

Review

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Molecular genetics of Ewing sarcoma, model systems and finding novel (immuno-) therapeutic targets

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How to cite this article: van der Ent W, Sand LGL, Hogendoorn PCW. Molecular genetics of Ewing sarcoma, model systems and finding novel (immuno-) therapeutic targets. *J Transl Genet Genom* 2018;2:10. <https://doi.org/10.20517/jtgg.2018.09>

Received: 11 May 2018 **First Decision:** 15 May 2018 **Revised:** 20 May 2018 **Accepted:** 21 May 2018 **Published:** 16 Jul 2018

Science Editor: David N. Cooper **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Ewing sarcoma (EWS) is a bone- and soft tissue tumour affecting primarily children and young adults. A quarter of patients present with metastases at the time of diagnosis and have a poor outlook in terms of overall survival. Efforts are made across the field to gain deeper insight in the genetics of this enigmatic neoplasm. EWS is characterized by presence of an oncogenic translocation gene, *EWSR1-ETS*. In addition, there are a limited number of known recurrent DNA copy number variations and mutations. Subsequent of the above, the epigenetic profile of EWS is subject of interest. In this review, we summarize the current available knowledge on the genetics underpinning EWS, explore the current knowledge of its epigenetic profile, discuss *in vitro* and *in vivo* model systems, and explore the unravelling knowledge of potential targets for treatment including recent insights into potential immunotherapy.

Keywords: Bone neoplasm, Ewing sarcoma, genetic translocation, DNA copy number variation, mutation

INTRODUCTION

Ewing sarcoma (EWS) is the second most common bone- and soft tissue sarcoma affecting children and young adults. It presents microscopically as a tumour consisting of rather uniform small blue round cells expressing CD99 on its cell membrane, usually with glycogen deposition in its cytoplasm, and is characterized by an *EWSR1-ETS* translocation. Peak incidence is between 10 and 20 years old, with a slight majority of male patients (1.4:1 ratio)^[1]. EWS was first described by James Ewing in 1921 as a “diffuse endothelioma of bone”^[2], however, the cell of origin of EWS has long been a subject of debate. Based on ultrastructural



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features and biomarkers, in the past it has been ascribed to endothelial, reticuloendothelial, hematopoietic, neural crest and mesenchymal cells^[3-5]. Currently, the view is that EWS arises from a progenitor or stem cell derived from neural crest or mesenchymal cells. The main sites of involvement are the long bones, pelvis and ribs (~85% of all cases), and lower extremities and the paravertebral region in cases of extra skeletal involvement (~15%)^[1,6].

Overall survival rates of EWS patients with localised disease have starkly improved since the introduction of systemic therapy to the treatment regime^[7]. Patients with localised disease currently have an overall event free survival of 60%-70%^[8]. In a quarter of patients, metastatic disease is observed at the time of diagnosis. These patients have an unfavourable prognosis, with overall event free survival of 20%-30%^[9-12]. Patients who solely have lung-metastases seem to fare somewhat better than patients with metastases in bone or bone-marrow^[13,14].

Standard protocol for treatment of EWS upon diagnosis is chemotherapy followed by surgical resection and/or radiotherapy^[15,16]. In Europe, the chemotherapeutic regime consists of multiple cycles of vincristine, ifosfamide, doxorubicin and etoposide^[16,17].

GENETIC ALTERATIONS

Chromosomal translocations

In 85% of all EWS cases, there is a reciprocal t(11;22)(q24;q12) translocation, merging ES Breakpoint region 1 (*EWSR1*) to Friend leukaemia virus integration site 1 (*FLI1*)^[18]. The fusion of *EWSR1* with *ERG*, t(21;22)(q11;q12), makes up for another 10% of EWS cases^[19,20]. Both *FLI1* and *ERG* are members of the erythroblast transformation-specific (ETS) transcription factor family and share a conserved DNA binding domain structure. Fusion of the N-terminal region of *EWSR1*, which contains a strong transcriptional activation domain^[21,22], and these DNA binding domains causes aberrant transcription of a multitude of genes. Additionally, *EWSR1*-ETS is known to affect epigenetic programs^[23,24], splicing^[25,26], and metabolic activity^[27] of EWS. Oncogenic fusions between *ETS* genes and transcriptional activators are not unique to EWS: in 50%-70% of prostate cancers, similar chromosomal rearrangements are found^[28].

The breakpoint position varies, and commonly occurs between exons 7-11 for *EWSR1* and exons 4-9 for *FLI1*, leading to variations in fusion types between patients with *EWSR1-FLI1*. The most common type is a fusion between *EWSR1* exons 1-7 and *FLI1* exons 6-9, a type 1 fusion, or *EWSR1* exons 1-7 and *FLI1* exons 5-9, a type 2 fusion. The effect of these variations in the oncogene on its function has been debated. While some studies observe differences in malignancy between fusion types^[29,30], a European prospective trial found no prognostic significance between them^[31]. Different fusion types were shown to have a different dependence on various proteins of the splicing machinery, so inhibition of splicing factors may have therapeutic value in some cases of EWS^[32].

As the only consistent genetic alteration, among the very few found in EWS in general, *EWSR1-FLI1* has been the main player in the attempts to develop *in vitro* and *in vivo* models for the disease. However, development of transgenic mouse-models is hampered by toxicity of *EWSR1-FLI1* to most cells^[33]. Additional genetic variations may be needed to create a permissive environment, implied by the bias of disease occurrence in Caucasians^[1]. A recent GWAS identified 3 EWS-associated single nucleotide polymorphisms (SNPs), near *EGR2*, *BMF* and *TARDBP* genes^[34]. At the locus near *EGR2*, the causative SNP links two GGAA repeat stretches^[35]. In addition to classic ETS transcription factor binding sites, *EWSR1-FLI1* preferentially binds to such stretches of GGAA repeats, where its activity increases with the increase of amount of repeats^[36,37].

In addition to the more common *EWSR1-FLI1* and *EWSR1-ERG* translocation events, other fusions between members of the TET family of proteins (including *EWSR1* and *FUS*) and members of the ETS transcription

Table 1. Rare chromosomal rearrangements found in Ewing sarcoma

Rearrangement types	Fusion gene	Chromosomal rearrangement	References
Rearrangement between TET and ETS family of genes	<i>EWSR1-ETV1</i>	t(7;22)(p22;q12)	Jeon <i>et al.</i> ^[40]
	<i>EWSR1-ETV4</i>	t(17;22)(q21;q12)	Urano <i>et al.</i> ^[41]
	<i>EWSR1-FEV</i>	t(2;22)(q35;q12)	Peter <i>et al.</i> ^[42]
	<i>FUS-ERG</i>	t(16;21)(p11;q22)	Shing <i>et al.</i> ^[43]
	<i>FUS-FEV</i>	t(2;16)(q35;p11)	Ng <i>et al.</i> ^[44]
Rearrangement between TET and non-ETS family of genes	<i>EWSR1-NFATC2</i>	t(20;22)(q13;q12)	Szuhai <i>et al.</i> ^[45]
	<i>EWSR1-POU5F1</i>	t(20;22)(p21;q12)	Yamaguchi <i>et al.</i> ^[46]
	<i>EWSR1-SMARCA5</i>	t(4;22)(q31;q12)	Sumegi <i>et al.</i> ^[47]
	<i>EWSR1-PATZ</i>	t(1;22)(p36.1;q12)[with inv(22)]	Mastrangelo <i>et al.</i> ^[48]
	<i>EWSR1-SP3</i>	t(2;22)(q31;q12)	Wang <i>et al.</i> ^[49]
Rearrangement between non-TET and non-ETS family of genes	<i>CIC-DUX4</i>	t(4;19)(q35;q13)	Kawamura-Saito <i>et al.</i> ^[38]
	<i>CIC-FOXO4</i>	t(X;19)(q13;q13.3)	Brohl <i>et al.</i> ^[50]
			Sugita <i>et al.</i> ^[51]
	<i>BCOR-CCNB3</i>	inv(X)(p11.4p11.22)	Pierron <i>et al.</i> ^[39]
	<i>BCOR-MAML3</i>	t(x;4)(p11.4;q31.1)	Specht <i>et al.</i> ^[52]
	<i>ZC3H7B-BCOR</i>		Specht <i>et al.</i> ^[52]
	<i>CRTC1-SS18</i>		Alholle <i>et al.</i> ^[53]

factor family have been identified. In some cases, tumours with histological, radiological and clinical features are identified without rearrangement between TET members and ETS members. These “Ewing-like” tumours do bear other chromosomal translocations, such as the *CIC-DUX4* or *BCOR-CCNB3*^[38,39]. An overview of these rarer types of translocation found in EWS is given in Table 1^[38-53].

Copy number alterations

Besides the characteristic *EWSR1-ETS* translocation, chromosomal copy number alterations have been described with various implications for clinical outcome. Trisomy of chromosome 8 occurs in about 35%-45% of all cases, gain of chromosome 12 in 25%-33%^[54-57]; these gains can occur together or separately, and are more likely to be found in relapses than in primary tumours. Chromosome 8 gain confers no significant prognostic value, while gain of chromosome 12 has been reported to associate with adverse effects in patients with localised disease^[58]. Gain of chromosome 2 is primarily found in localised tumours and may indicate a positive prognosis^[59].

An unbalanced der(16)t(1;16) translocation leading to partial tri- or tetrasomy of 1q and partial monosomy of 16 is found in 10%-30% of cases^[54,57,60,61]. Gain of 1q was repeatedly correlated to an adverse clinical prognosis. Overexpression of cell division cycle protein 2 (*CDT2*), encoding a protein involved in the ubiquitin ligase activity and DNA damage repair, is suggested to underlie this aggressive phenotype^[62].

After 16q, loss of 9p21 is most frequently observed, and results in a poor clinical outcome^[63]. A reason for this may be the loss of cyclin-dependent kinase Inhibitor 2A (*CDKN2A*), present at this locus.

Mutations

Studies investigating the genomic landscape of EWS highlight the paucity of recurrent somatic mutations in this cancer^[50,64,65]. Mutation or downregulation of *CDKN2A* is reported in 10%-30% of EWS cases^[66-70]. Encoding P16^{INK4A}, loss of *CDKN2A* has been shown to correlate with a poor prognosis^[67,68,71], though a later study has stated that it is not a reliable prognostic marker for localised EWS^[72]. *TP53* has also been shown to lead to a poor prognosis, although these mutations are infrequent (less than 15% of cases)^[73], and also a non-reliable prognostic marker for localised disease^[72].

Inactivating mutations in *STAG2* are reported in 9%-21% of EWS cases^[74,75], appear to be mutually exclusive with *CDKN2A*, while co-associating with *TP53* mutations and poor patient prognosis^[64]. *STAG2* is an oncogene recurrently mutated in various cancer types^[76]. As a member of the cohesion complex, it helps in

holding chromosome pairs together during cell division, and in the context of cancer also affects genome organization and transcriptional regulation^[77]. *STAG2* is functionally redundant with *STAG1*, and depletion of *STAG1* resulted in lethality in EWS cells with mutated *STAG2*, but not in cells with wildtype *STAG2*, making it an interesting target for therapeutics^[78].

Epigenetics

Despite the quite homogenous genetic make-up of EWS, disease course and presentation varies significantly between patients. This may be attributed to a heterogeneity in the epigenetic profile of EWS tumours. A recent study characterized epigenetic heterogeneity of EWS compared to other tumour types, between different EWS cases, and within individual tumours^[79]. Based on genome-wide methylation profiles, EWS can be separated from other cancer types and normal tissue. Between patients, no epigenetically defines subtypes are found: samples fall along a mid-to-high range of variation for methylation. Intra-tumour heterogeneity was is also high and varying.

The epigenetic profile of EWS has been directly linked to the activity of EWSR1-FLI1^[24,80]. EWSR1-FLI1 preferentially binds to sites with GGAA repeats, and classical ETS binding sites. At GGAA repeat sites, EWSR1-FLI1 acts as an enhancer and causes opening of the chromatin, while at ETS sites it displaces ETS transcription factors, deactivating their enhancer activity.

Efforts are being made to determine epigenetic changes specific to EWS, and the consequences they may have on disease development and progression^[81-83]. The methylation of *NPTX2* or *PHF11* has been linked to a less favourable prognosis^[82]. Another study showed that hypermethylation of *PTRF* leads to suppression of this tumour suppressor gene, and that reintroduction could lead to p53 activation and subsequent apoptosis^[83].

MODEL SYSTEMS

The relatively simple genetic make-up of EWS makes it an appealing tumour for which to develop a transgenic animal model. However, despite many attempts made, no research group as of yet has been successful^[33]. Introduction of the EWS oncogene *EWSR1-FLI1* in mice has led to embryonic lethality, or development of non-EWS-resembling cancer^[84,85]. For a comprehensive overview of the efforts made by six independent laboratories to generate a murine model for EWS, we refer to the 2016 publication by Minas *et al.*^[33]. In addition to murine models, development of zebrafish transgenic models for EWS has also been attempted. The transient expression of *EWSR1-FLI1* in zebrafish embryos led to mitotic defect in the developing embryo^[86]. A stable transgenic zebrafish line with expression of *EWSR1-FLI1* developed solid tumours with histologic features of small round blue cell tumours in a *TP53*^{-/-} background, but the model simultaneously gave rise to malignant peripheral nerve sheet tumours. Another zebrafish model showed that loss of one or both wildtype alleles of the zebrafish *EWSR1* orthologue *ewsa* led to an increase in tumour formation in a *TP53*^{-/-} background, suggesting that *EWSR1* deficiency may also contribute to the malignancy of *EWSR1*-translocation driven tumours^[87]. Given the lack of transgenic animal models, the EWS research community looks for different models in which to test potential novel therapeutics for the disease.

In vitro models

In order to test novel therapeutics, classic 2D culture is widely used. Though rapid and convenient, the contribution of the tumour microenvironment cannot be assessed in these assays, while this could alter drug response significantly. Cells undergo biomechanical stimulation from shear stress due to blood flow, or mechanical forces from surrounding muscle contractility or body impact, and alter their behaviour in response^[88,89]. EWS cells are no different, and several models have been reported taking this into account^[90-92]. Shear stress was applied to EWS cells by growing them on 3D scaffolds within a flow perfusion bioreactor. This resulted in an increased production of IGF1, and a shear-stress dependant alteration in response to IGF1-R inhibitors^[90]. Continued development of this model involves the addition of mesenchymal stem

cells (MSCs) to the 3D culture, to investigate cross talk between stromal cues and those from biomechanical forces. EWS and MSCs co-cultured in flow perfusion bioreactors stimulated each other's growth and led to altered responses to various inhibitors^[91]. Another model evaluates the effect of mechanical loading by culturing cells in 3D scaffolds and subjecting these to cycles of compression^[89]. This led to an upregulation of *RUNX2*, linked to drug resistance and poor prognosis when highly expressed in patients.

***In vivo* models**

To accurately model tumours in a 3D fashion, and assess the effect of the tumour microenvironment on drug response, good animal engraftment models are essential. Earliest xenotransplantation models were injected either subcutaneously or intravenously in nude or *NOD/scid* mice^[93-95]. Later murine models sought to more closely resemble the native tumour environment by performing orthotopic xenografts in rib bones^[96], femur^[97], pretibial space^[98], and gastrocnemius muscles^[99]. Not only new compounds are tested but also novel drug delivery methods, such as silk gel to effectuate a sustained release of chemotherapeutics locally^[100]. Ultrasound visualization is being used for more accurate tissue implantation, as well as identifying early response to treatment^[101,102].

In addition to murine models, several groups have also used zebrafish embryonic xenograft models to test novel therapeutics^[95,103-105]. Here, cells are injected in the yolk of the embryo, show intra- and extravasation and migration into muscles and fins within 4 days after implantation, making these models interesting for rapid initial screening of a large number of possible therapeutic compounds.

A recent development is the establishment of patient-derived xenograft (PDX) models. A limitation of established cell-lines is that they may have deviated from the original disease during years of culture. With PDX models, pieces of resected tumour are directly implanted in mice, where they will remain until the tumour has reached a size where they need to be resected again and implanted in new mice. This way, the artificial environment of *in vitro* culture can be avoided. Being a rare tumour, the development of EWS and Ewing-like PDX models is slow, but several models have been established already^[106,107].

THERAPEUTIC OPPORTUNITIES

A limited number of direct targets can be identified from genetics of EWS, with EWSR1-ETS being the most prominent one. Targeting the fusion protein directly has proven to be challenging due to its flexibility, but progress has been made and a phase I clinical trial (NCT02657005) is ongoing with a small molecule inhibitor^[108]. Aside from targeting the EWSR1-ETS fusion protein directly, inhibiting its dominant direct targets has been investigated as therapeutic alternative, as EWSR1-ETS is a key driver in the epigenetics^[23,24], transcription^[109], splicing^[25,26], and metabolic^[27] reprogramming of EWSR1-ETS.

Epigenetic remodelling inhibitors, including HDAC inhibitors and LSD1^[23], with success tested in the pre-clinical phase, are being tested in clinical trials. EWSR1-ETS influences the transcription at such a level that the cells become very sensitive to genotoxic agents, causing EWS to act like a BRCA1 deficient tumour with impaired homologous recombination, as shown in a recent study^[109]. The impaired homologous recombination opens therapeutic opportunities for PARP1 inhibitors, and these were effective in the induction of cell death in *in vitro* and xenograft models. If this translates into the clinic is currently being investigated, as clinical trials with combinations of genotoxic agents and PARP1 inhibitors are ongoing^[110,111]. Recently a study demonstrated the dysregulation of EWSR1-ETS at protein level and subsequent induction of a high unfolded protein response (UPR). Inhibiting an important protein in this pathway, IRE1 α -XBP1 led to reduced cell viability *in vitro* and *in vivo*.

IMMUNOTHERAPY IN EWS

The low mutation rate of EWS suggest that corrective apoptosis pathways, such as the TNF-related apoptosis

inducing ligand (TRAIL) pathway and the death receptor pathway, are still active^[112-117]. As a result, the tumour is sensitive to natural killer (NK) and cytotoxic T-cell driven activation of these pathways, leading to cell death. It has been shown that the presence of higher numbers of cytotoxic T-cells in the tumour micro-environment correlated with better overall survival^[118].

Considering the sensitivity to NK and cytotoxic T cells, immunotherapy might be a promising therapeutic intervention for EWS. NK clinical trials have started using immunotherapeutic strategies in the treatment of EWS^[119-122]. They involve treatment with donor NK cells after allogeneic haematopoietic cell transplantation, or after receiving lymphodepleting chemotherapy and IL2, in some cases with a follow-up NK treatment 35 days after haematopoietic cell transplantation. *In vitro* results show that histone deacetylase inhibitors increase expressions of NKG2D ligands on EWS cells, which can sensitize them for cytolysis via NK cells^[123].

Using primed or T cell receptor (TCR)-engineered T cells is a second approach for which EWSR1-ETS would be a very selective target. However, preclinical studies haven't been as successful *in vivo* as NK immunotherapy, or lacked further follow up^[124-127]. A limitation of this therapy is the dependency on MHC class I surface expression, which is downregulated by EWS cells^[128].

Chimeric antigen receptor (CAR) T cells or NK cells can act independent of MHC class proteins and can selectively be designed for all surface proteins, making it an interesting third approach. In leukaemia and lymphomas, CAR-T cells were very successful in the clinic^[129,130]. The main target investigated in EWS is the glycolipid GD₂, which is expressed in most EWS tumours at varying levels and against which CAR-T cells have been developed^[131]. Preclinical studies targeting GD₂ demonstrated a reduction in tumour volume and a clinical trial with the latest CAR-T cells has been initiated^[132]. As this therapy can be designed for every surface expressed antigen one can imagine that other targets might be worth investigating such as the recently identified surface protein LINGO1^[133].

A fourth approach of immunotherapy being investigated for clinical use are cancer vaccines. Higher numbers of CCL21-producing cells at the tumour site was correlated with an improved chemotherapy response and overall survival: this might imply that attraction of dendritic cells and CCR7-positive T cells by a cancer vaccine or a dendritic cell-based cancer vaccine would help to induce long term protection. In addition, cancer vaccines can change the suppressive immune environment by inducing a pro-inflammatory immune response or inhibiting production of immune inhibitory proteins, such as transforming growth factor β 1 (TGF- β 1)^[134-137]. The presence of a suppressive immune environment in EWS tumours is illustrated by low the number of infiltrating immune cells, and T cell infiltration induced upregulation of HLA-G, an immune inhibitory receptor^[138]. In addition, in xenografts a high number of myeloid-derived suppressor cells were detected in EWS tumours, leading to a reduction in CAR-T cell activity^[139]. A phase IIb with a TGF- β 1 inhibiting cancer vaccine is ongoing, but further insight in the immunosuppressive microenvironment of EWS would be beneficial to design an immunotherapy which homes to the tumour site and has long-term tumour specific cytotoxic activity.

CONCLUDING REMARKS

EWS has a fairly stable genome with a low number of somatic mutations. Aside from the characteristic EWSR1-ETS translocation, only a limited number of recurrent mutations and copy number alterations are found. Deeper insight into the underlying biology of the disease is slow to be obtained, due to its rarity. However, owing to various collaborative efforts and use of state-of-the-art technologies in the field, the needed headway is being made in order to develop novel therapeutics, including potential for immunotherapeutic approaches. Current *in vitro* and *in vivo* models including zebrafish models are helpful in the process for better understanding of the molecular genetics of these tumours and maybe more importantly, can act as systems for exploring new therapeutic approaches.

DECLARATIONS

Authors' contributions

Literature research and manuscript writing: van der Ent W, Sand LGL, Hogendoorn PCW

Manuscript editing: van der Ent W, Hogendoorn PCW

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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