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Clinicopathological characteristics and genetic features of young and senior Ewing sarcoma patients

Jiali Li[1] and Yuan Ji[1*]

Abstract

Background Ewing sarcoma (EwS) is a highly malignant and heterogeneous tumor. Exploring clinicopathological characteristics and genetic features of EwS is critical for prognosis and treatment regimen.

Methods Clinicopathological characteristics and genetic features of young (≤ 30y) and senior (> 30y) EwS patients were analyzed based on histology, phenotype, and next-generation sequencing (NGS) detection.

Results The young group (18/36) presented nontypical EwS histological morphology, whereas the senior group (18/36) presented typical morphology. The prognosis of the young group was found to be worse compared with the senior group for patients without metastasis at the initial diagnosis. DNA- and RNA-based NGS was conducted on 20 extraosseous EwS patients. 16/20 samples demonstrated EWSR1-FLI1 fusion and 4/20 demonstrated EWSR1-ERG fusion. However, 13/16 EWSR1-FLI1fusions were detected both in DNA- and RNA-based NGS, 1/16 was detected only at the DNA level, and 2/16 were detected only at the RNA level. An analysis of the genetic profiles of the EWSR1-FLI1 cases revealed that the young group was inclined to couple with more copy number variations (CNV), such as CCND1, CDK4 amplification, and fusion variations, such as CHEK1-EWSR1, SLIT2-EWSR1, and EWSR1-FAM76B fusion. The senior group was more likely to have SNV or Indel mutations, such as EPHA3 and STAG2 mutations. Moreover, patients with more CNV abnormalities had a worse prognosis than those with predominantly SNP variants. In addition, compared with the senior group, the young group had significantly higher CyclinD1 protein expression.

Conclusion Clinicopathological characteristics and genetic features in young and senior EwS patients differed significantly. Targeting cell cycle dysregulation based on age subgroup may be a potential therapeutic strategy for Ewing sarcoma.

Keywords Ewing sarcoma, EWSR1-FLI1, CNV, Prognosis, Cell cycle

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Introduction

Ewing sarcoma (EwS) is a highly aggressive small round-cell sarcoma typically characterized by deeply stained nuclei, unremarkable cytoplasm, pseudo-rosette structure, finely granular chromatin, and lack of nucleolus. Frequent sites of EwS are diaphysis and metaphysis of the long bones and pelvis. Approximately 12% of the patients have been reported with incidence of EwS in anatomical sites such as soft tissue and small intestine. Treatment options such as complete surgical resection and adjuvant radiotherapy have been known to achieve a 5-year survival rate of approximately 65–70% for localized EwS, however, at the initial diagnosis itself, most patients show presence of localized infiltration or even systemic metastases, leading to poor prognosis with a 5-year survival rate of < 30% [1, 2]. Currently available incomplete statistics indicate that approximately 80% of patients suffering from EwS are children and adolescents, and incidence of EwS in patients above 30 years old is comparatively rare and more often is detected in soft tissues. To date, the biological behavior of pediatric and adult Ewing sarcoma patients remains controversial. Relevant studies dealing with age stratification in EwS patients are scarce. A study conducted by Verrill et al. reported no difference between pediatric (≤ 18 years) and adult (> 18 years) EwS patients [3]. However, an analysis of the SEER (surveillance, epidemiology, and end results) database demonstrated that adult patients with EwS have a significantly worse prognosis compared with pediatric and adolescent patients [4]. A study by Lee et al. indicated adult age to be a poor prognostic factor for overall survival for patients with EwS [5]. Therefore, an in-depth analysis of the clinicopathological and molecular biological characteristics of EwS based on age stratification is urgently needed for better understanding of the clinical prognosis and to identify targeted therapies for patients with EwS.

The characteristic molecular alteration in EwS is the fusion of the FET gene family (often EWSR1) with members of the ETS family (FET-ETS). The most common fusion type observed in 85–90% of patients is the EWSR1-FLI1 fusion caused by the t(11;22) (q24;q12) translocation, and approximately 5–10% cases exhibit EWSR1-ERG fusion. In addition, there are other rare fusion partners such as ETV1, ETV4, and FEV [6]. Genetic studies have revealed that EwS is associated with high heterogeneity and low tumor mutational burden (TMB), and the prominent concomitant mutations identified so far include STAG2 (15–22%), CDKN2A (12%), and TP53 (7%) [7]. However, dearth of meaningful duplicate mutations for early monitoring, treatment, and prognostic prediction is a problem. In this paper, we retrospectively analyzed the clinicopathological characteristics of 36 patients with EwS in Zhongshan Hospital, Fudan University, and explored the gene profiles of EwS patients with EWSR1-FLI1 fusion mutations, hoping to provide clues for prognostic prediction and targeted therapy.

Materials and methods

Patients

Thirty six EwS patients who had tested positive for EWSR1 rearrangement, who had received curative resection from July 2015 to July 2018 at the Zhongshan Hospital, Fudan University, Shanghai, China were enrolled in the study. Clinicopathological data including age, gender, location, pathological diagnosis, treatment strategy, and follow-up data were collected. The study was approved by the ethical review board of Zhongshan Hospital, and informed consent was obtained from all patients or their guardians.

Histological and immunophenotypic testing

Hematoxylin–eosin staining was performed to observe the pathological morphology of formaldehyde-fixed paraffin-embedded tissue samples (FFPE). EnVison two-step immunohistochemical staining was used for CD99, NKX2.2, BCL2, Cyclin D1, and others, to aid in the diagnosis of EwS.

Fluorescence in situ hybridization (FISH) detection

The EWSR1 (22q12) gene breakage probe (F.01194-01, Ambipin, Guangzhou) was used for fluorescence in situ hybridization (FISH) detection. The experiment was conducted in accordance with the instructions provided with the kit. Accordingly, tumor cells with uniform nucleus size, intact nuclear boundaries, homogeneous DAPI (4′,6-diamidino-2-phenylindole) staining, isolated nuclei without overlapping, and clear signals were selected. 200 tumor cells were counted for each case. Two fused yellow signal dots (2F) or closely adjacent red and green signals (< two dots in diameter) were interpreted as negative for EWSR1 rearrangement. EWSR1 rearrangement positive signals included typical or atypical hybridization patterns, which could be one fused yellow signal and one separated red, one separated green signal (1F1R1G), one fused yellow and one separated red signal(1F1R), or one fused yellow and one separated green signal (1F1G). The cutoff value was set at 30%.

NGS sequencing

Nucleic acid co-extraction was used to extract DNA and RNA from formalin-fixed paraffin-embedded tissue samples (FFPE). The 654 gene panel whole exon detection kit from Berry Oncology was used to analyze the gene expression profiles of the patients.

Statistical analysis

Statistical analyses were performed in the R environment or using SPSS 16.0 for Windows. Categorical data was compared using Chi-square or Fisher's exact tests and Kaplan– Meier method was used to calculate the OS (overall survival). The log-rank test was used to analyze the differences. All tests were two-sided, and P-values < 0.05 were considered statistically significant.

Results

Clinicopathological analysis

A total of 36 patients pathologically diagnosed with EwS were included in the study, the age of diagnosis ranged from 15 years to 90 years (mean = 34.0, median = 32.0). The patients were divided into two groups: young-aged (≤ 30 years) and senior-aged (> 30 years). Male: female ratio was 1:1.6 in the young group and 1:2 in the senior-aged group. Statistical analysis indicated no statistically significant difference with regards to gender between these two groups (P = 0.73). Simultaneous comparison of the correlation between the site of onset and age revealed that EwS in four patients from the young-aged group originated in the bone, and in 14 cases, it originated from other anatomical sites outside the bone, however, all 18 cases in the senior-aged group were of extraosseous origin (P = 0.03). Besides, the two groups did not differ with regards to the incidence of metastasis and age. Furthermore, an analysis of the histological morphology of the two groups indicated that patients from the young age group showed more atypical EwS morphology features, such as obvious wrinkling of the nuclear membrane, irregular contour, obvious nucleoli, and thick granular nuclear chromatin. Pseudo-rosette clusters were rarely seen in the young group. However, senior-aged patients showed more typical EwS morphology features such as still-regular outline, obscure nucleoli, fine granular nuclear chromatin, and pseudorosette structures (Fig. 1; Table 1). In addition, some patients demonstrated significant necrosis, but there was no statistically significant difference with age (P = 0.63). Immunohistochemical staining which was performed to detect the expression of CD99, NKX2.2, BCL2, and CyclinD1 protein, revealed diffuse strong positive expression of CD99, NKX2.2, and BCL2 in all patients, which confirmed the histological diagnosis of EwS. Meanwhile, the positive expression rate of CyclinD1 protein in the young group was 89%, and that in the senior group was 56% (P = 0.03). EWSR1 rearrangement positivity was confirmed by FISH assay in all patients (Fig. 1). The details are given in Table 1.

Molecular features

We further analyzed the gene expression profiles of 20 patients with extraosseous EwS using whole-exome sequencing (WES), which demonstrated that a total of 16 patients were positive for the EWSR1-FLI1 fusion, in which 13/16 were detected both at the DNA and RNA levels, 1/16 was detected only at the DNA level, and 2/16 were detected only at the RNA level. In addition,

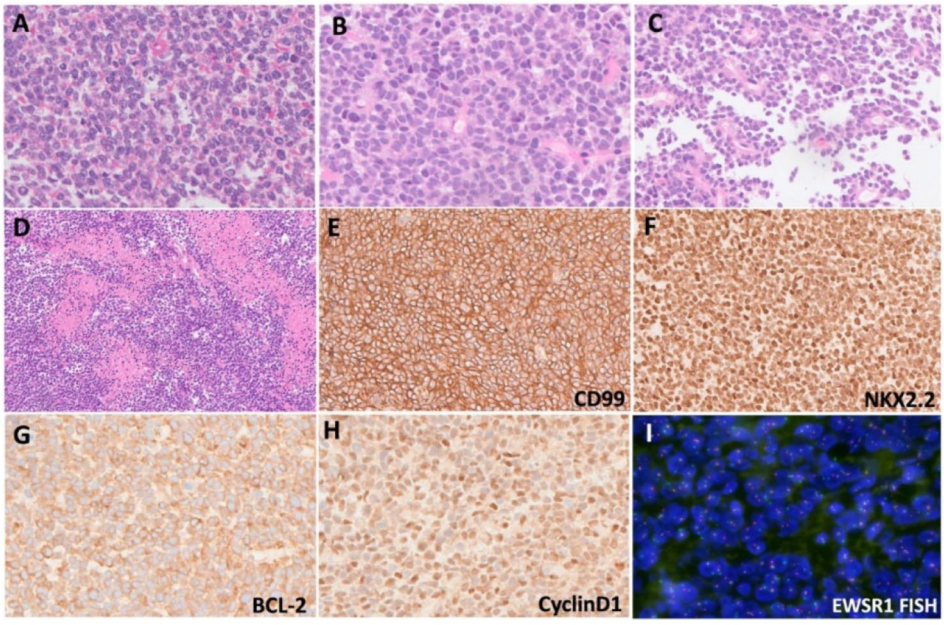

Fig. 1 **(A)** Wrinkling nuclear membrane, irregular contour, obvious nucleoli, and thick granular nuclear chromatin in ≤ 30y group; **(B)** Still-regular outline, obscure nucleoli, and fine granular nuclear chromatin in > 30y group; **(C)** Pseudorosette structures were easily detected in > 30y group; **(D)** Necrosis was observed in one ≤ 30y patients; **(E)** CD99 immunohistochemical staining showed diffuse strong positivity of the membrane; **(F)** NKX2.2 immunohistochemical staining showed diffuse strong positivity of the nuclear membrane; **(G)** BCL-2 immunohistochemical staining showed diffuse moderately strong positivity of the membrane; **(H)** CyclinD1 immunohistochemical staining in ≤ 30y group; **(I)** EWSR1 rearrangement positivity detected by FISH assay

Table 1 Comparison of clinicopathological characteristics between young and senior patients with Ewing sarcoma

Clinic-pathological characteristics		No. of patients		*P* value
		≤30y(18)	>30y(18)	
Sex	Female	11	12	0.73
	Male	7	6	
Location	Bone	4	0	**0.03***
	Extraosseous	14	18	
Nucleolus	Yes	13	3	**0.0008***
	No	5	15	
Chromatin	Regular	3	14	**0.0002***
	Irregular	15	4	
Pseudorosettes	Yes	2	13	**0.0002***
	No	16	5	
Necrosis	Yes	2	3	0.63
	No	16	15	
Metastasis	Yes	12	11	0.73
	No	6	7	
CyclinD1	Yes	16	10	**0.03***
	No	2	8	

four patients with EWSR1-ERG fusion were detected at both DNA and RNA levels (Table 2). Furthermore, concomitant mutations were detected in 9/13 patients with EWSR1-FLI1 fusion at both DNA and RNA levels. By analyzing the concomitant mutations, it was found that CDK4, CCND1, and MYC amplification were separately detected in two patients (2/9, 22%) of the ≤30 years old group. Other cell cycle regulators such as CCND2, MCL1, MYCN, and TERC copy number amplification, as well as ATM, CDH1, and CHEK1 copy number deletion, were detected in the young group. Also, three rare EWSR1 fusion types, CHEK1-EWSR1, SLIT2-EWSR1, and EWSR1-FAM76B, were detected in three young patients. In contrast, EPHA3 and STAG2 point mutations were detected in two patients (2/9, 22%) separately in the >30-year- old group. Moreover, KLF4, NTHL1, EP300, RYBP, BCL11B, DAXX, MUTYH, RET, TIPARP, ABL1, MTOR, PBRM1, PRKAR1A, SLX4, TP53, CYP2B6, PTPRS, NCOA, EWSR1, PQLQ2, ARID1A mutation, and insertion/deletion mutations including CALR, MGA, ERF, and VEGFA were also detected. However, CNV and fusion mutations were rarely found in the senior group (Fig. 2). Our results showed that copy number variants and fusion variants were more common in young patients, whereas senior-aged patients were more often accompanied by point mutations or insertion/deletion mutations.

Follow-up and prognosis

All 36 patients were initially diagnosed in our hospital, and 13/36 patients had local or distant metastases at the initial diagnosis. According to the follow-up data, all patients were treated with the VAC/IE regimen of chemotherapy and simultaneous radiotherapy after initial surgical resection. We attempted stratification with morphology and molecular findings to see if these correlate with survival. The results showed that the remarkable nucleolus was a predictor of poor prognosis of EwS patients. Moreover, patients with more

Table 2 The molecular profiles of 20 EWSR1-FLI1/ERG fusion Ewing sarcoma patients

Case	Sex	Age	Fusion partner	NGS	Accompanying Molecular Variation	Variant Type
1	F	25	EWSR1-FLI1	DNA+RNA	SLIT2-EWSR1 Fusion, CCND1/CCND2/CDK4/MYC/MCL1/TERC/MYCN Amplication	CNV、Fusion
2	F	28	EWSR1-FLI1	DNA+RNA	EWSR1-FAM76B Fusion, CCND1/MYC Amplication，ATM/CDH1/CHEK1 Deletion	CNV、Fusion
3	M	20	EWSR1-FLI1	DNA+RNA	CHEK1-EWSR1 Fusion	Fusion
4	F	16	EWSR1-FLI1	DNA+RNA	CDK4 Amplication, TBX3/KMT2D/KMT2C Mutation	CNV、SNP
5	M	29	EWSR1-FLI1	DNA+RNA	CCND1/MCL1/MYC/SOX2/TERC Amplication，EGFR/ESR1 Mutation	CNV、SNP
6	F	21	EWSR1-FLI1	RNA	PTBP1-PALM Fusion、VAX1-KIAA1598 Fusion	Fusion
7	M	32	EWSR1-FLI1	DNA	N.A	N.A
8	M	40	EWSR1-FLI1	DNA+RNA	GLI1 Amplication，CDKN2A/CDKN2B/MTAP Deletion	CNV
9	F	33	EWSR1-FLI1	DNA+RNA	ARID1A/NRTK1/BRCA2/TSC2/CREBBP	SNP
10	M	49	EWSR1-FLI1	DNA+RNA	EPHA3	SNP
11	F	38	EWSR1-FLI1	DNA+RNA	BCL11B/DAXX/ERF/MUTYH/RET/STAG2/TIPARP	SNP
12	M	66	EWSR1-FLI1	DNA+RNA	CYP2B6/PTPRS Mutation, SMAD4 Deletion	SNP、CNV
13	F	37	EWSR1-FLI1	DNA+RNA	NTHL1/EPHA3/KLF4/STAG2	SNP
14	M	39	EWSR1-FLI1	DNA+RNA	MTOR/SLX4/PRKAR1A/TP53/TP53/DOT1L/PBRM1/VEGFA/ABL1	SNP
15	F	57	EWSR1-FLI1	RNA	BCL2/CHEK1/FLT1/HDAC2/PTEN, CDK4 Amplication	SNP、CNV
16	F	37	EWSR1-FLI1	DNA+RNA	ARID1A/CALR/EP300/MGA/RYBP	SNP
17	F	44	EWSR1-ERG	DNA+RNA	BRAF、MCL1 Amplication	SNP、CNV
18	F	68	EWSR1-ERG	DNA+RNA	N.A	N.A
19	F	19	EWSR1-ERG	DNA+RNA	RAD51B/ABL1/STAG2/TSHR	SNP
20	F	54	EWSR1-ERG	DNA+RNA	N.A	N.A

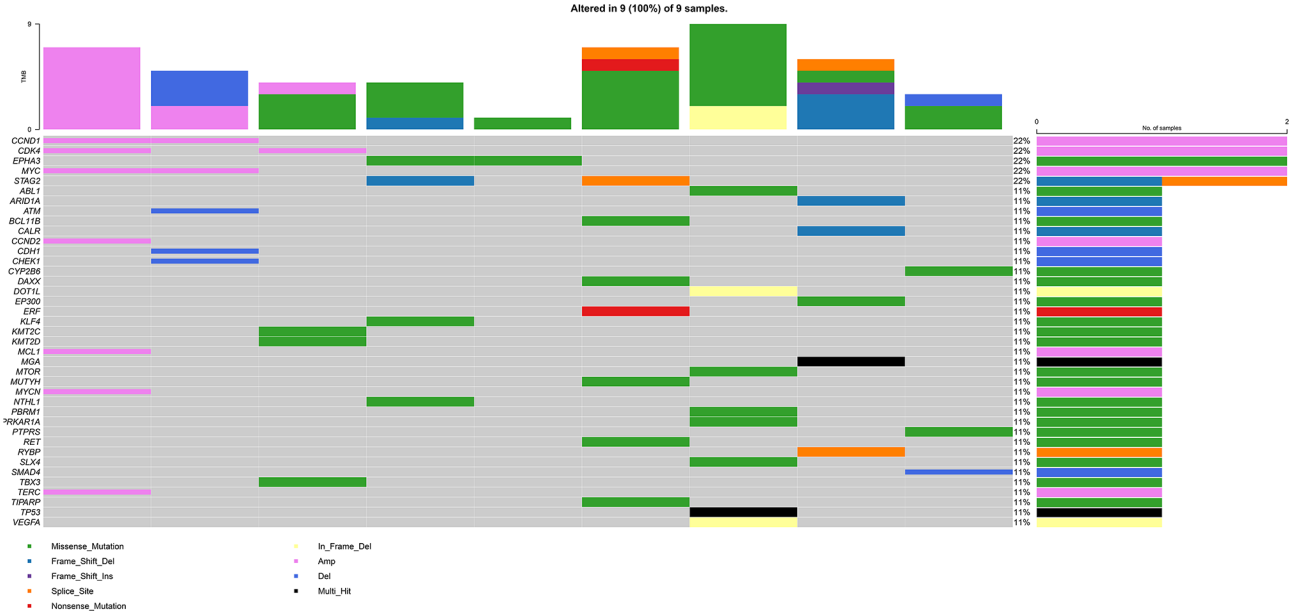

Fig. 2 Oncoplot showed the gene profile for nine EWSR1-FLI1 fusion patients confirmed by both DNA- and RNA-based NGS. The first 3 columns were ≤ 30 years old group

CNV abnormalities had a worse prognosis than those with predominantly SNP variants ($p<0.05$) (Fig. 3A-B). Besides, an independent comparison of the prognosis of the patients with or without detectable metastases at first diagnosis indicated that in patients without local infiltration and distant metastases, the survival rate was significantly lower in the ≤30 years old group (≤30 without-meta) compared with the >30 years old group (>30 without metastases) ($p<0.05$). However, no statistically significant difference in survival was observed between the two groups of patients in whom metastases were detected at initial diagnosis (Fig. 3C-D).

Discussion

In this study, we analyzed the clinicopathological features and molecular characteristics of young (≤30 years old) and senior (>30 years old) EwS patients and found significant differences in the histological morphology, prognosis and genotype of the two groups. Young patients showed a greater incidence of the morphological features of atypical EwS, whereas senior patients had more typical Ewing sarcoma histological morphology. With regards to the prognosis of patients with or without metastasis, the 5-year overall survival of young EwS patients without metastasis was found to be lower than that of seniors. Nevertheless, the overall survival of the young age and senior patients with metastasis detected at initial diagnosis showed no difference. The fact was, all of the younger patients suffered relapse or metastasis after surgical resection, which resulting in the overall survival was 0% during the follow up. Whereas, in the senior ones, the overall survival was about 60% of patients without metastasis, which indicated that the prognosis of young EwS patients was probably significantly worse than that of senior-aged patients without metastasis.

Previously, Verma et al. and Lee et al. found adults had worse prognosis than the pediatric EwS patients which directly contradict our findings. They took 18 years-old as cut-off line to compare the characteristics of pediatric (≤18 years) vs. adult (>18 years) ESFTs, which mainly include US population and minority populations, and the majority of the adult cohort in their studies were predictably skewed toward younger patients, with few who were of middle and advanced age. However, our research analyzed the Asian population in the single center, took 30 years-old as cutoff value, compared the clinicopathological and genetic features between the young (≤30y) and senior (>30y) patients. Currently, there are no studies using population-based databases that examine differential clinical factors between pediatric and adult cohorts, also, the effect of age on survival has long been a subject of debate, with different studies reporting conflicting results. Moreover, Verma et al. found the osseous and extraosseous EwS are not associated with overall survival in pediatric patients. While, the primary tumor site are associated with overall survival in adult patients, which demonstrated that the age and primary tumor site are the critical factors influenced the EwS outcome. In our study, the majority of the cohort inclined to the middle and older aged patients, the youngest patient is 15-year old. Correspondingly, 32/36 (89%) of the cases were of extraskeletal origin. We thought the target population and age-based subgroup were the critical factor which resulting in the different findings. Besides, with

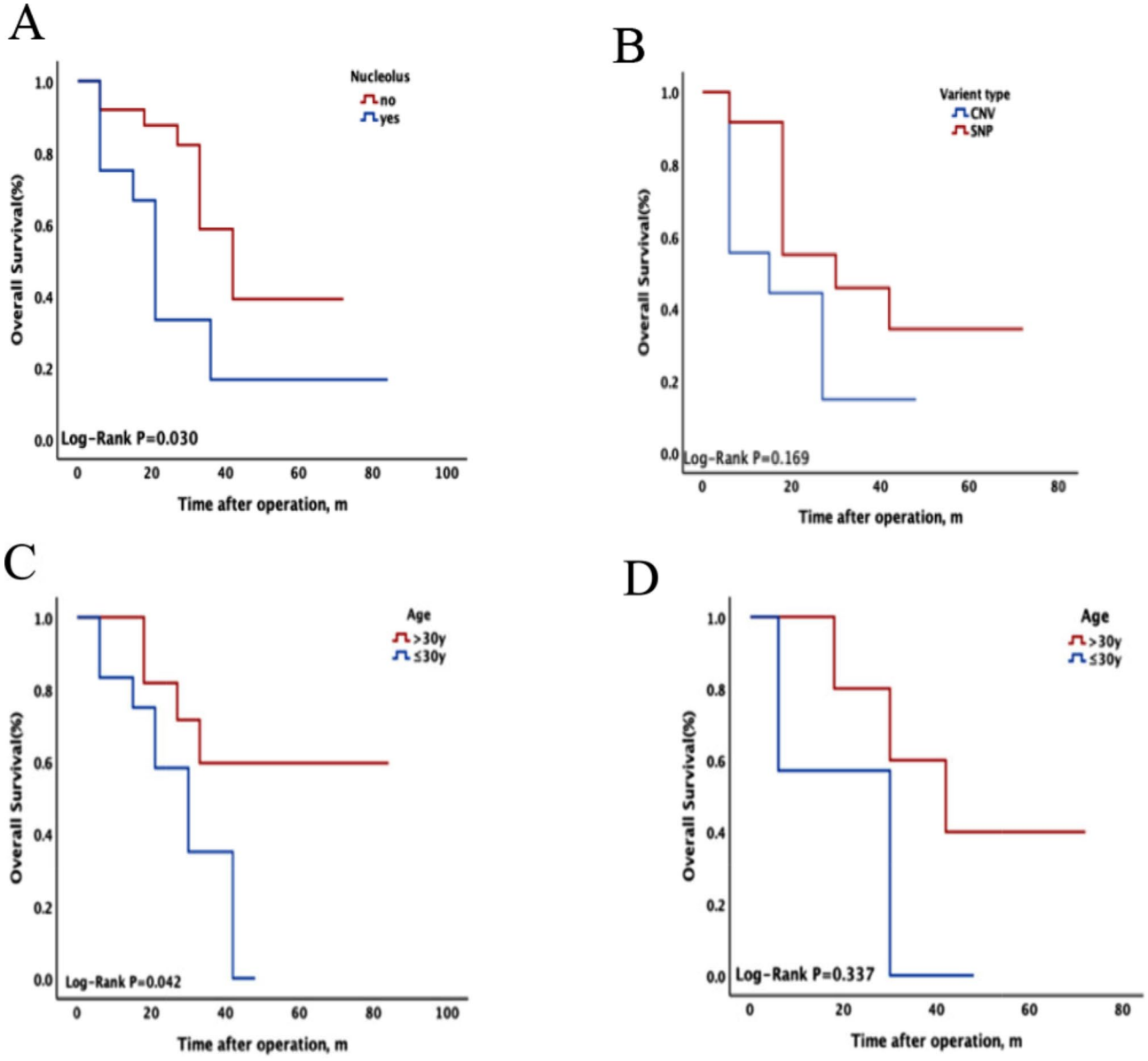

Fig. 3 **(A)** Kaplan–Meier survival analysis showing overall survival with or without nucleolus in all 36 patients. **(B)** Kaplan–Meier survival analysis showing overall survival with variant types in 20 patients. **(C)** Kaplan–Meier survival analysis showing overall survival of ≤ 30 years old group and > 30-year-old groups across all 23 patients without metastasis. **(D)** Kaplan–Meier survival analysis showing overall survival of the ≤ 30 years old group and > 30-year-old group across all 13 patients with metastases

the advancement of ES treatment, the modern treatment paradigms is of great necessity to determine that the results presented herein are accurate and representative. Indeed, due to a tiny percentage of patients of EwS, our analyses and conclusions are limited by the single-center nature and further validation is necessary for clinical utility.

Young patients with EWSR1-FLI1 fusion were more likely to have molecular abnormalities of copy number variation and fusion variation, mainly referring to cell cycle regulatory molecules, such as CCND1, CCND2, CDK4, MCL1, MYC, MYCN and TERC, than in the senior patients. Simultaneously, rare EWSR1 fusion types, namely the CHEK1-EWSR1 fusion, SLIT2-EWSR1 fusion, and EWSR1-FAM76B fusion, were detected in three young-aged patients. The role of these fusion types has not been reported in the literature. In contrast, point mutations and insertion/deletion mutations, including EPHA3 and STAG2 are predominant in senior patients. EPHA3 mutations are widely found in various cancers and sarcomas, including breast, lung, colorectal, and gastric cancers, mesenchymal tumors, and subgroups of melanoma, and are highly expressed [8]. EPHA3 mutations have been found to increase the rate of apoptosis

and G0/G1 phase cell cycle block in tumor cells in small cell lung cancer, thereby reducing resistance to chemotherapeutic agents [9]. In addition, abnormal activation of EPHA3 leads to activation of the AKT signaling pathway, thereby promoting cell growth and survival. Therefore, some studies have proposed that targeting EPHA3 as well as downstream signaling pathways (e.g., Akt) as therapeutic targets might benefit tumor patients with EPHA3 mutations [10]. The above findings indicate that cell cycle dysregulation could be a key factor in the development of EwS, but the types of mutations differ significantly between young and senior patients. In addition, studies have reported that tumor patients with more copy number variation have more unstable genomes, resulting in worse outcomes [11–14]. This might be the reason why the prognosis of young patients is worse than that of senior patients.

CyclinD1 is a critical protein in cell cycle regulation, which is expressed in some high-grade sarcomas but not in low-grade sarcomas [15]. Fagone et al. reported that the positive rate of CyclinD1 was up to 91% in pediatric and adolescent EwS patients [16], indicating that CyclinD1 could serve as an indicator for the differential diagnosis of EwS, compared with other small blue round-cell sarcomas and might serve as an important therapeutic target for EwS [17]. In this study, comparison of the protein expression levels of CyclinD1 in two groups of patients revealed that the expression rate of CyclinD1 protein was as high as 89% in young patients, but only 56% in senior patients indicating that high CyclinD1 expression in young patients might be an important factor responsible for poorer prognosis in this group of patients. However, there was no significant correlation between CyclinD1 protein expression and *CCND* gene amplification. In recent years, CDK4/6-targeted inhibitors have become potential antitumor drugs, and their efficacy has been demonstrated in a variety of tumor therapies [18, 19]. Cyclin D1 can bind to CDK4 or CDK6 and phosphorylate Rb proteins, thereby releasing the transcription factor E2F that binds to Rb, which in turn promotes cell proliferation and contributes to the transition of the cell cycle from the G1 phase to the S phase. Cyclin D1 amplification was attributed to enhanced CDK4/6 activity in breast cancer [20], but no relevant studies have been reported in EwS. Further investigations are needed to determine whether CyclinD1 amplification or high expression can predict the therapeutic effect of CDK4/6 inhibitors and enhance the activity of CDK4/6 inhibitors.

In summary, our study confirmed that the clinicopathological features and gene expression profiles of EwS significantly differ in young and elderly patients. Recognition of clinicopathological features in patients of different ages can enable determination of the prognosis and administer targeted therapy regimen for EwS patients. Also, based on age stratification, targeting cell cycle signaling pathways, such as CCND1-CDK4 and EPHA3-AKT, could serve as a targeted therapeutic strategy for EwS patients. Of course, the relatively small sample of Ewing sarcoma patients enrolled in our study was a limitation of the study, and further investigation using expanded sample is needed.

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Author contributions
Jiali Li-Implementation of the experiment; investigation, data curation, writing paper; Yuan Ji-review and supervision. All authors have read and agreed to the published version of the manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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