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# The Clinical Heterogeneity of Round Cell Sarcomas with *EWSR1/FUS* Gene Fusions. Impact of Gene Fusion Type on Clinical Features and Outcome

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## Abstract

The genetic hallmark of classic Ewing sarcoma is a recurrent fusion between *EWSR1* or *FUS* gene with a member of the ETS transcription factor family. In contrast, tumors with non-ETS gene partners have been designated until recently 'Ewing-like sarcoma', as a provisional molecular entity, as their clinical and pathologic features were still evolving. However, this group was reclassified as 'round cell sarcoma with *EWSR1*-non-ETS fusions' in the latest 2020 WHO classification. Moreover, round cell sarcomas with either *CIC* or *BCOR* gene abnormalities, initially classified under Ewing family of tumors, are now regarded as stand-alone pathologic entities based on their distinct features. In this study we investigated the clinical characteristics of 226 confirmed Ewing sarcoma patients [*EWSR1-FLI1* (n=176), *EWSR1/FUS-ERG* (n=35), *EWSR1/FUS-FEV* (n=12), *EWSR1-ETV1/4* (n=3)] and 14 round cell sarcoma patients with *EWSR1*-non-ETS fusion [*EWSR1/FUS-NFATC2* (n=10), *EWSR1-PATZ1* (n=3), *EWSR1-VEZF1* (n=1)]. The impact on overall survival (OS) was assessed in 90 patients with available follow-up, treated between 2011–2018. Patients with fusions involving *FEV* and *NFATC2* genes showed an older median age at diagnosis, compared to those with *EWSR1-FLI1* (p=0.005), while extraskeletal location was more common in tumors with non-canonical *EWSR1-FLI1* fusions (p=0.001). Axial and pelvic primary sites were more common in patients with *EWSR1-FLI1* (72%), while tumors with *NFATC2* fusions were more frequent in the limb (78%, p=0.006). The 3-year OS in patients with *EWSR1-FLI1* was 91%, compared to only 60% in patients with alternative fusions (p=0.037); the latter group showing a higher rate of metastases at presentation. However, this OS difference was not significant in patients with localized tumor (p=0.585). Our

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study demonstrates significant correlations between fusion subtype and age at presentation, primary tumor sites, and OS, in both conventional Ewing sarcoma and round cell sarcoma with *EWSR1*-non ETS fusions patients. Larger studies are needed to determine survival differences in localized tumors.

**Keywords**

Ewing sarcoma; Ewing-like sarcoma; *EWSR1*; *FUS*; gene fusions

## 1 INTRODUCTION

Ewing sarcoma is the prototypical round cell sarcoma which occurs with predilection in the bone of children and young adults. The majority of conventional Ewing sarcomas are characterized genetically by recurrent fusions involving *EWSR1* and *FLI1*, the latter encoding a member of the ETS transcription factor family, resulting in an oncogenic transcriptional program[1]. In less than 10% of cases, the gene fusions of Ewing sarcoma involve other *ETS* gene members, such as *ERG*[2], *FEV*[3], *ETV1*[4], or *ETV4*[5]. *EWSR1* and *FUS* are members of the FET family of RNA binding proteins, have similar functions and are interchangeable in translocation driven sarcomas[6].

In addition to the *EWSR1-ETS*-positive classic Ewing sarcoma, a rare subset characterized by *EWSR1*-non-ETS fusions, which was until recently provisionally termed as 'Ewing-like sarcoma' tumors has emerged, being characterized by fusions between *EWSR1* or *FUS* with non-ETS partners, such as *PATZ1*[7], *SP3*[8], *NFATC2*[9,10], and *SMARCA5*[11]. Although preliminary gene expression and epigenetic profiles suggest that at least some of these molecular subsets encountered in Ewing-like sarcomas are distinct from the canonical *EWSR1/FUS-ETS* fusions[12], large clinicopathologic studies are lacking. In this study, taking advantage of our large dataset of Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusion cases with well-characterized gene fusions, we sought to correlate the impact of various fusion subtypes on clinical and pathologic findings, as well as survival outcomes.

## 2 MATERIAL AND METHODS

### 2.1 Patient selection

The MSKCC files and personal consultation files of the senior author (CRA) were searched for the diagnosis of Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusions with confirmed gene fusion information or with available tissue to determine the molecular subtype. A total of 240 patients diagnosed between 1986 and 2019 were included, showing the following break-down fusions (Fig. 1): *EWSR1-FLI1* (n=176), *EWSR1/FUS-ERG* (n=35), *EWSR1/FUS-FEV* (n=12), *EWSR1/FUS-NFATC2* (n=10), *EWSR1-ETV1/4* (n=3), *EWSR1-PATZ1* (n=3), or *EWSR1-VEZF1* (n=1). Most of the fusion types (n=179) were determined by FISH assay using custom BAC probes for all known gene partners and fusion variants. In a small subset, the translocation partners were obtained from a variety of other molecular methods, including RT-PCR (n=20), karyotype (n=2), targeted RNA sequence (n=12), or MSK-IMPACT assay (n=27). Patients with only evidence of *EWSR1/FUS*

rearrangement but no available tissue for further investigation of the gene partner were excluded from the study (n=37).

For survival analysis, patients with available follow-up data diagnosed between 2011 and 2018 were selected, with a minimum follow-up of one year (n=90). Single patients with rare fusion variants, such as one patient with *EWSR1-ETV4,* were excluded from further survival analyses. The dose and period of chemotherapy were adjusted based on their age, co-morbidities and toxicities. All patients were followed up according to a standard protocol[13].

Hematoxylin and eosin-stained slides and immunohistochemical stains were re-reviewed. The tumors were assessed for growth pattern, cytomorphology (round, oval, spindle, epithelioid, plasmacytoid/rhabdoid phenotype), nuclear features including nuclear contour, chromatin pattern and presence of nucleoli, mitotic activity, necrosis, type of stroma and myxoid change. Review of the CD99 immunohistochemical staining patterns was also assessed. The patients' charts were retrospectively reviewed. The following clinical data were retrieved: age, gender, primary tumor site, stage at diagnosis (primary versus distant metastasis at diagnosis), tumor size, modality of initial therapy, recurrence, vital status and survival time. In a few patients, information on gender (n=2), skeletal or extraskeletal primary site (n=9), and limb or axial location (n=6) were not available due to the consultation cases. The study was approved by the Institutional Review Board.

### 2.2 Fluorescence in situ hybridization (FISH)

FISH was conducted for *EWSR1/FUS*, *FLI1*, *ERG*, *FEV*, *NFATC2*, *ETV1*, *ETV4*, or *PATZ1*. FISH for break-apart assay was applied on formalin-fixed and paraffin-embedded 4-micron sections as previously described[14]. Custom probes using bacterial artificial chromosomes (BACs) covering and flanking each gene were utilized[15]. The BAC clones were selected according to the UCSC genome browser (http://genome.ucsc.edu) and obtained from the BACPAC sources of Children's Hospital of Oakland Research Institute (CHORI) (Oakland, CA) (http://bacpac.chori.org). DNA from individual BACs was isolated in line with 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. Two hundred tumor nuclei were evaluated using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Newton, MA). A cut-off of >20% nuclei showing a break-apart signal was considered to be positive for rearrangement. Nuclei with incomplete set of signals were omitted from the score.

### 2.3 Other molecular methods to determine the fusion type

In a smaller subset of cases the gene fusion was determined either by RT-PCR (n=20), karyotype (n=2), MSK-IMPACT assay (n=27), or targeted RNA sequencing (n=12), as previously described[16–18]. Targeted RNA sequencing was performed either by using an Archer™ FusionPlex™ platform[19,20] or a TruSight RNA Fusion Panel (Illumina, San Diego, CA) on an Illumina MiSeq platform[21], using standard protocols. Reads were independently aligned with STAR (version 2.3) against the human reference genome (hg19) and analyzed by STAR-Fusion.

### 2.4 Statistical analysis

Kaplan-Meier curves were used to estimate overall survival (OS). OS was defined as the time from the diagnosis to death and was censored at the date of the latest follow-up. Categorical variables were compared between groups using chi-square tests. Numerical variables were compared using Kruskal-Wallis Test. Statistical analyses was performed using SPSS version 21 (IBM), with significance set at two-tailed $p < 0.05$.

## 3 RESULTS

### 3.1 Correlation between fusion transcript type and clinical parameters

A total of 240 patients diagnosed with Ewing sarcoma or round cell sarcoma *EWSR1*-non-ETS fusions with complete gene fusion information were included in the study. The most common fusion type was *EWSR1-FLI1* (n=176, 73%), followed by tumors with *ERG* gene rearrangements (n=35, 15%), either *EWSR1-ERG* (n=26, 11%) or *FUS-ERG* (n=9, 4%). Other less common fusions were identified, including: *EWSR1/FUS-FEV* (n=12), *EWSR1/FUS-NFATC2* (n=10), *EWSR1-ETV1/4* (n=3), *EWSR1-PATZ1* (n=3), or *EWSR1-VEZF1* (n=1)(Fig. 1).

The median age at diagnosis for the entire group was 23 years, with a wide age range of 1–78 years (Table 1). Ewing sarcoma patients with canonical fusions had a median age at diagnosis of 23 years (range, 1–75) in the *EWSR1-FLI1*-positive cohort, and a median age of 20 years (range, 1–64) in the *EWSR1/FUS-ERG* group. The Ewing sarcoma patients harboring *EWSR1-ETV1/4* fusions had the youngest median age at diagnosis, of 8 years old (range, 1–12) and the single case with *EWSR1-VEZF1* fusion occurred in a 12 year-old patient (Fig. 2). In fact, one-third of cases positive for *EWSR1-ETV1/4* fusion occurred in very young children (aged <2 years), while only one case each occurred in the more common *EWSR1-FLI1* and *EWSR1/FUS-ERG* molecular groups. In contrast, patients with *EWSR1/FUS-FEV* and *EWSR1/FUS-NFATC2* fusions had an older median age at presentation of 35 (range, 5–61) and 43 (range, 27–78) years, respectively. The distribution of pediatric patients (aged <18 years) was higher in the molecular subsets of *EWSR1-ETV1/4* (3/3) and *EWSR1-VEZF1* (1/1), compared to *EWSR1/FUS-ERG* (43%, 15/35) and *EWSR1-FLI1* (34%, 63/176) groups. In contrast, the proportion of older adult patients (aged >40 years) was highest in *EWSR1/FUS-NFATC2*-positive group (50%) compared to that of the canonical *EWSR1-FLI1* group (24%).

Only 39% of patients with *EWSR1-FLI1* canonical fusion presented at extraskeletal sites, in contrast to all patients harboring *EWSR1/FUS-FEV, EWSR1-ETV1/4, EWSR1-PATZ1*, and *EWSR1-VEZF1* fusions (Fig 3). The limb location was a frequent primary site in the round cell sarcoma group with *EWSR1*-non-ETS fusion patients, encompassing all cases with *EWSR1-PATZ1* and *EWSR1-VEZF1* fusions, and 7/9 *EWSR1/FUS-NFATC2*-positive cases (Fig 3). In contrast, axial and pelvis presentation was more common in patients with canonical fusions such as *EWSR1-FLI1* (72%) and *EWSR1/FUS-ERG* (54%). Overall, statistically significant differences with fusion type were detected in age at presentation (p=0.005), skeletal versus extraskeletal location (p=0.001), and limb versus axial primary sites (p=0.006).

### 3.2 Morphologic Features and Immunohistochemistry

Although all tumors included in this comprehensive series were classified as round cell malignancies, only Ewing sarcomas with the canonical *EWSR1/FUS-FLI1* and variant *EWSR1/FUS-FEV* showed the classic features typically recognized in Ewing sarcoma. The latter features including solid sheets of uniform round cells with ill-defined cell borders, scant, often clear, cytoplasm, and monomorphic round nuclei, with smooth contours and fine chromatin. Tumors in these 2 molecular groups were uniformly positive for CD99, while Ewing sarcoma cases with canonical fusions also showed consistent nuclear positivity for FLI1. However, FLI1 reactivity was not limited to this genetic subset being positive in other *ETS*-related fusions. In contrast, tumors with *EWSR1/FUS-ERG* fusions showed in general a more variable spectrum of histologies, which spanned from round, ovoid, short spindle and even epithelioid cells (Fig 4). Immunohistochemically, this group was consistently positive for CD99 and ERG markers (Fig 4). Rare *EWSR1-ETS* fusion variants included cases with unusual histologic features. One of the *EWSR1-ETV4* tumor occurring in an infant, showed extensive tumoral calcifications, while the single case harboring an *EWSR1-VEZF1* fusions showed a primitive round cell phenotype with an increased myxoid and fibrous stromal components (Fig. 4). Moreover, round cell sarcomas with *EWSR1*-non-ETS fusions with either *NFATC2* or *PATZ1* gene abnormalities showed increased variability (Fig 5), compared to the uniform cytomorphology or immunoprofile of tumors with the *EWSR1-FLI1* canonical fusion.

The CD99 immunohistochemical findings showed strong and diffuse membranous immunoreactivity for all cases with *EWSR1-FLI1*, *EWSR1/FUS-ERG*, *EWSR1/FUS-FEV*, and *EWSR1-ETV1/4* fusions. Moreover, all *EWSR1-NFATC2* tumors with available material were positive for CD99, however, in 2 of the 6 cases the reactivity was only focal, while remaining showed diffuse strong membranous staining. The single case with *EWSR1-VEZF1* was negative for CD99. Desmin and MyoD1 were positive in one tumor with *EWSR1-PATZ1.*

### 3.3 Comparison of fusion type with overall survival

Ninety patients with available follow-up, treated during 2011 and 2018, were selected for survival analysis. Patients characteristics are shown in Table 2. The fusion distribution among this smaller cohort of patients was the following: *EWSR1-FLI1* (n=67, 74%), *EWSR1/FUS-ERG* (n=16, 18%), *EWSR1/FUS-FEV* (n=4, 4%), and *EWSR1/FUS-NFATC2* (n=3, 3%), which mirrored the overall distributions in age and primary location observed in the entire cohort (Table 2). The median age at diagnosis for this smaller group of patients was 23 years of age, which was identical to the group of patients with *EWSR1-FLI1* canonical fusion (range, 2–78); while for the *EWSR1/FUS-ERG* group was 19 years (range, 2–64). In contrast, patients with *EWSR1/FUS-NFATC2* fusion had an older age at diagnosis (median 61 years, range, 27–78). Extraskeletal primary sites were more common in the *EWSR1/FUS-FEV* (100%) and *EWSR1/FUS-NFATC2* (67%) group of tumors, compared to that of the *EWSR1-FLI1* group (45%). Similar to the entire cohort, all cases in this selected subset with *EWSR1/FUS-NFATC2* fusion presented in the limb, while axial and pelvis location was more common in patients with *EWSR1-FLI1* fusions (66%).

Overall, treatment for the primary disease included surgical resection in 61 of 90 (68%) patients. Of 59 patients presenting with localized disease, 48 patients underwent definitive surgical resection for the primary tumor, while 10 patients presenting with localized disease underwent radiation therapy as a definitive treatment for the primary tumor and one patient died during preoperative chemotherapy. Among the 59 patients presenting with localized disease, 54 (92%) patients underwent neoadjuvant and/or adjuvant chemotherapy. The majority of patients (n=48, 89%) received a chemotherapy regimen including vincristine, doxorubicin, cyclophosphamide with ifosfamide and etoposide (VDC/IE) ± irinotecan and temozolomide. Patients with localized disease harboring rare fusion variants, such as *EWSR1-FEV* (n=1) or *EWSR1-NFATC2* (n=2), were also treated with neoadjuvant/adjuvant chemotherapy involving VDC/IE. Of 28 patients who received neoadjuvant/adjuvant chemotherapy with available data, 12 (43%) patients had more than 90% chemotherapy-induced necrosis in their resected specimens. The proportions of patients with favorable chemotherapy response, defined as > 90% necrosis, were not significantly different among the different fusion types (*EWSR1-FLI1,* 42%, 10 of 24; *EWSR1/FUS-ERG,* 67%, 2 of 3, p=0.482, however, this analysis did not have enough power due to small sample size. One such example of favorable pathologic response was encountered in a 1-year-old female who presented with a left pelvic sidewall soft tissue tumor harboring an *EWSR1-ETV4* fusion (Fig. 3). The patient was treated with neoadjuvant/adjuvant chemotherapy with VDC/IE and irinotecan/temozolomide per our Ewing sarcoma regimen, as well as with radiation therapy after surgical resection. The patient remains alive with no evidence of disease 60 months follow-up.

Thirty-one patients (34%) presented with metastatic disease at diagnosis. The proportion of patients with metastasis at presentation was higher in patients with *EWSR1/FUS-ERG* (44%), *EWSR1/FUS-FEV* (75%) and *EWSR1/FUS-NFATC2* (33%), compared to *EWSR1-FLI1* (30%).

At the time of last follow-up, 73 patients (81%) were alive, including 41 (47%) without evidence of disease and 32 (34%) alive with disease. Fifteen patients (17%) died of disease and two patients (2%) experienced deaths of other causes. The 3-year and 5-year OS for the entire cohort were 85.7% (95% CI: 74.7% to 92.2%) and 69.5% (95% CI: 50.0% to 82.6%), respectively (Fig 6). Overall, the OS was significantly different among fusion types (p=0.042, Fig. 7A). The classic Ewing sarcoma group (*EWSR1-FLI1*, *EWSR1/FUS-ERG*, and *EWSR1/FUS-FEV*) showed a better outcome compared to round cell sarcoma with *EWSR1*-non-ETS fusions (*EWS/FUS-NFATC2)*, with a 3-year OS of 86.6% vs 50.0%, respectively, however, the difference was not statistically significant (p=0.657). OS was also not significantly different between Ewing sarcoma patients with *EWSR1-FLI1* versus *EWSR1/FUS-ERG* canonical fusions (p=0.167). However, when patients with *EWSR1-FLI1* positive tumors were compared to all other fusion types in this cohort (*EWSR1/FUS-ERG*, *EWSR1/FUS-FEV*, and *EWSR1/FUS-NFATC2*) this subset was associated with a significantly better OS (3-year OS, 91.4% vs 60.5%, p=0.037, Fig. 7B).

The overall 3-year and 5-year OS of the patients presenting with localized disease at diagnosis (n=59) were 93.2% (95% CI: 79.7%−97.8%) and 80.7% (95% CI: 55.0%−92.6%), respectively. In this clinical subset, no significant differences in OS were noted when

comparing Ewing sarcoma (*EWSR1-FLI1*, *EWSR1/FUS-ERG*, and *EWSR1/FUS-FEV*) and round cell sarcomas with *EWS/FUS-NFATC2* fusion (p=0.450), nor comparing *EWSR1-FLI1* and *EWSR1/FUS-ERG* fusion variants (p=0.872). Although the 3-year OS appeared to be longer in the *EWSR1-FLI1* genetic group compared to other fusion variants overall (*EWSR1/FUS-ERG*, *EWSR1/FUS-FEV*, and *EWS/FUS-NFATC2)*, the difference was not statistically significant (95.3% vs 66.7%, respectively, p=0.585). In 36 patients with available *EWSR1-FLI1* exonic composition, there was no significant difference in OS among type 1 (*EWSR1* exon7 fused to *FLI1* exon6) versus other variants (3-year OS, 100% vs 90.8%, respectively, p=0.377).

## 4 DISCUSSION

The advances and wide application of molecular studies in routine clinical practice have allowed a detailed genetic subclassification of Ewing sarcoma and round cell sarcomas with *EWSR1*-non-ETS fusions based on their recently described gene fusions[9–12]. As these novel molecular subsets are quite rare, large studies assessing their clinical presentation, pathologic features and survival are unavailable. Although methylation classifiers or/and gene expression clustering have suggested that lesions formerly known as Ewing-like sarcoma subsets, such as tumors with *EWSR1-NFATC2* and *EWSR1-PATZ1,* are epigenetically and genomically distinct from conventional Ewing sarcoma with canonical fusions[12,22,23], clinical and survival comparisons of these two genetic subgroups are lacking. Moreover, it is likely that a number of prior (pre-NGS) clinical studies of Ewing sarcoma, selected based on *EWSR1* and *FUS* gene rearrangements by FISH, may have combined Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusion patients under one group, and thus the clinical features and survival information might be biased.

Our results provide information regarding the incidence of round cell sarcoma with *EWSR1*-non-ETS fusions among a large group of Ewing family of tumors, which accounts for 6% (14/240) of the patients. Among the classic Ewing sarcoma group, the two most common fusion variants were *EWSR1-FLI1* (73%; 176/240) and *EWSR1/FUS-ERG* fusions (15%; 35/240), followed by *EWSR1/FUS-FEV* (5%; 12/240), and *EWSR1-ETV1/4* (1%; 3/240).

By comparing the clinical and pathologic features with the gene fusion type in this large cohort of Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusion patients some interesting correlations emerged particularly related to age at diagnosis and primary anatomic site. Patients with round cell sarcomas harboring *EWSR1/FUS-NFATC2* fusion had an older median age at diagnosis (43 years), while sarcoma patients harboring *EWSR1-ETV1/4* fusions had the youngest median age at diagnosis, of 8 years old (range, 1–12). In fact, one-third of cases positive for *EWSR1-ETV1/4* fusion occurred in very young children (aged <2 years). The single case with *EWSR1-VEZF1* fusion occurred in a 12 year-old patient. Other smaller series have suggested similar correlations, with one infant patient harboring an *EWSR1-ETV4* fusion[5], and four patients with *EWSR1/FUS-NFATC2* showed relatively increased median age (38 years), all presenting in the extremity[24].

Patients with rare variant fusions such as *EWSR1/FUS-FEV, EWSR1-ETV1/4, EWSR1-PATZ1*, and *EWSR1-VEZF1* showed the highest incidence of extraskeletal sites. In contrast

Ewing sarcoma with canonical fusions showed a predilection for skeletal sites (58%) and pelvic and trunk (68%). Other smaller series have shown tumors with *EWSR1-ETV4, EWSR1-PATZ1*, or *EWSR1-VEZF1* mainly arise from extraskeletal sites[5,23,24]. Previous studies of Ewing sarcoma with canonical fusions demonstrated a prevalence for skeletal primary sites in 73% of cases and of axial location in 62% of cases[25,26], which was consistent with our results.

Our study highlights certain differences in the overall survival among various gene fusion groups, likely attributable to the higher metastatic rate at presentation in the patients with non-*EWSR1-FLI1* fusions. Specifically, patients with *EWSR1/FUS-ERG*, *EWSR1/FUS-FEV*, and *EWSR1/FUS-NFATC2* fusions were associated with higher rates of metastasis at presentation and worse overall survival. Prior data from the literature reveal discrepant results between outcome and fusion type/fusion transcript[25–28]. Although retrospective studies initially suggested that the *EWSR1–FLI1* type 1 variant (*EWSR1* exon 7 fused to *FLI1* exon 6) was associated with better survival[26,27], two subsequent prospective studies did not validate this observation[25,26]. In keeping with latter prospective data, patients with tumors harboring *EWSR1-FLI1* type 1 variant was not associated with better survival outcome in our small dataset. In the de Alava study[29], the median survival regardless of stage in the group of patients with fusion transcripts other than type 1 was 27 ± 3.8 months compared to 113 ± 2.2 months for the group with type 1 transcripts. In contrast, the survival data of our cohort demonstrates a significant improvement and the difference in outcome based on fusion type has diminished, presumably due to the improved chemotherapeutic regimens applied. Moreover, previous reports showed that Ewing sarcomas with either *EWSR1–FLI1* or *EWSR1–ERG* have similar survival outcomes[29]. Restricting our survival analysis on patients presenting with localized disease at diagnosis, our results did not show a significant difference in overall survival between fusion subsets, although the number of evaluated patients in each group was relatively limited. Further studies using a large/international cohort are needed to determine a survival difference based on fusion type, particularly in patients with localized disease.

Previous studies have demonstrated that older age at diagnosis was associated with a worse outcome in Ewing sarcoma[30,31]. Whether this disparity in survival outcomes is attributable to differences in treatment modalities or sensitivity, tumor biology, or host microenvironment remains an area of active investigation. Moreover, our results point to a different layer of complexity, that of a variable predilection of certain fusion types for certain age groups, which may translate into different tumor biology and survival differences among age groups.

Some of the potential biological differences among various molecular Ewing sarcoma subsets have been investigated in a few recent studies[12,23,32]. Using a comprehensive whole-genome and transcriptome sequencing analysis, one study demonstrated that Ewing sarcomas with *ERG* gene rearrangements occur through an unbalanced, chromoplexy pattern of fusion, likely due to the opposite directions of transcriptions of the *EWSR1* and *ERG* genes[32]. In that study, Ewing sarcoma patients harboring gene fusions through the chromoplexy process were more likely to relapse and had a high incidence of *p53* mutations, compared to patients showing balanced *EWSR1-FLI1* fusions[32]. These results correlate with

our findings of a higher incidence of metastatic rate at presentation and worse OS in *EWSR1-ERG* fusions. Moreover, recent molecular studies described that *EWSR1-NFATC2* or *EWSR1-PATZ1* positive round cell sarcomas have distinct genomic and/or methylation signatures[12,23] Unsupervised hierarchical clustering of DNA methylation data revealed a homogeneous methylation cluster for undifferentiated small round cell sarcoma with *EWSR1-NFATC2* fusion, which clearly segregated from canonical Ewing sarcoma[12]. In addition, copy number profiles of *EWSR1-NFATC2* cases showed characteristic recurrent losses on chromosome 9q and segmental gains on 20q13 and 22q12 involving the *EWSR1* and *NFATC2* loci, respectively[12]. In *EWSR1-PATZ1*-positive sarcomas, secondary driver mutations in cell-cycle genes, in particular *CDKN2A* (71%), were common[23]. Our current results, showing distinct clinicopathologic features of these two molecular subsets from classic Ewing sarcoma, provide further support in favor of them being stand-alone pathologic entities.

Our study had several limitations, most importantly the relatively small number of patients and events in some of the molecular subsets, which precluded conducting a multivariate survival analysis and hampered our ability to make definitive conclusions. Moreover, the patient cohort was limited to a single quaternary institution, which is likely to infer a referral bias towards more rare molecular subsets or patients with more aggressive behavior. Despite these limitations, the study's strengths rely in providing detailed clinical and therapeutic information of a large, molecularly well-characterized, cohort of Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusion group.

In summary, we outline a number of important correlations between the *EWSR1*/*FUS* gene fusion types and clinicopathologic features. Our data reveal a less favorable overall survival in patients with non-canonical fusions, such as *EWSR1/FUS-ERG*, *EWSR1/FUS-FEV* and *EWSR1/FUS-NFATC2*. This survival difference is likely due to the higher incidence of metastatic spread at presentation in these less common subsets. Larger, multi-institutional studies are needed to definitively evaluate if these differences in survival outcomes may persist in patients with localized disease.

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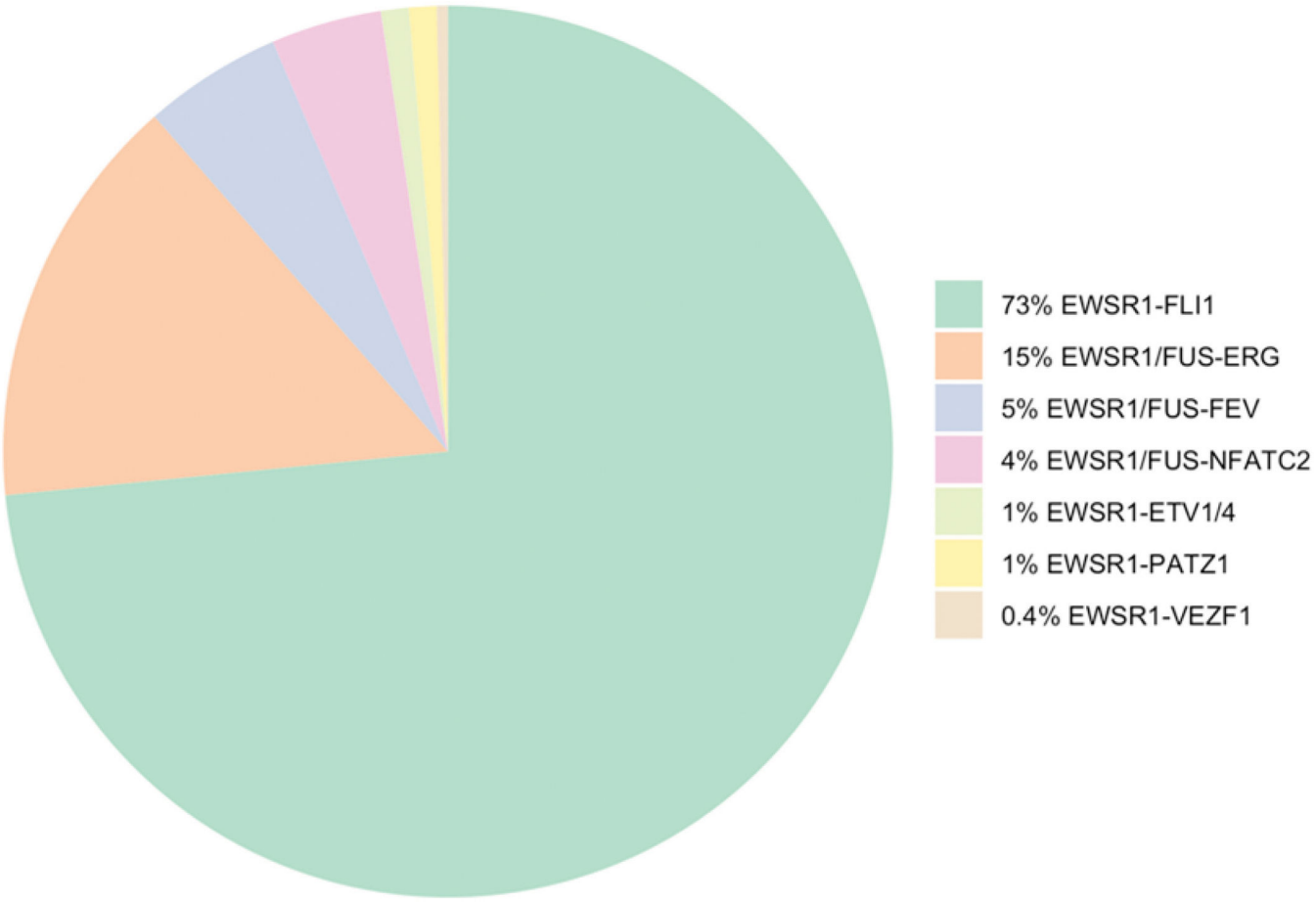


**Figure 1.**
Pie chart showing the distribution of gene fusions in Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusion.

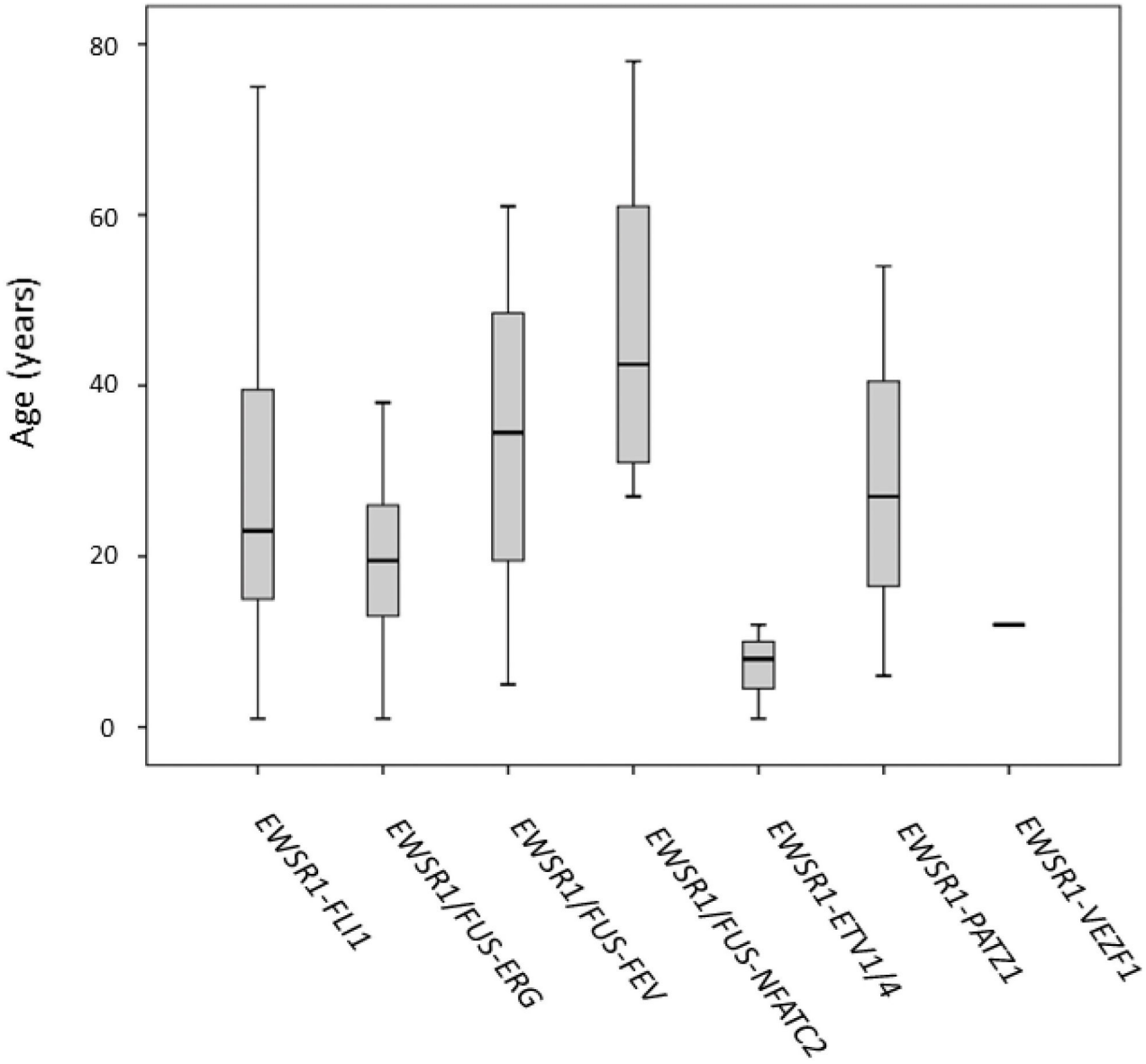


**Figure 2.**

Box plot showing age distribution of patients with round cell sarcomas with *EWSR1/FUS* gene rearrangements in relationship to gene fusions types.

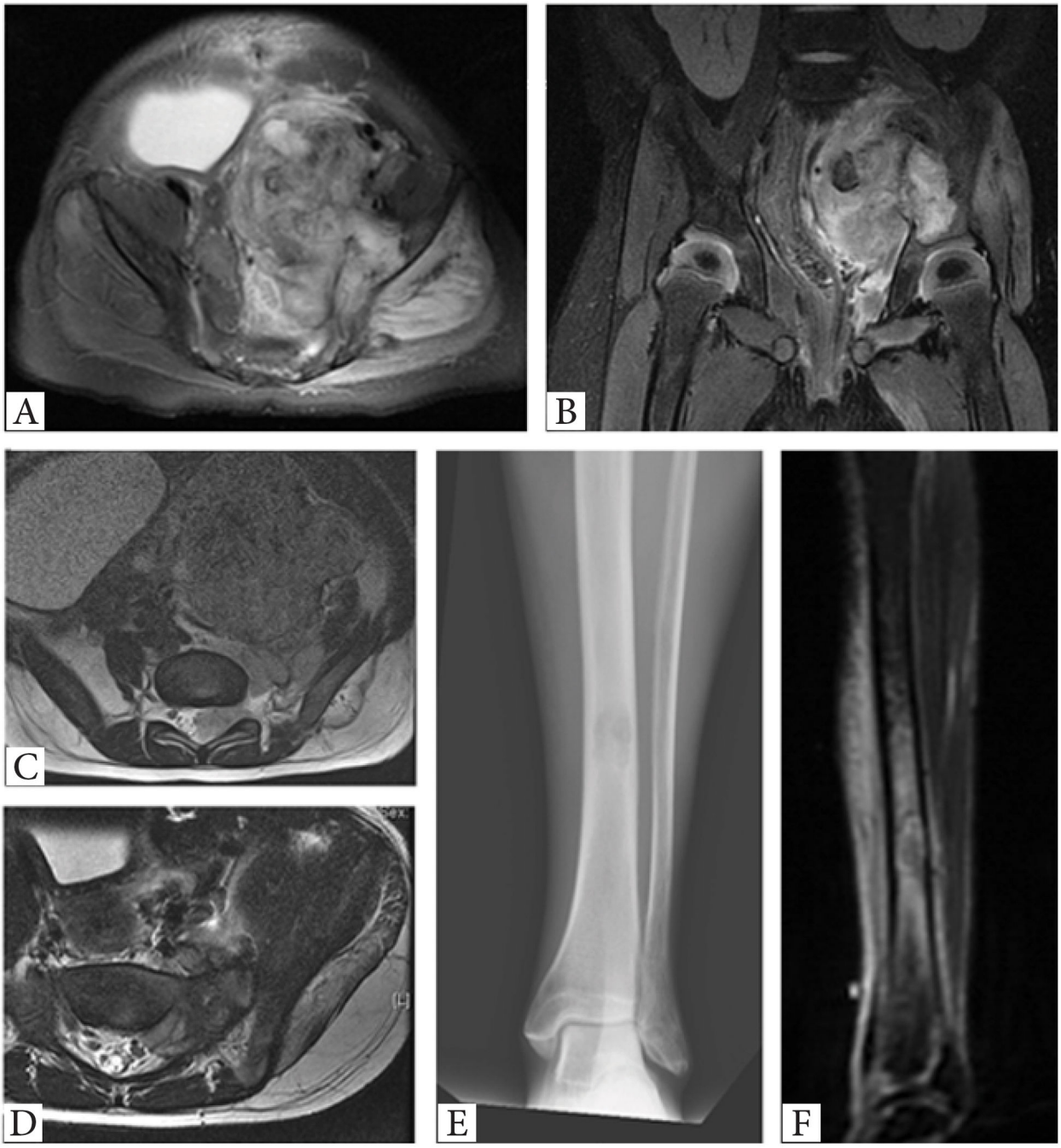


**Figure 3. Radiographic findings in rare Ewing sarcoma and round cell sarcoma with *EWSR1*-non-ETS fusion subsets.**

**A-D.** Large paraspinal/pelvic soft tissue mass in a one-year old female with a rare *EWSR1-ETV4* fusion, showing a favorable chemotherapy response to Ewing sarcoma regimen. **A,B**. MRI T2 weighted image showing a left pelvic side wall soft tissue mass extending to paraspinal area. **C**. MRI T1 weighted image showing tumor extension to the left L5/S1 neural foramen. **D**. MRI T2 weighted image showing a substantial decrease in tumor size

after preoperative chemotherapy. **E,F.** Permeative tibial lesion in a 78 year-old male with a round cell sarcoma with an *EWSR1-NFATC2* fusion.

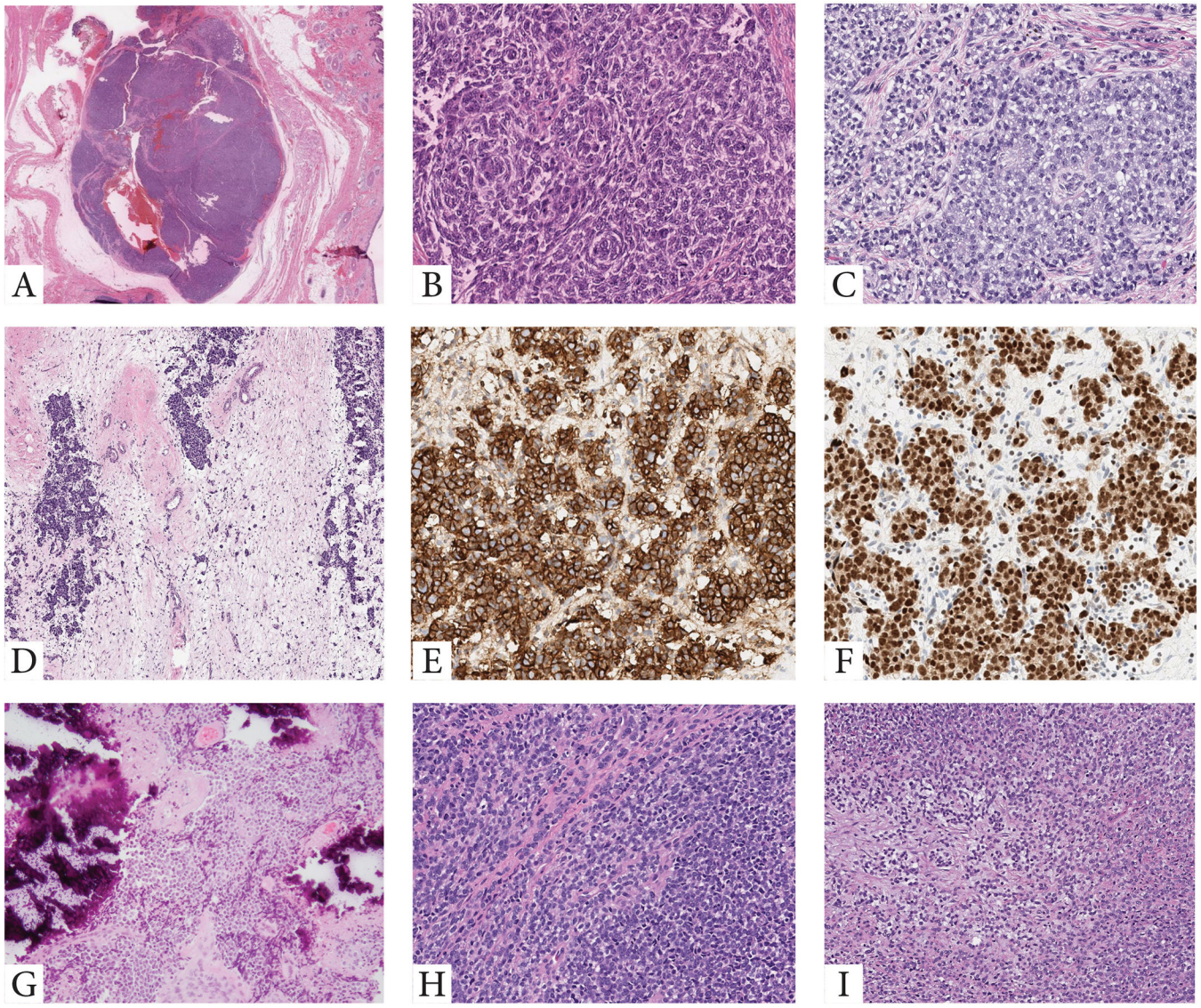


**Figure 4. Morphologic features of tumors with variant fusions in the Ewing sarcoma family.**

**A-F.** Morphologic spectrum of tumors harboring *EWSR1-ERG* fusions. **A,B.** Superficial nodule within the subcutis showing at high power primitive round to ovoid cells with a peculiar whorling-like growth (9/M, orbit). **C.** Omental Ewing sarcoma in a 21 year-old female showing solid sheets of round to epithelioid cells with moderate amount of clear to amphophilic cytoplasm. **D-F.** Post-therapy lesion showing islands of residual viable tumor cells within the fibrotic marrow spaces of the rib. Viable cells remained strongly and diffusely positive for CD99 (E) and ERG (F). **G-I.** Less common *EWSR1-ETS* variants in Ewing sarcomas. **G.** A paraspinal lesion with extensive calcifications arising in an infant with *EWSR1-ETV4* (same patient as in Fig 3). **H**. Primitive round cells in solid sheets in a tumor with *EWSR1-FEV* (10/M). **I**. Round cell malignancy with increased myxoid or fibrous stromal component in a tumor positive for *EWSR1-VEZF1* (12/F, calf).

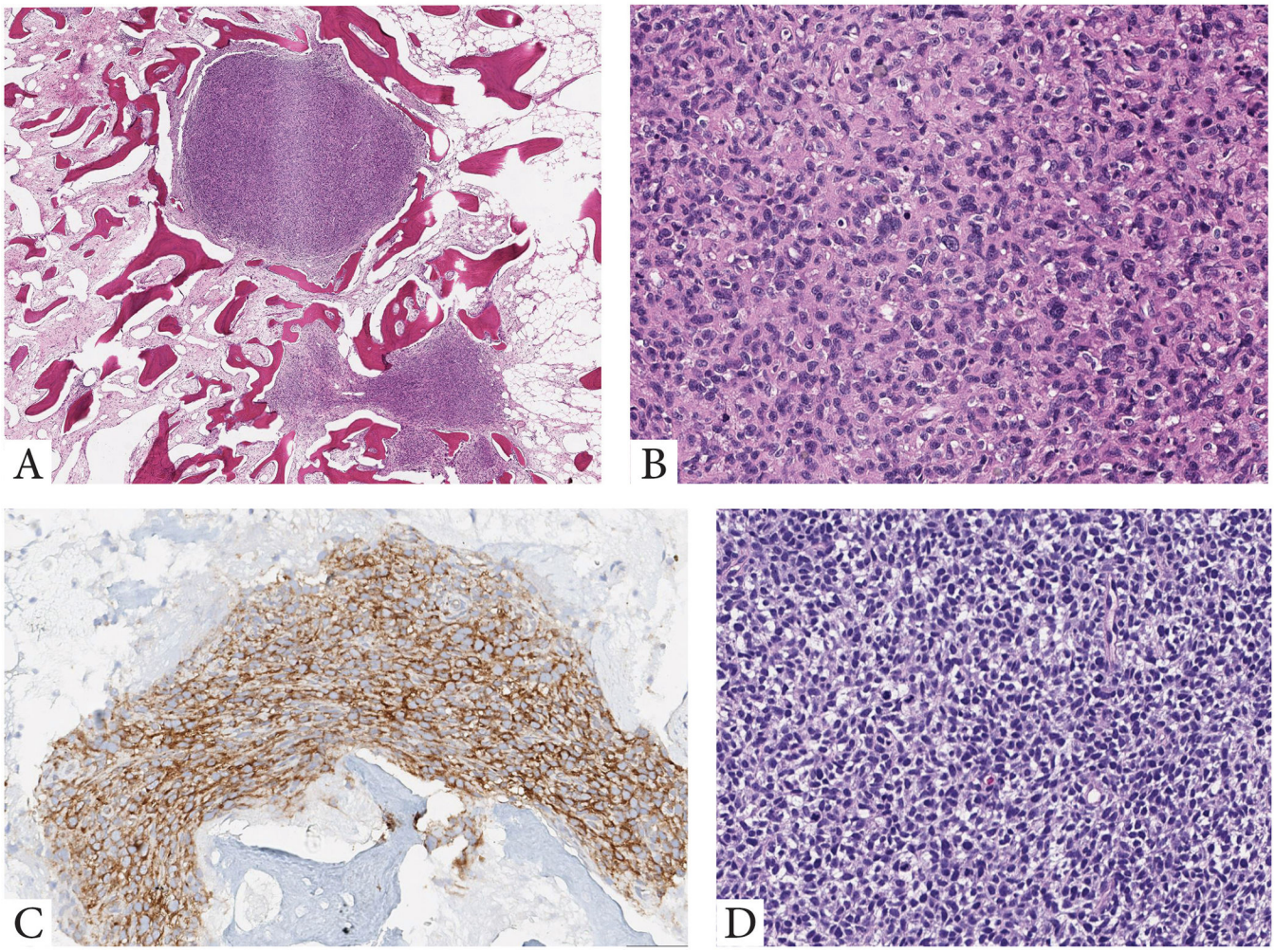


**Figure 5. Histologic spectrum of round cell sarcoma with *EWSR1*-non-ETS fusion.**

**A-C.** Intra-osseous nodules of primitive cells harboring an *EWSR1-NFATC2* fusion, which at high power were composed of a mixture of round, ovoid and scattered slightly pleomorphic cells with enlarged nuclei, and ill-defined cell borders. The tumor cells were diffusely positive for CD99 (membranous pattern) (78/M, tibia, same patient as in Fig 3). **D.** Primitive round cell tumor with an *EWSR1-PATZ1* fusion (37/M, shoulder).

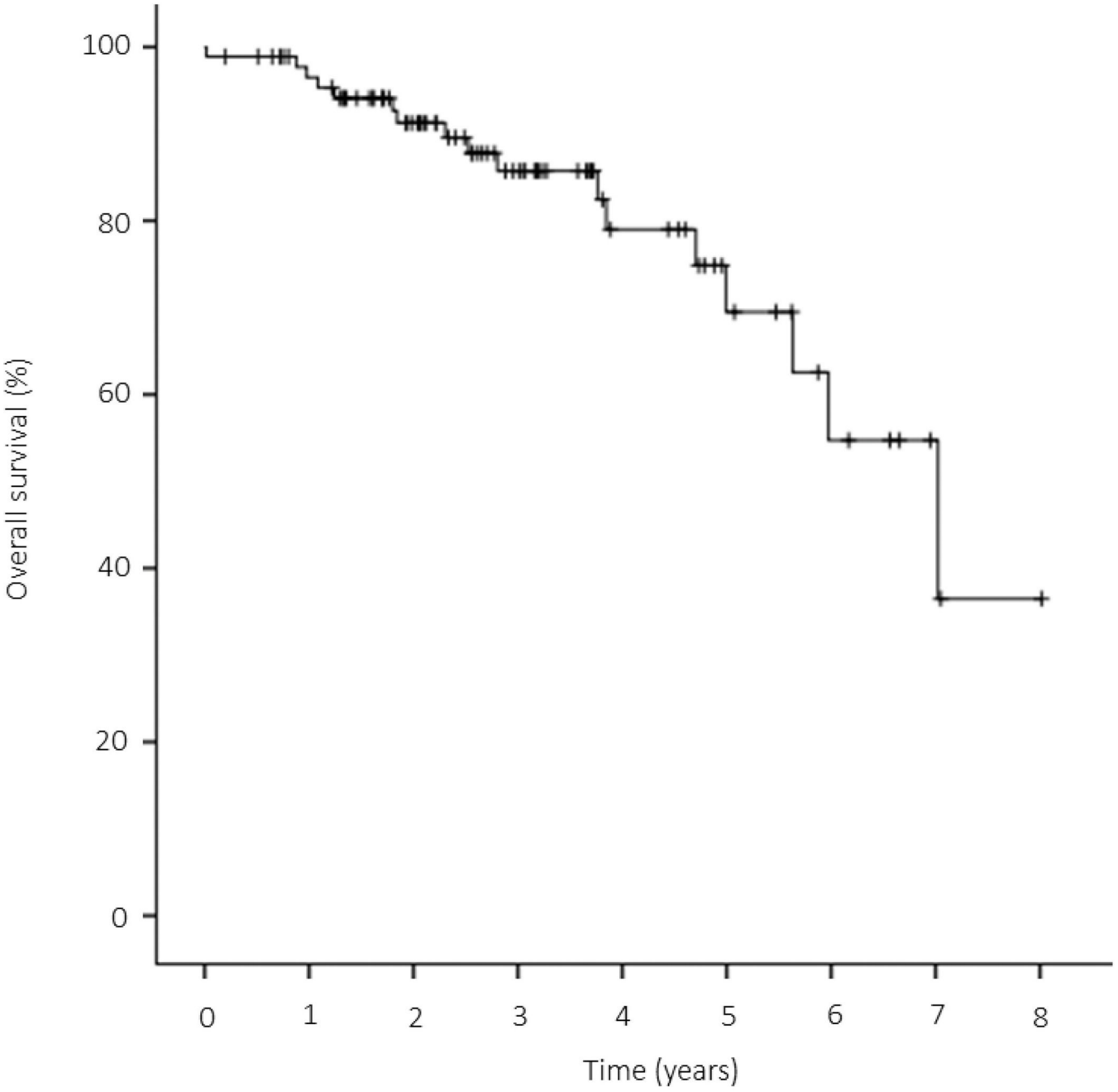


**Figure 6.**
Kaplan-Meier curve depicting the overall survival of our cohort.

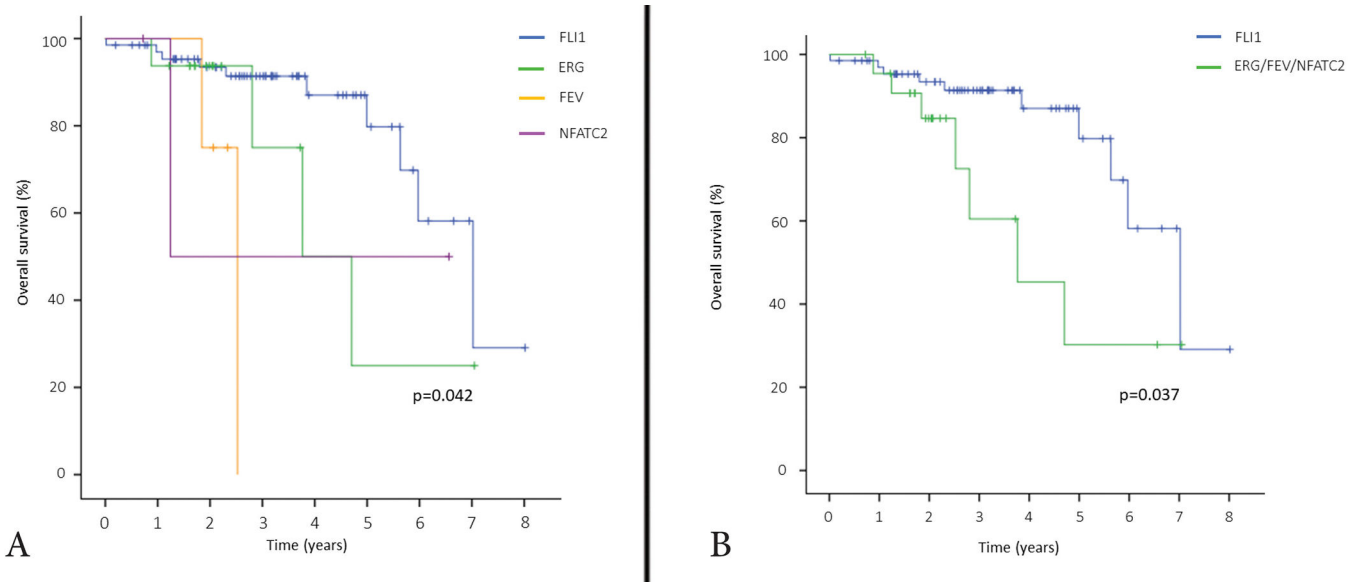


**Figure 7. Kaplan-Meier curve illustrating the overall survival stratified by gene fusion type.**
**A.** *EWSR1-FLI1* (Blue); *EWSR1/FUS-ERG* (Green); *EWSR1/FUS-FEV* (Orange); *EWSR1/FUS-NFATC2* (Purple). **B.** *EWSR1-FLI1* (Blue); other fusion types including *EWSR1/FUS-ERG*, *EWSR1/FUS-FEV*, and *EWSR1/FUS-NFATC2* (Green).

**Table 1.**

Demographic and primary site correlations with gene fusion type

| | | Total | % | *EWSR1-FLI1* | % | *EWSR1/FUS-ERG* | % | *EWSR1/FUS-FEV* | % | *EWSR1/FUS-NFATC2* | % | *EWSR1-ETV1/4* | % | *EWSR1-PATZ1* | % | *EWSR1-VEZF1* | % | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total (n = 240) | | 240 | | 176 | 73 | 35 | 15 | 12 | 5 | 10 | 4 | 3 | 1 | 3 | 1 | 1 | 0.4 | |
| Age (median, range, years) | | 23 (1–78) | | 23 (1–75) | | 20 (1–64) | | 35 (5–61) | | 43 (27–78) | | 8 (1–12) | | 27 (6–54) | | 12 (NA) | | **0.005** |
| Sex (n =238) | Male | 144 | 60 | 108 | 61 | 17 | 50 | 8 | 67 | 6 | 67 | 2 | 67 | 3 | 100 | 0 | 0 | **0.482** |
| | Female | 94 | 40 | 68 | 39 | 17 | 50 | 4 | 33 | 3 | 33 | 1 | 33 | 0 | 0 | 1 | 100 | |
| Primary site (n = 231) | Skeletal | 127 | 55 | 104 | 61 | 19 | 54 | 0 | 0 | 4 | 44 | 0 | 0 | 0 | 0 | 0 | 0 | **0.001** |
| | Extraskeletal | 104 | 45 | 67 | 39 | 15 | 46 | 12 | 100 | 5 | 56 | 3 | 100 | 1 | 100 | 1 | 100 | |
| Primary site (n = 234) | Limb | 79 | 34 | 48 | 28 | 16 | 46 | 4 | 33 | 7 | 78 | 2 | 67 | 1 | 100 | 1 | 100 | **0.006** |
| | Axial and pelvis | 155 | 66 | 125 | 72 | 19 | 54 | 8 | 67 | 2 | 22 | 1 | 33 | 0 | 0 | 0 | 0 | |

NA, not available

**Table 2.**

Demographic, clinical characteristics and survival correlations with fusion type

| | | Total | % | *EWSR1-FLI1* | % | *EWSR1/FUS-ERG* | % | *EWSR1/FUS-FEV* | % | *EWSR1/FUS-NFATC2* | % | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total | | 90 | | 67 | 74 | 16 | 18 | 4 | 4 | 3 | 3 | |
| Age (median, range, years) | | 23 (2–78) | | 23 (3–64) | | 19 (2–64) | | 28 (16–40) | | 61 (27–78) | | 0.099 |
| Sex | Male | 49 | 56 | 40 | 60 | 6 | 38 | 3 | 75 | 2 | 67 | 0.346 |
| | Female | 39 | 44 | 27 | 40 | 10 | 62 | 1 | 25 | 1 | 33 | |
| Primary site | Skeletal | 45 | 51 | 37 | 55 | 7 | 44 | 0 | 0 | 1 | 33 | 0.150 |
| | Extraskeletal | 43 | 49 | 30 | 45 | 9 | 56 | 4 | 100 | 2 | 67 | |
| Primary site | Limb | 31 | 35 | 23 | 34 | 6 | 38 | 0 | 0 | 3 | 100 | 0.052 |
| | Axial and pelvis | 57 | 65 | 44 | 66 | 10 | 62 | 4 | 100 | 0 | 0 | |
| Tumor size (median, range) | | 8 (1–26) | | 8 (1–18) | | 7 (3–22) | | 8 (5–18) | | 3 (3–26) | | 0.889 |
| AJCC stage at diagnosis* | IIa | 28 | 33 | 22 | 35 | 4 | 29 | 0 | 0 | 2 | 67 | 0.226 |
| | IIb | 24 | 29 | 20 | 32 | 3 | 21 | 1 | 25 | 0 | 0 | |
| | IVa | 18 | 21 | 14 | 22 | 2 | 14 | 1 | 25 | 1 | 33 | |
| | IVb | 14 | 17 | 7 | 11 | 5 | 36 | 2 | 50 | 0 | 0 | |
| Metastasis at presentation | | 31 | 34 | 20 | 30 | 7 | 44 | 3 | 75 | 1 | 33 | 0.245 |
| Definitive surgery | | 61 | 69 | 47 | 70 | 12 | 75 | 1 | 25 | 3 | 100 | 0.149 |
| (Neo)adjuvant chemotherapy | | 58 | 66 | 46 | 69 | 9 | 56 | 2 | 50 | 2 | 67 | 0.722 |
| Survival status | DOD | 15 | 17 | 8 | 12 | 4 | 25 | 2 | 50 | 1 | 33 | |
| | DOOD | 2 | 2 | 2 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | |

DOD, dead of disease; DOOD, dead of other diseases

*AJCC stage is not shown in six patients due to lack of size data