphology, of which one *EWSR1-ZNF444*, two *EWSR1-POU5F1*, and one each showed *EWSR1* or *FUS* rearrangement alone after an exhaustive work-up for all other known gene partners.

Conversely, some of the tumors that were initially diagnosed as MET based on morphologic findings, immunophenotype and/or *EWSR1/FUS* gene rearrangements, were excluded and/or reclassified as other mesothelial or mesenchymal neoplasms based on the subsequent NGS or FISH results of variant *EWSR1* gene fusions, involving other gene partners such as *CREM*, *ATF1*, *FLI1*, and so forth, typically seen in other neoplasms. Our results further emphasize the significant challenges in diagnosing myoepithelial tumors, especially at the

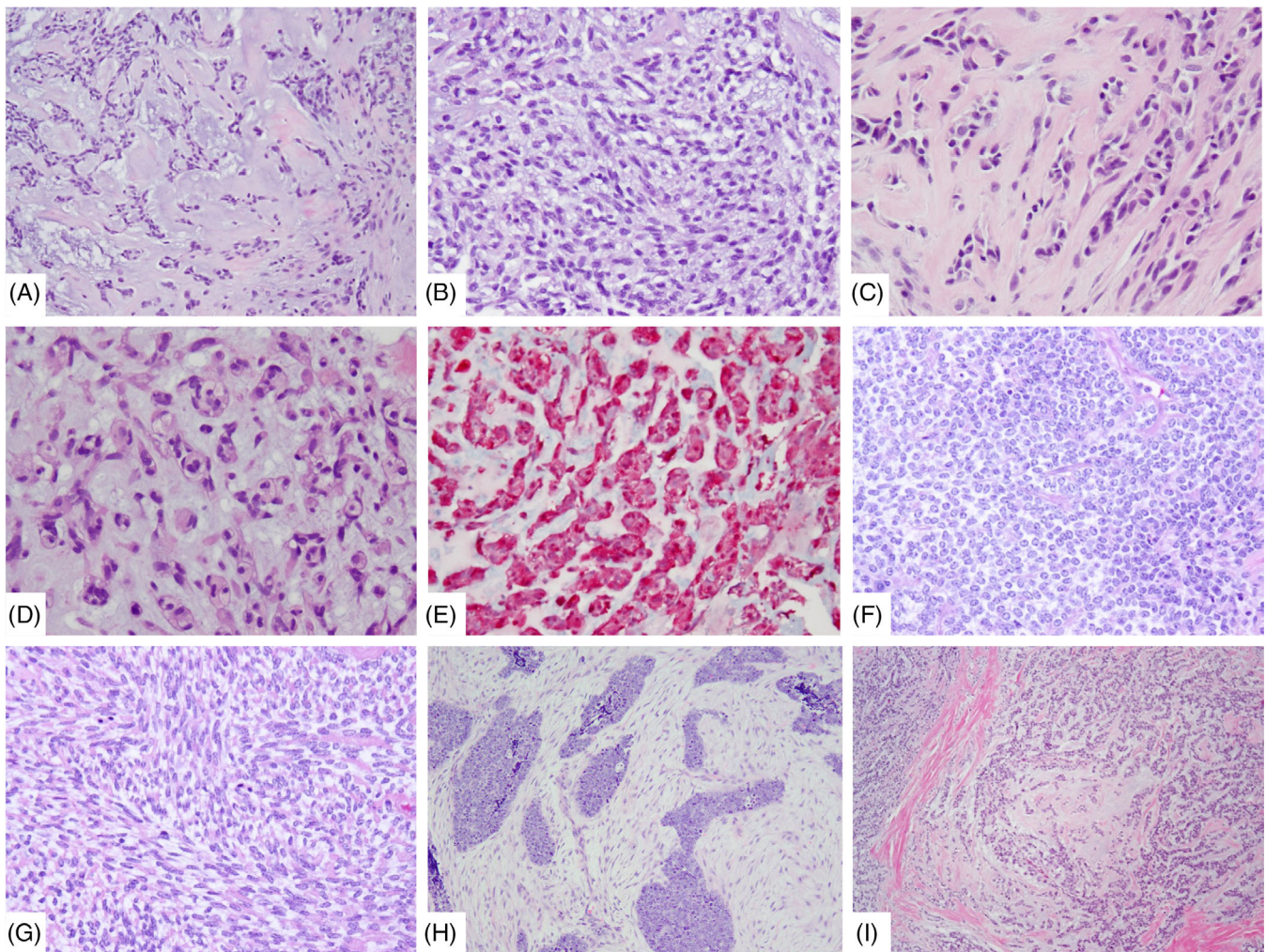


FIGURE 4 Morphologic appearances of rare genetic subtypes of MET. *FUS-KLF17*-positive MET shows often a diffusely myxoid or fibromyxoid stromal component with ovoid to epithelioid cells arranged in cords and a reticular pattern, reminiscent of chordoma phenotype (parachordoma; A-D). These tumors are often S100 protein-positive (E). MET with *EWSR1-ZNF444* are often malignant and may display an undifferentiated round to spindle cell phenotype (F,G), with variable stromal component, ranging from very scant to prominent and desmoplastic (H). A rare tumor with *EWSR1-KLF15* fusions showing cords of epithelioid cells embedded in a variably myxoid or fibromyxoid stroma (I) [Color figure can be viewed at wileyonlinelibrary.com]

malignant/high-grade end of the spectrum, without a comprehensive molecular analysis. A particular pitfall is an overlap with the increasing family of round cell sarcomas, in particular with Ewing sarcoma-like tumors, which now encompass variant morphologies and immunoprofiles.¹⁶ In fact, three tumors occurring in children were initially misinterpreted as Ewing sarcoma at the outside institution based on an *EWSR1* gene rearrangement positive result and treated with Ewing sarcoma regimens.

Although some morphologic-genotypic correlations were observed in our initial molecular study,⁸ our current larger investigation was able to draw more robust associations. First, MET with *EWSR1-POU5F1* fusions represents the most common molecular subset (28%), being prevalent in children or young adults, presenting in the deep soft tissues of the extremities. Morphologically, tumors often displayed nested epithelioid morphology with clear cytoplasm, and thin fibrous septa. The majority of MET with *EWSR1-POU5F1*

fusions (11/15; 73%) showed microscopic features in keeping with malignant behavior. Second, most MET with *EWSR1-PBX1/3* (12/15, 80%) consisted of benign-appearing spindle cells organized in fascicles or embedded in hyaline sclerotic stroma. This subset of pure spindle cell MET expressed S100 and EMA, but typically lacked cytokeratin expression. *PBX1/3* fusions were most prevalent among skeletal MET, found in more than half of bone tumors. Third, chordoma-like morphology (parachordoma) was observed in 6/8 MET with *FUS-KLF17* fusions, of which three had a benign appearance and three had features of malignancy.

In addition, there were three MET with *EWSR1-ZNF444* fusions. Notably, all three tumors had malignant microscopic features. Two cases represented soft tissue tumors with undifferentiated round cell morphology that lacked myoepithelial markers; these presented in the flank of a 44-year-old woman and the temple of a 2-year-old girl. The third case was a visceral lung MET in a 64-year-old woman, which

showed malignant epithelioid histology, expression of CK and S100, and an aggressive clinical behavior with multiple metastatic implants over a 10-year period, as illustrated in a previous publication.⁸

Other rare fusion genes were *EWSR1-KLF15* and *EWSR1-KLF17*. *EWSR1-KLF15* was detected in two MET, both occurring in children, aged 7 and 12 years. The first case concerned a thigh mass with an unusual biphasic malignant phenotype consisting of cords of epithelioid cells and nests of undifferentiated round cells. The second case was a benign visceral MET of the urinary bladder with classic morphology. Both *EWSR1-KLF15* fused MET expressed myoepithelial markers. Three other malignant MET with *EWSR1-KLF15* fusions and myoepithelial marker expression have been described in the literature. Strikingly, like our first case, these three MET had variable biphasic malignant morphology with solid sheets of malignant epithelioid cells and areas with undifferentiated and small blue round cell morphology. One case was a parotid tumor in a 20-year-old woman that had metastasized to the lung,¹⁷ whereas the other two cases were large renal childhood tumors (in girls aged 4 and 6 years) that also developed lung metastases.¹⁸ These data indicate that MET with *EWSR1-KLF15* mainly occurs in children and are strongly associated with undifferentiated round cell morphology and clinically malignant behavior. The single MET with *EWSR1-KLF17* was described in an earlier publication by our group.¹¹ This tumor presented in the foot of a 74-year-old male and showed benign but classic histology.

Several soft tissue and bone tumors with overlapping morphologic features, including trabecular cords of epithelioid cells in myxoid, myxohyaline or sclerotic stroma, and co-expression of S100 and/or CK/EMA, enter the differential diagnosis of MET. One of the closest mimics is extraskeletal myxoid chondrosarcoma (EMC), which shows bland ovoid to epithelioid cells interconnected in trabecular and cribriform networks in an ample myxoid stroma. EMC does not have a specific immunoprofile, but expression of S100 and EMA is found in 20% to 40% of cases, whereas it usually lacks cytokeratin expression.¹⁹ EMC diagnosis relies on the identification of its characteristic *EWSR1-NR4A3* or *TAF15-NR4A3* fusion genes.²⁰ Another group of look-alike tumors are chordoma and chordoma periphericum, which represent true notochordal related neoplasms. Due to striking overlapping morphologic features and immunoprofile (co-expression of CK, EMA, and S100), the distinction between chordomas and soft tissue MET, previously designated as parachordomas, has been problematic in the past. Chordomas typically occur in the axial skeleton, while rare extra-axial tumors have been reported at any site.²¹ It was later recognized that only the true notochordal lesions are positive for the transcription factor brachyury, while MET (parachordomas) are not.²² Ossifying fibromyxoid tumor (OFMT) is another tumor closely resembling the morphology and immunoprofile of MET. OFMTs are typically composed of cords and trabeculae of uniform ovoid cells with bland nuclei set in variable collagenous and myxoid stroma. OFMTs often show a thick fibrous capsule and a peripheral shell of ossification. By IHC, OFMT may show expression of S100 (60%-70%) or desmin (50%), whereas CK and EMA expression is less common.²³ The large majority of OFMT show recurrent *PHF1* gene rearrangements which can confirm the diagnosis in challenging cases.^{24,25} Epithelioid MPNST

(EMPNST) is another rare soft tissue tumor with lobular architecture, cords of epithelioid cells with eosinophilic cytoplasm, within a collagenous stroma. EMPNST typically exhibits diffuse immunostaining for S100 and SOX10, and variably positivity for CK, while showing loss of SMARCB1 expression in the majority of cases.²⁶ However, SOX10 expression and loss of SMARCB1 do not exclude MET. Some of the deceptively bland and fibrotic MET may mimic either sclerosing epithelioid fibrosarcoma (SEF) or a low-grade fibromyxoid sarcoma (LGFMS). A further pitfall can occur if a positive *EWSR1* or *FUS* gene rearrangement is documented. However, MUC4 is usually negative in MET, while it is diffusely and strongly expressed in most SEF (75%) and all LGFMS.²⁷ As our results highlight, 5 (8%) MET cases showed an undifferentiated round cell morphology, closely resembling a round cell sarcoma, including Ewing family of tumors or occasionally desmoplastic round cell tumor. Of interest, the first reported case of an *EWSR1-POU5F1* positive tumor, occurring in the pelvis in a 39-year-old woman, showed an undifferentiated round cell phenotype,²⁸ mimicking a lesion in the Ewing sarcoma family. The challenge also stems from the overlapping *EWSR1/FUS* gene abnormalities detected in both tumor categories. In these cases, establishing their fusion gene partners by additional molecular techniques that complement FISH break-apart assays for *EWSR1* and *FUS* is recommended for a more definitive subclassification.

In conclusion, the histopathologic classification of MET presenting in soft tissue, bone, and visceral organs remains challenging, as these tumors show remarkable heterogeneity in morphology, rather nonspecific IHC marker expression, and high variability in clinical presentation and behavior. In this expanded series of 66 MET, several genotype-phenotype correlations are emerging, which provide diagnostic utility in daily practice and may serve as future roadmap for potential therapeutic target discovery. Of particular importance is the recognition that rare MET subsets with undifferentiated (small blue) round cell morphology may harbor uncommon fusion genes, for example, *EWSR1-ZNF444* or *EWSR1-KLF15*. Our results further show a good but imperfect concordance between the so-called "myoepithelial immunoprofile", used currently as the diagnostic mainstay, and the presence of fusion gene alterations. In fact, 12 (20%) cases lacked this immunoprofile, including seven with classic morphologic features and either characteristic *EWSR1/FUS-POU5F1* or *PBX1/3* related fusions. In contrast, the remaining five cases had an atypical phenotype, composed of undifferentiated round cell morphology, and being associated with variable gene fusions, including *EWSR1-POU5F1* and *EWSR1-ZNF444*. Further studies are needed to establish the relationship of these undifferentiated tumors harboring so-called "myoepithelial gene fusions" with other round cell sarcomas in the family of Ewing sarcoma-like spectrum.

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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