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A Subset of Malignant Mesotheliomas in Young Adults are Associated with Recurrent *EWSR1/FUS-ATF1* Fusions

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Abstract

Malignant mesothelioma (MM) is a rare, aggressive tumor often associated with asbestos exposure and characterized by complex genetic abnormalities, including deletions of chromosome 22. A gene fusion involving *EWSR1* and *YY1* gene on 14q32 has been reported in 2 patients over the age of 60 with peritoneal MM. However, the incidence of *EWSR1* rearrangements in MM and the spectrum of its fusion partners remain unknown. We recently encountered 2 MM cases with *EWSR1-ATF1* fusions and sought to investigate the prevalence and clinicopathologic features associated with this abnormality. As both index cases occurred as intra-abdominal tumors in young adults, we searched our files for pleural and peritoneal MM occurring in adults younger than age of 40. All cases were tested by FISH using custom BAC probes for *EWSR1, FUS* and *ATF1* genes. When available, immunohistochemistry for BAP1 was performed. A total of 25 MM from patients aged 40 or less were screened, either from peritoneum (n = 13) or pleura (n = 12), with a median age of 31 (range 7–40). Two additional *ATF1*-rearranged tumors were identified at pleural and peritoneal sites with *EWSR1* and *FUS* as fusion partners, respectively, for a total of 4 cases (16%, 4/25). The fusion positive cases displayed classic epithelioid morphology, immunoreactivity for cytokeratins and WT1, and negativity for S100. BAP-1 expression was retained in the 3 fusion-positive cases with available material, and in 80% (12/15) of the fusion-negative cases. Our results expand the spectrum of tumor types harboring *EWSR1/FUS-ATF1* gene fusions to include a subgroup of conventional epithelioid MM. Other features of this unique MM subset include young age at presentation, lack of asbestos exposure and retained BAP1 expression.

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https://wwwn.cdc.gov/eworld/Data/Malignant_mesothelioma_Number_of_deaths_by_sex_race_age_group_and_median_age_at_death_US_residents_age_15_and_over_20012010/434

Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Mortality - All COD, Aggregated With County, Total U.S. (1990–2010) - Linked To County Attributes - Total U.S., 1969–2011 Countries.

Keywords

EWSR1; *FUS*; *ATF1*; gene fusion; mesothelioma; young adults

INTRODUCTION

Malignant mesothelioma (MM) is a rare and aggressive tumor of mesothelial lining, being more prevalent in the pleura than peritoneum (1). A causal relationship with occupational exposure to asbestos is well established (2). As most cases occur after long-term exposure to this carcinogen (3), MM patients are typically diagnosed in their 8-th decade of life (median age of 73 years old in USA; www.cdc.gov). However, a small subset of MM occurs in patients without significant asbestos exposure history and a younger age, lacking well-defined risk factors. Germline mutations in *BRCA1 associated protein-1 (BAP1)* predisposing to MM may account for a small portion of these cases (4), and have also been implicated in increasing sensitivity to asbestos carcinogenesis (5).

Due to the low incidence of MM, few comprehensive genomic studies have been performed to date, which have identified abnormalities in a number of cancer related genes, such as: *CDKN2A, NF2, SETD2, TP53, DDX3* and *BAP1* (6, 7). Furthermore, MM were previously shown to exhibit complex chromosomal copy number variations, including frequent losses in chromosomes 1p, 4q, 9p, 13q, 14q and 22q, either by conventional karyotype and comparative genomic hybridization (8). Despite frequent alterations involving chromosome 22, specific gene rearrangements involving *EWSR1* (22q12) have been reported only recently in 2 peritoneal MM, harboring an *EWSR1-YY1* fusion (9).

We recently encountered 2 similar peritoneal MM cases exhibiting *EWSR1* rearrangements, but being fused instead to the *ATF1* gene, and sought to investigate the prevalence and clinicopathologic features associated with this abnormality. As both cases occurred as intra-abdominal tumors in young adults and displayed epithelioid morphology, we searched our files for pleural and peritoneal MM occurring in adults younger than age of 40.

METHODS

Index Cases and Extended Cohort Selection

The first index case was a 21 year-old man who presented with a bulky and widely invasive peritoneal tumor at an outside institution. As the initial clinical diagnostic consideration was a desmoplastic round cell tumor, FISH for *EWSR1* was performed and revealed a break-apart signal, while the RT-PCR was negative for the *EWSR1-WT1* canonical fusion. Available formalin fixed paraffin embedded (FFPE) tissue sections were further investigated for other potential fusion partners of *EWSR1.* Thus, FISH analysis revealed an *ATF1* gene rearrangement.

The second case was that of a 33 year-old female with a peritoneal tumor with histologic features of epithelioid MM, which was investigated by the institutional hybrid-capture based targeted next-generation sequencing assay (MSK-IMPACT) for additional molecular characterization. The results showed an *EWSR1-ATF1* fusion candidate.

To further expand the cohort, we searched the files of Department of Pathology at Memorial Sloan Kettering Cancer Center and personal consultations of CDF and CRA for the diagnosis of MM, in patients under the age 40, with available tissue for molecular analysis, from a two-decade period (1995–2016). Review of Hematoxylin and Eosin (H&E) was done for each case. Previously performed and submitted immunohistochemical stains were reviewed and complementary markers were performed if needed in a subset of cases. Clinical follow-up data were obtained from review of medical charts. The relationship between tumor location and age, gender, smoking status or asbestos exposure was assessed using two-sided Fisher/s exact test. The study was approved by the Institutional Review Board.

Fluorescence In Situ Hybridization

Four μm-thick sections from formalin-fixed paraffin-embedded (FFPE) tissue were prepared to perform FISH applying custom probes using bacterial artificial chromosomes (BAC; Supplementary Table 1), as previously described (10). Probes were designed to cover *EWSR1, FUS, ATF1*, and *SMARCB1*, chosen according to USSC genome browser and obtained from the Children`s Hospital of Oakland Research Institute (CHORI; Oakland, CA, USA; http://bacpac.chori.org). DNA from individual BACs was isolated according to the manufacturer's instructions, labeled with different fluorochromes in a nick translation reaction, denatured, and hybridized to pretreated slides. Slides were then incubated, washed, and mounted with DAPI in an antifade solution. The genomic location of each BAC set was verified by hybridizing them to normal metaphase chromosomes. Scoring was performed on two hundred successive nuclei using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Newton, MA). At least 20% of the nuclei showing a break-apart signal were required to interpret as a positive score, and nuclei with an incomplete set of signals were omitted.

All cases were first tested for *EWSR1* gene abnormalities. The *EWSR1*-rearranged tumors were then evaluated for break-apart signal using the *ATF1* BACs. The *EWSR1* negative tumors were then tested for *FUS* break-apart, as *FUS* gene has previously been shown to substitute for *EWSR1* in gene fusions involving members of the CREB transcription factors, including ATF1 (11, 12). As deletions of 22q have been reported as a common finding in MM, we have also examined abnormalities of this region by FISH using *EWSR1, SMARCB1* and a 22q11 reference (RP11–960P211 and RP11–81B3), as previously described (13). A monosomy pattern (or large deletion) was defined if one allele copy of both *EWSR1* and *SMARCB1* genes were lost, with a ratio of 1:1. In MM2 index case, FISH fusion assay was also performed to confirm the association of the *EWSR1* and *ATF1* genes. For the FISH fusion assay the BAC probes were labeled as follows: centromeric EWSR1 (red) and telomeric ATF1 (green). A positive result was interpreted when the red and green signals came-together as one signal (yellow).

Next-Generation Sequencing

One patient was consented for molecular testing with the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT). This hybridization capture-based next-generation sequencing (NGS) assay detects somatic single

nucleotide mutations, small indels, copy number alterations and selected structural variants in several cancer-related genes (410 for the version used) (14). Briefly, DNA extracted from FFPE tumor sample was prepared in libraries and sequenced on an Illumina HiSeq2500, using patient`s blood DNA as a reference to ensure the somatic nature of the variant calls.

Immunohistochemistry

When FFPE material was available, IHC was performed on FFPE tissue sections using a fully automated system (Benchmark ULTRA; Ventana Medical Systems, Tucson, AZ). The following antibodies were used: BAP1 (Santa Cruz, 1:50), Calretinin (Ventana, SP65), Cytokeratin-Pan (DAKO, M3515, 1:1600), CK5/6 (Ventana, 790–4554), Desmin (Ventana, 760–2513), EMA (Ventana, E29), ER (Leica, 6F11), Myogenin (Cell Marque, 760–2832), OCT4 (Cell Marque, 760–4392), PAX8 (Proteintech, 10336-I-AP, 1:100), S100 (Z0311, 1:8000, Dako), Vimentin (Ventana, 790–2917), and WT-1 (Leica, PA0562).

RESULTS

Clinico-pathologic and Molecular Findings of Index Cases

The first index case (MM1) was a 21 year-old man, who presented with symptoms of acute appendicitis, and upon resection showed an extensive peritoneal tumor, infiltrating omentum and involving loco-regional lymph nodes. Morphologically, the tumor showed sheets of epithelioid cells with focally papillary architecture, abundant eosinophilic cytoplasm, small nuclei with well-defined borders, open chromatin and inconspicuous nucleoli (Fig. 1A–C). Psammoma bodies were present in moderate number (Fig. 1B). On immunostaining, the tumor revealed diffuse positivity for AE1/AE3, EMA and WT-1, focal positivity for calretinin, but negativity for PAX8, Vimentin, OCT3/4 and S100. FISH studies performed revealed *EWSR1* break-apart signal and a concurrent gene rearrangement of *ATF1* (Fig. 2).

The second case (MM2) occurred in a 33 year-old female with a mesenteric tumor and retroperitoneal lymphadenopathy. Microscopically, the tumor showed typical features of an epithelioid MM, with predominantly solid sheets of eosinophilic cells with focal gland-like and papillary structures (Fig. 1 D–E). The tumor cells exhibited immunostaining for AE1/AE3, CK5/6, WT-1 (diffuse) and calretinin (focal). A focal area of desmin reactivity was observed in the absence of myogenin staining in the solid epithelioid component (Fig. 1I). MOC-31, PAX8, S100 and SOX10 were negative in the tumor cells. The tumor was subjected to further molecular testing by a hybrid-capture based targeted next-generation sequencing assay (MSK-IMPACT), with a median sequencing coverage of 897X. This revealed fusion of *EWSR1* exons 1–14 (NM_013986) to *ATF1* exons 5–7 (NM_005171) (Fig. 3). No somatic mutations, copy number alterations or other structural variants were identified. The rearrangement of both genes was also confirmed by FISH (Fig. 2C).

Further FISH Screening in Young Patients Identifies an Additional Pleural MM with *EWSR1-ATF1* fusion

As both index cases were identified in young patients, we conducted our screen for additional MM cases in this age population. From 1995–2016, 23 additional cases were identified in patients of age 40 or less, 11 from the peritoneum and 12 from the pleura. The

clinical and pathologic findings of the entire cohort (n = 25) are listed in Table 2. Briefly, the cohort had a median age of 31 years (range 7–40) at diagnosis, with a gender ratio varying based on the anatomic site involved (p = 0.047). Most (77%) peritoneal tumors occurred in males, while only 33% of the thoracic tumors presented in male patients. Among the 16 patients with information available, none had a history of direct asbestos exposure, although 3 patients with fusion-negative tumors had a possible secondary (indirect) exposure through relatives. Smoking history was available for 14 (56%) of patients, with only 5 patients being identified as heavy-smokers.

FISH screening identified an additional positive case (MM3; Fig. 2 A–B) for the *EWSR1-ATF1* fusion. The tumor occurred in a 34-year-old female and originated from the pleura (Table 1). Like the prior 2 index cases, it displayed epithelioid cells with eosinophilic cytoplasm and round nuclei (Fig. 1F). The tumor cells were positive for cytokeratins and WT1, while negative for S100.

FISH Screening Allows Detection of a *FUS-ATF1* Rearranged Peritoneal MM

Fusion-negative tumors were also further tested for *FUS* gene abnormalities by FISH. Among the 22 tumors tested, one revealed a *FUS-ATF1* rearrangement (1/22) (Supplementary Fig. 1), for a total of 4/25 (16%) *EWSR1 or FUS-ATF1* fusion positive MM. The *FUS-ATF1* fusion positive tumor occurred in a 25-years-old woman with widely extensive peritoneal tumor. Tumor exhibited epithelioid morphology with clear cell changes, and was immunohistochemically positive for AE1-AE3, WT1, D2/40 (focal) and CK5/6, while negative for calretinin, Ber-EP4, MOC31, B72.3, PAX8, ER, inhibin, desmin, OCT3/4, GATA3 and S100. Electronic microscopy performed at an outside institution reported the presence of long and thin cellular projections, consistent with the non-intestinal microvilli of mesothelioma.

Additionally, among the 21 fusion-negative MM, 5 (24%) cases showed large deletions by FISH of *EWSR1* and *SMARCB1*, with a 1:1 ratio (Fig. 2D).

All patients with fusion positive MM had advanced stage at presentation and progression of disease after debulking surgery and radio-chemotherapy occurred in three patients who had clinical follow up data available (Table 2).

MM with FUS/*EWSR1-ATF1* fusion show retained BAP1 Immunoexpression

BAP-1 immunostaining was assessed in 18 tumors with available FFPE material. Expression was retained in 15/18 (83%) cases and only 3 pleural MM showed loss of BAP1 staining (Table 2). The *FUS-ATF1* fusion-positive and two of the *EWSR1-ATF1* rearranged tumors showed retained expression of BAP1 (Table 1 and Fig. 1I). The third *EWSR1-ATF1* case had no remaining material for immunotesting. In the fusion negative tumors, nuclear expression of BAP1 was lost in only 3/15 (20%).

DISCUSSION

We report 4 cases of conventional epithelioid MM exhibiting recurrent *EWSR1 or FUS-ATF1* gene fusions among a comparatively large cohort of 25 children and young adults,

under the age of 40. These findings further expand understanding of the pathogenesis of MM, as well as enrich the growing list of pathologic entities harboring fusions of *EWSR1* with members of the CREB family of transcription factors. All 4 cases presented in young adults, lacking history of asbestos exposure, and microscopically displayed a typical epithelioid phenotype, with retained expression of BAP1, suggesting a novel MM subset.

EWSR1 (22q12) encodes a protein with a carboxy-terminus RNA binding domain, which has roles in mitosis, microtubule processing, DNA repair and cellular ageing (15). *EWSR1* has gained the infamous reputation of providing a promiscuous 5` gene partner in many different fusions involved in mesenchymal neoplasia and beyond (16, 17). Particularly complex and heterogeneous is the spectrum of pathologic entities recently described as sharing fusions between *EWSR1* and one of the c-AMP dependent transcription factor member of the CREB-ATF1 family (18). An *EWSR1-ATF1* fusion transcript has been described in several tumor types without an unifying cell lineage, morphology or behavior, spanning both benign and malignant tumors of either mesenchymal or epithelial differentiation (19). These include clear cell sarcoma (20), angiomatoid fibrous histiocytoma (21, 22), GI clear cell sarcoma (23), hyalinizing clear cell carcinoma (24), myoepithelial carcinoma (25), primary pulmonary myxoid sarcoma (19) and more recently in a myxoid mesenchymal tumor with intracranial predilection (26). To our knowledge, *EWSR1/FUS-ATF1* fusions have not been previously described in a MM case. Two studies involving whole genome sequencing did not reveal rearrangements of *EWSR1* or *ATF1* in 99 and 22 MM cases, respectively (6, 27). Data available from the Cancer Genomic BioPortal (www.cbioportal.com; 01/2017) (28) showed no evidence of further rearrangement of *FUS* or *CREB1* in these cohorts. Our index case 2 represents the single MM case with alterations in either *EWSR1, FUS* or *ATF1* genes from the MSK-IMPACT cohort, which contains genomic data for 131 patients with MM (data not shown). In the series reporting 2 MM cases with *EWSR1-YY1* fusion (9), a control cohort consisting of 14 MM patients, ranging from 43–78 years (median 66), was studied but no additional cases with *EWSR1* rearrangement were found. Recently, a biphasic pleural tumor with features of mesothelioma and undifferentiated round cell harboring *EWSR1* rearrangement was reported, but the gene partner was not identified (29).

Taken together, our data suggest that *EWSR1/FUS* rearrangements are rare events in the general population of MM. The 4 *EWSR1/FUS-ATF1* fusion positive cases were remarkably identified among the cohort of 25 young patients, suggesting that this genetic alteration has a higher prevalence in this rare clinical subset of MM. As most of the previous genomic studies included either none or only a minute fraction of patients under the age of 40, this likely explains why this abnormality was not previously identified in MM. Indeed, MM occurring in adults younger than age of 40 is estimated to be quite rare (2% of cases) (30). A recent review suggests that some clinical features might be unique to this subset, including a more even ratio of pleural to peritoneal tumors and more importantly a better prognosis compared to MM in older patients (30). Recently, an *ALK*-related fusion was reported in a pediatric peritoneal MM case (31), further support that a subset of MM in children and young adults is driven by alternative pathogenetic mechanisms. As expected, none of the young patients in our cohort, including the fusion-positive cases, had a history of direct exposure to asbestos. Risk factors for MM in patients without significant exposure to

asbestos, accounting for 20% of all MM, are not well defined, but include radiation exposure, non-asbestos mineral fibers, as well as genetic predisposition such as *BAP1* germline loss (30). While the germline status of *BAP1* remains unknown in our cohort, retained immunohistochemical expression of BAP1 in most cases was observed. This argues against a germline *BAP1* predisposition in these patients, given that germline cases have been found to exhibit the loss of BAP1 expression (4).

Indeed, loss of BAP1 expression was found in only 3/18 tested cases (17%) in our overall cohort of mesotheliomas in patients under 40. This is a lower frequency than reported in different series (27–70%) using negative BAP1 expression by IHC as a surrogate for *BAP1* mutation status (32–35). Noteworthy, the median age of patients in these studies is either unknown or > 60 years old, sharply contrasting with ours. Although limited by the size of our cohort, our findings suggest that BAP1 loss might be less common in younger patients with MM, including the *EWSR1*-rearranged cases. Both young age and loss of BAP1 expression were independently associated with better prognosis in one of these studies (35). However, a more definitive conclusion regarding the clinical behavior of *EWSR1/FUS*-rearranged MM is precluded due to the low number of cases, but the information available in our cases suggests an advanced stage at diagnosis (3/3 peritoneal; 0/1 pleural) and subsequent recurrence (3/3 with follow up).

Concordantly with the frequent deletions of chromosome 22 observed in MM, including band 22q12 harboring *EWSR1* (8, 36), 5/21 of fusion-negative MM from our screening cohort showed loss of one *EWSR1* or *SMARCB1* allele by FISH. In contrast, copy number alterations in chromosome 12, where *ATF1* is located, appear to be less frequent in MM. However, one case showing a translocation involving chromosomes 12 and X has been described in a patient with MM lacking a history of asbestos exposure (37).

Several entities with *EWSR1-ATF1* fusions are considered in the differential diagnosis of epithelioid malignancies that could occur in abdomen or thorax of young patients, though given classic morphology and immunoprofile of fusion-positive MM these entities are discussed for general reference. Amongst them is the GI clear cell sarcoma, a tumor of children and young adults, with epithelioid morphology arranged in solid nests and occasionally pseudo-papillary growth, and with rare multinucleated, osteoclast-like giant cells (38). In contrast to MM, the GI clear cell sarcoma show diffuse immunostaining for S100 and SOX10, but lacks cytokeratin reactivity as well as reactivity for mesothelial markers (39). A single case report of an *EWSR1-ATF1* positive myoepithelial tumor was described in the pelvis, exhibiting myxoid background and focal EMA reactivity, as well as S100 positivity (25). Although rare in the thoracic or abdominal cavity, angiomatoid fibrous histiocytomas have been reported at these sites, harboring *EWSR1-ATF1* fusions (40). Additionally, rare cases with endobronchial or pulmonary presentation have been described (41, 42). However, angiomatoid fibrous histiocytomas typically stain for desmin, while being consistently negative for cytokeratin. Although one of our *EWSR1-ATF1* positive MM showed patchy desmin reactivity in the solid epithelioid component it also co-expressed diffuse cytokeratin and WT1 in keeping with the diagnosis of MM. Hyalinizing clear cell carcinoma, described initially as a distinct salivary gland tumor with *EWSR1-ATF1* fusion (24), was subsequently reported in the bronchus (43), but so far not in abdominal locations.

The tumor is characterized by bland epithelioid cells exhibiting at least focally clear cytoplasm, arranged in cords, sheets, small nests and trabeculae. They are often surrounded in a background of hyalinized fibrous tissue and show reactivity for CK7, CK5/6, but also for squamous cell markers (p63/p40). Primary pulmonary myxoid sarcoma is a rare pulmonary/endobronchial tumor with lobulated architecture, consisting of spindle to epithelioid cells arranged in solid and reticular patterns, within a diffusely myxoid stroma (42). The tumor is typically negative for cytokeratins and harbors the related *EWSR1-CREB1* fusion (42); while no example with *EWSR1-ATF1* fusion has been yet reported. Finally, a group of unclassified myxoid mesenchymal neoplasms with predilection for the intracranial compartment and characterized by fusions of *EWSR1* with members of the CREB gene family, including *ATF1*, was recently described (26). From this series of 5 tumors occurring in young patients (range: 12–23 years), one presented in the pelvic soft tissues. These tumors were characterized by lobulated growth, with variably myxoid to solid areas of round to ovoid cells, in addition to distinctive amianthoid collagen fibers forming rosette-type structures. None of the cases showed cytokeratin expression.

From a morphologic perspective, the main differential diagnosis of epitheloid peritoneal MM in young patients is with serous carcinoma, although they tend to occur in females of older age (44). In contrast to high grade serous carcinoma, MM typically reveal a non-hierarchical papillary growth, with mild to moderate atypia and a relatively low mitotic activity. Immunohistochemically, serous carcinomas express MOC31, Ber-EP4 as well as PAX8, which is only rarely expressed in MM (45). Although our cohort showed a male predominance in the peritoneal location, PAX8 was tested in all peritoneal tumors occurring in female patients and was negative, arguing against a serous carcinoma diagnosis. A less common diagnostic consideration was mesonephric adenocarcinoma occurring in the peritoneum of young patients. These tumors show mixed growth patterns, variable degree of atypia and brisk mitotic activity. While exhibiting immunolabelling for cytokeratin and PAX8, they are typically negative for WT1 (46). To exclude this rare diagnostic consideration, we have tested 4 cases from our files but no *EWSR1/FUS* gene rearrangements were found by FISH (data not shown).

In summary, we identified 4 MM cases harboring *EWSR1-ATF1* (3) and *FUS-ATF1* (1) fusions from a large cohort of 25 MM patients under the age of 40. Although the number of positive cases is relatively small, this is the largest MM series to date focusing on this age group. This finding expands the spectrum of heterogeneous entities harboring *EWSR1/FUS-ATF1* fusions, to include MM, among other mesenchymal and epithelial malignancies. Taken together, our findings suggest that fusions involving *EWSR1* or *FUS* and *ATF1* are rare events in mesothelioma, which appear to be restricted to younger patients without significant exposure to asbestos or BAP1 germline or somatic loss, and indistinguishable from conventional MM. In keeping with the simple genomic profile of other translocation-associated neoplasms, one of the index cases tested by targeted NGS showed that the *EWSR1-ATF1* fusion was the sole genetic abnormality identified, without other mutations or copy number changes. Whether the genetic profile of MM occurring in children and young adults is similarly heterogeneous to the older age group will require further study, but our findings suggest that at least a small subset harbors abnormalities such as losses of 22q and BAP1 at chromosomal and immunohistochemical levels, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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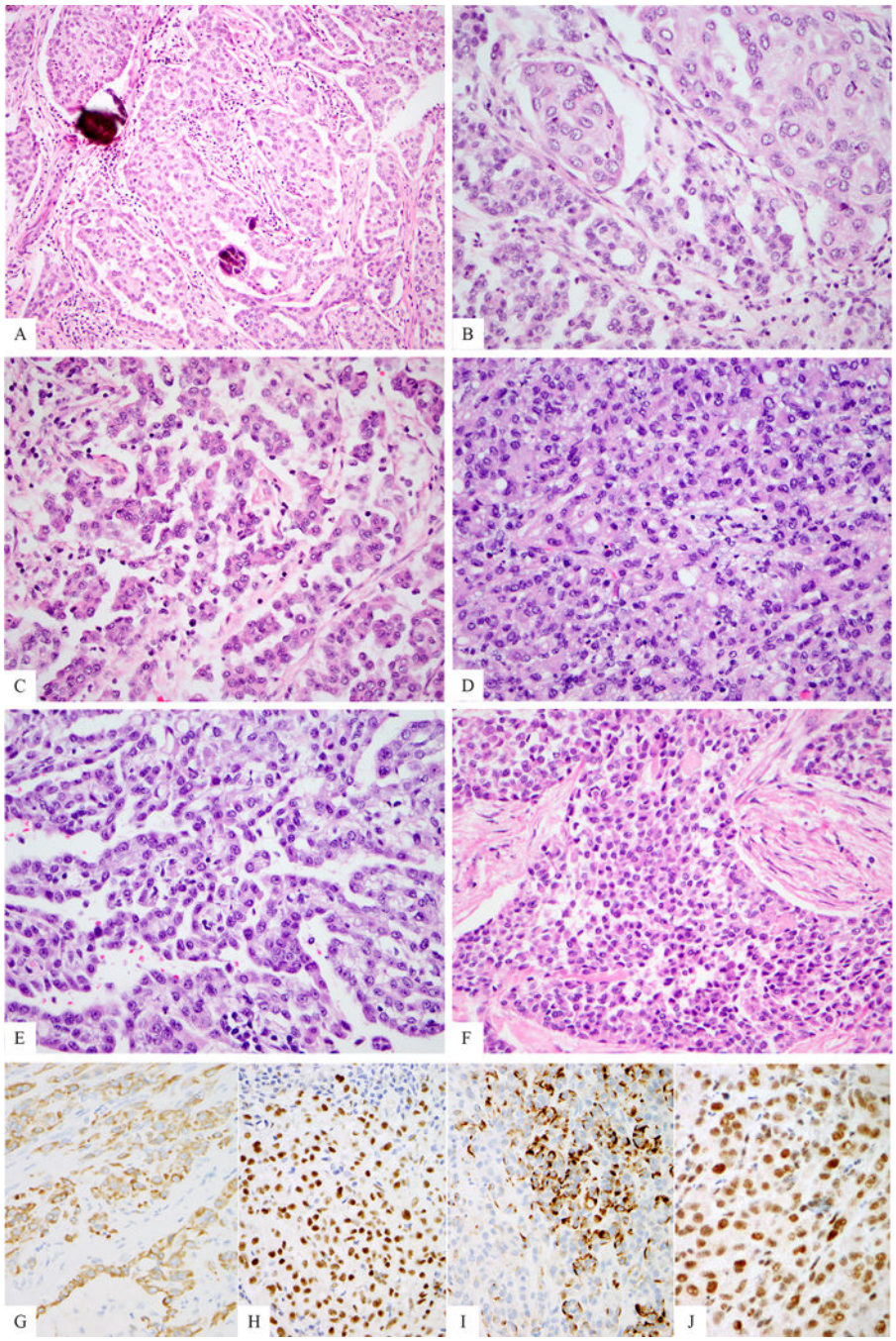

Figure 1. Histologic features of *EWSR1-ATF1* fusion positive mesotheliomas

Medium power view of peritoneal index case (MM1) exhibiting focal psammoma bodies (A). The tumor displayed a conventional epithelioid morphology with focal papillary architecture, as well as abundant eosinophilic cytoplasm and open chromatin (B, C). MM2 showed a predominantly solid growth (D), with only focal papillary architecture (E). MM3 exhibited a predominant round cell phenotype with scant cytoplasm (F). All cases were immunohistochemically positive for AE1/AE3 (G, MM2), WT1 (H, MM2) and focal desmin

expression was observed in a single case (I, MM2). Immunohistochemical expression of BAP1 was retained in the 3 fusion positive cases tested (J, MM2).

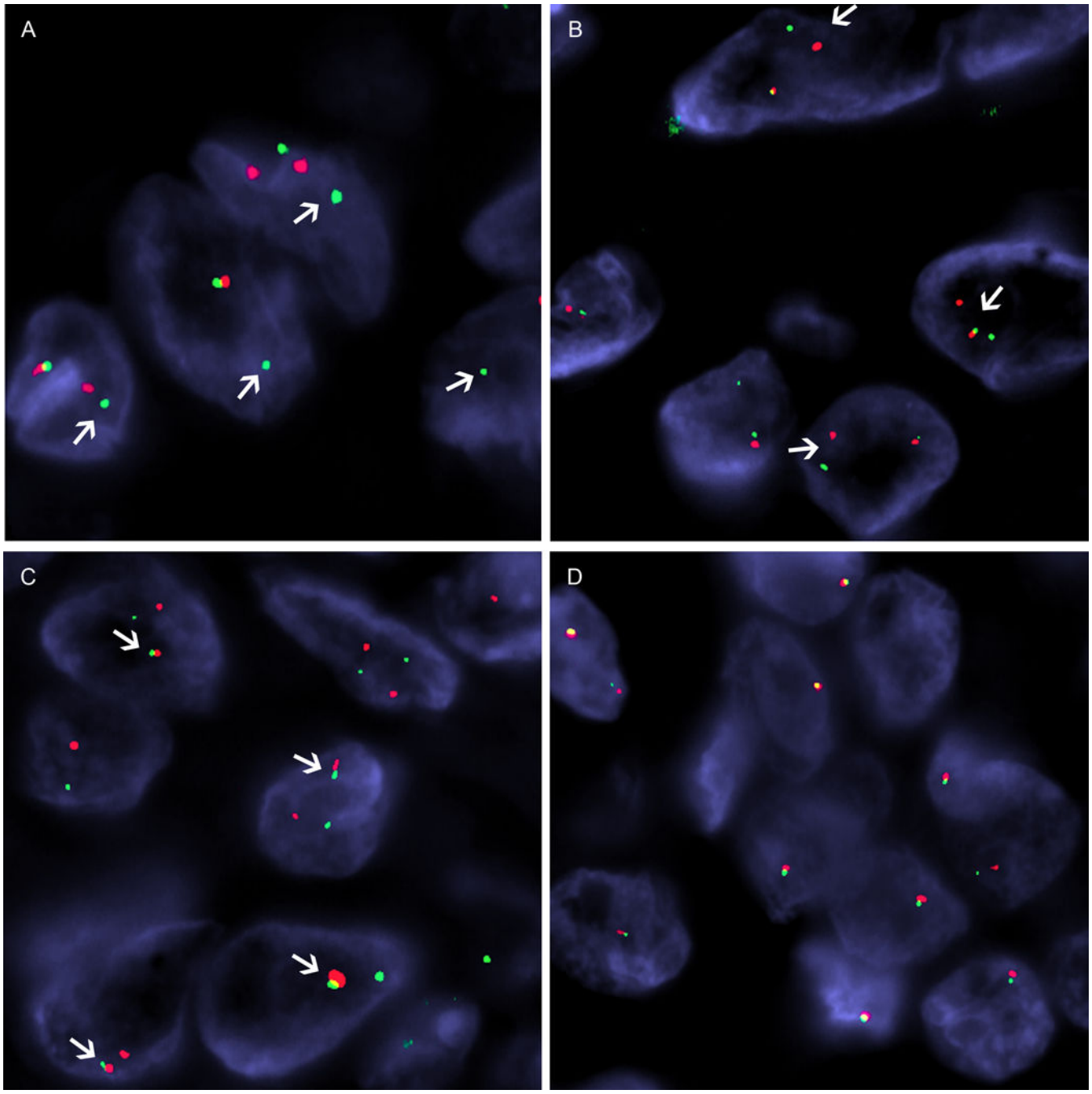

Figure 2. Fluorescence in situ hybridization showing EWSR1

(A, red, centromeric; green, telomeric) **and ATF1** (B, red, centromeric; green, telomeric) **break-apart signals** (arrows) in MM3. C) FISH fusion assay in MM2 illustrates the come-together signals (arrows) between centromeric *EWSR1* (red) and telomeric *ATF1* (green), confirming the fusion result detected by next-generation sequencing assay (MSK-IMPACT). D) Large deletion or monosomy of 22q12 showing only one copy of *EWSR1* (red, centromeric, green telomeric) in a fusion-negative pleural mesothelioma from a 37 years-old patient.

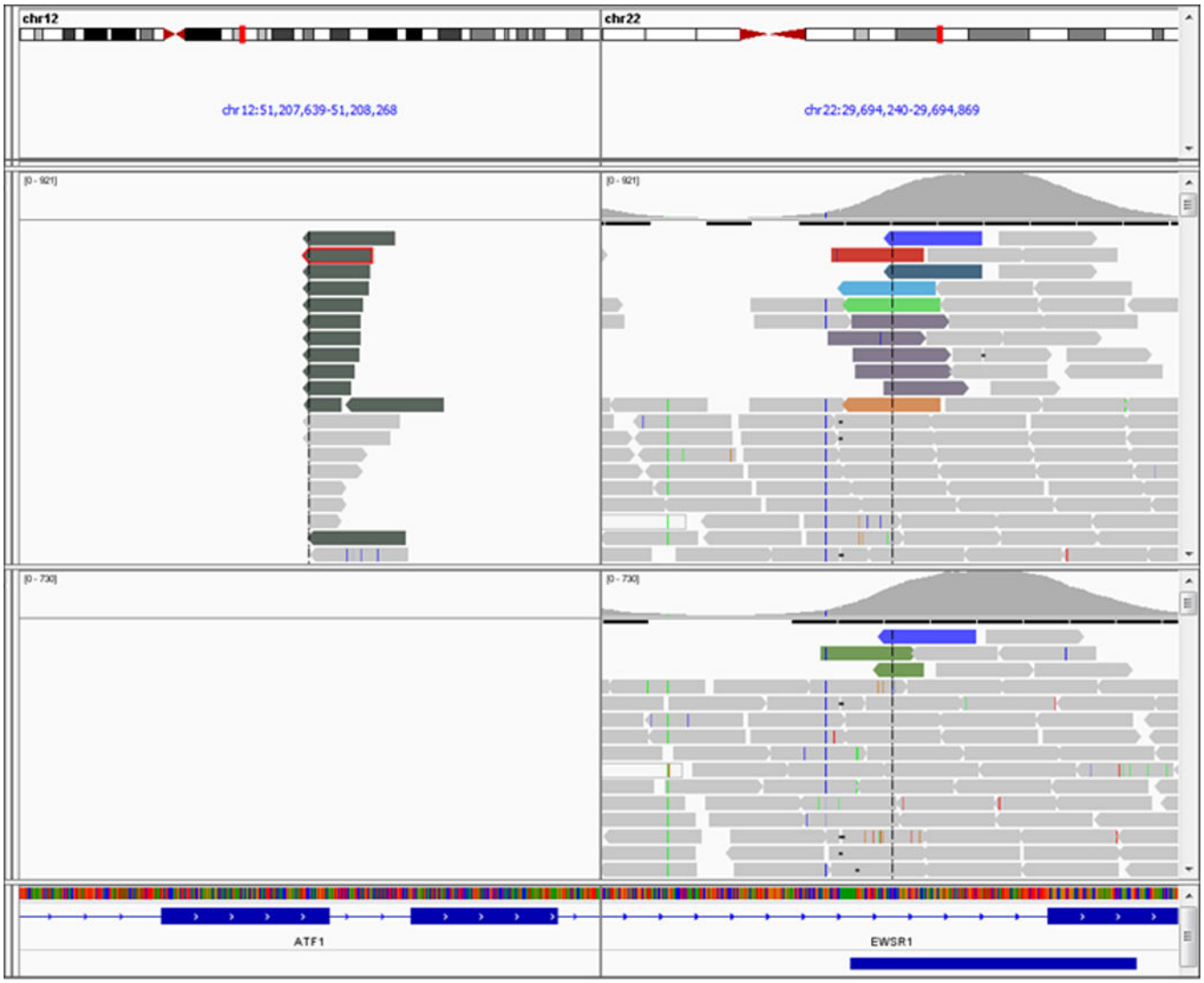

Figure 3.

Representation of the *EWSR1-ATF1* fusion in Integrated Genome Viewer for MM2, where each bar represents a single sequenced read. The sets of reads (on top) map to *EWSR1* on chromosome 22, corresponding paired end reads map to *ATF1* on chromosome 12, supporting a somatic fusion involving both genes. Upper panel: tumor; lower panel: normal blood control.

Table 1

Clinicopathological Features of *EWSR1/FUS-ATF1* Fusion Positive Malignant Mesotheliomas

Case	Age/Sex	Location	Asbestos exposure	Smoking	Morphology	Stage	Follow up	BAP-1 IHC	Fusion	Method
MM1	21/M	Peritoneal	N/A	N/A	Epithelioid	Advanced, pN1	N/A	Failure	EWSR1-ATF1	FISH
MM2	33/F	Peritoneal	None	Never smoker	Epithelioid	Advanced, cM1	POD	Retained	EWSR1-ATF1	FISH + targeted NGS
MM3	34/F	Thoracic	None	10 pack-year	Epithelioid	pT2N0/II	POD	Retained	EWSR1-ATF1	FISH
MM4	25/F	Peritoneal	None	Never smoker	Epithelioid	Advanced	POD	Retained	FUS-ATF1	FISH

IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; POD progression of disease; NGS: Next-Generation Sequencing; stage: c: clinical, p: pathologic

Table 2

Clinicopathological Features of Malignant Mesotheliomas in the Screening Cohort

Characteristics	Localization		
	Peritoneal n = 13 (52%)	Thoracic n = 12 (48%)	Total n = 25
Age, years; median (range)	30 (7–40)	33 (8–38)	31 (7–40)
Sex, n (%)			
Male	10 * (77)	4 (33)	14 (56)
Female	3 (23)	8 (67)	11 (44)
Asbestos exposure, n (%)			
None	6 (46)	7 (58)	13 (52)
Potential Indirect	0 (0)	3 (25)	3 (12)
N/A	7 (54)	2 (17)	9 (36)
Smoking			
Never smoker	3 (31)	2 (17)	6 (24)
Light smoker	0	3 (25)	3 (12)
Heavy smoker	1(8)	4 (33)	5 (20)
N/A	8 (61)	3 (25)	11 (44)
Morphology			
Epithelioid	13 (100)	11 (92)	24 (96)
Sarcomatoid	0	0	0
Biphasic	0	1 (8)	1 (4)
BAP1 IHC, n (%)			
Retained	6 (46)	9 (75)	15 (60)
Loss	0	3 (25)	3 (12)
Failure/N/A	1/6 (54)	0/0	7 (28)
EWSR1/FUS-ATF1 fusion, n (%)			
Positive	3 (23)	1 (8)	4 (16)
Negative	10 (77)	11 (92)	21 (84)

* male: female ratio statistically significant between thoracic and peritoneal localization (p = 0.047); N/A: not available; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization