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Review Article

Molecular and biologic biomarkers of Ewing sarcoma: A systematic review

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HIGHLIGHTS

- Numerous diagnostic, prognostic, and predictive biomarkers were found.
- It is recommended to search for a panel of biomarkers due to higher sensitivity and specificity.
- Markers involved in proliferation/inflammation are associated to poorer prognosis.
- Predictive markers have three different mechanisms of action.

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ABSTRACT

With an annual incidence of less than 1%, Ewing sarcoma mainly occurs in children and young adults. It is not a frequent tumor but is the second most common bone malignancy in children. It has a 5-year survival rate of 65–75%; however, it has a poor prognosis when it relapses in patients. A genomic profile of this tumor can potentially help identify poor prognosis patients earlier and guide their treatment. A systematic review of the articles concerning genetic biomarkers in Ewing sarcoma was conducted using the Google Scholar, Cochrane, and PubMed database. There were 71 articles discovered. Numerous diagnostic, prognostic, and predictive biomarkers were found. However, more research is necessary to confirm the role of some of the mentioned biomarkers.

1. Introduction

Primary bone cancer is responsible for around 5% of all childhood and adolescent cancers [1]. While there are numerous subtypes, Ewing's sarcoma (ES) is one of the most common primary bone tumors occurring in adolescents and young adults, affecting between 300 and 560 people in the United States each year [2]. Diagnosis of ES can be made with a combination of imaging, histology, and more recently, molecular techniques that highlight chromosomal translocations that are specific to ES [3]. It is treated in a multidisciplinary way using chemotherapy, radiotherapy and/or surgical resection [4]. ES is a highly aggressive tumor, with 20–25% of patients presenting metastases at the time of diagnosis [5]. Therefore, it is classified as a tumor with unfavorable prognosis and a poor overall survival, especially in metastatic or relapsed disease [6]. Despite the numerous advances in treatment regimens and the overall improvement of its prognosis in the last few years, ES remains an aggressive tumor; a more effective and specialized approach is therefore required. In the era of precision oncology, it is essential to study tumor heterogeneity, which could pave the way for a more tailored approach in cancer care. Biomarkers have recently revolutionized patient care in oncology and have largely contributed to the improvement of patient outcome. These biomarkers could be diagnostic (confirm the disease), prognostic (predict disease evolution) or predictive (identify individuals who could respond to certain treatments) [7]. In this systematic review, we highlight the main biomarkers of ES in order to create a base for a new perspective of care for ES as well as improve the survival of patients diagnosed with this tumor.

2. Material and methods

To obtain the maximum number of articles containing data on complex genomic profiling of Ewing sarcoma, an extensive search of the

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literature was conducted in Google Scholar (pages 1–20), the Cochrane database using the keywords "Ewing" and "Sarcoma", and in the PubMed database until March 2023. Using Boolean Operators, the Mesh Terms "High-Throughput Nucleotide Sequencing" and "Biomarkers, Tumor" were used alongside the keywords "Comprehensive genome profiling" or "Comprehensive genomic profiling" and "Ewing sarcoma". Fifteen articles were added through manual search.

A total of 898 articles were extracted. Titles and abstracts of retrieved articles were screened for eligibility, and then entire texts were analysed. The main point is to include studies in English that contain data on both molecular and biologic biomarkers of Ewing sarcoma. Articles focusing on Ewing sarcoma without significant information on biomarkers or genes, or papers discussing unusual locations or presentations of this tumor, as well as books or reviews that are older than one year were excluded. The 71 papers that respond to the objectives will be part of this review. The process is summarised in the PRISMA diagram (Fig. 1).

3. Results

Biomarkers can be classified as either diagnostic, prognostic, or predictive. Diagnostic biomarkers typically assist in determining the intended pathology's diagnosis. There were 24 papers that discussed ES diagnostic biomarkers. Patients are categorized based on risk and outcomes using prognostic biomarkers. Prognostic biomarkers for ES were presented in 36 papers. Predictive biomarkers often have the function of identifying patients who will benefit most from a particular course of treatment. There were 12 articles that reported predictive biomarkers for ES (Table 1).

3.1. Diagnostic markers of Ewing sarcoma

3.1.1. Molecular

The reciprocal translocation between chromosomes 11 and 22 was found to be a characteristic and pathognomonic diagnostic biomarker for ES, but it is not very sensitive [8-10]. There were no articles in the literature discussing other potential molecular biomarkers for the diagnosis of ES.

3.1.2. Biologic

Hormones: Reubi et al. found that proCCK (procholecystokinin) has a high plasma concentration in patients with ES and could be used to diagnose ES at an early stage [11]. Another hormone, ProGRP (Pro-gastrin releasing peptide) was found to have high serum levels in patients with ES, making it a potential diagnostic biomarker [12]. Honda et al. showed that the levels of ProGRP correlate with the volume of ES, and this finding is consistent during treatment. It can also be used for the differential diagnosis of ES and is more specific than NSE (neuron specific enolase) [13].

NKX2.2 and CD99: Shibuya et al., Machado et al., and Yoshida et al. demonstrated that although NKX2.2 immunohistochemistry (a transcriptional target of EWSR1-FLI1) could be used for the diagnosis of ES, its combination with CD99 makes this test highly specific [14-16]. When used alone, Hung et al. showed that it was poorly specific and that NKX2.2 was present in both ES and Ewing like sarcomas [17]. Also, when studying CD99 with the EWSR1 rearrangement, these two tests together had higher sensitivity and specificity than each alone [18]. Ahmed et al. found that when alone, FLI-1 was a useful marker for ES diagnosis, but when combined with CD-99, its positivity was more dependable [19].

However, Xiao et al. showed that ZBTB16 immunohistochemistry was a highly sensitive and specific biomarker for ES, even more sensitive than CD99 [20]. Along with NKX2.2, ETV4 and BCOR immunohistochemistry can also be helpful to distinguish ES from CIC-rearranged or BCOR-associated sarcomas [15]. Moreover, Russel-Goldman et al. also showed that NKX2.2 can be used to distinguish ES from other tumors [21]. A study by McCuiston et al. showed that when ES is located in the sinonasal tract, NKX2.2 can be a very sensitive biomarker for ES but not so specific [22].

PAX7: A study by Toki et al. showed that PAX7 immunohistochemistry was positive in 90% of cases of ES, but its diagnostic potential needs to be further evaluated before it can be used as a diagnostic biomarker [23]. However, Fernandez-Pol et al. showed that in decalcified bone marrow, PAX7 is a useful marker that can confirm ES after finding the Ewing sarcoma rearrangement [24].

Others: Krumbholz et al. showed that a correlation was found between the kinetics of EWSR1 fusion sequence copy numbers in the plasma and variations of the tumor volume in patients with localized

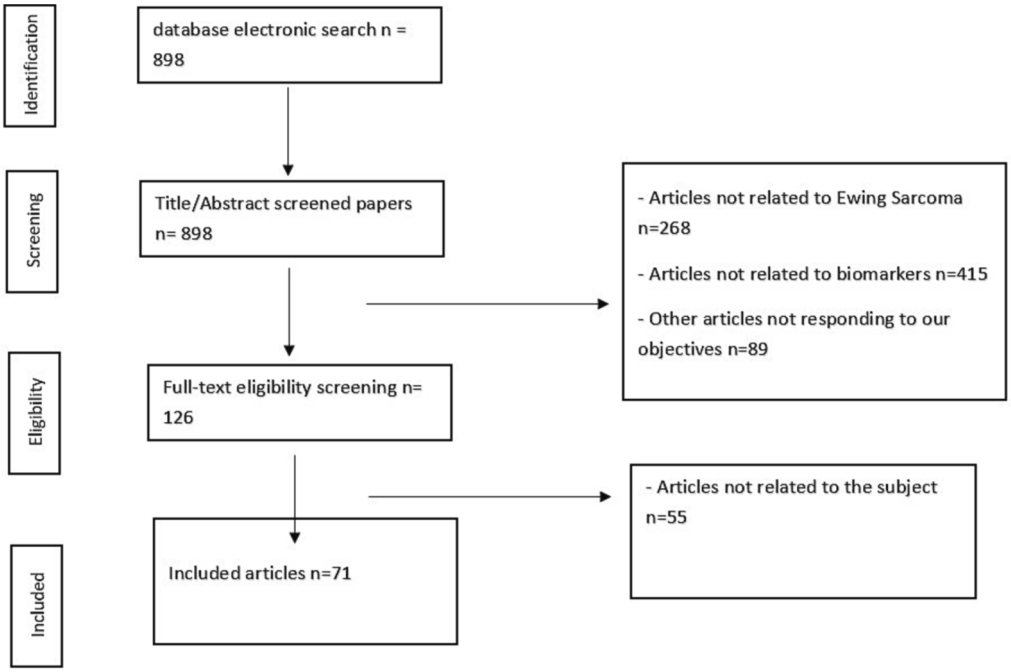

Fig. 1. PRISMA flowchart for article selection process.

Table 1
Summary of the biomarkers found in this systematic review.

Reference	Type of study	N	Biomarker	Type	Outcome
Turc-Carel et al, 1983	Retrospective	4	t(11;22)	molecular	t(11;22)(q24;q12) was observed in 4 out of 5 cell lines
Aurias et al, 1983	Retrospective	4	t(11;22)	molecular	Chromosome 22 is involved 6 times, and chromosome 11 is involved 3 times in 4 Ewing's sarcoma tumors
Prieto et al, 1984	Case report	1	t(11;22)	molecular	t(11;22) was seen in all cells studied
Zhao et al, 2021	Experiment	N/A	*NCAPG*, *KIF4A*, *NUF2* and *CDC20*	molecular	*NCAPG*, *KIF4A*, *NUF2* and *CDC20* genes may play an important role in ES prognosis
Perbal et al, 2009	Retrospective	170	*CCN3*	molecular	Low expression of the *CCN3* gene was associated with a better prognosis when event-free or overall survival was considered
Huang et al, 2004	Retrospective	60	*TP53*, and *p16/p14ARF*	molecular	*TP53* mutation and *p16/p14ARF* deletion were associated with worse overall survival
Yin et al, 2018	Meta-analysis	N/A	*PYGM*, *MEF2C*, *TRIM63*, *BUB1B*, and *RACGAP1*	molecular	*PYGM*, *MEF2C* and *TRIM63* genes were downregulated in ES, upregulation improved prognosis *BUB1B* and *RACGAP1* were upregulated in ES, upregulation worsened prognosis
Ren et al, 2020	Experiment	N/A	*CRLF3, ECD,FABP4, FGF6, GNRH2, NDRG1, PAK2, PLTP, PTGDS, RBP1,* and *ZC3HAV1*	molecular	The 11-gene signature (*CRLF3, ECD,FABP4, FGF6, GNRH2, NDRG1, PAK2, PLTP, PTGDS, RBP1,* and *ZC3HAV1*) strongly predicts ES prognosis
De Alava et al, 1999	Retrospective	55	*TP53*	molecular	*TP53* alteration in ES predicts poor outcome
Abrahao-Machado et al, 2018	Retrospective	112	*MTAP*	molecular	Loss of *MTAP* expression in ES is associated with poor overall survival
Liu et al, 2018	Retrospective	99	higher mutation burden in the genome (*TP53, STAG2*…)	molecular	A high mutation burden was associated with low overall survival and time to progress Mutations in STAG2 or TP53 are associated with a higher mutation burden
Mendoza-Naranjo et al, 2013	Experiment	N/A	*ERBB4*	molecular	*ERBB4* expression increases ES metastasis and correlates with disease progression
Tsuda et al, 2020	Retrospective	10	FEV gene rearranegment	molecular	FEV rearrangements in patients correlated with more extraskeletal sites and a poorer outcome compared to EWSR1-FLI1 or EWSR1-ERG
Lerman et al, 2015	Retrospective	112	*TP53*	molecular	*TP53* mutations did not significantly affect event-free survival
Le deley et al. 2010	Prospective study	565	rearrangements	molecular	EWS-ERG and EWS-FLI1 rearrangements had no impact on prognosis
Cidre-Aranaz et al., 2022	Experiment	N/A	TCF7L1	molecular	low expression of the TCF7L1 is associated with poor overall survival
Musa et al., 2019	Experiment	N/A	MYBL2	molecular	High *MYBL2* predicts response to CDK2 inhibitors
Li et al., 2021	Experiment	N/A	PRC1/PLK1	molecular	Upregulation of PRC1 promotes poor survival but increases sensitivity to PLK1 inhibition
Mackintosh et al., 2012	Retrospective	105	Chromosome 1q gain/CDT2	molecular	Chromosome 1q gain and CDT2 overexpression were associated with poor overall and disease-free survival
Funk et al., 2022	Experiment	N/A	Chromosome 8 gain/ EIF4EBP1	molecular	Chromosome 8 gain and EIF4EBP1 overexpression were associated with poor survival
Tirode et al., 2014	Retrospective	112/ 299	STAG2, among others	molecular	STAG2 alteration is of negative prognostic value
Shulman et al., 2022	Retrospective	135	STAG2	molecular	STAG2 alteration carries poor prognosis
Marino et al., 2014	Retrospective	109	miR-34A	biologic	miR-34A expression is associated with higher event-free and overall survival miR-34A expression was lower in metastases compared to local tumors, and is inversely correlated to Cyclin D1 and Ki-67
Satterfield et al., 2017	Experiment	N/A	miR-130b	biologic	miR-130b is overexpressed in ES cells that are more invasive and aggressive, via negative regulation of ARHGAP1
He et al., 2017	Experiment	N/A	LRWD1	biologic	High expression of LRWD1 mRNA is associated with poor survival
Shulman et al., 2018	Retrospective	94	ctDNA	biologic	Detectable ctDNA was associated with worse event-free survival, overall survival and death
Sannino et al., 2019	Retrospective/ Prospective	189/ 141	SOX2	biologic	High SOX2 mRNA or protein levels were associated with poorer survival
Moore et al., 2017	Experiment	N/A	miR-193b and ErbB4	biologic	MiR-193b suppresses growth in ES by inhibiting ErbB4
Agelopoulos et al., 2015	Retrospective	116	FGFR1	biologic	FGFR1 TKI significantly reduced 18-FDG-PET activity
Reubi et al., 2004	Retrospective	12	proCCK	biologic	proCCK has higher concentrations in ES patients
Yamaguchi et al., 2015	Retrospective	9	ProGRP	biologic	5 out of 9 ES patients had high ProGRP levels
Honda et al., 2019	Retrospective	16	ProGRP	biologic	ProGRP levels correlate with tumor volume in ES patients, even during treatment. ProGRP is more specific than NSE
Shibuya et al., 2014	Retrospective	46	NKX2.2 and CD99	biologic	NKX2.2 is a useful diagnostic marker for ES, and its specificity is higher when combined with CD99
Machado et al., 2017	Retrospective	237	NKX2.2 and CD99	biologic	The combination of NKX2.2 and CD99 positivity is a very reliable diagnostic marker for ES
Yoshida et al., 2012	Retrospective	30	NKX2.2	biologic	NKX2.2 is a valuable diagnostic marker for ES
Hung et al., 2016	Retrospective	40	NKX2.2	biologic	NKX2.2 is sensitive but not very specific to ES

(*continued on next page*)

Table 1 (*continued*)

Reference	Type of study	N	Biomarker	Type	Outcome
Louati et al., 2018	Retrospective	41	CD99 and EWSR1	biologic	CD99 analysis combined with FISH for EWSR1 detection has high sensitivity and specificity
Ahmed et al., 2016	Retrospective	50	FLI-1	biologic	FLI-1 is a useful diagnostic marker for ES, but its positivity is more dependable when combined with other markers
Xiao et al., 2019	Retrospective	12	ZBTB16	biologic	ZBTB16 is a highly specific and sensitive biomarker for ES
Russel-Goldman et al., 2018	Retrospective	10	NKX2.2	biologic	NKX2.2 is very sensitive, but only moderately specific in ES diagnosis
McCuiston et al., 2018	Retrospective	7	NKX2.2	biologic	NKX2.2 is a useful and highly sensitive marker for ES of the sinonasal tract, but it is not entirely specific
Toki et al., 2018	Retrospective	30	PAX7	biologic	PAX7 is a sensitive marker for ES, further evaluation required
Fernandez-Pol et al., 2018	Retrospective	10	PAX7	biologic	PAX7 is a very useful marker for ES in decalcified bone marrow biopsies
Krumbholz et al., 2016	Retrospective	20	EWSR1	biologic	EWSR1 copy numbers in plasma correlate closely wih tumor volume in localized and metastatic disease
Solooki et al., 2013	Retrospective	14	sCD30 and sCD40L	biologic	Serum concentrations of sCD30 and sCD40L are elevated in ES and could serve as a potential diagnostic marker
Fagone et al., 2015	Experiment	N/A	Cyclin D1	biologic	Cyclin D1 is highly expressed in ES cells compared to rhabdomyosarcoma or normal cells, making it a potential diagnostic biomarker
Machado et al., 2010	Retrospective	236	Ezrin	biologic	Ezrin is expressed heterogenously in bone tumors and does not offer any clues in their differential diagnosis
Machado et al., 2013	Retrospective	217	Galectin-1	biologic	Galectin-1 has poor expression in ES compared to other tumors, it can be used to distinguish between them Galectin-1 is not related to ES prognosis
Garcia-Monclús et al., 2018	Experiment	N/A	EphA2	biologic	EphA2 phosphorylation at serine 897 was associated with higher invasivness of ES cells
Luo et al., 2018	Experiment	N/A	PPP1R1A	biologic	ES cells are more aggressive with PPP1R1A phosphorylation
Toretsky et al., 2001	Prospective	111	IGF-1 and IGFBP-3	biologic	IGFBP-3 to IGF-1 ratios were higher in patients with metastatic disease
Na et al., 2014	Retrospective	61	CXCL16 and CXCR6	biologic	CXCL16 and CXCR6 were highly expressed in ES cells, and their expression was associated with the occurrence of lung metastis and poorer prognosis
Roundhill et al., 2021	Retrospective	24	Neurexin-1	biologic	High expression of neurexin-1 and low levels of regulators of its activity were associated with poor survival
Ohmura et al., 2021	Experiment	N/A	RRM2	biologic	High expression of RRM2 is associated with poor survival and metastatic disease
Machado et al., 2018	Retrospective	370	PD-1, PDL-1 and CD8	biologic	Absence of PD-1 in ES is associated with poor prognosis PDL-1 and CD8 are not related to ES prognosis
Machado et al., 2022	Retrospective	N/A	PAX7 and NKX2.2	biologic	Absence of either PAX7 or NKX2.2 is associated with a poorer prognosis
Ash et al., 2011	Retrospective	46	CD56	biologic	CD56 can be a good prognostic marker for ES, as low expression is associated with better survival and prognosis
Lai et al., 2006	Retrospective	49	STAT3	biologic	High activation of STAT3 correlated with better survival in ES
Park et al., 2006	Retrospective	71	Id2	biologic	Id2 levels were observed in most ES cases and could be a marker of poor prognosis
Mahmoud et al., 2022	Retrospective	109	survivin	biologic	High survivin expression was associated with poor overall and event-free survival
Jiang et al., 2021	Retrospective	32	CCT6A	biologic	High CCT6A expression was associated with low overall survival and poor prognosis
Aryee et al., 2002	Retrospective	29	KAI1	biologic	KAI1 is not relevant in ES prognosis
Rajabalian et al., 2010	Experiment	N/A	ER	biologic	ER may be involved in drug resistance and could be a therapeutic target
Ye et al., 2003	Retrospective	13	HER2	biologic	HER2 is not a relevant biological or therapeutic pathway in ES
Anderson et al., 2016	Clinical Trial	144	IGF-1R	biologic	IGF-1R could be a therapeutic target in ES
D Tap. Et al. 2012	Clinical Trial	35	IGF-1R	biologic	IGF-1R could be a therapeutic target in ES
de Hooge et al., 2007	Retrospective	18	PI-9	biologic	PI-9 may have an impact in chemotherapy sensitivity
Kennedy et al., 2015	Experiment	N/A	Cyclin D1/CDK4	biologic	ES cells depend on the cyclin D1/CDK4 pathway and its inhibition could be therapeutic
Town et al., 2016	Experiment	N/A	LINGO1	biologic	LINGO1 is expressed in ES tumors but not in other somatic tissue making it a potential therapeutic target
Baldauf et al., 2016	Experiment	N/A	BCL11B and GLG1	biologic	BCL11B and GLG1 are highly specific and can be used to diagnose ES
Orth et al., 2020	Experiment	N/A	BCL11B and GLG1	biologic	BCL11B and GLG1 are highly specific and can be used to diagnose ES

and metastatic disease. Also, during initial chemotherapy, a rapid reduction of ctDNA was found in the majority of patients, and relapse development was signalized by the recurrence of increasing ctDNA levels [25].

Solooki et al. reported high serum levels of sCD30 and sCD40L in ES, which shows potential significance for the diagnosis [26]. Another marker, cyclin D1, was also found in high expression in ES cells [27].

Machado et al. showed that Ezrin immunohistochemistry expression has no role in the differential diagnosis of bone tumors [28]. Later on, Machado et al. found another marker, Galectin-1, that can be used as a biomarker to eliminate ES diagnosis when suspicious. This is because it is not present in ES but is positive in most cases of small cell osteosarcoma [29].

Baldauf et al. additionally showed that BCL11B and GLG1 detection

could be an effective and cost-efficient way of diagnosing ES [30]. This was also further demonstrated by Orth et al., who showed that BCL11B and GLG1 are highly specific for ES especially in conjunction with CD99 [31].

3.2. Prognostic markers of Ewing sarcoma

3.2.1. Molecular

Gene-signatures: Ren et al. discovered that when compared to other prognostic biomarkers, this 11-gene signature (*CRLF3, ECD, FABP4, FGF6, GNRH2, NDRG1, PAK2, PLTP, PTGDS, RBP1,* and *ZC3HAV1*) has a higher predictive value of poor prognosis [32].

A meta-analysis of data by Yin et al. found that *PYGM*, *MEF2C*, and *TRIM63* were also downregulated, and their expression was positively associated with survival rates in ES [33]. However, *BUB1B* (a member of spindle assembly checkpoint protein) and *RACGAP1* (a component of central spindle and essential for cytokinesis induction), both upregulated in ES, were negatively associated with survival rates of ES. Newly discovered potential markers of poor prognosis are *NCAPG*, *KIF4A*, *NUF2* and *CDC20* found by Zhao et al. [34].

Copy number variants: Numerous studies investigating copy number variants (CNV) in ES showed reoccurring abnormalities affecting whole chromosomes or segments [35]. For instance, chromosome 1q gain was associated with poorer overall and disease-free survival by Mackintosh et al. [36]. *CDT2*, a gene located in 1q, was found to contribute to this phenotype, and its overexpression was also labeled of negative prognostic value [36]. 16q loss was also associated with poor overall survival [35].

Another frequent somatic alteration found in ES is chromosome 8 gain. It was demonstrated by Funk et al. that chromosome 8 gain was associated with a worse overall survival, with overexpression of *EIF4EBP1* being responsible for most of its effects, and showing tight clinical correlation with a poor outcome in ES patients [37].

Phosphorylation: Garcia-Monclus et al. showed that ES cells are more aggressive when they have higher levels of phosphorylation of EphA2 at serine 897 [38]. Furthermore, Luo et al. showed that ES that have high levels of phosphorylated PPP1R1A also tend to be more aggressive [39].

MicroRNA: Marino et al. reported that tumor-related deaths and adverse events were higher in patients having ES with absent expression of miR-34a, and both 5-year event-free-survival and 5-year overall-survival were higher in patients with miR-34a expression [40]. Also, miR-34a was lower in metastases when compared to primary tumors [40]. Another microRNA, miR-130b, was found to be overexpressed in ES, promoting invasion, migration, proliferation in vitro, and has a higher metastatic potential in rats. It does that by promoting the activation of an oncogenic CDC42/PAK1/JNK by negatively regulating ARHGAP1 [41].

CCN3: Another marker of bad prognosis was found by Perbal et al. [42]. When considering either event-free or overall survival, the low expression of the gene *CCN3* is associated with a better prognosis. Even further, the less the NH3 domain of the *CCN3* gene is detected, the better the prognosis [42].

Genomic stability and rearrangements: Liu et al. found that the more the genome was unstable, the more aggressive the sarcoma [43]. Also, when considering gene rearrangements, Tsuda et al. showed that the presence of the FEV gene rearrangement was associated with a poorer prognosis when compared to EWSR1-FLI1 or EWSR1-ERG [44]. Moreover, a study by Le Deley et al. showed that there was no difference in the prognostic outcome when comparing EWS-ERG, type 1, type 2, and non-type1/2 EWS-FLI1 fusions [45].

P53: De Alava et al. found in 2000 that alterations of *TP53* appeared to have an impact on the prognosis of patients with ES, making it worse [46]. Huang et al. found that the deletion of *p16/p14ARF* alone had a low negative influence on prognosis, but when combined with a *TP53* mutation, the combination becomes the most crucial factor in determining outcomes, followed by the stage [47]. However, Lerman et al. showed that *TP53* mutation and *CDKN2A* deletion are unreliable prognostic biomarkers in localized Ewing sarcoma [48].

LRWD1: He et al. showed that high expression of LRWD1 (Leucine rich repeats and WD repeat Domain containing 1) mRNA is associated with poor survival. PLS3 (actin-binding protein plastin 3) and DLX2 (distal less homeobox 2), which were downregulated when LRWD1 was depleted, however, were not linked to poor prognosis [49].

TCF7L1: Cidre-Aranaz et al. were able to demonstrate, after thorough analysis of clinically significant gene expression in ES, that TCF7L1 (transcription factor 7 like 1) was a regulator of metastasis, and that low expression of the TCF7L1 gene was associated with poor overall survival in ES patients [50].

PRC1/PLK1: In another experiment, Li et al. found that upregulation of PRC1 (protein regulator of cytokinesis 1) promotes tumor growth and correlates with poor survival and clinical outcome. However, it was also shown that high PRC1 expression renders the cells vulnerable to PLK1 (polo-like kinase 1) inhibition, leading to cell death even in chemo-resistant cells [51].

ctDNA: An association between ctDNA (circulating tumor DNA) and both tumor volume and metastasis in ES was found, making their presence associated with an inferior outcome. It was also found that the higher their detection, the higher the risk of events and death [52].

Others: Machado et al. found that when *MTAP* expression was lost in ES, the overall survival was shorter [53]. Naranjo et al. found that high *ERBB4* expression is associated with metastatic disease in ES patient samples [54]. Also, high mRNA/protein expression of SOX2 was found to be associated with poor outcomes in ES [55].

3.2.2. Biologic

IGF: Toretsky et al. demonstrated that IGF-1 levels alone were not correlated to survival. High IGFBP-3: IGF-1 identified more clearly metastatic patients. Although not statistically significant, it was found that high IGFBP-3:IGF-1 ratios (between the 75th and 90th percentile) improved survival in metastatic patients [56].

CXCL16 and CXCR6: A study by Na et al. reported that immunohistochemistry of CXCL16 and CXCR6 showed high expression in ES cells when compared to normal or osteosarcoma cells with CXCR6 being more specific to ES, thus their involvement in tumorigenesis. ADAM 10 and 17 were also present, and a relationship between the expression of both ADAM 10 and CXCL16 was found [57]. Also, CXCL16 and CXCR6 were constitutively expressed in peritumoral lymphocytes and histiocytes, and the expression of these two was associated with lung metastasis, thus the poor prognosis.

Neurexin-1: Roundhill et al. found that when highly expressed in localized ES, neurexin-1 is associated with metastasis and relapse. Moreover, the low expression of APBA1 and NLGN4X (neurexin-1 binding partners) is associated with poor clinical outcomes [58].

RRM2: Ohmura et al. found that on immunohistochemistry, the high expression of RRM2 (ribonucleotide reductase regulatory subunit M2) which along with RRM1 forms RNR (ribonucleotide reductase), is associated with poor overall survival, metastatic disease at diagnosis and metastatic or local relapse. [59].

Others: Machado et al. found that the absence of PD-1 on immunohistochemistry in ES is related to a poor prognosis [60]. It was also demonstrated in another study that the absence of either PAX7 or NKX2.2 immunoreactivity in ES is associated with a poor prognosis [61].

Another marker of poor prognosis found using flow cytometry, CD56, was found by Ash et al. [62]. The absence of this marker can identify a subgroup of patients with excellent prognosis, in whom treatment reduction could be carefully considered [62]. Also, the constitutively high activation of STAT3 on immunohistochemistry was found to be related to less aggression of ES [63]. Moreover, ID2, a helix-loop-helix protein that was found to be highly expressed on immunohistochemistry of ES cells, is suggested to be a marker of poor prognosis [64].

Newly found markers included survivin which was associated with worse overall survival and event-free survival [65], and CCT6A due to its involvement with low overall survival [66].

3.2.3. Unrelated

Machado et al. showed that Galectin-1 is not related to ES prognosis [29]. In another study, he also found that both CD8 expression in infiltrating lymphocytes and PD-L1 were not related to prognosis [60]. Another marker unrelated to ES prognosis is KAI1, found by Aryee et al. [67].

3.3. Predictive markers of Ewing sarcoma

3.3.1. Molecular

miR-139b and ErbB4: Moore et al. showed that the micro-RNA miR-139b, which is usually downregulated in ES, suppresses the growth of the tumor by inhibiting ErbB4, making it a potential therapeutic target [68]. Moreover, Naranjo et al. found that lapatinib treatment demonstrated modest decreases in *ERBB4* activity; this may also be used to treat or prevent metastatic ES [54].

CCN3: Perbal et al. showed that the reduced expression of *CCN3* and of the NH3 domain in *CCN3* was associated with better outcomes. This prognostic significance is even better in radiotherapy-treated patients and insignificant in surgery-treated patients [42]. Thus, the possible link between *CCN3* and radiotherapy sensitivity.

FGFR1: Agelopoulos et al. showed that FGFR1 therapy in patients with ES reduced 18-FDG-PET uptake, making it a viable therapeutic target for ES [69].

MYBL2: Musa et al. successfully demonstrated in an experiment that binding of the EWSR1-FLI1 fusion transcription factor to a polymorphic enhancer like DNA element controls expression of *MYBL2*, a transcription factor that has variable expression between tumors. High *MYBL2* expression was found to be predictive of the use of CDK2 inhibitors, which inhibit the upstream pathway of *MYBL2* [70].

3.3.2. Biologic

ER and HER2: In ER+ (Estrogen Receptor) ES, ER is overexpressed, and this expression is involved in drug resistance, making it a potential therapeutic target [71]. However, when it comes to HER2, Ye et al. showed that it is not a major therapeutic target in ES [72].

IGF-1R: Anderson et al. showed that Robatumumab, a human antibody that binds and inhibits IGF-1R, can have a potential therapeutic role in ES [73]. Tap et al. also showed that Ganitumab, another antibody with the same mechanism, was efficient in managing ES [74].

Others: Hooge et al. showed that PI-9 (protein inhibitor-9) may have an impact on the sensitivity to chemotherapy [75]. Furthermore, Kennedy et al. showed that in ES cells, the cyclin D1/CDK4 pathway is activated, and the cells are sensitive to chemical inhibition of CDK4 and cyclin D1 [76]. An interesting finding was LINGO1, a surface protein found on ES cells in most cases, making it a potential drug target [77].

4. Discussion

4.1. Diagnostic markers of Ewing sarcoma

Diagnostic markers are an important asset used to detect early ES before the prognosis gets poorer. Through an extensive review of the literature, many diagnostic markers have been found. The translocation between chromosomes 11 and 22 remains the most important genetic characteristic of this tumor. However, hormones can actually be used to diagnose ES such as proCCK and proGRP [11,12]. ProCCK being elevated in ES does not affect the tissues targeted by CCK [11], and it would be safe to assume that the same can be said for proGRP. Actually, both hormones can be used not only to diagnose ES, but also to monitor treatment efficacy and detect a relapse [11,13].

Other biomarkers can also be used to diagnose ES, but what may be more efficient is a combination of those, due to the fact that some markers may not be as effective alone as when they are combined with other factors. NKX2.2 is an example because, alone, it is not as highly specific as when used with CD99 [15]. However, ZBTB16 was found to be more sensitive than CD99 [20], making an association between NKX2.2 and ZBTB16 more desirable as a diagnostic method. A reason why ZBTB16 was more sensitive than CD99 is that ZBTB16 is upregulated as a result of the EWS-FLI1 fusion [20]. Also, it is beneficial to use NKX2.2 in the diagnostic panel as it can be very sensitive for ES when the latter is located in the sinonasal tract since tumors in this location are of immunohistochemical heterogeneity [22]. PAX7 is another factor that can also be used to confirm ES after a positive test for EWSR1 rearrangement [24].

Other markers may be useful not only for qualitative but also quantitative diagnosis. Cell-free tumor DNA (ctDNA) including the characteristic and causative EWSR1-FL1 and EWSR1-ERG rearrangements, demonstrated its importance in monitoring tumor burden at diagnosis, therapeutic response, and disease relapse, since there was a correlation between this marker and the tumor volume [25].

Markers that are not found in ES can also be used for the elimination of ES, such as Galectin-1, a biomarker highly positive in small cell osteosarcoma which is a differential diagnosis, but not found in ES [29].

When suspecting ES, there are now other ways than testing to see the translocation between chromosomes 11 and 22. A combination of ZBTB16 and NKX2.2 would be a good initial test due to the high sensitivity and specificity of ZBTB16 and the sensitivity of NKX2.2 for ES, even when the latter is in the sinonasal tract. When doubting between small cell osteosarcoma and ES, the better way to find the diagnosis is searching for Galectin-1. BCL11B and GLG1 could also serve as a cost-effective way to diagnose ES due to their high specificity. After treatment of ES, an early diagnostic test for relapse would be the serum value of ctDNA and proGRP. It would be better to search for both to make the test more specific and sensitive. Markers such as sCD30, sCD40L, cyclin D1, PAX7, proCCK and proGRP need to be studied more to test for their added value as diagnostic biomarkers.

4.2. Prognostic markers of Ewing sarcoma

Prognostic biomarkers are important in ES workup because they help in risk stratification and management. These markers can be associated with a better or worse prognosis. Not only molecules and genes are associated with prognosis, but also the level of phosphorylation, such as the level of phosphorylation of EphA2 at serine 897 [38] and PP1R1A [39], which are associated with a poor prognosis the more they are phosphorylated. In fact, the phosphorylation of EphA2 at serine 897 correlates with the migratory capacity of ES cells, and its silencing is associated with decreased cell viability, clonogenic capacity, tumor growth, decreased cell migration, invasion and metastasis in vivo [38]. Also, when EphA2 was silenced, 4 genes (CCL2, ADAM19, PIK3CG, and PTPN21), associated with tumor progression, were downregulated and 2 genes (PCDH8 and LUM), considered as tumor suppressors, were upregulated [38]. This could lead to the conclusion that markers associated with a poor prognosis are usually involved in promoting proliferation, whereas those associated with a good prognosis are usually involved in suppressing it. Another explanation can be that markers of poor prognosis can be genes associated with inflammation, such these 2 genes FABP4 and NDRG1 found by Ren et al. in 2021 [32]. FABP4 was found to be positively correlated with macrophages, and NDRG1 is negatively correlated with Th2 cells, which can explain why both of them are markers of poor prognosis [32].

Also, the qualitative significance of a biomarker can be as good as its quantitative significance. One example is ctDNA, the presence of which has been linked to a poor outcome. Another important discovery is that the higher the levels of ctDNA, the higher the risk of events and death, making this biomarker an important one for patient stratification [52].

It was found by Liu et al. that genomes that have a higher mutational

burden are associated with a poor prognosis, making alterations in genes such as STAG2 and TP53 factors of poor outcomes [43].This was also confirmed by Tirode et al., who additionally showed that STAG2 is one of the most frequent somatic alterations in ES [78], and by Shulman et al., who showed that STAG2 alterations carried poor prognosis [79]. However, Lerman et al. demonstrated that the TP53 mutation is an unreliable biomarker for prognosis in localized Ewing sarcoma [48]. Thus, more research is needed on this topic to identify any influence on prognosis.

After diagnosing ES, prognostic markers are needed to stratify the patients into risk categories. The presence of this 11-gene signature (CRLF3, ECD, FABP4, FGF6, GNRH2, NDRG1, PAK2, PLTP, PTGDS, RBP1, and ZC3HAV1), a higher phosphorylation of EphA2 at serine 897 or of PP1R1A, higher rate of mutations due to mutated genes such as TP53 etc., FEV gene rearrangement, high expression of LRWD1, overexpression of CXCL16 or/and CXCR6, high expression of neurexin-1 or downregulation of its binding partners (APBA1 or NLGN4X), high expression of RRM2, BUB1B, RACGAP1, ctDNA, SOX2, or ErbB4, and absence of PD-1, PAX7, NKX2.2, MTAP, or Brachyury is associated with a poorer prognosis. In such cases, clinical trials are needed to see how to therapeutically address this cancer. Furthermore, it was found that the overexpression of CXCL16, CXCR6, neurexin-1, ErbB4, ctDNA, and RRM2 is associated with metastasis, making the search for these markers important to prevent the occurrence of such metastasis. Another interesting feature of ctDNA is the correlation between its level and the risk of events and death, making the values of this marker a potential guide for patient stratification and the implementation of an algorithm of management.

However, when there is an overexpression of miR-34a, PGYM, MEF2C, TRIM63, or STAT3, or a reduced expression of CCN3 and its NH3 domain, or CD56, the prognosis is said to be better, and after evaluation of each case by itself, a potential and well-studied reduction of treatment can be considered, making the patient able to benefit from a reduced treatment-related toxicity.

More research is required to investigate the relationship between miR-130b, PAK1, IGFBP-3: IGF-1, ID2, NCAPG, KIF4A, NUF2, CDC20, CCT6A, and survivin and the prognosis of ES.

4.2.1. Predictive markers of Ewing sarcoma

Predictive markers are important to make a management choice because they can predict sensitivity to a certain line of treatment based on its presence or absence. Some, if not most, of these biomarkers are involved in the tumorigenesis and proliferation of ES. miR-139b and ErbB4 are an example of such biomarkers [54,68]. In fact, the treatment is most of the time a drug that can inhibit the progression of said biomarker such as lapatinib, which decreases the activity of ErbB4 [54].

Other markers, while possibly involved in ES, may not serve as predictive markers, such as HER2, which is moderately present on ES cells but trastuszumab had no effect on ES [72]. However, some markers may even be surface proteins, but what makes them good predictors of treatment is their consistent presence on ES cells and their action. An example would LINGO1, a surface protein consistently found on ES cells, that internalizes via the endosome-lysosome pathway when bound by anti-LINGO1 IgGs antibodies, allowing it to deliver cytotoxic drugs and induce cell death [77].

Some predictive markers can act by making the tumor more sensitive to a certain treatment. An example is the CCN3 gene and the NH3 domain in the latter, which showed that their reduced expression was associated with better outcomes. This relationship was even stronger in radiotherapy and absent in surgery, which leads to the conclusion that these markers may be involved in radiation sensitivity [42].

It is suggested to start searching for an overexpression of ErbB4 in ES. If the latter is present, lapatinib can then be used as a treatment [54]. When faced with the choice between surgery and radiotherapy, it is better to see if there is a reduced expression of CCN3 and its NH3 domain. If so, then radiotherapy would be the better option. When PAK1 is overexpressed, a good treatment would be PAK1 inhibitors, such as IPA3 and Frax597. Most of the time, it would be beneficial to use chemicals that inhibit the CDK4 and cyclin D1 pathways, since they are activated in ES cells. More studies are needed to test for the potential therapeutic use of miR-139b, ER, FGFR1, IGF-1R and LINGO1. However, trials are needed to confirm these suggestions before they can be implemented in the treatment arsenal for ES.

5. Strengths and limitations

This study presents with some strengths, mainly the number of included studies, and the extensive search method used and included going through three databases. However, a lot of the studies included are still experimental and may not benefit the clinical management of ES in their actual state.

6. Conclusion

In conclusion, the identification and use of biomarkers for ES can provide valuable insights into the diagnosis, prognosis, and treatment of this aggressive tumor, especially when used in combination or as a panel. While there is still much to learn about the underlying mechanisms of this disease, we have identified several promising biomarkers in this review that have shown potential as diagnostic tools, as well as predictors of response to therapy and overall survival. Further research in this area is required to demonstrate the clinical utility of some of the biomarkers discussed, but holds great promise for improving outcomes for patients with ES and advancing our understanding of this complex disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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