Ewing sarcoma

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Abstract | Ewing sarcoma is the second most frequent bone tumour of childhood and adolescence that can also arise in soft tissue. Ewing sarcoma is a highly aggressive cancer, with a survival of 70–80% for patients with standard-risk and localized disease and ~30% for those with metastatic disease. Treatment comprises local surgery, radiotherapy and polychemotherapy, which are associated with acute and chronic adverse effects that may compromise quality of life in survivors. Histologically, Ewing sarcomas are composed of small round cells expressing high levels of CD99. Genetically, they are characterized by balanced chromosomal translocations in which a member of the *FET* gene family is fused with an *ETS* transcription factor, with the most common fusion being *EWSR1–FLI1* (85% of cases). Ewing sarcoma breakpoint region 1 protein (EWSR1)–Friend leukaemia integration 1 transcription factor (FLI1) is a tumour-specific chimeric transcription factor (EWSR1–FLI1) with neomorphic effects that massively rewires the transcriptome. Additionally, EWSR1–FLI1 reprogrammes the epigenome by inducing de novo enhancers at GGAA microsatellites and by altering the state of gene regulatory elements, creating a unique epigenetic signature. Additional mutations at diagnosis are rare and mainly involve *STAG2*, *TP53* and *CDKN2A* deletions. Emerging studies on the molecular mechanisms of Ewing sarcoma hold promise for improvements in early detection, disease monitoring, lower treatment-related toxicity, overall survival and quality of life.

Ewing sarcoma is a malignant bone tumour (occurring predominantly in the pelvis, femur, tibia and ribs) or soft-tissue tumour (occurring predominantly in the thoracic wall, gluteal muscle, pleural cavities and cervical muscles) that mainly affects children, adolescents and young adults (AYAs) with ~1.5 cases per million children and AYAs globally. Approximately 20–25% of patients present with metastases at diagnosis that are often resistant to intensive therapy[1]. Standard of care for Ewing sarcoma consists of a multimodal treatment regimen including surgical resection and/or local radiotherapy as well as intensive multi-agent chemotherapy[2]. Ideally, diagnosis and treatment of Ewing sarcoma should be carried out in a sarcoma reference centre by a multidisciplinary team that includes radiologists; pathologists; paediatric, medical and radiation oncologists; orthopaedic and general surgeons; and specialized nurses. Despite proven effectiveness for treatment of localized disease, the long-term survival of patients with metastatic or relapsed Ewing sarcoma remains unacceptably low[1,3].

Historically, the group 'Ewing sarcoma family of tumours' encapsulated lesions on the basis of morphological and immunophenotypical features and the presence of chromosomal translocations and included extraosseous Ewing sarcoma, peripheral primitive neuroectodermal tumours and Askin tumours[4]. However, the 2013 WHO classification of sarcomas uniformly defined these tumours as 'Ewing sarcoma' (REFS[5,6]), which are characterized by pathognomonic *FET–ETS* gene fusions[7,8]. The WHO classification also includes the term 'Ewing-like sarcomas', which are small round cell sarcomas with morphologically similar appearances to Ewing sarcoma but are characterized by different fusion genes and clinical and pathological features (BOX 1). Recent RNA profiling data indicate that these rare, non-*FET* and/or non-*ETS* fusion-positive tumours are biologically distinct from *FET–ETS* Ewing sarcoma[7]. Here, we focus on *FET–ETS*-positive Ewing sarcoma.

Almost 100 years after the first description of Ewing sarcoma[9], its cell of origin is still a matter of debate[10]. Regardless of this histogenetic uncertainty, Ewing sarcoma is genetically well characterized: its main driver mutations are specific chromosomal translocations that fuse a member of the FET family of proteins (encoded by *FUS*, *EWSR1* and *TAF15*), which are RNA-binding proteins involved in transcription and splicing, with different members of the ETS (E26-specific) family of transcription factors, which are involved in cell proliferation, cell differentiation, cell-cycle control, angiogenesis and apoptosis — most commonly *FLI1* (85% of cases)[11] (FIG. 1).

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In a permissive cellular context, these chimeric fusion proteins act as aberrant transcription factors with neomorphic functions that deregulate hundreds of genes (for example, genes involved in cell-cycle regulation, cell migration and proliferation) by binding DNA at either *ETS*-like sequences that contain a single GGAA motif or at GGAA microsatellites (which are composed of multiple iterative GGAA motifs)[12–14].

As additional genetic alterations apart from *FET–ETS* fusions are exceedingly rare[15–18], Ewing sarcoma constitutes a prototypic model to study the oncogenic process in a simplified context, which has stimulated sustained interest in this disease. New discoveries on the molecular mechanisms underlying Ewing sarcoma offer the opportunity to overcome current clinical challenges by identifying effective targeted therapies and prognostic and predictive biomarkers. This Primer condenses our current knowledge on Ewing sarcoma, focusing on the epidemiology, mechanisms, diagnosis, management and clinically relevant therapeutic advances.

Epidemiology

Demographics

Ewing sarcoma mainly affects children and AYAs with a peak incidence at the age of 15 years; boys and men are slightly more affected than girls and women (sex ratio of 3:2)[19]. In 1970, striking disparities in Ewing sarcoma incidence were reported across populations[20,21], which have been reinforced by data from the US Surveillance Epidemiology and End Results (SEER) registry[19]. Specifically, Ewing sarcoma is predominantly observed in populations of European descent with an estimated incidence of ~1.5 cases per million children and AYAs[19]. The estimated incidence in populations of Asian and African descent is substantially lower, with annual rates of ~0.8 and ~0.2 cases per million children, respectively, implying that genetic variants specific to European ancestry could influence Ewing sarcoma risk[19,20,22]. Interestingly, the incidence of Ewing sarcoma in African Americans remains low as compared with Americans of European ancestry, indicating that the rarity of Ewing sarcoma in Africans and African Americans is based on a genetic germline component rather than environmental or lifestyle factors[20,22]. Despite the rarity of Ewing sarcoma, infrequent and anecdotal instances of familial clustering of Ewing sarcoma in siblings or cousins have also been reported, further suggesting an important genetic component to Ewing sarcoma[23]. No environmental risk factors for Ewing sarcoma have been identified other than in a single Australian national case–control study that linked farm exposure with an increased probability of developing Ewing sarcoma[24].

Currently, patients with localized disease generally have a 5-year overall survival of ~70–80%, which may be lower in patients presenting with pelvic tumours, large tumours and/or incomplete tumour regression after neoadjuvant or adjuvant chemotherapy. Patients presenting with metastasis at diagnosis generally have a significantly lower overall survival of <30%, whereas patients with isolated pulmonary metastasis have an overall survival of ~50%[1]. No robust statistical tool is currently available to estimate the life expectancy or longevity of survivors of Ewing sarcoma.

Genetic risk factors

Ewing sarcoma is a genetically well-characterized disease (see below). Low-to-moderate risk alleles (FIG. 2) may promote tumorigenesis, possibly through functional interaction with the most commonly occurring fusion protein in the disease, Ewing sarcoma breakpoint region 1 protein (EWSR1)–Friend leukaemia integration 1 transcription factor (FLI1)[25,26]. Germline sequencing studies have revealed that ~13% of patients with Ewing sarcoma harbour rare inactivating variants or mutations in DNA damage repair genes, including the same genes that are enriched in hereditary breast cancer (for example, *BRCA1*)[27,28] (FIG. 2). However, although most of these variants are known polymorphisms[27,28], their pathogenetic roles remain unclear. Moreover, Ewing sarcoma is rarely observed among the ~120 cancer predisposition syndromes described to date[29]; thus, whether the occurrence of these variants indicates recommending genetic counselling for patients with Ewing sarcoma and their families remains unclear.

Additionally, genome-wide association studies (GWAS) have identified six candidate susceptibility loci in which common variants may affect the expression of nearby genes, including *TARDBP* (which is structurally similar to *EWSR1*)[25], known EWSR1–FLI1 target genes (*EGR2* and *NKX2-2*), members of core EWSR1–FLI1 regulatory circuitries (*RREB1*) and genes involved in centrosome stabilization (*KIZ*) and apoptosis (*BMF*) (T.G.P.G., D.S., H.K., O.D. and U.D., submitted) (FIG. 2). Mechanistically, for example, a common variant in linkage disequilibrium with the GWAS association signal at the 10q21.3 locus alters the structure of an EWSR1–FLI1 response element (a GGAA microsatellite), which increases EWSR1–FLI1 binding to the response element, *EGR2* expression and proliferation of Ewing sarcoma cells[26]. Similar mechanisms could operate at the other candidate susceptibility loci, as the identified lead GWAS signals are significantly closer to GGAA microsatellites than can be expected by chance (T.G.P.G., D.S., H.K., O.D. and U.D., submitted). Notably, most GWAS risk alleles show striking differences in their frequencies across populations, which might partly account for the variable incidence of Ewing sarcoma[25,26]. In line

Box 1 | **Ewing-like sarcomas**

Ewing-like sarcomas (ELSs) are a heterogeneous group of small round cell sarcomas that are histologically and clinically subtly different from Ewing sarcoma, although these tumours were considered as Ewing sarcomas until approximately 2010. ELS entities typically lack the key Ewing sarcoma *FET–ETS* gene fusions but harbour other recurrent specific gene fusions and/or rearrangements.

***CIC*-fused sarcomas**
This group of ELSs includes sarcomas with *CIC–DUX4*, *CIC–FOXO4* and *CIC–NUTM1* fusions[206,207].

***BCOR*-rearranged sarcomas**
This group of ELSs includes sarcomas with *BCOR–CCNB3*, *BCOR–MAML3* and *ZC3H7B–BCOR* fusions and sarcomas with *BCOR* internal duplications[7,208].

***NFATC2* sarcomas**
This group of ELSs includes sarcomas with *EWSR1–NFATC2* fusions, which commonly show an *EWSR1* amplification pattern on fluorescence in situ hybridization[209].

The functional role of these gene fusions and rearrangements is currently being elucidated, and an active search for therapeutic targets is being carried out. Patients with ELS are usually enrolled in Ewing sarcoma clinical trials because many of these ELS entities do not have specific clinical trials available. This reality may have important implications as recent data showed that patients with *BCOR*-rearranged sarcomas have relatively similar clinical outcomes to those with Ewing sarcoma, whereas those with *CIC*-fused sarcomas have poorer outcomes[208].

with this concept, a germline sequencing study found significant length differences in a GGAA microsatellite located in the promoters of *NR0B1* and *CAV1* in people of European descent as compared with Africans[30]. In addition, germline polymorphisms located in the *CD99* gene, which encodes an important membranous glycoprotein regulated by EWSR1–FLI1 that is routinely used for Ewing sarcoma diagnosis and that contributes to Ewing sarcoma metastasis[31,32] (for review, see REFS[33,34]), were reported to be associated with Ewing sarcoma risk[35].

Mechanisms/pathophysiology

Genetic alterations

FET–ETS fusions. Ewing sarcoma is characterized by a recurrent balanced chromosomal translocation[36], most commonly t(11;22)(q12;q24). This translocation results in the fusion of the *FET* family gene *EWSR1* with the *ETS* family gene *FLI1* (REF.[11]). Depending on the position of breakpoints within the *EWSR1* and *FLI1* genes, different subtypes of *EWSR1–FLI1* transcripts have been described[37]. In the 15–20% of Ewing sarcomas that are negative for *EWSR1–FLI1* fusions, variant fusions between *EWSR1* and other members of the *ETS* family occur, most commonly *ERG* (encoding transcriptional regulator ERG)[37,38] (FIG. 1). The *EWSR1–ERG* fusion provided the first example of different but related gene fusions occurring in phenotypically and clinically similar human solid tumours[39], suggesting the concept of genetic redundancy in driver lesions in solid tumours. Subsequently, *ETV1* (REF.[40]), *ETV4* (REF.[41]) or *FEV*[42] fusions to *EWSR1* were identified. Similarly, some variant fusions were described between *ETS* genes and *EWSR1* paralogues of the *FET* gene family (namely, *FUS* and *TAF15*)[7,43] (FIG. 1).

The clinical significance of these fusion genes has been investigated. Although a retrospective study found different *EWSR1–FLI1* transcript subtypes to be associated with different patient outcomes[44], two prospective studies could not validate this observation[45,46]. In addition, Ewing sarcomas harbouring *EWSR1–FLI1* or *EWSR1–ERG* have similar clinical phenotypes[39]. Variant non-*FET* and/or non-*ETS* fusions that define tumours being distinct from *FET–ETS* Ewing sarcoma will not be discussed here but have been extensively reviewed elsewhere[8].

Additional protein-coding mutations. As in most developmental cancers, additional recurrent mutations in Ewing sarcoma are rare. For example, *STAG2* and *TP53* mutations are detected at diagnosis in 15–21% and 5–7% of cases, respectively[15–18]. *STAG2* mutations can evolve from a subclonal to a clonal population during disease progression[16,18]. Cohesin subunit SA2 (STAG2) is part of the cohesin complex, which is a ring-like structure that holds sister chromatids together during mitosis and shapes the 3D chromatin structure[47]. Loss-of-function *STAG2* mutations can drive aneuploidy in cancer[17]. Interestingly, inhibition of the STAG1 cohesin component in the context of *STAG2* mutations evokes synthetic lethality in Ewing sarcoma and offers possible therapeutic perspectives[48]. Alterations in *STAG2* alone or combined with *TP53* mutations are associated with poor outcome[18].

Other mutations have been identified in *EZH2* (encoding a methyltransferase), *ZMYM3* (which is involved in cell morphology and cytoskeletal organization) and *BCOR* (encoding a transcriptional co-repressor)[18]. Furthermore, *CDKN2A* (encoding a cyclin-dependent kinase that regulates cell proliferation) is deleted in 10–22% of cases[15,16,18,49,50]. However, little is known about genetic intratumoural heterogeneity in Ewing sarcoma, its subclonal genetic architecture and how these factors relate to clinical outcome. Moreover, whether the genomic landscape in Ewing sarcoma at relapse is different is also unknown. These questions will be addressed by ongoing initiatives aiming at prospectively characterizing genomic alteration in recurrent or refractory solid tumours[51,52].

Copy number variations. Cytogenetic and comparative genomic hybridization studies have identified recurrent chromosomal abnormalities in Ewing sarcoma[53–55], usually involving whole chromosomes or chromosome arms. Chromosomal gains include chromosome 8 (50% of cases), chromosome 2 and chromosome 1q (25% of cases) and chromosome 20 (10–20% of cases). Chromosome 1q gain[18,56] is frequently associated with chromosome 16q loss as a result of an unbalanced translocation (t(1;16)). Copy number variation studies by the PROVABES consortium using samples derived from the EURO-E.W.I.N.G.99 (EE99) and EWING 2008 trials showed that chromosome 1q gain and possibly chromosome 16q loss define patients with poor clinical outcome. An attractive candidate gene that possibly contributes to the poor clinical outcome of patients with chromosome 1q gain is *CDT2* (REF.[56]), a gene involved in cell-cycle control for which gene 'dosage' may increase proliferation rates in Ewing sarcoma harbouring chromosome 1q gains. Fluorescence in situ hybridization (FISH) analysis with specific probes for chromosome 1q

FET part	*ETS* part	Fusion gene	Chromosomal translocation	Frequency
FUS	*FEV*	*FUS–FEV*	t(2;16)(q35;p11)	<1%
	ERG	*FUS–ERG*	t(16;21)(p11;q22)	<1%
EWSR1	*FLI1*	*EWSR1–FLI1*	t(11;22)(q24;q12)	≈85%
	ERG	*EWSR1–ERG*	t(21;22)(q22;q12)	≈10%
	ETV1	*EWSR1–ETV1*	t(7;22)(p22;q12)	<1%
	ETV4	*EWSR1–ETV4*	t(17;22)(q21;q12)	<1%
	FEV	*EWSR1–FEV*	t(2;22)(q33;q12)	<1%
	ETV5 (?)	*EWSR1–ETV5*	?	?
TAF15	?	?	?	?

Fig. 1 | ***FET–ETS* fusion oncogenes in Ewing sarcoma.** Oncogenic translocations in Ewing sarcoma encompass a member of the *FET* gene family (*FUS*, *EWSR1* or *TAF15*) in combination with different members of the *ETS* family of transcription factors (such as *FLI1*, *ERG*, *ETV4* and *FEV*). *EWSR1–ETV5* and *TAF15–ETS* gene fusions in Ewing sarcoma have not been described to date but could be possible on the basis of the high structural similarity of the *FET* and *ETS* family members.

and chromosome 16q might be useful for routine detection of the respective gains and losses. The most frequent deletions involve chromosome 9p and *CDKN2A* and are associated with poor prognosis[15,16].

Aberrant transcription

The various *FET–ETS* gene fusions result in very similar consequences at the protein level, creating a chimeric peptide that fuses the amino-terminal, low-complexity domain of the FET partner to the DNA-binding domain of the ETS protein. The *FET–ETS* fusion gene is always expressed as it is driven by the ubiquitously active promoters of the FET family members such as that of *EWSR1*. In most cases, a reciprocal *ETS–FET* fusion gene is also created as the result of the balanced chromosome translocation. However, this reverse fusion is usually not or is only lowly expressed as it is driven by the tissue-restricted *ETS* promoter, which is usually inactive in Ewing sarcoma[37].

The majority of functional studies have focused on the most frequent fusion, *EWSR1–FLI1*. *EWSR1–FLI1* encodes a dominant oncoprotein[57] that binds DNA and acts as an aberrant transcription factor[58,59], regulating a variety of different genes involved in cell-cycle regulation, cell migration, signal transduction, chromatin architecture, telomerase activity and many other cell functions[60]. Specifically, EWSR1–FLI1 can bind to two types of binding motif of transcriptional targets. One motif is similar to those of the corresponding wild-type ETS family transcription factors and includes the GGAA core sequence[13,14]. The other motif seems to be specific to the fusion protein; it consists of tandem GGAA repeats that form microsatellite sequences[13,14]. Recent data show that these motifs and additional characteristics of target gene promoters, including their epigenetic regulators, might account for observed gene repression or activation functions of EWSR1–FLI1 (REFS[61–63]). The second most common fusion protein, EWSR1–ERG, has similar functions to EWSR1–FLI1 as FLI1 and ERG are structurally comparable and have almost identical DNA-binding domains[38]. Indeed, the clinical outcome for patients harbouring either type of fusion is comparable[39].

A recent study has shown that gene transcription mediated by EWSR1–FLI1 leads to the frequent formation of R loops, which are three-stranded structures composed of a DNA:RNA hybrid and an associated non-template single-stranded DNA. These R loops might sensitize Ewing sarcoma cells to poly(ADP-ribose) polymerase (PARP) inhibitors, possibly via sequestration and inactivation of breast cancer type 1 susceptibility protein (BRCA1)[64], which has a central role in DNA damage repair. Future studies will continue to add layers of complexity to the function of these chimeric oncoproteins.

Epigenetic alterations

Given the relevance of epigenetic mechanisms in controlling cellular identity and the few genetic alterations detected in Ewing sarcoma tumours[15,16,18], the epigenome likely plays a critical part in Ewing sarcoma initiation and progression. Several of the rare recurrent somatic mutations observed in Ewing sarcoma affect genes that encode epigenetic regulators, most notably *STAG2* (TABLE 1). Comprehensive epigenome profiling has revealed that EWSR1–FLI1 drives widespread epigenetic reprogramming by inducing de novo enhancers (Ewing-specific enhancers) and by repressing enhancers that are active in many cell types, including those of mesenchymal origin[61,62,65].

Enhancers are cell-type-specific and dynamically used regulatory elements that control the temporal and spatial activation of gene expression. The enhancer genome-wide signature observed in Ewing sarcoma is highly unique[63] and is functionally linked to the oncogenic transformation by EWSR1–FLI1. Indeed, Ewing-sarcoma-specific enhancers are enriched in GGAA microsatellites[61,66,67], and EWSR1–FLI1 seems to act as a pioneer transcription factor that can overcome the closed chromatin state of these GGAA microsatellites in Ewing sarcoma cells, increasing DNA accessibility to other transcription factors, chromatin modifiers and remodelling complexes[61,67]. Specifically, the prion-like domain of *EWSR1* fused to *FLI1* has been reported to interact with subunits of the BRG1–BRM-associated factor (BAF) chromatin-remodelling complexes, which results in recruitment of BAF complexes at GGAA microsatellites[67]. This recruitment drives chromatin remodelling, establishes de novo enhancer elements (FIG. 3) and activates the Ewing sarcoma transcriptional programme[67].

By contrast, how EWSR1–FLI1 represses the activity of certain enhancers is less clear. EWSR1–FLI1 does not bind the majority of repressed enhancers, suggesting that repression of these elements occurs indirectly. For example, it has been suggested that EWSR1–FLI1 interferes with the activating role of transcription factors such as AP-1 (activator protein 1)[62,63] and TEAD (TEF transcription factors)[68] and/or drives chromatin-repressive complexes to these loci[69] (TABLE 1). Ewing sarcoma tumours harbouring *EWSR1–ERG* have been shown to be similar to those harbouring *EWSR1–FLI1* regarding their transcriptome[7], epigenome[63] and clinical behaviour[70].

Epigenetic regulation in Ewing sarcoma cells might also be conducted by non-coding RNAs. Non-coding RNAs,

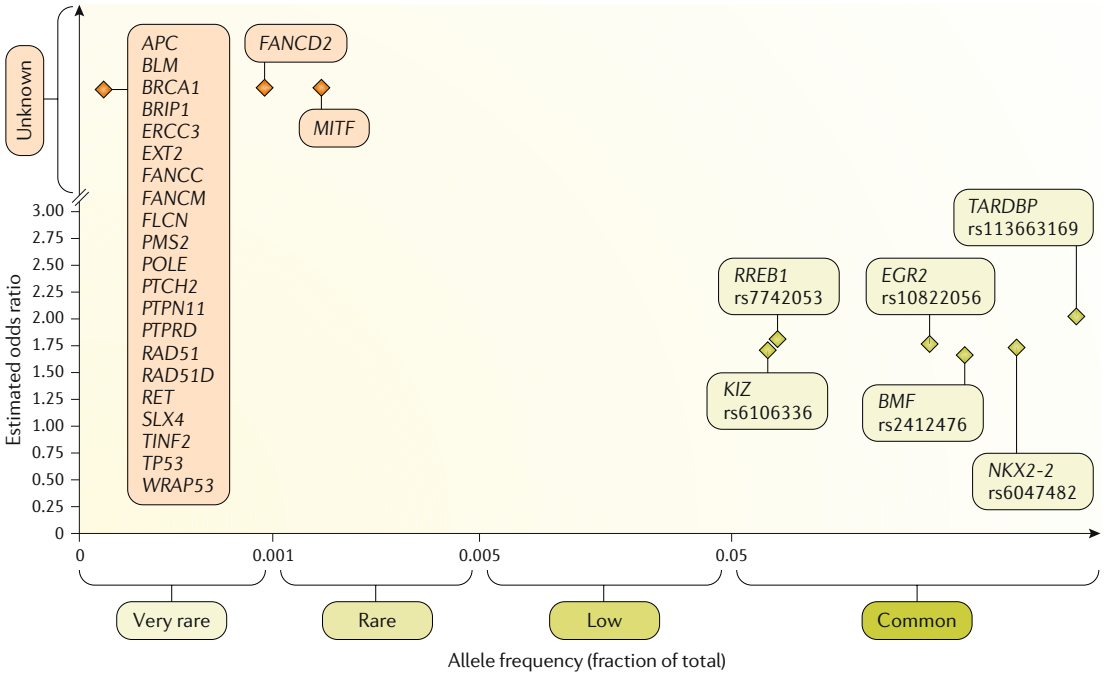

Fig. 2 | **Genetic predisposition to Ewing sarcoma.** Germline sequencing studies have identified very rare likely deleterious polymorphisms or mutations in DNA damage repair genes (such as *BRCA1* and *FANCC*; orange), the precise pathogenicity and genetic effect size of which remain currently unknown[27]. By contrast, genome-wide association studies have identified common variants that might alter the expression levels and/or function of nearby genes (green) that could individually have small effects on tumour initiation but could cooperate with the somatically acquired *EWSR1–FLI1* fusion oncogene to promote tumorigenesis (T.G.P.G., D.S., H.K., O.D. and U.D., submitted)[25]. For most genes, except for *EGR2* (REF.[26]), experimental proof of their involvement in Ewing sarcoma susceptibility is not available yet. Ongoing work is identifying additional rare or low-frequency alleles that might explain the susceptibility of people of European descent to Ewing sarcoma. Mechanisms of epistatic interaction of these risk alleles remain to be defined. Data from REFS[25–27].

including microRNAs and long non-coding RNAs, modulate gene expression and are, accordingly, considered epigenetic modifiers. At the same time, the expression of non-coding RNAs can be regulated by other epigenetic mechanisms, such as histone post-translational modifications and DNA methylation[71]. Indeed, a number of non-coding RNAs have been reported to be regulated by EWSR1–FLI1 and to be key players in Ewing sarcoma tumorigenesis[72] (TABLE 1), potentially providing novel biomarkers and therapeutic approaches.

Cell of origin

The lack of genetic subtypes suggests that Ewing sarcoma is derived from a single cellular lineage with variable developmental timing of oncogenic conversion accompanied by impaired differentiation potential[73]. However, the developmental origin of Ewing sarcoma is still an enigma owing to its undifferentiated phenotype, epigenome structure and gene expression pattern[62,63,74]. The neomorphic functions and transcriptomic and epigenetic consequences of FET–ETS fusion proteins render the identification of the Ewing sarcoma cell of origin extremely difficult; as >80% of Ewing sarcomas arise in bone, candidate progenitors may reside in the developing bone mesenchyme, comprising mesodermal and neural-crest-derived cell types.

Indeed, neural-crest-derived stem cells and bone-marrow-derived mesenchymal stem cells (MSCs), as well as osteochondrogenic progenitors, are susceptible to EWSR1–FLI1-mediated immortalization[75–79]. In these cells, chromatin regions, including GGAA microsatellites, might serve as binding regions for EWSR1–FLI1 (REF.[80]). In support of a neural crest stem cell of origin, tumour gene expression patterns in the presence of *EWSR1–FLI1* resemble those of neural-crest-derived stem cells[77,81]. However, knockdown of *EWSR1–FLI1* drives the Ewing sarcoma transcriptome towards that of MSCs[73], suggesting a mesenchymal origin of Ewing sarcoma. Finally, an epithelial origin has also been proposed, which is consistent with the expression of cell–cell adhesion molecules such as claudin 1 and tight junction protein ZO1 in Ewing sarcoma[82].

Interactions with the microenvironment

As in other cancers, Ewing sarcoma cells must often contend with harsh microenvironmental conditions to remain viable (FIG. 4). Numerous reports link stress adaptation to aggressive behaviour in Ewing sarcoma. For example, growth factor deprivation or hypoxia both trigger enhanced extracellular matrix (ECM) degradation[83] and increase CXC-chemokine receptor 4 (CXCR4) expression[84] to enhance the invasiveness of Ewing sarcoma. Hypoxia also induces neuropeptide Y (NPY), which promotes growth and bone invasion in Ewing sarcoma[85]. In addition, hypoxia influences EWSR1–FLI1 expression and its downstream targets, including

Table 1 | **Epigenetic regulators in Ewing sarcoma**

Regulator	Function	Implication in Ewing sarcoma
BRG1–BRM-associated factor (BAF) complex	Also known as the SWI/SNF complex, an ATP-dependent chromatin-remodelling complex consisting of 15 subunits that are encoded by 29 genes[220]	BAF recruitment to GGAA microsatellites by the EWSR1–FLI1 fusion protein establishes de novo Ewing-sarcoma-specific enhancers and contributes to the Ewing sarcoma transcriptional programme[67]
BCL-6 co-repressor (BCoR)	• BCoR functions as a co-repressor when tethered to DNA and, when overexpressed, can potentiate BCL-6 repression • BCoR also interacts with class I and II HDACs[221]	• Somatic mutations within *BCOR* have been detected in Ewing sarcoma (2.7% of cases)[18] • A BCoR fusion (BCoR–CCNB3) has been described in Ewing-like sarcomas[222]
BCL-6 co-repressor-like protein 1 (BCoRL1)	• BCoRL1 functions as a co-repressor when tethered to DNA at promoter regions • The encoded protein can interact with class II HDACs to repress transcription[223]	Rare, somatic mutations in *BCORL1* have been detected in Ewing sarcoma, but without statistical significance[16]
Bromodomain and extraterminal domain (BET) family proteins	• This family of proteins (including BRD2, BRD3, BRD4 and BRDT) recognize acetylated chromatin and regulate gene expression • BRD4 associates with active promoters and active enhancers in the genome of normal and cancer cells[224]	Multiple studies suggest that Ewing sarcoma cells are susceptible to BET inhibitors such as JQ1 (REFS[225,226])
Cohesin subunit SA2 (STAG2)	Subunit of the cohesin complex that regulates chromatin architecture[227]	• Most common recurrent somatic mutations in Ewing sarcoma (~20% of cases) are in *STAG2* (REFS[16,18]) • STAG2 displays synthetic lethal interaction with its paralogue STAG1 in Ewing sarcoma cell lines[48]
Histone-lysine *N*-methyltransferase EZH2	A functional enzymatic component of PRC2 that catalyses the addition of methyl groups to histone H3 at lysine 27, leading to transcriptional repression[228]	• Somatic mutations in *EZH2* have been detected in Ewing sarcoma (2.7% of cases)[18] • EZH2 expression is induced by EWSR1–FLI1 (REFS[109,229]) • EZH2 has been shown to block the endothelial and neuro-ectodermal differentiation of Ewing sarcoma cells[109]
Ewing sarcoma associated transcript 1 (EWSAT1)	A previously uncharacterized long non-coding RNA (LINC00277)	EWSAT1 is a downstream target of EWSR1–FLI1 that facilitates the development of Ewing sarcoma via the repression of target genes[230]
HOX transcript antisense RNA (HOTAIR)	• A long non-coding RNA that interacts with PRC2 and the histone demethylase KDM1A • HOTAIR regulates chromatin state[231]	HOTAIR primes Ewing sarcoma cells for tumorigenesis via epigenetic dysregulation involving LSD1 (REF.[232])
Histone-lysine *N*-methyltransferase 2D (KMT2D) and 2 C (KMT2C)	These mixed-lineage leukaemia proteins are histone methyltransferases that methylate lysine 4 of histone H3 (H3K4me)[233]	Rare, somatic mutations in *KMT2D* and *KMT2C* have been detected in Ewing sarcoma[16]
Histone-lysine *N*-methyltransferase PRDM9	A zinc-finger protein with histone methyltransferase activity that trimethylkates lysine 4 of histone H3 (H3K4me3)[234]	Rare, somatic mutations in *PRDM9* have been detected in Ewing sarcoma[18]
Histone-lysine *N*-methyltransferase SETD2	A histone methyltransferase that specifically trimethylates lysine 36 of histone H3 (H3K36me3)[235]	Rare, somatic mutations in *SETD2* have been detected in Ewing sarcoma[18]
Lysine-specific demethylase 3 A (KDM3A)	A JmjC domain-containing protein, which specifically demethylates monomethyl H3K9 and dimethyl H3K9 (REF.[236])	Expression is induced by EWSR1–FLI1-mediated suppression of hsa-miR-22, contributing to the clonogenicity, anchorage independence and metastatic potential of Ewing sarcoma cells[237]
Lysine-specific histone demethylase 1 A (KDM1A)	• Also known as LSD1, a histone demethylase that demethylates both lysine 4 (H3K4me) and lysine 9 (H3K9me) of histone H3, acting as a co-activator or a co-repressor, respectively • Associated with the Mi-2/NuRD complex[238]	• Suggested as an important factor in transcriptional regulation mediated by EWSR1–FLI1 (REF.[69]) • KDM1A inhibition by the small molecule HCI-2509 is currently being tested as potential targeted therapy for Ewing sarcoma and a clinical trial will soon be initiated[239]
miR-34a	A microRNA tumour suppressor in multiple types of cancer that suppresses multiple targets[240]	• Suggested to predict survival of patients with Ewing sarcoma[241] • miR-34a inhibits proliferation of Ewing sarcoma cell lines and increases sensitivity to doxorubicin and vincristine[241]
miR-145	A microRNA regulator of *OCT4*, *SOX2* and *KLF4* expression that facilitates the differentiation of embryonic stem cells by repressing the core pluripotency factors[242]	• miR-145 functions with EWSR1–FLI1 in a mutually repressive feedback loop[76,243] • miR-145 is a key player in Ewing sarcoma tumorigenesis and cell differentiation[76,243], and its expression is regulated by RISC-loading complex subunit TARBP2 in cancer stem cells, which disturbs the clonogenicity of Ewing sarcoma cells[244]
NAD-dependent protein deacetylase sirtuin 1 (SIRT1)	A class III HDAC with a role in transcriptional regulation, SIRT1 can induce histone deacetylation and methylation, DNA methylation and deacetylation of proteins[245]	High SIRT1 expression is associated with Ewing sarcoma metastasis and poor prognosis[104]

Table 1 (cont.) | **Epigenetic regulators in Ewing sarcoma**

Regulator	Function	Implication in Ewing sarcoma
Nucleosome remodelling deacetylase (Mi-2/NuRD) complex	A nucleosome remodelling and histone deacetylase–demethylase complex that is involved in transcriptional regulation and chromatin assembly[238]	NuRD interacts with EWSR1–FLI1 and contributes to transcriptional repression programmes in Ewing sarcoma[69]
Polycomb complex protein BMI1	A ring-finger protein and a major component of PRC1, which is a transcriptional repressive complex[246]	Expression is induced by EWSR1–FLI1 (REF.[77]) and may have a vital role in Ewing sarcoma tumorigenesis[247]
RE1-silencing transcription factor (REST)	Also known as a neuron-restrictive silencer factor (NRSF), REST acts as a hub for the recruitment of multiple chromatin-modifying enzymes[248]	• Expression of *REST* is regulated by EWSR1–FLI1 and is high in Ewing sarcoma[249] • REST inhibits neuronal phenotype development in Ewing sarcoma cells[69]
Zinc-finger MYM-type protein 3 (ZMYM3)	• Chromatin-interacting protein that promotes DNA repair by homologous recombination[250] • Component of a KDM1A-transcription repressor complex[251]	Somatic mutations in *ZMYM3* have been detected in Ewing sarcoma (2.7% of cases)[18]

BCL-6, B cell lymphoma 6; BRD, bromodomain-containing protein; BRDT, bromodomain testis-specific protein; CCNB3, G2/mitotic-specific cyclin B3; EWSR1, Ewing sarcoma breakpoint region 1 protein; FLI1, Friend leukaemia integration 1 transcription factor; HDAC, histone deacetylase; LSD1, lysine-specific histone demethylase 1A; PRC, Polycomb repressive complex.

enhanced expression of metastasis-related proteins and soft agar colony formation (an assay to evaluate cellular transformation in vitro) under low oxygen[86]. Ewing sarcoma cells suppress detachment-induced cell death (anoikis), in part by upregulating the tyrosine kinase v-erb-b2 avian erythroblastic leukaemia viral oncogene homologue 4 (ERBB4), which drives an RACα serine/threonine-protein kinase (AKT)–focal adhesion kinase 1 (FAK)–Ras-related C3 botulinum toxin substrate 1 (RAC1) signalling axis to increase metastatic capacity in vivo[87]. Furthermore, interactions with the immune system likely have key roles in stress adaptation (BOX 2).

Genomic studies in epithelial tumours posit that mutational changes lead to clonal selection of stress-adaptive cells, resulting in acquisition of chemoresistance and metastatic capacity[88]. However, as Ewing sarcoma tumours are genomically 'quiet' (REFS[16,18,89]), other mechanisms must be invoked to explain stress-adaptive responses in these tumours, such as epigenetic[63] or mRNA translational reprogramming[90]. With respect to translational reprogramming, under acute stress, tumour cells suppress overall mRNA translation and protein synthesis to save energy but selectively translate major stress-adaptive mRNAs, the protein products of which are key to cell survival under stress[90]. In Ewing sarcoma cells, increased expression of the nuclease-sensitive element-binding protein 1 (YB1; also known as YBX1) RNA-binding protein leads to translational activation of hypoxia-inducible factor 1α (HIF1α)[91]. HIF1α transcriptionally enhances vascular endothelial growth factor (VEGF) expression (and, consequently, enhances angiogenesis) and augments metastatic capacity in vivo[91]. Under diverse stresses, YB1 also induces formation of cytosolic stress granules, which silence sequestered mRNAs but enhance translation of selected messages to facilitate survival. Stress granules enhance metastatic capacity of sarcoma cells, including Ewing sarcoma[92].

In addition to the propensity of tumour cells to avoid, bypass or overcome the consequences of microenvironmental stresses, their ability to communicate with the various cell types populating the tumour niche affects Ewing sarcoma pathogenesis. This two-way process involves microenvironmental cell signals being relayed to the tumour cells via ligand–cell-surface receptor interactions; reciprocally, tumour cells educate the microenvironment via the shedding of exosomes[93,94]. In the bone marrow niche, the microenvironmental signals include insulin-like growth factor (IGF), which is required for EWSR1–FLI1 transformation, Ewing sarcoma growth[95,96] and angiogenesis[97]; platelet-derived growth factor (PDGF), which is required for chemotaxis and migration[98]; fibroblast growth factor 2 (FGF2), which is needed for Ewing sarcoma motility and invasion[99,100]; and IL-6, which is required for apoptosis resistance in the tumour cells[101]. In turn, EWSR1–FLI1 modulates the responsiveness of Ewing sarcoma cells to transforming growth factor-β (TGFβ)[102] and NOTCH ligands[103].

Metastasis

Metastasis is the most powerful adverse prognostic factor in Ewing sarcoma. However, its underlying mechanisms remain poorly understood, although molecular pathways that correlate with metastasis have been identified through comparison of primary and metastatic Ewing sarcoma cell lines and tumours, including the aforementioned stress pathways (FIG. 4).

Although no clear unifying model of Ewing sarcoma metastasis exists, a number of compelling studies point to key metastatic drivers in this disease, many of which seem to influence stress-adaptive pathways. For example, ERBB4 is more highly expressed in metastatic Ewing sarcoma cell lines and contributes to metastasis by activating the phosphoinositide 3-kinase (PI3K)–AKT and FAK pathways[87], which cooperate to increase survival of tumour cells detached from the ECM (to suppress anoikis); ERBB4 also increases local tumour invasive capacity[87]. The class III deacetylase SIRT1 (NAD-dependent protein deacetylase sirtuin 1), a metabolic sensor, is more highly expressed in Ewing sarcoma metastases than in primary tumours[104]. Differential transcriptomic analyses between pairs of primary tumours and metastases have highlighted inactive tyrosine-protein kinase transmembrane receptor ROR1 and its putative Wnt family member 5 A (WNT5A) ligand, which is known to increase cell motility, as candidate regulators of cell migration in Ewing sarcoma[105].

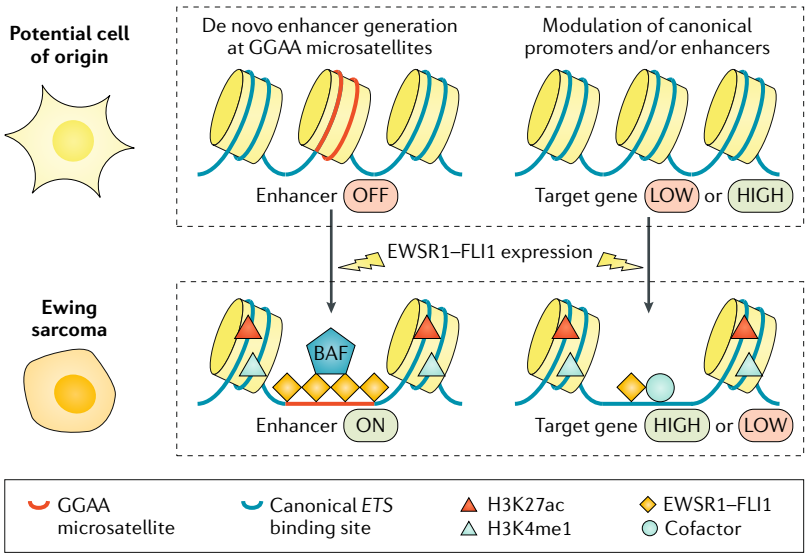

Fig. 3 | **EWSR1–FLI1-mediated epigenetic remodelling of regulatory elements.** The fusion transcription factor Ewing sarcoma breakpoint region 1 protein (EWSR1)–Friend leukaemia integration 1 transcription factor (FLI1) binds to GGAA microsatellites and acts as a pioneer transcription factor. EWSR1–FLI1 recruits the BRG1–BRM-associated factor (BAF) chromatin-remodelling complex, which drives chromatin remodelling and establishes de novo enhancers at these GGAA repetitive elements. Active enhancers are enriched in H3K27ac and H3K4me1. EWSR1–FLI1 also changes the epigenetic state of gene regulatory elements, such as promoters and putative enhancers, possibly in cooperation with cofactors such as transcription factor E2F[218], activator protein 1 (AP-1) complex[62] and TEF transcription factors (TEAD)[68]. 'HIGH' and 'LOW' refer to gene expression levels.

Activation of the pro-migratory protein RAC1 via FGF2 signalling by the bone microenvironment favours invasiveness and metastasis in Ewing sarcoma[99].

Bone-marrow-derived MSCs may also promote Ewing sarcoma metastasis by migrating to the lungs and differentiating into endothelial cells and pericytes to promote tumour blood vessel formation within metastases[106]. Ewing sarcoma cells, when stressed by growth factor deprivation or hypoxia, upregulate CXCR4, leading tumour cells to migrate towards stroma-produced CXCR4 ligand stromal cell-derived factor 1 (SDF1) at distant sites to mediate metastasis[84]. In hypoxic conditions, YB1 directly enhances translation of mRNAs encoding HIF1α to promote metastasis in Ewing sarcoma, potentially by enhancing angiogenesis in these tumours[91,92]. In addition, hypoxia modulates EWSR1–FLI1 transcriptional signatures, leading to HIF1α induction[86]; one of these EWSR1–FLI1 targets may be Dickkopf-related protein 2 (DKK2)[107], which is also able to induce HIF1α[107] and is reported to participate in metastatic spread of Ewing sarcoma. Additional transcripts or proteins regulated by EWSR1–FLI1 (such as *CAV1* (REF.[108]), *EZH2* (REF.[109]), *GPR64* (also known as *ADGRG2* (REF.[110])), *NPY*[85], *TRIP6* (REF.[111]) and *CD99* (REFS[31,32])) also influence migration, invasion and/or metastatic colonization in Ewing sarcoma. Similarly, increases in reactive oxygen species and an oxidative stress phenotype have been linked to expression of the metalloreductase STEAP1 (a transcriptional target of EWSR1–FLI1), increasing Ewing sarcoma invasiveness[112].

Although considered a mesenchymal tumour, a process resembling an epithelial-to-mesenchymal transition has been reported to occur in Ewing sarcoma and could be a critical mechanism driving metastasis[113]. In this model, cells with high expression of EWSR1–FLI1 (EWSR1–FLI1$^{[high]}$) represent the major population of Ewing sarcoma and account for its proliferation phenotype. These cells also display high cell–cell adhesion propensity (FIG. 4). However, rare EWSR1–FLI1$^{[low]}$ cells can acquire a mesenchymal-like phenotype and metastasize[114–116]. Indeed, EWSR1–FLI1$^{[low]}$ cells switch towards a cell–matrix adhesion phenotype, which correlates with higher migration, invasion and metastasis[115]. Heterogeneity in EWSR1–FLI1 fusion protein levels may be either stochastic or driven by molecular pathways that are as yet unidentified. For example, an EWSR1–FLI1$^{[low]}$ phenotype may be linked to antagonist effects on cytoskeleton regulation through direct competition for chromatin occupation between EWSR1–FLI1 and MKL/myocardin-like protein 2 (MKL2; also known as MRTFB), a Rho–F-actin pathway transcriptional effector[68]. The adhesion plaque protein zyxin, α5 integrin and the transcriptional inhibitor zinc-finger E-box-binding homeobox 2 (ZEB2) also have roles in this cytoskeleton transition process[114,117]. Activation of WNT/β-catenin and derepression of *TNC* (a gene that is typically repressed by EWSR1–FLI1 and encodes an established promoter of metastasis) also promote the transition towards a metastatic, EWSR1–FLI1$^{[low]}$ cell state[116].

Diagnosis, screening and prevention

Patients with Ewing sarcoma can present with localized disease or clinically overt metastases. The majority of patients present with a history of locoregional pain, which may be intermittent, sometimes nocturnal and worsens over time. Pain is often mistaken for 'bone growth' or injuries resulting from sports or daily life activities (such as tendinitis, muscle pain, muscle injuries or osteomyelitis). In a substantial number of patients, pain is followed by a palpable soft-tissue mass, which may be indiscernible for a long time in patients with pelvic, chest wall or femoral tumours. Pain without an adequate event to explain the symptoms and pain lasting >1 month should prompt further investigation[118].

As initially nonspecific 'B symptoms' (such as moderate fever, night sweats and loss of appetite) are mostly absent except in advanced stages or metastatic disease, diagnosis of Ewing sarcoma can be delayed; the median time to diagnosis is 3–9 months[119]. However, time to diagnosis is not associated with outcome in Ewing sarcoma[120]. As Ewing sarcoma usually arises in the diaphysis of virtually any bone or in soft tissues, symptoms vary depending on the affected site. Additionally, tumour sites vary with age; an analysis focusing on children and AYA patients with bone Ewing sarcoma[121] showed that older AYA patients (20–24 years of age) had more pelvic and axial primary tumours, larger tumours and worse outcomes than children (0–9 years of age)[121]. In addition, Ewing sarcomas in older patients tend to occur more frequently in soft tissues[122].

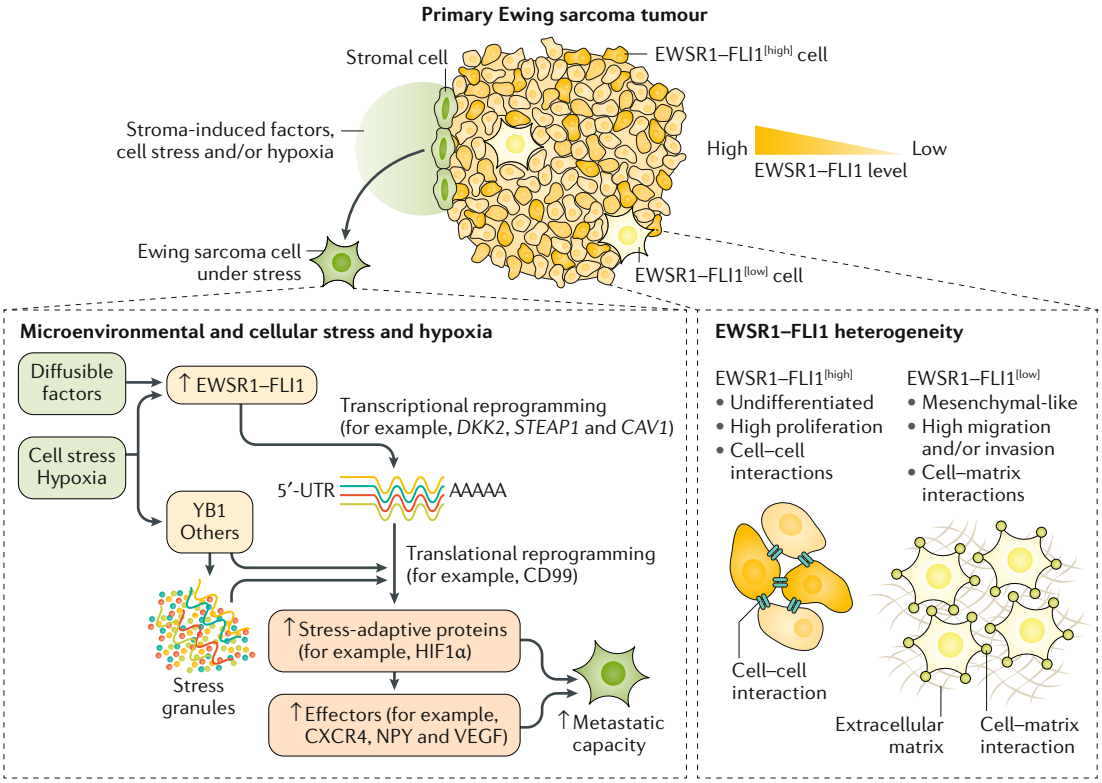

Fig. 4 | **Mechanisms of metastasis in Ewing sarcoma.** Metastasis can occur through various complementary and/or concomitant processes in Ewing sarcoma. Stochastic events might account for the presence of rare metastasis-prone cells with low expression of Ewing sarcoma breakpoint region 1 protein (EWSR1)–Friend leukaemia integration 1 transcription factor (FLI1) (EWSR1–FLI1[low]). Diffusible factors from the microenvironment, such as fibroblast growth factor 2, may also control EWSR1–FLI1 levels in Ewing sarcoma[219]. In addition, harsh conditions and hypoxia within the tumour microenvironment can lead to cell stress in Ewing sarcoma, which induces the synthesis of stress-adaptive proteins via two processes. First, primary Ewing sarcoma cells alter EWSR1–ETS protein levels to favour transcription and expression of EWSR1–FLI1 targets such as *DKK2*, *STEAP1*, *CAV1* and CD99. Alternatively, primary tumours increase expression of RNA-binding proteins such as nuclease-sensitive element-binding protein 1 (YB1)[92], which either directly enhances translation of stress-adaptive messages, such as hypoxia-inducible factor 1α (HIF1α), or indirectly enhances translation of stress-adaptive messages through stress granule (dense aggregations in the cytosol composed of proteins and RNAs) formation and translational reprogramming. Both of these pathways result in enhanced synthesis of stress-adaptive proteins, potentially leading to cells with increased metastatic capacity. CXCR4, CXC-chemokine receptor 4; NPY, neuropeptide Y; UTR, untranslated region; VEGF, vascular endothelial growth factor.

Diagnostic work-up

The diagnostic work-up of someone suspected of having Ewing sarcoma should include the medical history, with a focus on the onset of symptoms. Furthermore, a brief family history is indicated as Ewing sarcoma is associated with very rare germline mutations and variants in cancer predisposition genes (FIG. 2) — namely, in *TP53*, *RET* and *PMS2* (REF.[123]). A careful physical examination of the patient, including inspection and palpation of the tumour and organ function tests, should be performed to check the eligibility for systemic treatment (BOX 3).

Diagnostic laboratory testing. Currently, no blood or urine markers are available for the routine diagnostics of Ewing sarcoma. Nonspecific markers of bone involvement such as elevated alkaline phosphatase may be detected. High lactate dehydrogenase (LDH) is usually correlated with tumour burden and has been shown to be associated with inferior outcome[118].

Radiographic imaging and metastatic evaluation. An effective evaluation for the staging and treatment of patients with Ewing sarcoma relies mainly on the correct identification of the primary tumour extension and the accurate detection of metastatic disease. Tumour imaging and metastatic evaluation include an initial radiological evaluation, successive CT of the lungs and bone scintigraphy for the detection of metastases[124]. On radiographs, Ewing sarcoma shows tumour-related osteolysis as a destructive mass arising from the diaphyseal–metaphyseal bone with a multilayered appearance (‘onion skin’) (FIG. 5a,b). Additional CT scans enable the detection of small lesions in the lungs (FIG. 5c) and the assessment of bone destruction and periosteal involvement[125].

MRI provides higher definition images to evaluate the extent of the disease and is used if the tumour does not arise in the bone (FIG. 5d). Additionally, bone scintigraphy and PET alone or in combination with CT could be used to evaluate the presence of metastasis and/or the response to treatment[126] (FIG. 5c,e). Metabolism-based

Box 2 | **Interaction of Ewing sarcoma cells with the immune system**

In the context of the microenvironment, interactions with immune cells have only recently been appreciated as potential exogenous mediators of tumour cell behaviour, including plasticity. In this arena, increased $CD8^+$ T cell infiltration correlates with reduced Ewing sarcoma progression[210], suggesting a role for adaptive antitumour immunity to prevent tumour progression. However, this finding has been disputed[170] and, histologically, Ewing sarcoma tumours are typically depleted of immune and other inflammatory cell infiltrates[5]; these tumours are considered as 'immune deserts' or 'cold'. Whether Ewing sarcoma tumours actively suppress inflammatory cell infiltration to mediate immune escape, or whether other mechanisms of immune privilege are invoked in this disease, remains poorly understood.

Ewing sarcoma typically shows low human leukocyte antigen (HLA) expression, particularly in metastatic lesions[164], suggesting a link between low HLA levels and immune escape. However, T cells bearing human allorestricted transgenic T cell receptors are a promising immunotherapy in Ewing sarcoma[168]. An attractive — but not mutually exclusive — possibility to explain immune evasion is that altered cytokine and chemokine profiles influence the immune response (or lack thereof) in Ewing sarcoma, such as through the actions of specific stromal cells[211]. Indeed, $CD68^+$ tumour-associated macrophage (TAM) infiltration has been correlated with poor outcome in Ewing sarcoma, possibly via increased angiogenesis[212]. Whether TAMs contribute to altered cytokine profiles remains unknown. However, increased IL-6 expression in the tumour stroma correlates with metastatic progression in Ewing sarcoma; IL-6 and other yet-to-be-described key cytokines might be generated by local stromal cells[101]. Whatever the mechanism, immune evasion might also explain why Ewing sarcomas generally lack high expression of immune checkpoint molecules, such as programmed cell death protein 1 (PD1) and programmed cell death 1 ligand 1 (PDL1)[170,171].

methods, such as ^{18}F-fluorodeoxyglucose (FDG) PET–CT, which measures the uptake of FDG by the lesion, can be used to determine tumour regression or progression before the identification of morphological alterations by anatomical imaging methods such as CT and MRI[127]. Finally, it has recently been shown that FDG PET–CT can assess the presence of metastases in the bone marrow[128] in addition to bone marrow biopsies. Indeed, two recent US studies suggested that invasive bone marrow aspiration can be omitted in standard-risk patients with Ewing sarcoma if there is no evidence of bone marrow involvement on FDG PET–CT[129,130].

Pathology. Ewing sarcoma belongs histologically to the heterogeneous group of small round cell sarcomas, which are morphologically very similar to each other. However, recent advances in molecular pathology have helped to decipher Ewing sarcoma with *FET–ETS* fusions from morphological mimics, which are often referred to as 'Ewing-like sarcomas' (BOX 1). In fact, some small round cell sarcomas that were previously considered as 'histological variants' of Ewing sarcoma later proved to be genetically and clinically distinct entities and include *CIC*-fused and *BCOR*-rearranged sarcomas[7,131] (TABLE 2).

The definitive diagnosis of Ewing sarcoma should be made (or reviewed) at a sarcoma reference centre by biopsy, providing sufficient material for conventional histology, immunohistochemistry, molecular pathology and biobanking[132]. Gross examination of untreated Ewing sarcoma specimens is now uncommon because induction chemotherapy is now standard (see below, Management). Nevertheless, the cut surface is grey-white, soft and frequently includes areas of haemorrhage and necrosis. Histologically, conventional Ewing sarcoma has a solid pattern of growth and is composed of monomorphic small cells with round nuclei[5] (FIG. 6a,b). The chromatin is finely stippled, and nucleoli are usually not observable. Usually, extensive deposits of glycogen are observed in the cytoplasm; periodic acid–Schiff staining is positive in half of tumours, especially in well-fixed specimens. Reticulin stains are negative because Ewing sarcoma lacks matrix among tumour cells. A 'large cell', or 'atypical', variant of Ewing sarcoma has been reported[133]; the main difference of these cells from conventional Ewing sarcoma are larger-size nuclei with irregular contours. Conspicuous nucleoli can be seen and periodic acid–Schiff stains are usually negative in this variant (FIG. 6c).

In Ewing sarcomas showing more neural differentiation (that is, peripheral primitive neuroectodermal tumour), the tumour cells cluster in ill-defined groups of up to ten cells that orient towards a central space (FIG. 6d). They show higher expression of neuron-specific enolase and neuroectodermal markers such as Leu-7 (CD57) than conventional Ewing sarcoma[5]. After induction chemotherapy, Ewing sarcoma cells show a variable degree of necrosis and are replaced by loose connective tissue[5]. Histopathological assessment of tumour necrosis after therapy correlates with overall survival. The prognostically relevant cut-off value is 10% residual viable tumour cells[1].

CD99 is a cell-surface glycoprotein and a relevant diagnostic marker for Ewing sarcoma[134]. Strong, diffuse membranous expression of CD99 is evident by immunohistochemistry in ~95% of Ewing sarcomas[5] (FIG. 6e). However, CD99 expression is not specific for Ewing sarcoma and occurs in a large group of normal tissues and tumour types, including other round cell sarcomas and lymphoblastic lymphoma and leukaemia. Immunohistochemical detection of FLI1 is more specific for Ewing sarcoma than is CD99. However, the specificity of FLI1 is limited by its expression in lymphoblastic leukaemias and lymphomas and several soft-tissue sarcomas; its sensitivity is reduced by the occurrence of variant translocations not involving *FLI1* (REF.[135]). Caveolin 1 expression has been shown to be useful to diagnose Ewing sarcomas negative for CD99 expression[136]. Similarly, expression of homeobox protein NKX-2.2, another protein related to the pathogenesis of Ewing sarcoma, is specific for this entity[137]. Lastly, innovative genomics-based combinations of immunohistochemical markers such as B cell lymphoma/leukaemia 11B (BCL-11B) and Golgi apparatus protein 1 (GLG1) have been described as being highly specific for Ewing sarcoma but require validation in prospective studies[131].

Currently, a diagnosis of Ewing sarcoma can be confirmed only by molecular pathology, which is mandatory when cases have unusual clinical and pathological features. FISH-based detection of *EWSR1* rearrangements (FIG. 6f) and/or reverse transcription PCR (RT-PCR), detection of *FET–ETS* gene fusions specific for Ewing sarcoma have been used for the past 25 years as a diagnostic tool[138]. A reference laboratory for small round cell tumour diagnosis should offer FISH analysis and/or RT-PCR[139]. Several commercial

Box 3 | **Diagnostic procedures for Ewing sarcoma**

- Medical history
- Physical examination
- Laboratory tests should include complete blood count, blood serum chemistry (including lactate dehydrogenase (LDH) level), erythrocyte sedimentation rate, coagulation test, pregnancy test (if applicable) and virology (according to national or institutional guidelines)
- Imaging should include MRI of the full bone or compartment of the suspected primary tumour, CT or ^{18}F-fluorodeoxyglucose (FDG) PET–CT of the lungs, whole-body imaging (FDG PET, FDG PET–CT or FDG PET–MRI) and echography for evaluation of organ function
- In patients with diagnosed pulmonary metastases, pulmonary function tests
- Pathology should include molecular pathology (detection of *FET–ETS* fusions and rearrangements) by fluorescence in situ hybridization (FISH) and/or reverse transcription PCR (RT-PCR), (targeted) RNA sequencing for rare gene fusions in some patients, immunohistochemistry (staining for CD99), haematoxylin and eosin staining and periodic acid–Schiff staining

sources for *EWSR1* break-apart probes are available; however, assays using *EWSR1* break-apart probes do not detect *EWSR1–ETS* fusions per se. Rather, the assays detect *EWSR1* gene rearrangements, which are important for differential diagnosis with other sarcoma subtypes that harbour *EWSR1* fusions with genes other than *ETS* (for example, desmoplastic small round cell tumours). The use of next-generation sequencing (NGS) is advisable for small round cell sarcomas in which FISH and/or RT-PCR cannot confirm the Ewing sarcoma diagnosis. In molecular pathology laboratories in which molecular confirmation of Ewing sarcoma is mainly based on FISH analysis, RT-PCR and NGS help to avoid the potential pitfall of relying only on FISH-based assays to detect *EWSR1*-containing fusions (Ewing sarcomas with *EWSR1–ERG* fusions are in many cases negative for *EWSR1* rearrangement detection by FISH)[140].

Staging and risk classification

The clinical stage at diagnosis is one of the major predictors of survival. The accurate determination of tumour burden at diagnosis is a critical factor in planning treatment and predicting outcome (BOX 4). Imaging studies are the central tool for detection of metastases (FIG. 5).

Ewing sarcoma predominantly spreads via the bloodstream. The most common metastatic sites are the lungs, bones or bone marrow, whereas other sites are rare. For the detection of bone marrow metastases, occurring in ~10% of patients, bone marrow aspirates and trephine biopsies from sites that are tumour negative by imaging are typically performed[141,142]. However, modern imaging techniques such as FDG PET–CT scans have the potential to replace invasive bone marrow aspirates and trephine biopsies[129,130].

Tumour volume (TV) is measured by sectional imaging using the following formula: $TV = a \times b \times c \times F$, where a, b and c represent the maximum tumour dimensions in three planes, with $F = \pi/6 = 0.52$ for spherical tumours or $F = \pi/4 = 0.785$ for cylindrical tumours. Large tumours >200 ml in volume have been associated with worse outcome[2].

Screening and prevention

Ewing sarcoma is a very rare disease; robust screening markers for estimating the risk of Ewing sarcoma development are presently not available. In addition, serum markers that would enable screening approaches are not available. Except for one Australian national case–control study, which found an association of Ewing sarcoma with farm exposure[24], no precise exogenous noxae have been identified that would enable a systematic prevention of Ewing sarcoma. Early detection of Ewing sarcoma can be accelerated by creating awareness among physicians to include Ewing sarcoma in the differential diagnosis of pain that is otherwise unexplained, nonspecific and persistent in young people.

Management

The management of patients with Ewing sarcoma requires a multidisciplinary team that includes paediatric, medical and radiation oncologists; orthopaedic and general surgeons; and nurses. Patients with newly diagnosed localized standard-risk Ewing sarcoma can expect a survival of 70–80%. However, this high survival emerges from intensified cytotoxic drug regimens, which are associated with late effects. Furthermore, primary disseminated disease and relapse are associated with extremely poor outcomes and novel treatment strategies are urgently required, which is why Ewing sarcoma is frequently included in multi-histology phase I and phase II sarcoma trials. Global variations in the diagnosis and management of Ewing sarcoma are summarized in TABLE 3.

Types of treatment

Induction chemotherapy. Patients with newly diagnosed Ewing sarcoma are treated with a combination of multi-agent cytotoxic chemotherapy and local control measures (surgery and/or radiotherapy). Induction chemotherapy is given before local treatment to reduce the size of the primary tumour and address micrometastatic disease because micrometastatic disease is expected in all patients.

Cooperative group clinical trials have demonstrated that multidrug treatment and treatment intensity are important factors of therapy and that intensity of chemotherapy is important for outcome. Modern protocols consist of intense induction chemotherapy with vinca alkaloids, alkylating agents and anthracyclines. For example, the EE99 trial used an intensive multidrug induction chemotherapy regimen containing vincristine, ifosfamide, doxorubicin and etoposide (VIDE); this intense regimen was tolerated by the patients at intervals of 21–28 days[143]. Typically, long consolidation chemotherapy is an important element in the treatment of Ewing sarcoma to destroy

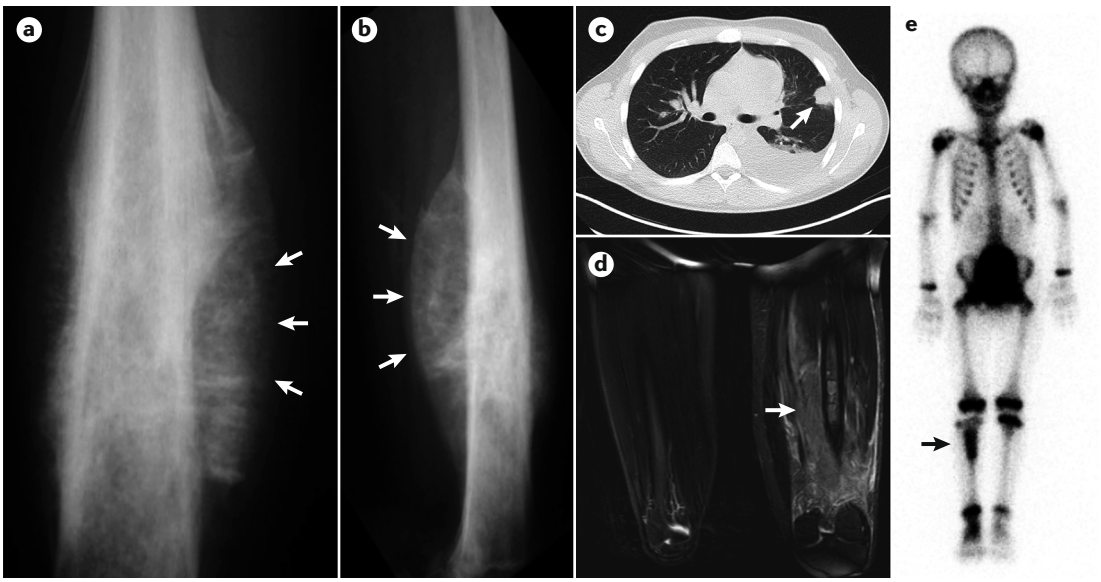

Fig. 5 | **Radiological presentation of Ewing sarcoma.** Anteroposterior (panel **a**) and lateral (panel **b**) X-ray images showing an osteolytic lesion of a Ewing sarcoma and involvement of the periosteal soft tissue (arrows) in the right femur of a 14-year-old boy. CT scan of the lungs showing a pulmonary metastasis (arrow) in a 19-year-old male patient (panel **c**). MRI scan (T2-weighted) showing the primary tumour in the right femur with accompanying soft-tissue oedema (arrow) of the patient shown in panel **a** (panel **d**). Bone scintigraphy of a 6-year-old male patient highlighting a Ewing sarcoma tumour mass comprising his right tibia (arrow; panel **e**).

slowly proliferating remaining tumour cells. The EE99 trial showed that standard-risk patients (BOX 4) do not differ in outcome if cyclophosphamide-based or ifosfamide-based consolidation chemotherapy is used[70]. High-risk localized patients (localized disease, poor histological response and large tumours) in the EE99 and EWING 2008 trials benefited more from high-dose busulfan and melphalan chemotherapy followed by transplantation of autologous haematopoietic stem cells (event-free survival (EFS) at 3 years of 67%) than from an eight-cycle standard dose of vincristine, actinomycin D and ifosfamide consolidation chemotherapy (3-year EFS 53%)[144]. Accordingly, high-dose busulfan and melphalan chemotherapy has been chosen as the high-dose regimen of choice on the basis of international transplant registry data[145], and results are supported by similar findings of the Italian and Scandinavian sarcoma groups[146].

The US Children's Oncology Group (COG) assessed the value of dose intensity by comparing 3-weekly (so-called uncompressed regimen) versus 2-weekly (compressed) regimens of vincristine, doxorubicin and cyclophosphamide alternating with ifosfamide and etoposide. The 5-year EFS was significantly better in patients who received the compressed regimen (73% versus 65%)[147]. A randomized comparison of the intense VIDE induction regimen and this compressed regimen is currently being performed in Europe[148] (ISRCTN92192408). International trials are also evaluating the value of an add-on treatment with zoledronic acid, an inhibitor of both osteolysis and tumour cell proliferation and invasion, in patients diagnosed with localized disease[148]. A recent US trial, the results of which are pending, investigated the combination of vincristine, topotecan and cyclophosphamide for non-metastatic disease given their high level of activity in the relapse setting[2].

Metastatic disease. Patients with metastatic Ewing sarcoma are treated either following regimens utilized in the care of patients with localized disease[149] or on randomized clinical trials seeking to improve outcomes for this group of patients. Early studies in patients with Ewing sarcoma and pulmonary metastases by the European Inter-Group Cooperative Ewing studies (EICESS-92) showed that whole lung irradiation (WLI) could improve a 5-year EFS compared with no WLI (49% versus 36%)[150]. In parallel, other studies have demonstrated a possible benefit from high-dose busulfan and melphalan chemotherapy and autologous stem cell transplantation (SCT) for metastatic disease[151]. Indeed, a collaborative European and US trial compared treatment with standard chemotherapy of vincristine, dactinomycin and ifosfamide plus WLI and high-dose busulfan and melphalan chemotherapy in a randomized fashion. The trial demonstrated no difference between the two therapeutic approaches, with 3-year EFS of 51% and 55%, respectively[151].

The COG is currently investigating the addition of monoclonal antibodies against the IGFI receptor (IGF1R) to dose-compressed chemotherapy (NCT02306161). An international study also investigated the value of adding high-dose chemotherapy using treosulfan and melphalan to VIDE and a regimen of vincristine, dactinomycin and cyclophosphamide chemotherapy[152]. Finally, a Brazilian group has showed that addition of platinum-containing chemotherapy to a regimen of vincristine, doxorubicin and cyclophosphamide alternating with ifosfamide and etoposide is of no value in patients with high-risk (BOX 4) Ewing sarcoma[153].

Table 2 | **Comparison of typical *FET–ETS*-driven Ewing sarcoma with 'Ewing-like' sarcomas**

Fusion type[a]	*n*	Age distribution (%)[b]	Sex ratio (male:female)	Tumour location (%)	5-year survival (%)	Morphology	Immunohistochemistry	Refs
FET–ETS	>1,000	• Children (35) • Adolescents (40) • Adults (25)	1.5:1	• Bone (88) • Soft tissue (10) • Visceral (2)	70–80[c]	Monomorphic small cells with round nuclei	• Strong and diffuse staining membrane expression of CD99 in >95% of tumours. • Positive staining for NKX2-2 in 100% of cases and caveolin 1 in 96% of cases • High BCL-11B and/or GLG1 expression has a specificity for Ewing sarcoma of >96%	19,131,136,137
CIC fused	139	• Children (8) • Adolescents (16) • Adults (76)	1.2:1	• Bone (3) • Soft tissue (87) • Visceral (10)	43	Myxoid stromal change, cell spindling, multifocal nuclear atypia and clear-cell cytology	• 90% of tumours express WT1 and 100% express ETV4 • CD99 expression is strong and diffuse in 23% of cases and focal in 61% of cases	206,207
BCOR rearranged	49	• Children (35) • Adolescents (41) • Adults (24)	5.1:1	• Bone (64) • Soft tissue (32) • Visceral (4)	72	Round to spindle-shaped cells and occasional myxoid stroma	• Positive staining for BCoR, TLE1 and cyclin D1 • Positive staining for CCND3 (in *BCOR–CCND3* tumours) • Focal staining for CD99 (42% of tumours)	208
EWSR1–NFATC2	13	• Children (0) • Adolescents (33) • Adults (77)	12:1	• Bone (83) • Soft tissue (17) • Visceral (0)	Insufficient data	Lymphocyte infiltration, scattered nuclear atypia and a nesting pattern of growth	Strong staining for CD99 (100% of tumours)	209,252

BCoR, BCL-6 co-repressor; BCL-11B, B cell lymphoma/leukaemia 11B; CCND3, G1/S-specific cyclin D3; ETV4, ETS translocation variant 4; GLG1, Golgi apparatus protein 1; TLE1, transducing-like enhancer protein 1; WT1, Wilms tumour protein. [a]Ewing-like sarcomas with *FET*, non-*ETS* gene fusions (for example, *EWSR1–SMARCA5*, *EWSR1–PATZ1* and *EWSR1–SP3*) are excluded owing to scant information available to date. [b]Children are considered 0–13 years of age, adolescents 14–18 years of age and adults >18 years of age. [c]Five-year survival may be lower for patients presenting with metastatic disease, pelvic tumours, large tumours and/or incomplete tumour regression after neoadjuvant or adjuvant chemotherapy.

Local control. Local control in Ewing sarcoma comprises surgery and/or radiotherapy. Surgery may consist of resection without reconstruction — for instance, if reconstruction is not feasible (amputation) or not necessary (for example, tumours in the fibula or scapula). Tumour resection with reconstruction can be performed using an endoprosthesis (an implant), allogeneic or autologous bone graft or rotationplasty (in which the remaining limb below the resected portion is rotated and reattached). In a combined modality setting, radiotherapy may be applied before or after surgery.

As no randomized prospective studies on the reconstruction method are available yet, the optimal mode of local control is debated. Indeed, treatment choice may depend on tumour location and volume as well as on the specialization of the given medical centre. However, two independent retrospective studies from serial cooperative group trials showed that patients who received only radiotherapy had inferior outcomes compared with patients who received surgery[154,155]. Nevertheless, both studies showed that the group of patients selected for definitive radiotherapy was enriched for adverse prognostic factors, such as pelvic primary site and larger tumour volume, which may bias the findings[154,155]. Another study by the COG demonstrated a significantly higher risk of local failure in patients treated with radiotherapy alone than in patients treated with surgery, but EFS and overall survival were similar[154].

The European cooperative trial group assessed the effect of postoperative radiotherapy using multivariate models on local control in 599 patients with localized Ewing sarcoma and good histological response to chemotherapy (>90%). The risk of local recurrence was statistically significantly lower in patients treated by surgery and radiotherapy than in patients treated by surgery alone in all studied subgroups, which included origin, site, size and histological response of the tumours as well as patients' age and sex. The benefit of postoperative radiotherapy was highly statistically significant for tumours >200 ml in volume at diagnosis and that had 100% tumour necrosis of the tumour after induction chemotherapy. However, no significant difference in survival among the subgroups was observed[156].

Owing to the specific features of pelvic anatomy, local treatment is often challenging. A European study retrospectively investigated factors associated with local recurrence and survival in localized Ewing sarcoma of the pelvis, prospectively registered in the aforementioned EE99 trial[143]. Here, large tumour volume (>200 ml) was not identified as an adverse risk factor, but poor histological response to induction chemotherapy was a major

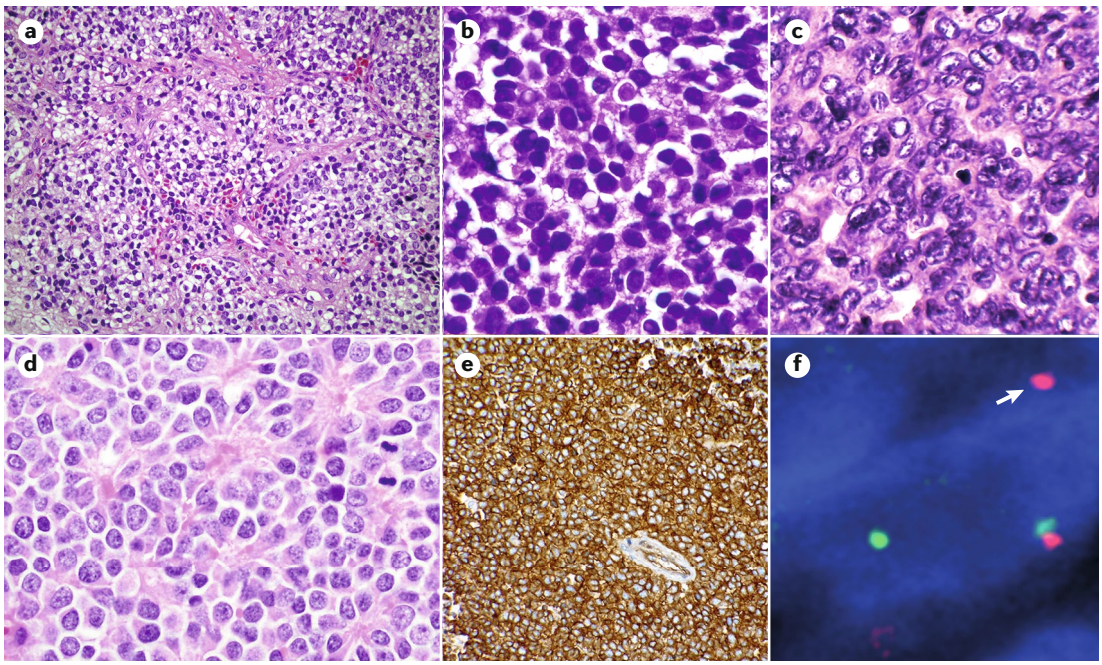

Fig. 6 | **Histomorphology of Ewing sarcoma.** Conventional Ewing sarcoma shows a diffuse and vaguely lobular appearance at low-power magnification (panel **a**, magnification ×10) with a fairly monotonous arrangement of small round cells (panel **b**, magnification ×40). Cases with atypical morphology have larger and darker nuclei (panel **c**, magnification ×40) than conventional cases. A rosette-like arrangement of tumour cells (panel **d**) provides evidence of neural differentiation in a minority of cases (magnification ×40). Panels **a**–**d** are haemotoxylin and eosin stained sections. Biopsy specimen of a 16-year-old boy showing diffuse and intense plasma membrane expression of CD99 by immunohistochemical staining (panel **e**, magnification ×10) and *EWSR1* gene rearrangements by fluorescence in situ hybridization (arrow) with a break-apart *EWSR1* probe (panel **f**, magnification ×600).

risk factor. Definitive radiotherapy (that is, radiotherapy as the only local treatment) in sacral tumours was comparable to combined surgery and radiotherapy in terms of local relapse and overall survival, whereas the combined local treatment in non-sacral tumours was associated with an improved local relapse and overall survival compared with surgery alone[157]. Indeed, an overall survival benefit was observed even in the subgroup of patients with wide surgical margins and a good histological tumour response to induction treatment. Thus, combined modality local treatment is recommended in non-sacral Ewing sarcoma of the pelvis.

Another main site of Ewing sarcoma is the thoracic wall, where local treatment may be challenging owing to the proximity to vital organs. Several retrospective studies have shown that the surgical outcome is better in patients who received induction chemotherapy[158], and additional radiotherapy does not consequently improve survival[159]. Benefit from postoperative radiotherapy was observed in patients with marginal or intralesional resections[159]. Thus, patients with narrow margins should receive postoperative radiotherapy.

Myeloablative therapy and SCT. High-dose myeloablative chemotherapy, which destroys bone marrow cells including tumour cells, is used to intensify the chemotherapy in patients with (very) high-risk Ewing sarcoma (metastatic disease, refractory disease or at relapse). This procedure is normally followed by autologous haematopoietic SCT to reconstitute the bone marrow. An early study of high-dose chemotherapy and total body irradiation in patients with newly diagnosed disseminated Ewing sarcoma was conducted by a cooperative trial group in the United States. The 2-year EFS was 20% for the entire cohort and 24% for patients who received myeloablative therapy[160]. Additionally, a European study retrospectively investigated the role of total body irradiation in two groups of patients. One group was treated with high-dose chemotherapy plus total body irradiation, and the second group was treated with tandem high-dose chemotherapy. The 5-year EFS was similar in both groups (22% versus 29%)[161]. Another European study included patients with metastatic disease involving lung, pleura or single-bone metastasis. All patients were treated with multimodal chemotherapy, local control and high-dose chemotherapy. The 5-year EFS for the full cohort was 43% in this more favourable group of patients[146]. The EE99 trial included a nonrandomized arm for patients with extrapulmonary metastatic disease in which all patients were planned to undergo high-dose chemotherapy. Of the 281 patients, 60% underwent high-dose chemotherapy; the 3-year EFS was 27%[142]. The study identified age >14 years, large primary tumours and more than one metastatic site as risk factors for poor outcome[142]. In patients with relapsed Ewing sarcoma, high-dose chemotherapy may be of some benefit if the patients respond well to a relapse chemotherapy regimen[162].

To date, no randomized study has reported on the value of high-dose chemotherapy followed by autologous haematopoietic SCT in Ewing sarcoma, although

Box 4 | **Risk groups in Ewing sarcoma**

Staging of malignant disease in Ewing sarcoma is of general importance as the stage at diagnosis is one of the major predictors of survival. With the advent of structured clinical trials for the treatment of Ewing sarcoma, prognostic factors are used as stratification criteria with consecutive adjustment of treatment intensity[213], including metastases, histological response to chemotherapy, tumour volume and age at diagnosis[119].

Standard risk

- Patients with localized disease, small tumours and good histological response (<10% vital tumour cells)
- Patients with small tumours <200 ml in whom histological response cannot be assessed
- In European trials, histological response to induction chemotherapy has been reported as a significant biomarker in the group of patients with localized disease[70,214–216]

High-risk localized

- Unfavourable histological response
- More than 10% vital tumour cells
- Patients with large tumours (≥200 ml) in whom histological response cannot be assessed are also stratified in the high-risk group in European trials[70,144,217]
- The North American study groups do not substratify patients with localized disease[147]

Very high-risk metastatic

- Disseminated disease
- Patients with lung metastases have been reported to have a better outcome than patients with other metastases[142]

cooperative trial groups have investigated this approach in patients with primary disseminated Ewing sarcoma. In the international EWING 2008 trial (NCT00987636), patients with primary disseminated disease are participating in a randomized comparison between ongoing conventional chemotherapy or ongoing conventional maintenance (standard-dose) chemotherapy plus high-dose chemotherapy with autologous stem cell rescue (TABLE 3). It should be noted that SCT is neither being tested in North America nor is it standard of care[2].

Some approaches tried to evaluate a possible graft versus Ewing sarcoma effect. In these approaches, allogeneic SCT was performed after high-dose chemotherapy. A retrospective analysis of 87 patients registered in three international registries for patients with haematopoietic SCT who were transplanted from human leukocyte antigen (HLA)-matched or haploidentical donors showed that high-intensity conditioning was more likely than reduced-intensity conditioning to lead to death of complications. Allogeneic SCT did not improve survival, with a 5-year overall survival of only 10–15%[163]. Additional total body irradiation has also been shown to be of no benefit with allogeneic or haploidentical SCT, leading to an even more unfavourable outcome[161].

Immunotherapy and targeted therapy. Given the weak immune invasion of Ewing sarcoma (BOX 2), investigation of immunotherapy for Ewing sarcoma lags behind other solid tumours. However, $CD8^+$ T cell infiltration correlates with better outcome[164] and some putative targets have been identified, such as GD2, which is expressed occasionally in Ewing sarcoma[165] (but more frequently in neuroblastoma and osteosarcoma[166,167]). Studies with anti-GD2 antibodies or chimeric antigen receptor (CAR) T cells are currently being performed in paediatric malignancies (NCT03356782 and NCT02159443). A pilot study used HLA-A*02:01/chondromodulin 1 peptide-specific allorestricted $CD8^+$ T cells, showing a response in one of three treated patients[168].

In another study, 'FANG' immunotherapy used autologous tumour cells as a source of neoantigen presentation. In that study, the cells were transfected with granulocyte–macrophage colony-stimulating factor (GM-CSF) to recruit dendritic cells, and furin-mediated TGFβ1 and TGFβ2 expression was blocked with short hairpin RNA[169]. A phase I trial of this approach is currently active (NCT01061840). Finally, programmed cell death protein 1 (PD1) and programmed cell death 1 ligand 1 (PDL1) are expressed only in a fraction of Ewing sarcoma cells[170]. Thus, checkpoint inhibitors may not evoke sufficient immune response and would have to be complemented with further strategies[171].

The EWSR1–FLI1 fusion protein itself is currently not druggable because it lacks any enzymatic activity. An indirect approach to targeting this transcription factor would comprise disruption of its transcriptional complex. RNA helicase A (RHA) is a crucial part of the transcriptional complex and blockage of its binding to EWSR1–FLI1 by a small-molecule inhibitor, YK-4-279, has been shown to effectively reduce proliferation of Ewing sarcoma cells[172]. An analogue of YK-4-279 is currently under investigation in a first-in-human phase I clinical trial (NCT02657005).

Relapsed Ewing sarcoma

Ewing sarcoma relapse is associated with very poor outcomes; patients who relapse within 24 months after diagnosis have a 5-year survival of <10%[3]. Favourable prognostic factors at relapse are local relapse, younger age, isolated pulmonary recurrence and low LDH levels[173]. Additionally, a structured follow-up imaging protocol in which patients undergo routine imaging may enable a longer overall survival in recurring patients[174].

A potential source of relapse may be a highly resistant clone of tumour cells that either existed a priori or developed under anticancer treatment. Investigations to identify and characterize such clones are actively being pursued. Few clinical trials on relapsed Ewing sarcoma have been completed (TABLE 4). The vast majority of the value of different therapeutic approaches in relapsed Ewing sarcoma is drawn from retrospective analyses[162,175]. The first large investigator-initiated clinical trial in relapsed Ewing sarcoma was initiated in the European consortium and compares standard chemotherapy regimens in terms of survival, safety and quality of life[176].

Special disease complications

Patients with Ewing sarcoma may present with pathological fracture or develop pathological fractures during treatment (14% of patients). Fractures in patients with Ewing sarcoma are the result of bone destruction and are more likely to occur in those with larger tumours (≥200 ml or ≥8 cm in the largest dimension). The main site for a pathological fracture is the femur, followed by the tibia (>50%). Despite these complications, survival in patients with pathological fractures is similar to those

Table 3 | **Global variation in diagnosis and management of Ewing sarcoma**

Feature	Country or region				
	Australia and New Zealand	Europe	Japan	North America	South America
Mandatory molecular diagnosis	Yes	Yes	NA	No	No
Stratification by LDH level	NA	No	No	No	Yes
Stratification by metastases	Yes	Yes[a]	No	No	No
Stratification by tumour size	Yes	Yes[a]	No	No	No
Stratification by tumour site	NA	No	No	NA	Yes
Stratification by histological response	Yes	Yes[a]	No	No	No
General recommendation of local treatment	NA	NA	NA	NA	NA
Surgery preferred[c]	Yes	Yes	Yes	Yes	Yes
Radiotherapy preferred[c]	No	No	No	Yes (pelvis)[253]	No
Surgery and radiotherapy preferred[c]	Yes	Yes	Yes	Yes	Yes
High-dose chemotherapy in high-risk localized disease recommended	Yes	Yes[2]	NA	No[2]	NA
High-dose chemotherapy in metastatic disease recommended	NA	• No[b] (REFS[2,152,254]) • Yes (France)[255]	NA	No[2]	No[153]
Randomized clinical trials being conducted	Yes[152]	Yes[149,153]	No	Yes[256]	Yes[153]

LDH, lactate dehydrogenase; NA, not applicable or no information available. Data from REFS[2,152] (Australia and New Zealand), REFS[1,2,149,152] (Europe), REFS[13,49,160,257] (North America) and REFS[153,190,258] (South America). [a]Not in the EWING 2012 protocol[149]. [b]Randomized in the EWING 2008 protocol. [c]The preferred local treatment modality is dependent on various factors, including site and size of the tumour, stage of disease or the patient's choice. In some patients, the preferred local treatment is not feasible or would not produce meaningful results; these patients may receive radiotherapy only.

without[177]. Approximately 6% of patients are diagnosed with Ewing sarcoma of the spine; thus, spinal cord compression may occur and warrants immediate decompression in most of the patients to prevent long-term neurological sequelae. In patients without severe neurological symptoms, systemic treatment plus steroids may be used to reduce tumour burden. Decompression at diagnosis positively affects the outcome in patients with Ewing sarcoma of the spine[178].

Quality of life

Patients with Ewing sarcoma are at risk of substantial disease-related and treatment-induced acute and long-term or late toxicity (BOX 5). Although prospective studies on late effects with a large and well-documented cohort of patients are still lacking, the chemotherapy agents commonly used in the management of Ewing sarcoma are known to be associated with a number of late effects in survivors of other cancer types. Chemotherapy regimens widely used in the treatment regimen of Ewing sarcoma mainly rely on anthracyclines, alkylating agents and etoposide. Cardiomyopathy, renal insufficiency, renal Fanconi syndrome and reduced fertility have been described[179].

Furthermore, patients are at risk of secondary malignancies due to chemotherapy and/or radiotherapy[141]. Secondary malignancies occur in ~9% of Ewing sarcoma survivors[179,180]. Etoposide-containing chemotherapy and high-dose chemotherapy were identified as risk factors for secondary malignancies[141]. As expected, radiotherapy is associated with an important risk of developing secondary cancers, mainly comprising osteosarcoma, acute myeloid leukaemia, breast cancer and thyroid cancer[180]. A recently published report from the Childhood Cancer Survivor Study (CCSS) described chronic health impairment in 70% of the patients 35 years after diagnosis of Ewing sarcoma[179]. The British CCSS reported that survivors have a 12.7 higher death rate than the general population, mainly owing to second malignancies during 25 years of follow-up, which argues for lifelong follow-up[181].

All local treatment modalities put Ewing sarcoma survivors at risk of treatment-related late sequelae in the form of neuromusculoskeletal complications and reduced functional capacity. However, a recent study showed that — independently of the different treatment modalities — the majority of patients had active lifestyles without major limitations decades after treatment[182].

Outlook

The outlook for Ewing sarcoma relies on the establishment of adequate animal models that recapitulate the characteristics of the disease and a deeper understanding of the genetic basis and epigenetic alterations that are essential for Ewing sarcoma initiation and progression. New detection methods should also be implemented, including biomarkers and liquid biopsy, and targeted therapeutic approaches should be developed.

Disease models

A large number of Ewing sarcoma cell lines have been described[15,16,18]. Some of these cell lines were used to perform xenograft experiments in immunodeficient mice[183] or zebrafish embryos[104,115] to investigate biological mechanisms and test drug efficacy. Indeed, as the microenvironment is critical in understanding Ewing sarcoma, intrafemoral or paraosseous orthotopic xenograft models can address these aspects[184,185]. In addition, allografts

Table 4 | **Treatment protocols for relapsed Ewing sarcoma and outcomes**

Treatment	n	Outcomes	Comments	Refs
Robatumumab (SCH 717454)	116	2-year overall survival: 9%	• Six patients with unresectable Ewing sarcoma stayed in remission for > 4 years • Long-term anti-IGF1R administration was well tolerated	204
Figitumumab	106	• Partial response: 14.2% • Median overall survival time: 8.9 months	Circulating IGFI levels positively correlated with outcomes	259
R1507	109	• Complete or partial response: 10% • Median overall survival time: 7.6 months	Bone tumour, general performance and high IGFI levels at start of treatment and at week 6 correlated with better outcomes	257
FANG autologous immunotherapy	12	• Partial response: 8% • 1-year overall survival: 75%	• Well tolerated • A tumour-specific systemic immune response was observed in all patients • Phase II trial completed and phase III trial initiated	260
Dendritic cell vaccination[a]	43[b] (24 of whom had late recurrence)	5-year overall survival: 63%	• Complete remission after standard relapse chemotherapy was a prerequisite for a long-lasting response or survival after vaccination • More favourable group of patients with late relapse	261
Dendritic cell vaccinationa with or without recombinant human IL-7	19 with metastatic disease			
Ontuxizumab	4	Phase I dose finding	Well tolerated in children	262
Temsirolimus, irinotecan and temozolomide	7	Phase I dose finding	Well tolerated in children	263

IGFI, insulin-like growth factor I; IGF1R, IGFI receptor. [a]Autologous lymphocytes, tumour lysate or keyhole limpet haemocyanin-pulsed dendritic cell vaccinations. [b]Patients with Ewing sarcoma or rhabdomyosarcoma; exact number of patients with Ewing sarcoma is not available.

of embryonic osteochondrogenic progenitors or MSCs expressing EWSR1–FLI1 can give rise to tumours that feature Ewing sarcoma molecular characteristics[75,78,79]. However, a faithful genetically engineered mouse model (GEMM) is still lacking owing to a paucity of knowledge of Ewing sarcoma histogenesis and the very restricted spatial–temporal tolerance to the expression of this oncogene in development (reviewed in REF.[186]).

The EWSR1–FLI1 transgenic mouse and zebrafish models described hitherto require either concomitant p53 deficiency or forced expression of anti-apoptotic BCL-2 family members to overcome oncogene toxicity and to efficiently develop sarcomas[187–189]. These models partially recapitulate some EWSR1–FLI1 transcriptional targets. However, the shortage of Ewing sarcoma GEMMs may be due to poor cross-species conservation of GGAA microsatellites[61,62]. Orthotopic patient-derived xenograft models were reported to recapitulate genomic alterations of several types of paediatric tumour[190]. Indeed, the utility of patient-derived xenograft models as a faithful preclinical sarcoma model was recently tested in various Ewing sarcoma and other sarcoma models showing promising results[183,191]. Moreover, the Pediatric Preclinical Testing Program of the US National Cancer Institute (NCI) and the European intersectoral consortium ITCC-P4 are developing various Ewing sarcoma patient-derived xenograft models, which will be used for preclinical drug testing.

Additional expectations for Ewing sarcoma patient-derived xenograft models rely on their capability to faithfully mimic metastatic disease or to recapitulate some heterogeneity found in Ewing sarcoma (such as EWSR1–FLI1[high] or EWSR1–FLI1[low] populations or *STAG2* subclonal evolution). Whether patient-derived xenograft models will enable modelling of metastatic disease without loading cells to the bloodstream at the time of xenografting remains an important open technical question.

Epigenetic alterations

Elucidating how EWSR1–FLI1 interacts with and reprogrammes the epigenome is likely to identify new drug targets and may yield biomarkers that can improve the design of future clinical trials. For example, patients with *STAG2* mutations have worse outcome[16,18], possibly related to an enhancer signature that is close to that of pluripotent stem cells[63]. This finding opens future opportunities for the development of stratified therapies.

Genetic risk factors for Ewing sarcoma have also been identified and may have links to the epigenome. For example, it has been shown that a single-nucleotide polymorphism within an interrupted GGAA microsatellite increases the number of consecutive GGAA repeats and the activity of the corresponding Ewing-sarcoma-specific enhancer[26]. The functional validation and further characterization of the Ewing-sarcoma-specific enhancers promises to be particularly illuminating. Not all enhancers regulated by EWSR1–FLI1 are expected to be involved in Ewing sarcoma development, and identifying epigenetic drivers and actionable enhancer elements is currently a major challenge. Finally, it is necessary to study the levels and impact of both genetic and epigenetic heterogeneity

Box 5 | **Effects of Ewing sarcoma and its treatments**

Acute effects

- Pain
- Pathological bone fractures
- Neurological symptoms (for example, palsy, paraesthesia and spinal cord compression syndrome)
- Frequent hospitalization
- Nausea, vomiting and risk of infection
- Mucositis
- Haematological toxicity
- Veno-occlusive disease
- Electrolyte imbalance
- Growth delay
- Social isolation
- Risk of toxic death

Late effects

- Impaired growth and poor weight gain
- Delayed or impaired puberty, infertility and/or premature menopause
- Cardiac toxicity
- Endocrine dysfunction
- Changes in skin structure or colour
- Weakening of the hair structure
- Chronic diarrhoea
- Pulmonary fibrosis
- Ongoing neurological impairment
- Scoliosis
- Dental abnormalities
- Benign neoplasms
- Secondary malignant neoplasm
- Ongoing risk of late Ewing sarcoma relapse
- Mutilation and handicaps
- Chronic kidney disease (that is, renal Fanconi syndrome)

in Ewing sarcoma, both across patients and over time within the same patient.

Biomarkers and liquid biopsies

Serial monitoring of tumour characteristics during and after treatment is particularly relevant in Ewing sarcoma because of its predominant localization at notoriously difficult-to-approach sites for biopsy. Novel techniques such as the detection of circulating tumour cells or targeted and whole-exome sequencing of exosomal or cell-free DNA will enable comprehensive serial profiling of tumours from blood. Liquid biopsies may constitute a simple clinical tool to faithfully recapitulate the individual tumour biology while guiding therapeutic decisions on the basis of a patient's risk profile. Indeed, detection of the informative genomic fusion sequence from a minimal amount of the patient's DNA has been established and can be used as a qualitative and quantitative parameter for Ewing sarcoma monitoring[192,193]. Additionally, detection of circulating tumour cells based on RT-PCR of EWSR1–FLI1 or EWSR1–ERG has been established and correlates with worse outcomes[194]. Also, vesicles with exosomal features have been identified containing Ewing-sarcoma-specific transcripts[93,94,195] that could potentially be used for diagnosis and disease monitoring via peripheral blood[93,195]. In addition, an innovative approach to detect $CD99^+$ Ewing sarcoma cells by flow cytometry in peripheral blood has been reported[196]. However, careful prospective validation is required before these techniques are to be used in clinical practice. Several international efforts, such as that of the PROVABES consortium, are ongoing to validate potential prognostic biomarkers in Ewing sarcoma, including STEAP1, CXCR4, EZH2, galectin 3 binding protein or PARP1 (REF.[197]).

Management and associated toxicity

Development of novel treatments for Ewing sarcoma is slow owing to the rarity of the disease. Patients with Ewing sarcoma have been included in clinical trials, but despite the conceptual promise, the effects of most of these strategies are quite modest[198]. However, a search for innovative targeted therapeutics is ongoing and includes small-molecule inhibitors, such as YK-4-279 (REFS[172,199]), which blocks protein–protein interactions with EWSR1–FLI1 or its downstream main targets. Moreover, several studies are focused on targeting the epigenetic deregulation present in Ewing sarcoma using inhibitors of lysine-specific histone demethylase 1A (LSD1; also known as KDM1A)[200], histone deacetylase (HDAC)[201] and DNA methyltransferases (such as 5-aza-2′-deoxycytidine)[202].

Additionally, PARP inhibitors have been preclinically explored as enhancers of drug sensitivity in combination with agents such as trabectedin[191] and/or temozolomide[203]. Moreover, although there is strong in vitro and in vivo evidence on the importance of the IGF1R pathway, clinical trials using IGF1R-blocking antibodies have proved not to be fully effective thus far[204]. Finally, new immunotherapeutic approaches using modified peptide-specific T cells against EWSR1–FLI1 yielded promising results in a preclinical model[205]. Collectively, these different approaches, although still under development, have the potential to advance Ewing sarcoma management with lower treatment-related toxicity than current strategies.

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