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REVIEW ARTICLE

Precision medicine in Ewing sarcoma: a translational point of view

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Abstract
Ewing sarcoma is a rare tumor that arises in bones of children and teenagers but, in 15% of the patients it is presented as a primary soft tissue tumor. Balanced reciprocal chimeric translocation *t*(11;22)(q24;q12), which encodes an oncogenic protein fusion (EWSR1/FLI1), is the most generalized and characteristic molecular event. Using conventional treatments, (chemotherapy, surgery and radiotherapy) long-term overall survival rate is 30% for patients with disseminated disease and 65–75% for patients with localized tumors. Urgent new effective drug development is a challenge. This review summarizes the preclinical and clinical investigational knowledge about prognostic and targetable biomarkers in Ewing sarcoma, finally suggesting a workflow for precision medicine committees.

Keywords Ewing sarcoma · Preclinical investigation · Prognostic biomarkers · Actionable pathways · Epigenomic targets · Precision medicine

Introduction

Ewing sarcoma (ES) is morphologically a round blue cell tumor. Differential diagnosis of immature tumors as ES has been a challenge in pathology laboratories for decades [1]. WHO established new diagnosis criteria for ES in 2013 [2]. Since then, the presence of a typical translocation confirms diagnosis, independently of primary tumor location and peculiar morphological data. Balanced reciprocal chimeric translocation *t*(11;22)(q24;q12), which encodes an oncogenic protein fusion (EWSR1/FLI1), is the most generalized and characteristic molecular event. *EWSR1* is a TET RNA-binding protein (like *TLS* and *TAF15*). *FLI1* is a member of the ETS family transcription factors (like *ERG, FEV, ETV1, E1AF*). Other gene fusions affecting the TET/ETS family members have been identified in ES and have diagnostic value as well [3]. ES typically arises in children's and teenagers' bone, but in 15% of the patients it is presented as a primary soft tissue tumor [4, 5]. Using conventional treatments (chemotherapy, surgery and radiotherapy), long-term overall survival rate is 30% for patients with disseminated disease and 65–75% for patients with localized tumors. Results have not improved during the last decades [6, 7]. Therefore, urgent new effective drug development is a challenge for the mankind.

Gene fusion *EWSR1/FLI1* acts as a transcription factor and has a main role in ES development, because it conditions an oncogenic transcriptional programme [8-15]. Moreover, it is suspected that *EWSR1/FLI1* determines an epigenetic dysregulation which has a remarkable position in Ewing biology. Many current and previous innovative efforts have been directed to recover this disrupted epigenetic programme. In addition, chromosomal copy number variations (CNV) are well described in Ewing tumors. There are some common CNV alterations in ES: gain of chromosome 1q, 8, 12 and loss of 9p21 and 16q [16-18]. The pathogenic role of these chromosomal aberrations is not well understood. Furthermore, as far as mutational status is concerned, ES is one of

Referees: Papo AS: alberto.pappo@stjude.org. Choy E: echoy@mgh.harvard.edu. Fox E: foxe@e-mail.chop.edu. All three are leaderships in translational medicine, and particularly in Ewing Sarcoma. They are from a different geographical area and we didn't meet them.

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the less mutated types of cancer [19, 20] and few mutations can be actionable with commercialized drugs.

Important personalized medicine projects for refractory tumors in pediatric oncology have been developed around the world in the past few years [21–27], aiming to transfer basic molecular information to the clinic. These investigational groups studied genomics and transcriptomics in a large patient series. Their interesting molecular data are enriching our previous knowledge about ES and other tumors in pediatric patients. Nevertheless, only few pediatric patients have benefited from this approximation, due to the fact that translation is a huge challenge in pediatric oncology, and many key points are still unknown [28]. In addition, a vast volume of data has been generated, and interpretation requires really important efforts.

This review summarizes the preclinical and clinical investigational knowledge about prognostic and targetable biomarkers in ES, finally suggesting a workflow for precision medicine committees.

Prognostic factors in ES

Clinical prognostic factors

The most important clinical prognostic factor at diagnosis is the presence of metastatic disease. This conditions a low survival rate [29, 30]. Other clinical prognostic factors have been described in the past few years: volume, tumor necrosis after neoadjuvant chemotherapy, age and axial location [29–32]. Comparing extraskeletal with skeletal located Ewing tumors, extraskeletal have better prognosis than those located in bone [33]. Patients that progressed while receiving initial therapy had worse prognosis than patients who relapsed later. Moreover, a short remission has bad prognosis [34, 35]. Nevertheless, this information is irrelevant when a patient affected by a refractory or relapsed ES tumor is forwarded to personalized medicine programmes [34, 35]. When patients are submitted to these programmes, general status, psychological burnout, and cumulative toxicity decrease the options of intensive interventions.

Biologic potential prognostic factors

Next to this hardly standarizable clinical data, biologic studies can enrich prognosis information at relapse. Some of them might be considered at diagnosis to stratify treatment (Table 1).

Translocation type

It was hypothesized that Ewing tumors with different translocations could have distinct prognosis in large cohorts [36, 37]. Fusion gene members have diagnostic importance, and must be taken into account when considering therapeutic alternatives. However, fusion type has not been demonstrated to be a prognostic marker [38–40].

Copy number variations (different to *chr9p21*, *CDKN2A* locus)

Some chromosomal CNV have been detected in Ewing tumors recurrently. These are gains of chromosome 1q, 8, 12 and loss of 9p21 and 16q. Chromosome 1q gain and chromosome 16q loss appear together because of an unbalanced translocation *t*(1;16) detected in this context.

It has been reported, in a short series, that patients with low copy number changes (≤ 3 copy number aberrations) have significantly better prognosis than patients with a high number of chromosomal alterations (in terms of event-free and overall survival) [41]. 1q gain and 16q loss have been associated with bad prognosis by several groups [17, 18, 42, 43]. No clear prognosis conclusions have been established about 8 and 12 chromosomal gains.

Table 1 Biological prognostic factors

Biologic data	Significance (prognosis)	References
1q gain and 16q loss	Bad	Roberts et al. [18]
STAG2 loss	Bad	Crompton et al. [57]
RASSF2 and/or *NPTX2* and/or *PHF11* gene methylation	Bad	Alholle et al. [58], Gharanei et al. [59]
High *CCL21* expression level	Good	Laurens et al. [152]
PD1/PD-L1(+) tumor stain	Bad	Kim et al. [63]
Eight and 12 chromosomal gain	Doubtful	Roberts et al. [18]
Translocation type	Doubtful	Le Deley et al. [38]
Homozygous deletion of *CDKN2A* locus	Doubtful	Lerman et al. [51]
TP53 mutations	Doubtful	Lerman et al. [51]

Copy number variations in *9p21* (*CDKN2A* locus)

CDKN2A (INK4A/ARF) homozygous deletion was first described by Kovar et al. in 1997 [44]. They observed *CDKN2A* deletions in 30% of ES tumors (8/27). This fact has been communicated later on several times [45–48]. Loss of this cell cycle arrest machinery component has been associated with worse prognosis [46, 47, 49]. Based on published data, the Children's Oncology Group (COG) committee established *CDKN2A* loss as a negative prognostic marker [50]. However, in the last years, Lerman D et al. [51] demonstrated in a large cohort that *CDKN2A* leak is not a prognostic factor in localized Ewing tumors. The work of Tirade et al. does not support that hypothesis [52]. Therefore, *CDKN2A* must not be considered an adverse prognostic factor by itself.

TP53 mutations

TP53 mutations have been proposed as a bad prognosis biological marker in ES during the last decades. Several retrospective studies were concordant with this suspicion. Different investigations sustained this hypothesis [53–56]. Their work suggested worse overall survival in the presence of *TP53* mutations. Recently, Lerman D et al. observed in a large series no significant differences in event-free survival of patients with *TP53* mutations, in the presence or not of *CDKN2A* deletions. Consequently, *TP53* mutations (present in around 10% ES patients) should not be considered as a bad prognosis factor when transferring personalized medicine study results to clinicians [51].

STAG2 (Stromal Antigen 2) mutations

STAG2 gene encodes for a component of cohesin complex (a complex required for sister chromatids cohesion after DNA replication) which is frequently mutated in ES (around 17% of tumors) [52]. *STAG2* mutations and *CDKN2A* losses are considered mutually exclusive [52]. Tirode et al. observed bad prognosis when *STAG2* and *TP53* mutations coexist. Brohl et al., in another huge sequencing effort, observed *STAG2* mutations in 21.5% of ES samples. They concluded that *STAG2* mutations may be associated with more advanced disease and a modest decrease in overall survival, independently of *TP53* status [20]. Crompton et al. described *STAG2* loss in over 15% of ES tumors. It mostly occurs by point mutation, but also by rearrangement, and this suggests that it is likely conducted by non-genetic mechanisms. *STAG2* losses were associated with metastatic disease, and therefore worse prognosis [57]. Taking into account the above, *STAG2* status may be used as a prognosis marker.

RASSF2, *NPTX2* and *PHF11* methylation pattern

The Farida Latif group, in two related papers, described poor overall survival when whichever of these genes was methylated [58, 59]. *RASSF2* (Ras Association Domain Family Member 2) codifies for one of the broad range of effector proteins that RAS protein family has [60]. RASSF2 protein interacts with KRAS [60]. RASSF2 and PAR4 (Proteinase-Activated Receptor 4) interact directly and this interaction is enhanced by activated KRAS [61]; PAR4–RASSF2 interaction allows tumor suppressor functions. When *RASSF2* is methylated, this function is lost, and maybe this contributes towards tumor development, as shown in prostate cancer [61]. In ES, an equivalent effect might happen. *NPTX2* (pentraxin II, neuronal) and *PHF11* (PHD Finger Protein 11) have an undiscovered role in cancer.

CCL21 expression levels

Laurens G. L et al. reported that higher RNA expression levels of *CCL21* condition an improved outcome. Therefore, it might be considered as a prognostic factor, although larger patient series are necessary to confirm this data [62].

PD1/PDL1 lymphocytes/ES expression

Kim et al. studied PD1 and PDL1 expression in a large cohort of ES patients. They concluded that patients with a PD1(+)/ PD-L1(+) pattern had the shortest survival time [63]. They also suggested that a PD1/PD-L1 positivity could be a criterion for a selection of patients susceptible of PD1-based immunotherapy.

Targetable biological markers

There are many molecular candidates postulated to become the best actionable target in Ewing sarcoma. However, there are very few that have gained importance (such as imatinib for GIST- or LMC-positive Philadelphia). In ES, there is still much to decide, and new candidates are being currently explored. These candidates are shown below.

EWSR1/FLI1 translocation

EWSR1/FLI1 fusion protein was described in 1992 [64]. The protein product results from *t*(11;22) and acts as an aberrant transcription factor [65]. During the last years, the oncogenic role of this fusion protein has been demonstrated in vitro and in vivo using different models [66–69]. It drives an oncogenic transcriptional programme, upregulating and downregulating thousands of genes [8, 9, 70]. Riggi et al. proposed the exact chromatin remodeling events leading to

gene activation and repression [15], and Bilke and others reported the binding sites of EWSR1/FLI1 fusion protein as GGAA microsatellite regions [71–75].

Due to *EWSR1/FLI1* suspected relevance in ES pathology, many therapeutic approaches have been directed to target the translocation or important proteins that present abnormal expression levels secondary to the fusion protein function.

A summary of them is presented.

Targeting EWSR1/FLI1 fusion protein

Targeting transcription factors directly has been a challenge for the scientific community until today. The most direct and specific approach to inactivate the EWSR1/FLI1 protein fusion is using oligonucleotides. They hybridize to specifically selected sequences and thereby break mRNA splicing and protein translation. Unfortunately, this strategy never reaches a complete absence of targeted proteins. Therefore, other strategies to block EWSR1/FLI1 activity have been developed.

Toretsky et al. described that RHA protein (RNA Helicase A) binds EWSR1/FLI1 and works in enhancing transcription signals in EWSR1/FLI1 target promoters [76]. Barber-Rotenberg et al. suggested that the small molecule YK-4-279 was able to disrupt the binding between EWSR1-FLI1 and RHA, blocking the transcriptional activity of EWSR1-FLI1 in vitro [77]. A more precise explanation for YK-4-279 mechanism of action was proposed by the same group: the disruption of EWSR1/FLI protein interactions within the spliceosome [78]. However, drug resistance was evident in some mouse cohorts [77, 79, 80]. The resistance mechanism has been studied by them, but no conclusive results have been obtained yet. Currently, clinical trials using this drug have not been registered.

Targeting downstream EWSR1/FLI1

EZH2 (enhancer of Zeste, *Drosophila*, Homolog 2)

EZH2 encodes a histone methyltransferase which methylates nucleosomal histone H3 at lysine-27 (H3-K27). It is part of the polycomb repressive complex-2 (PRC2). This complex initiates epigenetic silencing of genes involved in cell fate decisions [81, 82]. Richter et al. described an upregulation of *EZH2* in ES cells in vitro and in vivo, since EWSR1/FLI binds *EZH2* promoter to potentiate its expression. The high *EZH2* activity in ES suggests an important role in oncogenicity and immaturity of this tumor [83] and therefore, it is a potential target [84]. Pankita et al. have studied (in vitro) if tazemetostat (highly selective EZH2 inhibitor) potentiates or not chemotherapy agents (etoposide and irinotecan). Their preclinical results, not published yet, are hopefully in this direction [85]. Currently, one pediatric MATCH clinical trial is recruiting ES patients that are *EZH2* mutation carriers (NCT03155620) to receive tazemetostat, but it is not using EZH2 protein expression levels as a biomarker. In a small series, *EZH2* high expression has been suggested as a bad prognosis factor too [86]. More preclinical studies are necessary. The combination of EZH2 inhibitors with chemotherapy agents should be studied in a trial on ES in the near future.

BET proteins

BET proteins (BRD2, BRD3, BRD4) are bromodomain (BRD)-containing proteins [87] that recognize acetylated lysine residues on the tails of histone proteins. EWSR1/FLI1 fusion protein deregulates epigenetic programme and can lead to the creation of specific epigenetic marks [15, 88]. On this basis, BET inhibitors were tested and have demonstrated downregulation of genes involved in ES pathogenesis [89]. BET inhibitors drive apoptosis in ES cell lines too and, interestingly, the association of these drugs with PI3K/mTOR inhibitor *BEZ235* increases apoptosis [89]. Similar effects with EZH2 inhibitor combination are described for them. Several BET inhibitors have been tested in clinical trials, but none is recruiting ES patients yet.

LSD1 (lysine-specific demethylase 1)

Lysine-specific demethylase 1 was the first discovered histone lysine demethylase and can demethylate both H3K4me2/1 and H3K9me2/1 [90]. Due to the fact that ES fusion gene causes a deregulated epigenetic programme, it was hypothesized that this protein could be altered in ES tumors. Schildhaus et al. demonstrated high levels of LSD1 in different sarcoma types [91], and 1 year later Bennani-Baiti et al. confirmed the same phenomenon in ES, LSD1 inhibitors interfere with cell growth in ES (in vitro) [92]. Sankar et al. demonstrated that LSD1 inhibition with *HCI2509* modifies the downstream oncogenic phenotypes driven by EWSR1/ETS fusions both in vitro and in vivo ES models [93]. Reversing epigenetic modifications using LSD1 inhibitors were reproduced for other groups [94]. Currently, two phase I trials are recruiting ES patients to test LSD1 inhibitors: SP-2577 (NCT03600649) and INCB059872 (NCT03514407).

NKX2.2 (NK2Homeobox2)

The protein encoded by this gene contains a homeobox domain and its physiologic function as a transcription factor is only partially understood. It may be involved in the morphogenesis of the central nervous system and develops a role in the maintenance of *NEUROD1* expression in the

endocrine pancreas cells. Curiously, Smith et al. discovered a high expression of NKX2.2 in ES tumors when this gene had not been related with cancer previously [95]. This work established *NKX2.2* as a EWSR1/FLI target in ES. They showed that loss of *NKX2.2* expression via RNAi results in a loss of oncogenic transformation [95]. Afterwards, Owen et al. concluded that *NKX2.2* collaborates in oncogenic transformation via transcriptional repression (using repressor domains of NKX2.2), while the NKX2.2 transcriptional activation domain is dispensable [96]. Under these circumstances, *NKX2.2* could also be proposed as a therapeutic target for Ewing sarcoma. Nevertheless, targeting transcription factors is difficult. Successfully, other therapeutic strategies have demonstrated *NKX2.2* downregulation. In preclinical studies, treatment with HDAC inhibitor (vorinostat) depressed gene targets of EWSR1/FLI (*NKX2.2, BCL11B*). This effect is probably due to the fact that NKX2.2 recruits transcriptional corepressors and HDACs to gene promoters to develop its function [96]. Sampson et al. combined vorinostat with temozolomide, and irinotecan. Interestingly, they reported enhanced cytotoxicity in vitro when temozolomide is administered before vorinostat [97]. When targeting LSD1, similar effects to HDACs in ES models have been described [93].

Cyclin *D1* gene (*CCND1*) and cyclin *D4* gene (*CDK4*)

Starting from Riggi N. et al.'s study about chromatin remodeling mechanisms, Alyssa L. Kennedy et al. elaborated super-enhancer regions profiles. Super-enhancer regions of chromatin are regions of open chromatin with acetylated histones, transcription factors and transcriptional activators to promote transcription. Super-enhancer regions are corrupted in ES and they mark a tumor-specific gene expression programme [98]. They confirmed using this approach that ES was selectively dependent on cyclin D1 gene (*CCND1)* and cyclin D4 gene (*CDK4*) compared to other cancer cell lines. Moreover, they showed that ES cell lines are sensitive to pharmacological inhibition of CDK4/6, both in vitro and in vivo [98]. This study proposing CDK4/6 inhibitors is complementary to point 4 (see below).

PRKCB (protein kinase C beta)

Protein kinase C (PKC) family can be activated by calcium and second messenger diacylglycerol. PKC family members phosphorylate a wide variety of protein targets. Surdez et al. described that transcriptional activation of PRKCB (a member of PKC family) is directly regulated by the chimeric fusion *EWSR1/FLI1*. They detected that PRKCB phosphorylates histone H3T6 to permit global maintenance of H3K4 trimethylation at a variety of gene promoters involved in ES [99]. Nowadays, PKC inhibitors are in clinical trials, but none including ES patients.

HSP90 (heat shock 90 kDa protein)

HSP90 is a chaperone protein that promotes maturation, structural maintenance and proper regulation of specific protein targets involved in cell cycle control and signal transduction, among other functions. *EWSR1/FLI1* and *EWSR1/E1AF* fusion genes activate telomerase and up-regulate TERT (reverse transcriptase telomerase) in ES cell lines [100]. Ambatia et al. described that TERT is a client protein of the HSP90 chaperone complex and inhibition of HSP90 led to decreased TERT expression in cell lines [101]. They hypothesized that the combination of HSP90 inhibitors and bortezomib may exert more potent inhibitory effects [101].

FOXO1 (Forkhead box O1)

FOXO1 is a transcription factor that regulates differentiation, proliferation, tumor suppression, autophagy, and cell death [102]. *FOXO1* is transcriptionally repressed by EWSR1/FLI1 through direct promoter binding [102]. Interestingly, methylseleninic acid (MSA) increases *FOXO1* expression in the presence of EWSR1/FLI1, and induces massive cell death [103, 104]. This drug is not currently used in clinical trials.

Non-specific downstream effective drugs

Trabectedin is a marine alkaloid isolated from the Caribbean tunicate *Ecteinascidia turbinata* [105]. This drug interferes with the activity of EWSR1/FLI1 [10]. Grohar et al. described that trabectedin reverses a gene signature of induced downstream targets of *EWSR1/FLI1* in ES cell lines [106]. Until today, it has been approved for refractory adult sarcoma. Clinical trials with trabectedin in pediatric ES have been conducted. The trial of trabectedin in treating young patients with recurrent or refractory soft tissue sarcoma or Ewing's family of tumors (NCT00070109) has been completed. A number of eight patients received the drug: five progressed, two discontinued due to adverse events and one died. Amaral et al. published that trabectedin was able to strongly reduce EWSR1/FLI1 effects in vitro and in xenografts, but leading to IGF1R upregulation. The combination of trabectedin with IGFR1 inhibitors potentiates the efficacy of trabectedin in vitro and in vivo [107]. However, clinical trials are not underway yet. Lurbinectedin is a second-generation drug. Harlow et al. reported a complete reversal of EWSR1/FLI1 activity and elimination of established tumors in 30–70% of mice treated with this drug plus irinotecan [108]. This combination is being explored in clinical trials (NCT02611024).

Grohar et al. described ES preclinical sensitivity to mithramycin. They started a clinical trial but serious toxicities (hepatotoxicities) were detected and this limited its use [109]. The same group developed less toxic analogs EC-8105 and EC-8042. Both were less toxic than mithramycin in vivo but maintained suppression of EWS-FLI1 at similar concentrations [110]. None of them have been investigated in trials yet.

Midostaurin was also detected as a potential targeted therapy in ES during drug screening. This approach also found synergistic effect of simultaneous application of PKC412 (midostaurin) and IGF1R inhibitors [111].

IGF1/IGF1R

Autocrine and paracrine activated loop IGF1/IGF1R (insulin-like growth factor-I receptor) was postulated as a growing determinant in ES more than 20 years ago [112, 113], because *EWSR1/FLI-1* gene fusion expression disrupts the IGFIR signaling pathway [114]. Therefore, it could be considered as a downstream element of ES fusion proteins. However, the importance of IGF1R in ES deserves an independent commentary. Kang et al. showed in vivo that IGF1R activation was independent of ES fusion protein type. They demonstrated IGF1-induced expression in the presence of EWSR1/FLI1, EWSR1/ERG and FUS/ERG fusion proteins [115]. IGF1R inhibitors were developed and tested in preclinical models [116–118]. Several difficulties have been detected during preclinical efforts and clinical trials. Due to the similarity of the structures of IGF1R and INSR (insulin receptor), synthesis of selective inhibitors of IGF1R is very complex. Monoclonal antibodies directed to IGF1R have been the main strategy developed. R1507 [119], cixutumumab and [120] figitumumab [121] were well-tolerated drugs, but modest responses in monotherapy were detected when treating patients. Moreover, detecting predictors of response is also difficult. There was not an apparent correlation between response to cixutumumab and tumor expression of IGF1, IGF2, or IGF1R [120]. However, a strong association between IGF-1 serum levels pretreatment and survival benefit was identified by Juergens et al. [121].

As in other drugs, acquired resistance to IGF1R blockage is a major problem when targeting IGFIR in ES [122]. Combination of IGF1R inhibitors and mTor inhibitors or ERK inhibitors has been proposed as alternative, after studying resistance mechanism in monotherapy responding patients [123, 124]. In this way, hopeful results were obtained trialing cixutumumab plus temsirolimus (mTor1 inhibitor) [125]. Ambatia et al. proposed an association of HSP90 inhibitors with IGF1R blocking drugs, because HSP90 was upregulated in treated patients [101]. Nowadays, there are no clinical trials with cixutumumab or figitumumab in monotherapy or combination, recruiting ES patients.

With some difficulties, small molecules targeting IGF1R have been developed too.

OSI-906 (IGF1R and insulin receptor (INSR) inhibitor) has been tested in combination with erlotinib in solid tumors [126]. ADW742 induces apoptosis in ES cell lines, and synergizes with imatinib [127, 128]. Nevertheless, these molecules are not currently in clinical recruiting trials.

Despite these limitations, addressing IGF1R in a combined way is still of interest in ES. Efforts aimed to establish predictive response markers are essential for our patients.

PARP (Poly-ADP-ribose polymerase)

PARP is an enzyme that collaborates in base excision repair (BER) pathway. It catalyzes the poly-ADP-ribosylation of some acceptor proteins involved in chromatin architecture and in DNA metabolism when DNA damage is present. It is an essential step leading to reparation of DNA strand breaks. Soldatenkov et al. described that wildtype ETS transcription factors regulate positively *PARP* levels, but surprisingly, changes in EWSR1/FLI1 expression do not cause changes in *PARP* expression level. Therefore, it was hypothesized that the fusion protein acted directly on DNA repair pathway and allowed tumor cells to avoid apoptosis, independently of *PARP* [129].

Although the exact biological reasons are not well understood, *PARP* presents high levels in ES tumors [130]. The DNA break repair is defective in ES and tumor responses were seen with PARP inhibitors. At present, this is one of the most expanding fields in new therapies for ES. The reasons of response to PARP inhibitors were studied by Gill et al. They concluded that effectiveness in ES is not caused by a defect in homologous DNA repair, but through hypersensitivity to trapped PARP1–DNA complexes. This drives accumulation of DNA damage during replication, ultimately leading to apoptosis [131]. Stewart et al. described in vivo cytotoxicity with olaparib (PARP inhibitor) alone and higher in combination with temozolomide or irinotecan [132]. Norris et al. did not demonstrate synergism in vivo [133]. However, Engert et al. again, suggested synergism between PARP inhibitors and temozolomide and also cytotoxicity with doxorubicin, etoposide or ifosfamide [134]. Other PARP inhibitors showed similar effects [135]. Based on these preclinical approximations, clinical trials have been conducted. Olaparib in monotherapy was trialed without objective responses (no selection of patients based in biological markers was done) [136]. At this moment, ES patients have been recruited in two clinical trials with olaparib monotherapy (NCT03233204) or combined (NCT01858168); talazoparib has one recruiting trial (NCT02116777) and there is an active trial with niraparib (NCT02044120).

Another interesting focus is PARP inhibitors in combination with trabectedin. Ordóñez et al. described relevant

antitumor activity in preclinical models [137]. Smiths et al.'s proposal about synergic effects in apoptosis when associating radiotherapy with PARP inhibitors is also interesting [138].

Again, we are facing a huge problem: which biological markers should be used to select candidate patients for targeted therapy? Clinical trials and their published results are really required. We cannot personalize treatment in refractory patients without knowledge about these potential targetable biomarkers.

ATR (ataxia telangiectasia and Rad3-related protein)

ATR is an essential mediator of genomic integrity, replication-fork stability, cell cycle checkpoints, and DNA repair [139]. Activated oncogenes induce collapse of DNA replication forks, that generate replicative stress, double-strand breaks (DSBs) and therefore genomic instability [140]. High levels of replicative stress, facilitate tumoral cell death. ATR and CHEK1 are replicative stress response proteins. Targeting ATR leads to an increase in the levels of replicative stress that condition higher tumor toxicity. Nieto Soler et al. studied in ES cell lines that toxicity of ATR inhibitors correlated with CHEK1 and H2AFX expression levels (proportionally to the replicative stress) [141]. ATR inhibitors (VX-970, BAY1895344, M6620, AZD6738) are in clinical trials, but none are recruiting ES patients.

Homozygous *CDKN2A* deletion (9p21 deletion)

CDKN2A gene encodes 2 proteins: p16 (INK4), which is a cyclin-dependent kinase inhibitor, and p14 (ARF), which binds the p53-stabilizing protein MDM2 [142]. Wildtype p16 (INK4) arrests normal diploid cells in late G1 (p16-dependent cell cycle arrest is lost in cells lacking functional RB protein) [143, 144]. Promoting cell cycle arrest, p16 (INK4) interacts with CDK4 and inhibits its kinase activity [145]. Under these circumstances, CDK4 protein cannot phosphorylate RB protein, and this maintains cell cycle repression (RB mediated) [146].

Due to *CDKN2A* homozygous deletion in ES, the cell cycle is dysregulated and CDK4/CDK6 inhibitors could be useful targeted therapies, because they can mimic *CDKN2A* function at the cell cycle level. Marco Perez et al. investigated this in sarcomas (not exclusively ES) and their work supported the efficacy of CDK4 inhibitors against sarcomas displaying increased CDK4 levels, particularly in fibrosarcomas and MPNST (malignant peripheral nerve sheath tumor). High levels of p16 (INK4) indicated poor efficacy of CDK4 inhibitors [147]. This approximation hit depends on RB1 normal function. Unfortunately, *RB1* is lost in around 10% ES tumors [48]. Moreover, Hu et al. described in ES cell cultures, that only hypophosphorylated forms of pRband p130 are detectable after knockdown of EWS/FLI1, thus indicating activation of the pRB pathway in EWSR1/FLI1-positive ES tumors [148]. Schwentner et al. hypothesized that EWSR1/FLI1 mediates cell cycle activation due to the replacement of E2F3/pRB by constitutively expressed repressive E2F4/p130 [149]. Summarizing, cell cycle oncogenic activation because of *RB1* mutations and/or *EWSR1/FLI1* effects could be a mechanism of CDK4/6 inhibitors resistance in ES.

Furthermore, IGF1R has been proposed as a gene whose overexpression promotes drug escape to CDK4/6 inhibitors in ES tumors. Guenther et al. found elevated levels of fosfo-IGF1R in resistant cells. They suggest that dual targeting of CDK4/6 and IGF1R provides a candidate synergistic drug combination in ES [150]. Murakami et al. evaluated the efficacy of inhibitors of CDK4/6 and IGF1R inhibitors on ES patient-derived orthotopic xenograft mouse. Results were hopeful in that personalized study, but the model came from a *FUS/ERG*-positive tumor, and therefore not *EWSR1/FLI1*, as it is commonly found [151].

Nowadays, some trials with CDK4/6 inhibitors in ES are in course trying to reach conclusions. NCI-COG Pediatric MATCH study has two clinical trials using palbociclib in ES. Besides, abemaciclib has one clinical trial registered in ES (NCT02644460). In spite of that, many questions are unsolved: would certain levels of CDK4, p16 (INK4) and wildtype pRB be predictive of a response to CDK4/6 inhibitors? Could association of CDK4/6 inhibitors and *EWSR1/FLI1* targeted therapies be a therapeutic option? Will CDK4/6 inhibitors and IGF1R inhibitors association open new target therapies in ES? Can *EWSR1/FLI1*-negative ES tumors respond better to cyclin inhibitors?

Actionable mutated genes in ES

ES family tumors have few somatic mutations, but this low rate of mutations only exceptionally affects actionable genes [152]. Therefore, few pathogenic variants have been described in *BRCA2* [20], *KRAS* (G13N), *MET* (T1010I and N375S), *PIK3CA* [153] or *MLL2* [57]. Moreover, *EZH2* is mutated in around 2.5% of ES tumors [52]. Exceptionally, mutations in *BRAF* (BRAF V600E) are present in ES [154]. Additional difficulties are detected when targeted drugs as vemurafenib (BRAF V600E inhibitor) do not achieve the same effect as in other tumors (melanoma, low-grade gliomas, etc.). Gouravan et al. studied *BRAF* V600E mutated ES cell lines, and detected reduced phosphorylation of ERK during treatment with vemurafenib, suggesting that the MAPK pathway is active under this circumstances. This could suggest a bypass pathway to be targeted in association with BRAF inhibitors [154].

Although ES has a low rate of actionable mutations, a small number of patients could benefit from this

approximation. Therefore, next-generation sequencing should be a part of personalized focuses.

Tyrosine kinase receptors (c-KIT, PDGFR)

c-KIT and PDGFR are two class III receptor tyrosine kinases [155]. Scotlandi K et al. established that approximately 30% of patients expressed KIT in their primary ES tumor [156]. After that, Bozzi et al. provided evidence of constitutive KIT, PDGFRA, and PDGFRB expression/activation as a part of an autocrine/paracrine loop in ES (not activating mutations in tyrosine kinase domain of *KIT*, *PDGFRA*, and *PDGFRB* were detected) [157]. Both works propose imatinib (PDGFRA and KIT inhibitor) as a potential effective drug in this situation. Scotlandi et al. described that the drug acts additively with doxorubicin, inhibiting ES cell growth. Bozzi et al. combined imatinib with cisplatin in two chordoma patients and described a tumor response [157]. Yerushlami et al. studied that the effect of a combination of imatinib and cisplatin which produced a dose-dependent antiproliferative effect in ES cell lines, but there was no evidence of apoptosis [158].

On the basis of this preclinical information, imatinib has been investigated in ES clinical trials. Chao J et al. selected patients for tumor immunohistochemical expression equal or higher than 2 + /4 + . Only one patient with 3 + /4 + PDGFRA and 3 + /4 + KIT expression had a partial response. Other patients progressed under imatinib treatment [159]. Other concluded trials with imatinib in ES have not published their results yet. Imatinib–cisplatin and/or doxorubicin combinations have been scarcely investigated until today. Currently, clinical trials with regorafenib in monotherapy (NCT02048371) or combined with vincristine and irinotecan (NCT02085148) are ongoing. No other bimodal KIT and PDGFRA inhibitors (imatinib or pazopanib) are in trials.

VEGF/VEGFR(vascular endothelial growth factor and its receptor)

VEGF is highly expressed in ES tumors [160]. Endothelial development is mediated by VEGF-165 isoform [161]. Zhou et al. blocked VEGF receptor 2 (VEGFR-2) with antibodies and this suppressed tumor growth. This reduced tumor vessel formation in mice. The same group, based on previous knowledge about IGF1 function in VEGF-mediated angiogenesis, investigated a VEGFR2 inhibitor and a IGF1R inhibitor in mice with hopeful results [162, 163].

Cediranib (a pan-vascular endothelial growth factor receptor inhibitor) was used in 16 pediatric patients with solid tumors and one of three ES patients had a significant partial response [164]. Clinical trial NCT00516295 (with topotecan, vincristine, cyclophosphamide and bevacizumab) did not complete feasibility assessment. Multi-tyrosine kinase inhibitors (including VEGFR inhibitor) sorafenib and pazopanib have been tried and those trials were concluded. At present, regorafenib trials are ongoing (NCT02085148, NCT02048371, NCT02389244) (Table 2).

Based on adult tumors knowledge, some VEGF polymorphisms, VEGFR1 levels and VEGFR somatic mutations can be promising biomarkers for drug response in therapies targeting VEGF/VEGFR signaling [102].

Other interesting focuses

Winter et al. have published responses to tozasertib in ES cell lines. The combined inhibition of Aurora kinases A and B underlies its effect. Synergism with etoposide has also been proposed [165]. Alisertib did not complete phase I in ES patients [166].

STAT3 is a transcription factor that is activated by JAK proteins in the cytosol and upregulated in ES. It could be involved in sarcoma cell migration and invasion. Targeting it with a JAK-1/2 inhibitor, ruxolitinib might be an interesting investigation way in ES [167].

AKT phosphorylation level could serve as a biomarker of EGFR activity in ES and then it might be a biomarker of response to EGFR inhibitors. At the moment, this remains an untested hypothesis [168].

SIRT1 inhibitors are also under development. SIRT1 is overexpressed in ES tumors, participating in NOTCH pathway deregulation. ES patients may benefit from this approximation [169].

Targeting mutant p53 is a common goal for the scientist community. At the moment, APR-246 (PRIMA-1) is the most interesting drug. ES might benefit from clinical trials using this drug, but no data are available.

DKK2-SDF1α-CXCR4-Rac1 and ERBB4-PI3K-Akt-FAK-Rac1 pathways are potential drivers of metastasis in ES under exploration [170].

Immunotherapy

Targeted immunotherapy is in continuous development, and advances in this field must be taken into account when therapeutic decisions are being taken. Nevertheless, few biological markers can differentiate between potential responders and non-responders. About checkpoint inhibitors, phase I (nivolumab ± ipilimumab) studies in pediatric patients with ES have been completed. If PD1/PDL1 is a correct response marker, it is still under consideration [171]. Until more information is available, personalized programmes can use this immunohistochemical method to select immunotherapy candidate patients.

Table 2 Active clinical trials in Ewing sarcoma

Study title	Phase	Drug	Status	Clinical Trials.gov identifier
Targeted therapy directed by genetic testing in treating pediatric patients with relapsed or refractory advanced solid tumors, non-Hodgkin lymphomas, or histiocytic disorders (the pediatric match screening trial)	II	Tazemetostat (EZH2 inhibitor) and 12 more	Recruiting	NCT03155620
Clinical Trial of SP-2577 (seclidemstat) in patients with relapsed or refractory Ewing sarcoma	I	SP-2577 (LSD1 inhibitor)	Recruiting	NCT03600649
A study of INCB059872 in relapsed or refractory Ewing sarcoma	I	INCB059872 (LSD1 inhibitor)	Recruiting	NCT03514407
Pharmacokinetic study of PM01183 in combination with irinotecan in patients with selected solid tumors	I	Lurbinectedin (PM01183)	Recruiting	NCT02611024
Olaparib in treating patients with relapsed or refractory advanced solid tumors, non-Hodgkin lymphoma, or histiocytic disorders with defects in DNA damage repair genes (a pediatric MATCH treatment trial)	II	Olaparib	Recruiting	NCT03233204
Phase I study of olaparib and temozolomide for Ewing sarcoma	I	Olaparib	Recruiting	NCT01858168
Talazoparib and temozolomide in treating younger patients with refractory or recurrent malignancies	I/II	Talazoparib	Active, not recruiting	NCT02116777
ESP1/SARC025 global collaboration: a phase I study of a combination of the PARP inhibitor, niraparib and temozolomide and/or irinotecan or irinotecan and temozolomide in patients with previously treated, incurable Ewing sarcoma	I	Niraparib	Active, not recruiting	NCT02044120
Abemaciclib in children with DIPG or recurrent/refractory solid tumors	I	Abemaciclib	Recruiting	NCT02644460
SARC024: a blanket protocol to study oral regorafenib in patients with selected sarcoma subtypes	II	Regorafenib	Recruiting	NCT02048371
A phase I dose finding study in children with solid tumors recurrent or refractory to standard therapy	I	Regorafenib	Recruiting	NCT02085148
A phase II study evaluating efficacy and safety of regorafenib in patients with metastatic bone sarcomas	II	Regorafenib	Recruiting	NCT02389244

Discussion

Isolated advances in pediatric ES treatment arrived to clinic centers during the past 3 decades. Chemotherapy, radiotherapy and surgery are the main strategies to treat these patients [172]. Many recurrent or refractory ES patients are referred to personalized medicine programmes, in which therapeutic alternatives are studied and finally proposed. For this purpose, some biological data should be cautiously considered as potential biologic prognosis markers in ES (1q gain, 16q loss or *STAG2* mutations). Moreover, some point mutations in specific genes (as in *EZH2*), CNVs at specific chromosomal locus (as 9p21), information from immunochemistry staining and expression level of certain proteins could be used to decide the most appropriate compassionate use therapy or the theoretically more convenient clinical trial for each individual patient.

What we have gathered in this work constitutes the nucleus of knowledge on which medical decisions should rest in front of an ES patient in relapse. First, looking for clinical trials must be the first effort of every medical doctor when a patient relapses and no other conventional therapies are available. Second, in a personalized way, biological studies must be conducted to enrich knowledge about the individual disease. These efforts, already developed in reference centers, must keep in mind the urgency of translational investigation and consideration of clinical and preclinical recent advances. Sadly, due to the limited number of biological markers to predict responses taking decisions from pediatric personalized medicine committees, good responses are very difficult. Thus, we consider that carrying out molecular studies in patients is necessary, at the best by massive analysis technologies (NGS), to find new potentially actionable molecular targets. Otherwise, we should search for potential response markers and obtain relevant conclusions, so that all these efforts do not become useless.

Through this review, we would also like to highlight that, for personalized medicine, it is important to squeeze positive responses happened in several clinical trials of small groups of patients. Likewise, many of the mentioned drugs are actively being used in clinical trials with adult patients, without more biological basis than in children. Therefore, we support pediatric patients should be more actively considered for clinical trials, regarding benefits of

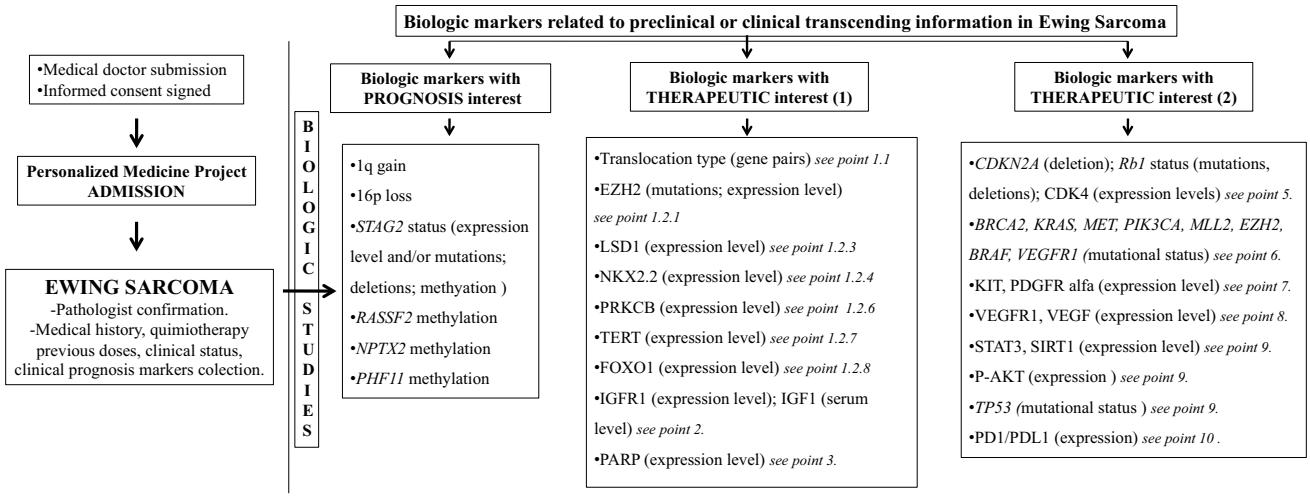

Fig. 1 Ewing sarcoma proposal workflow within personalized medicine projects

new targeted therapies. Indeed, running a certain degree of risk is the only option for many pediatric or young patients that do not have any other treatment options and a fatal outcome is expected.

Many of the approximations summarized in this review can only be used under compassionate use treatments and without enough molecular basis to choose the best option. Nevertheless, when clinical trials are not existing or recruiting and some biological markers suggest an alternative drug option, medical doctors should pursue target therapies in combination with conventional treatments, using preclinical data and recommendations of specialized precision medicine committees. Finally, as Vornicova et al. states, considering multimodal approximations is the unique way to get better results [173].

From our group and based on previous knowledge reported in this review, a proposal workflow for personalized medicine projects has been elaborated and is presented here (Fig. 1).

Author contributions All authors have participated in the research, but especially in revising it critically and all authors have approved the final article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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