

# Molecular detection and targeting of EWSR1 fusion transcripts in soft tissue tumors

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**Abstract** Soft tissue tumors are a heterogeneous group of tumors, traditionally classified according to morphology and histogenesis. Molecular classification divides sarcomas into two main categories: (a) sarcomas with specific genetic alterations and (b) sarcomas showing multiple complex karyotypic abnormalities without any specific pattern. Most chromosomal alterations are represented by translocations which are increasingly detected. The identification of fusion transcripts, in fact, not only support the diagnosis but also provides the basis for the development of new therapeutic strategies aimed at blocking aberrant activity of the chimeric proteins. One of the genes most susceptible to breakage/translocation in soft tissue tumors is represented by Ewing sarcoma breakpoint region 1 (EWSR1). This gene has a large number of fusion partners, mainly associated with the pathogenesis of Ewing's sarcoma but with other soft tissue tumors too. In this review, we illustrate the characteristics of this gene/protein, both in normal cellular physiology and in carcinogenesis. We describe the different fusion partners of EWSR1, the molecular pathways in which is involved and the main molecular biology techniques for the identification of fusion transcripts and for their inhibition.

**Keywords** EWSR1 partners · Fusion transcript detection · Fusion transcript inhibition

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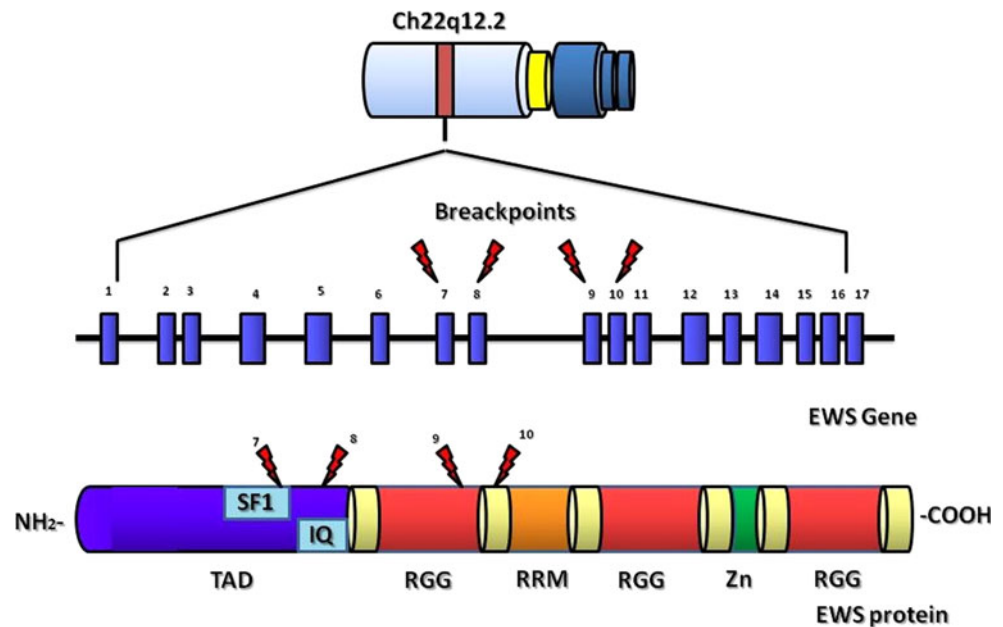
## EWS gene and protein structure

EWSR1 is located on 22q12.2 chromosome and spans about 40 kb of DNA with 17 exons. The first 7 exons encode the N-terminal domain and exons 11, 12 and 13 encode the putative RNA-binding domain. The protein consists of 656 amino acids and 68.5 kDa of size and is composed of several domains. TAD domain is a transactivation domain that contains a consensus sequence repeated SYGQ and two auto regulation regions, IQ and SF1. The domain IQ binds to calmodulin, inhibiting phosphorylation by protein kinase C (PKC) and favoring the activation of EWS. On the contrary, the binding with SF1 (splicing factor 1) inhibits the activation domain TAD, negatively regulating target genes of EWS. There are also three RGG domains rich in arginine and glycine, a domain RBD that binds single filament of RNA and DNA and a ring finger domain similar to the RAN-BP2 [1]. Regarding EWSR1 breakpoints, not all the regions composing the protein are susceptible to breakage in soft tissue tumors [2]. The areas mainly involved in translocation are EWSR1 exons 7 and 8, coding for the region SYGQ and exons 9 and 10, associated with regions protein RGG [3] (Fig. 1).

## EWS protein in normal cell physiology

EWS is a member of the TET (TIs, EWSR1, TafII) family of RNA-binding proteins, with FUS (TLS) and TAF15 (TAFII68). The codified protein is involved in transcriptional regulation for specific genes and in mRNA splicing. In particular, EWSR1 plays a role in transcription initiation. In fact, EWSR1 is able to link with the basal transcription factor TFIID and RNA polymerase II complex.

**Fig. 1** Schematic representation of EWS gene and protein domains with the main breakpoints illustrations



In normal cells, EWSR1 is phosphorylated by PRKC (protein kinase C) although its IQ domain, which inhibits RNA-binding EWSR1. EWSR1 is required for proper localization of aurora B during mitosis, also maintaining mitotic spindle integrity [4]. EWSR1 also associates with EP300 and CREBBP acting as coactivator of CREBBP-dependent transcription factors. EWSR1-EP300/CREBBP mediates FOS activation, as well as HNF4 genes activation [5, 6]. EWSR1 activates other transcription factors such as POU4F1 (or BRN3A, 13q13) [7], and POU5F1 (or OCT4, 6p21) genes which regulate differentiation of neuronal cells [7, 8]. EWSR1 and CCNL1 (cyclin L1) are also interacting partners of TFIP11 (tuftelin-interacting protein 11), a protein functionally related to the spliceosome and involved in pre-mRNA splicing [9]. In normal cell physiology, EWSR1 is required for cell survival in the central nervous system [4] and performs important functions in the regulation of genomic integrity and in RNA and micro RNA's maturation processes.

Regarding its localization, EWSR1 is ubiquity expressed [10, 11] with prevalent localization in the nucleus and more rarely in the cytoplasm and various subcellular compartments, depending on the methylation state of its RNA-binding domain [12]. Localization of EWSR1 in different subcellular compartments reflects its dynamic distribution during cell cycle [13].

### EWS translocation partners in cancer

Several gene families, mainly encoding for transcriptional regulators, can translocate with EWSR1. The chimeric proteins have an aberrant activity interfering with different

molecular pathways crucial for cell growth, differentiation and proliferation. These altered interactions are often responsible for the pathogenesis of soft tissue tumors.

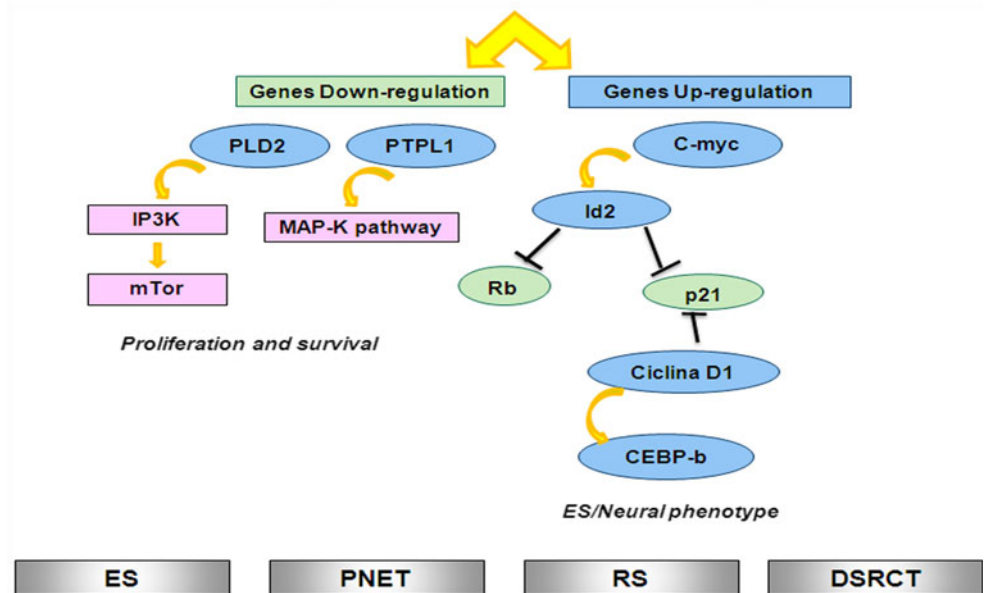
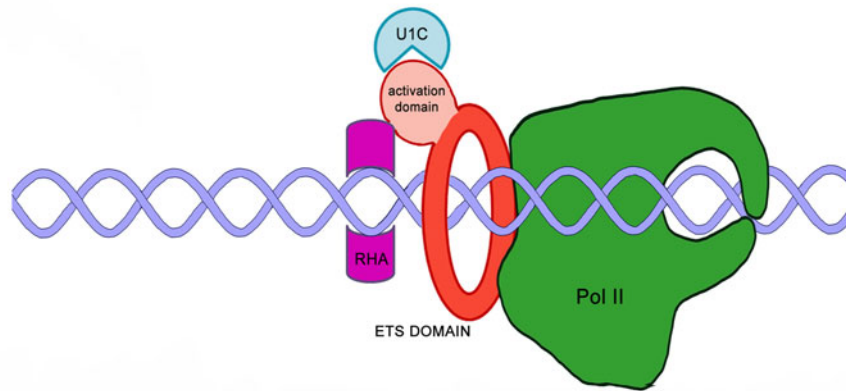
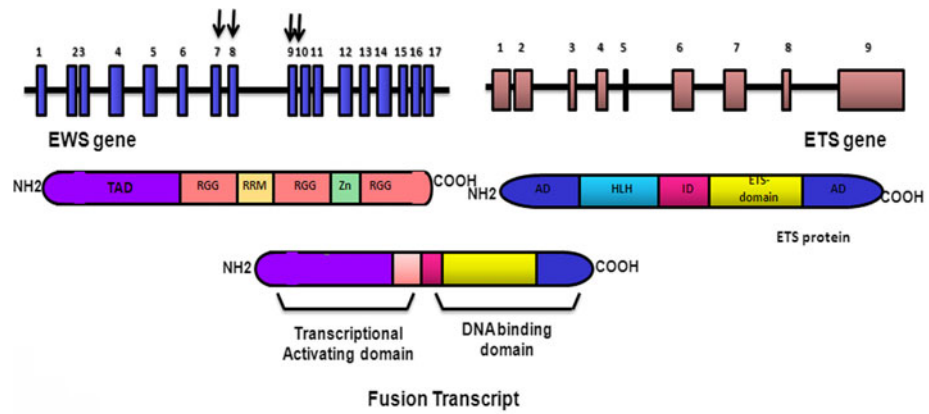
### ETS transcription factor family

The ETS (E twenty-six) family (Fig. 2) is one of the largest families of transcriptional factors involved in a wide variety of functions, including the regulation of cell differentiation, cell-cycle control, cell migration, cell proliferation, apoptosis and angiogenesis. All ETS family members are identified through a highly conserved DNA-binding domain, the ETS domain, which is a winged helix-turn-helix structure. The ETS family ETS domain is also involved in protein-protein interactions.

### *EWSR1/FLI1 (t(11;22)(q24;q12))*

Fli1 (Friend leukemia integration 1 transcription factor) was identified in the mouse genome as a viral integration site common to 2 retroviruses involved in virus-induced leukemias and lymphomas. The mouse Fli1 region is close to the centromere of chromosome 9 [14], while human FLI1 gene is located on chromosome 11q23-q24. It lies on a fragment flanked on the centromeric side, by the translocation breakpoint in acute lymphoblastic leukemia-associated t(4;11)(q21;q23) [15] and on the telomeric side in Ewing sarcoma-associated t(11;22)(q24;q12) breakpoint [16]. Several breakpoints have been described for EWSR1 and FLI1 in soft tissue tumors. The most common are EWSR1 exon 7 and FLI1 exon 6 (defined as Type I), EWSR1 exon 7 and FLI1 exon 5 (defined as Type II), EWSR1 exon 10 and FLI1 exon 6 (defined as Type III), and

**Fig. 2** Schematic representation of EWS translocation with ETS-genes family fusion partners: interaction with transcriptional complex on DNA and illustration of the main molecular pathways deregulated. *Gray squares* indicate the soft tissue tumors characterized by EWS/ETS translocations (ES: Ewing sarcoma; PNET: Primitive neuroectodermal tumor; RS: Rhabdomyosarcoma; DSRCT: Desmoplastic small-round-cell-tumor)



EWSR1 exon 7 and FLI1 exon 7 (defined as Type IV). Many other translocations have been described, such as EWSR1 exon 7 and FLI1 exon 8, EWSR1 exon 8 and FLI1 exon 6, EWSR1 exon 9 and FLI1 exon 4, EWSR1 exon 9 and FLI1 exon 7, EWSR1 exon 10 and FLI1 exon 5 and EWSR1 exon 10 and FLI1 exon 8 [3]. The fusion transcript EWSR1/FLI1, as well as many other chimeric proteins, interferes with several molecular pathways, influencing the

development and progression of this tumor. In particular, the interaction of the chimeric transcript with important mediators of cell cycle has been described. EWSR1/FLI1 results in an upregulation of c-Myc and a downregulation of p57KIP2 [17]. In addition, the fusion transcript is able to inhibit the transcriptional activity of p53 [18]. EWSR1/FLI1 also determines an upregulation of GLI, altering the molecular pathways associated with it [19]. It has been

recently demonstrated, using cellular models, that EWSR1/FLI1 is able to block the ability of Runx2 in order to induce osteoblast differentiation, responsible of Ewing tumor pathogenesis [20]. Recent studies have showed the strong association between chimeric transcripts and microRNA activity. EWS/FLI1 is able to modulate miRNA 145 controlling cell differentiation [21]. Additionally, deregulation of let7a is strongly related to EWS/FLI1 production [22]. The fusion transcript EWSR1/FLI1 is present in more than 90 % of the Ewing sarcomas, and in the related group of peripheral primitive neuroectodermal tumor (pPNET) [23], in rhabdomyosarcoma [24], in neuroblastoma [25] and in giant cell tumor of bone [26].

#### *EWS/ERG (t(21;22)(q22;q12))*

The transcription factor Erg is essential for definitive hematopoiesis and for the function of adult hematopoietic stem cells [27]. Chromosomal rearrangements involving ERG are found in acute myeloid leukemia, acute lymphoblastic leukemia, Ewing's sarcoma and more than half of all prostate cancers, but the normal physiological function of Erg is unknown. The chromosomal translocation t(21;22)(q22;q12) has been described in approximately 10 % of Ewing's sarcoma tumors. ERG shares 68 % overall amino acid identity with FLI and 98 % identity within their ETS DNA-binding domains. Considering the structural similarities of EWS/FLI and EWS/ERG fusions, it is likely that the two proteins act in order to deregulate similar target genes in Ewing's sarcoma. In fact, a retrospective study comparing EWS/ERG Ewing's sarcoma cases with EWS/FLI cases revealed no significant differences in pathological and clinical characteristics as well as overall survival. Several breakpoints have been described for EWSR1 and ERG in soft tissue tumors. The most common is EWS exon 7, which translocates to ERG exon 6, 7 and 9 [28]. Although the chimeric transcript EWSR1/ERG is able to interfere with different cellular pathways, for example, together with other EWS partners, it can suppress TGF-beta R activity [29]. Several ECM molecules are modulated by EWS/ERG, such as collagen COL11A2 [30], and laminin beta3 [31]. This fusion transcript can also be present in other peripheral primitive neuroectodermal tumor (pPNET) [32] and in desmoplastic small round cell tumor (DSRCT) [33].

#### *EWSR1/FEV t(2;22)(q33;q12); EWSR1/ETV1 t(7;22)(p22;q12); EWSR1/ETV4 t(17;22)(q21;q12)*

EWSR1/ETV1, EWSR1/ETV4 and EWSR1/FEV fusions occur in <1 % of Ewing sarcomas. FLI, ERG and FEV share 87 % identity and 98 % similarity, while ETV1 and ETV4 share 96 % identity and 100 % similarity in their

DNA-binding domains. Exon 7 of EWSR1 gene can translocate to FEV exon 2, ETV1 exon 11 and ETV4 exon 9 [34–36]. Many studies showed that EWSR1/FEV, EWSR1/FLI and EWSR1/ERG fusion proteins played similar roles. In fact, they induced oncogenic transformation of NIH3T3 cells and transcriptional repression of TGF-β receptor, whereas EWSR1/ETV1 and EWSR1/ETV4 were unable to induce oncogenic transformation in the same system. EWSR1/ETV1 and EWSR1/ETV4 in association with EWSR1/FEV play an important role in influencing the neoplasm localization. In fact, these translocations show a strong preference for extraskelatal primary sites. Their interference with important cellular pathways has also been described, for example, EWS/ETV1 is able to interact with TGF-beta pathway [29]. All three chimeric transcripts may be present in other peripheral primitive neuroectodermal tumor (pPNET) too [37, 38].

#### Homeodomain transcription factors family

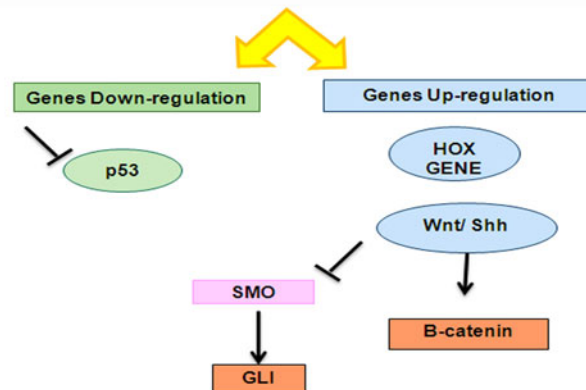
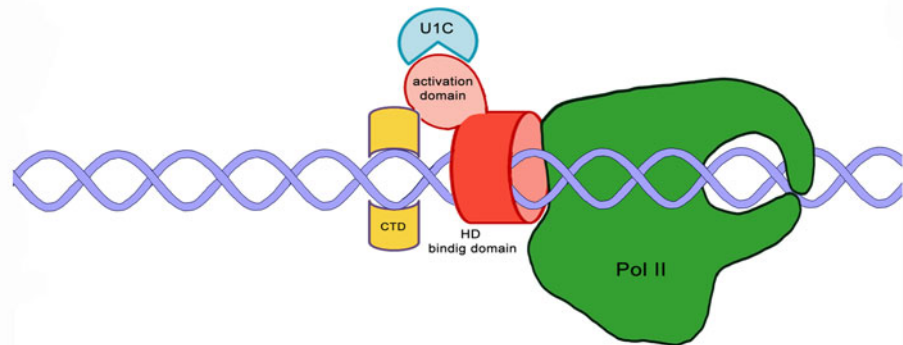
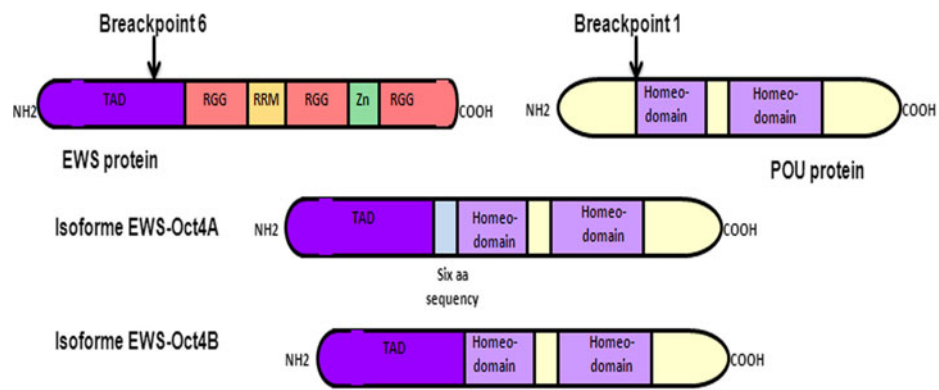
The homeobox gene superfamily (Fig. 3) encodes transcription factors that act as master regulators of development through their ability to activate or repress a range of downstream target genes. Numerous families exist within the homeobox gene superfamily. They are classified on the basis of the conservation of their homeodomains as well as on additional motifs that contribute to DNA binding and to the interactions with other proteins. There are many evidences, in literature, showing the strong involvement of many homeobox genes in human diseases, particularly in cancer.

#### *EWSR1/POU5F1 t(6;22)(p21;q12); EWSR1/PBX1 t(1;22)(q23;q12); EWSR1/DUX4 t(4;22)(q35;q12)*

POU5F1, known as OCT3/4, is a member of the POU family of transcription factors and is an important regulator of tissue-specific gene expression in lymphoid and pituitary differentiation and in early mammalian development. EWS exon 8 can translocate with POU5F1 breakpoint region, located in exon 1 [39]. EWSR1/POU5F1 translocation is described in myoepithelial tumor of bone, soft tissue, epithelium and myoepithelium [40]. Yamaguchi et al. [39] identified this type of translocation in tumor tissue derived from an undifferentiated sarcoma from the pelvic bone. Recently, using small-interfering RNA, the fundamental role of EWS-POU5F1 in tumorigenesis and tumor cell maintenance and its importance in the development and progression of sarcomas has been proved [41].

PBX1 (pre-B cell leukemia transcription factor 1) is involved in the regulation of osteogenesis and it is required for skeletal patterning and programming. A chromosomal translocation, t(1;19) involving this gene and TCF3/E2A

**Fig. 3** Schematic representation of EWS translocation with homeobox-genes family fusion partners: interaction with transcriptional complex on DNA and illustration of the main molecular pathways deregulated. *Gray squares* indicate the soft tissue tumors characterized by EWS/homeodomain translocations (RS: Rhabdomyosarcoma)



*Development and progression of sarcoma*



gene is associated with pre-B cell acute lymphoblastic leukemia. EWS–PBX chimeric protein was found in myoepithelioma with two different isoforms, one in frame and one, not pathogenetically important, out of frame [42].

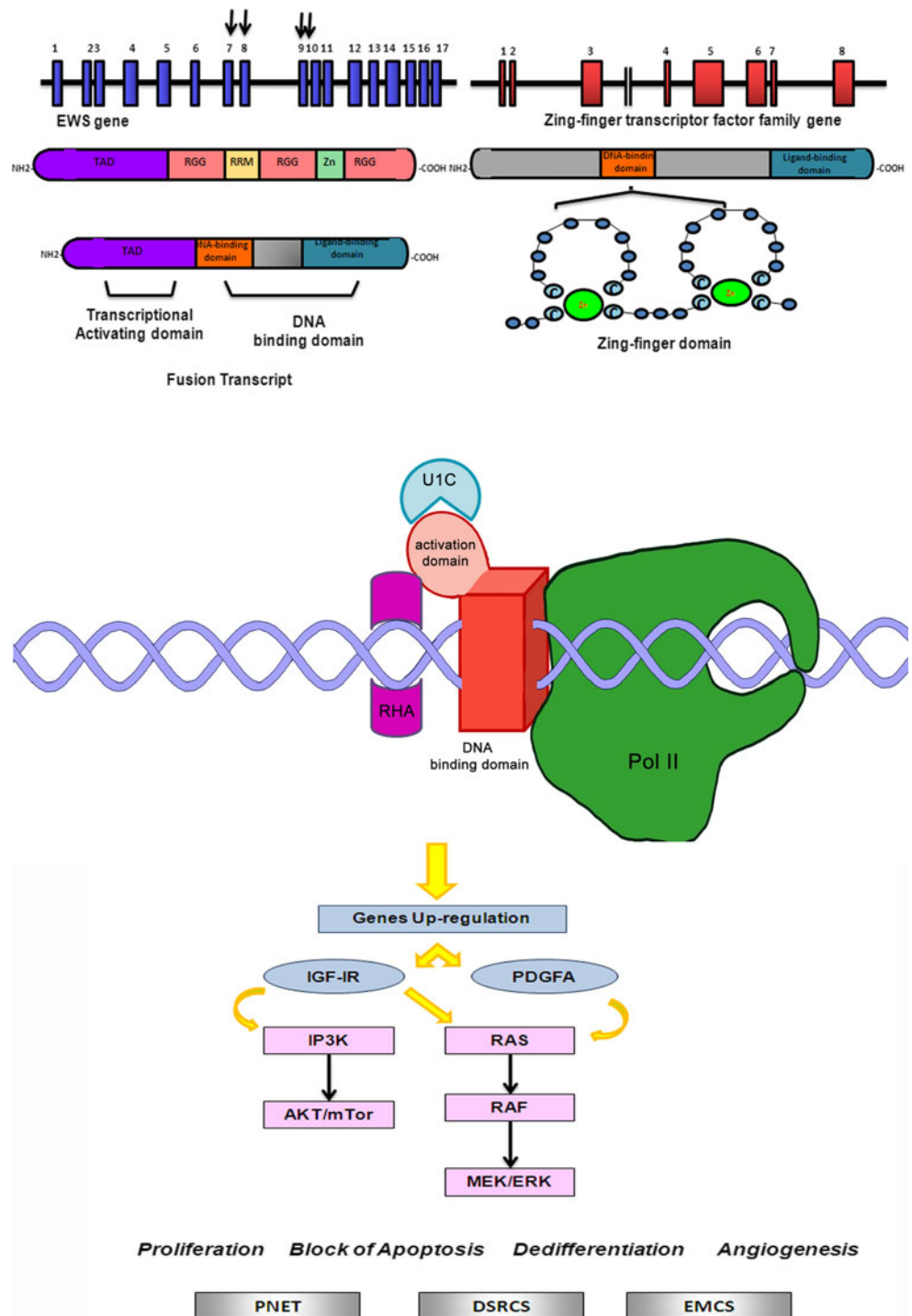
DUX4 (double homeobox, chromosome 4) contains two homeodomains similar in sequence to PAX3 and PAX7 homeodomains. It is involved in myogenic differentiation and cell-cycle control. EWS–DUX4 chimeric protein was found in rhabdomyosarcoma, interfering with normal

muscle cell proliferation [43] and in undifferentiated small blue round cell sarcomas [44].

Zinc finger transcription factor family

Zinc finger proteins (Fig. 4) are among the most abundant proteins in eukaryotic genomes. They have a characteristic motif of 28–40 amino acids (Cys–Cys–His–His). Proteins containing zinc fingers participate in DNA recognition,

**Fig. 4** Schematic representation of EWS translocation with zinc finger-genes family fusion partners: interaction with transcriptional complex on DNA and illustration of the main molecular pathways deregulated. *Gray squares* indicate the soft tissue tumors characterized by EWS/ZN translocations (PNET: Primitive neuroectodermal tumor; DSRCS: Desmoplastic small-round-cell-sarcoma; EMCS: Extraskeletal myxoid chondrosarcomas)



RNA packaging, transcriptional activation, regulation of cell death by apoptosis, protein folding and assembly and lipid binding [45]. Some zinc finger proteins are involved in the development of human diseases, particularly in cancer [46]. EWSR1 fusion transcripts with zinc finger proteins involved always EWS exon 8 and zinc finger-genes exon 1 [47].

*EWSR1/WT1*t(11;22)(p13;q12);*EWSR1/ZNF278* (*PATZ1*)t(22;22)(q12;q12);*EWSR1/ZNF384*(*CIZ/NMP4*) t(12;22)(p13;q12); *EWSR1/NR4A3* (*CHN*) t(9;22)(q22;q12)

*WT1* gene, deleted in individuals with Wilm’s tumor, encodes a zinc finger DNA-binding protein that acts as a

transcriptional activator or repressor, mainly regulating normal formation in the genitourinary system and mesothelial tissues. The chimeric transcript EWS/WT1 was only described in desmoplastic small round cell tumor [48, 49]. EWS/WT1 is able, in this neoplasia, to induce PDGFA expression [50] and trans-activation of IGF-IR gene [51].

*Patz1* encodes a zinc finger protein responsible repression of basal transcription as well as repression of RNF4-mediated activation and transcriptional activation of c-Myc. Deregulation of *Patz1* has been described in colorectal cancer [52] and in testicular tumors [53]. *PATZ1* gene is located 2 Mb distal to *EWS* gene and is transcribed in the opposite orientation. It was described as paracentric inversion of 22q12, generating the active EWS–ZNF278 fusion gene. The chimeric transcript has been described in small round cell tumor with multidirectional differentiation rather than pPNET [47].

Transcription factor ZNF384/CIZ/NMP4 plays a role in bone metabolism and spermatogenesis. It is recurrently involved in translocations in acute lymphoblastic leukemia, where it can produce a translocation transcript with *EWSR1* or with its homolog, *TAF15* [54].

CHN is a member of the steroid/thyroid receptor gene superfamily, with central bipartite zinc finger DNA-binding domain. The protein is implicated in the control of cell proliferation, differentiation and apoptosis, with a prevalent expression in the central nervous system. The specific chromosomal translocation *EWSR1/CHN* has been observed in extraskeletal myxoid chondrosarcoma [55, 56].

#### Leucine-zipper transcription factors family

Leucine-zipper transcription factors family consists of a positively charged segment linked with a sequence of heptad repeats of leucine residues (leucine zipper) (Fig. 5). Leucine-zipper transcription factors affect several developmental processes including dendritic cell development, myeloid differentiation and brain and ocular development.

#### *EWSR1/ATF1 t(12;22)(q13;q12)*

ATF1 is a transcription factor (c-AMP dependent) belonging to leucine-zipper proteins. The ability of this gene to produce a fusion transcript with *EWS* has been demonstrated in clear-cell sarcoma, defined in the past as malignant melanoma of soft parts (MMSP) [57, 58]. More recently, *EWS/ATF-1* has been described in soft tissue and angiomatoid fibrous histiocytoma [59–61], and in Hyalinizing clear-cell carcinoma (HCCC), a low-grade salivary gland tumor [62]. Finally, the most recent studies have shown the presence of *EWSR1–ATF1* fusion gene in a myoepithelial tumor [63] and in endobronchial pulmonary angiomatoid fibrous histiocytoma [64].

#### *EWSR1/CREB1 t(2;22)(q34;q12); EWSR1/CREB3L1 t(11;22)(p11;q12)*

CREB1 is a transcription factor (Fig. 5), member of the leucine-zipper family, that binds to the cAMP-responsive element. The protein is phosphorylated by several protein kinases, and induces transcription of several genes related to the cAMP pathway. Activation of CREB by phosphorylation has been implicated in the survival of mammalian cells and has been mainly involved in neuronal development. Moreover, many experimental evidences suggest that CREB1 plays a key role in mediating the malignant behavior of tumor cells. *EWS* is able to form a fusion transcript with CREB1 in several soft tissue tumors. It was also described as a recurrent variant fusion in clear-cell sarcomas [65, 66] and in angiomatoid fibrous histiocytoma [67]. More recently, a *EWS–CREB1* translocation has been described in a case of small blue round cell tumor of the interosseous membrane [68]. Another member of cAMP response element-binding proteins is CREB3, whose activity is related to herpes simplex virus (HSV) virion protein-16 (VP16), which has been described as translocation partner of *EWS* in small-cell osteosarcoma. In this tumor, CREB3 is fused in frame to *EWSR1* exon 11 [69].

#### *EWSR1/CHOP(dITT3) t(12;22)(q13;q12)*

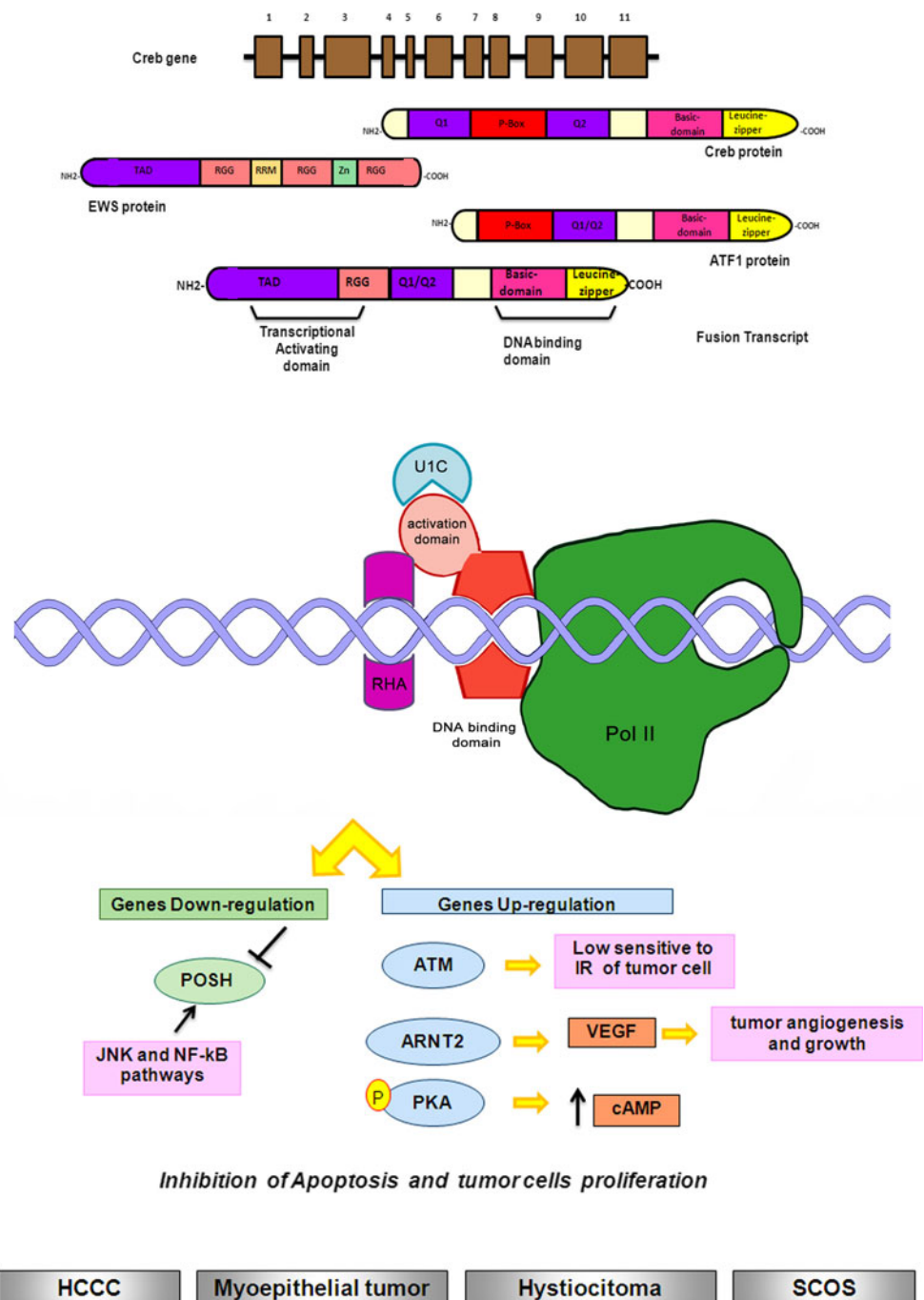
DDIT3/CHOP is a transcription factor characterized by a carboxy-terminal region formed by a DNA-binding basic domain and a leucine-zipper dimerization domain. DDIT3 negatively regulates C/EBP heterodimers formation, preventing their binding to C/EBP sequences in the DNA. DDIT3 is strongly implicated in adipogenesis, but also in erythropoiesis and in the induction of growth arrest [70]. CHOP is mainly implicated in cytogenetically alteration that characterizes myxoid liposarcomas, in which the gene translocates with *FUS* gene on chromosome 16p11. However, in this tumor, CHOP could produce another fusion transcript with N-terminal part of the *EWS* gene [71]. Several chromosomal sites are implicated in translocation (exons 1–7 of the *EWS* and exons 2–4 of the *CHOP* gene). It was subsequently identified a novel type of *EWS–CHOP* fusion gene that consisted of exons 1–10 of the *EWS* and exons 2–4 of the *CHOP* gene [72].

#### Other transcriptional regulators

#### *EWSR1/UQCRH t(1;22)(p34;q12)*

UQCRH (ubiquinol-cytochrome c reductase hinge protein) is a component of the ubiquinol-cytochrome c reductase complex associated with the mitochondrial respiratory chain, in which mediates the formation of the complex between cytochromes c and c1.

**Fig. 5** Schematic representation of EWS translocation with leucine-zipper-genes family fusion partners: interaction with transcriptional complex on DNA and illustration of the main molecular pathways deregulated. *Gray squares* indicate the soft tissue tumors characterized by EWS/leucine-zipper translocations (HCCC: Sarcomatous hepatocellular carcinoma; SCOS: Small-cell-osteosarcoma)



Deregulation of this gene has been described in several cancer cell lines, while a fusion transcript with EWSR1 has been detected in a panel of cancer cell lines of a small round cell sarcoma [73].

*EWSR1/NFATC2 t(20;22)(q13;q12)*

NFATC2 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2) is a transcription factor implicated

in control T-cell activation and function. Specifically, the transcription factor NFATc2 affects the regulation of cell differentiation and growth and plays a critical role in the development of colonic inflammation. Its deregulation was described in colon cancers [74], in pancreatic cancer [75] and in melanoma [76]. Recently, NFATc2 has been described as translocation partner of EWSR1 in Ewing sarcoma with breakpoints located in EWS exon 8 and NFATc2 exon 3 [77].



*EWSR1/SP3 t(2;22)(q31;q12)*

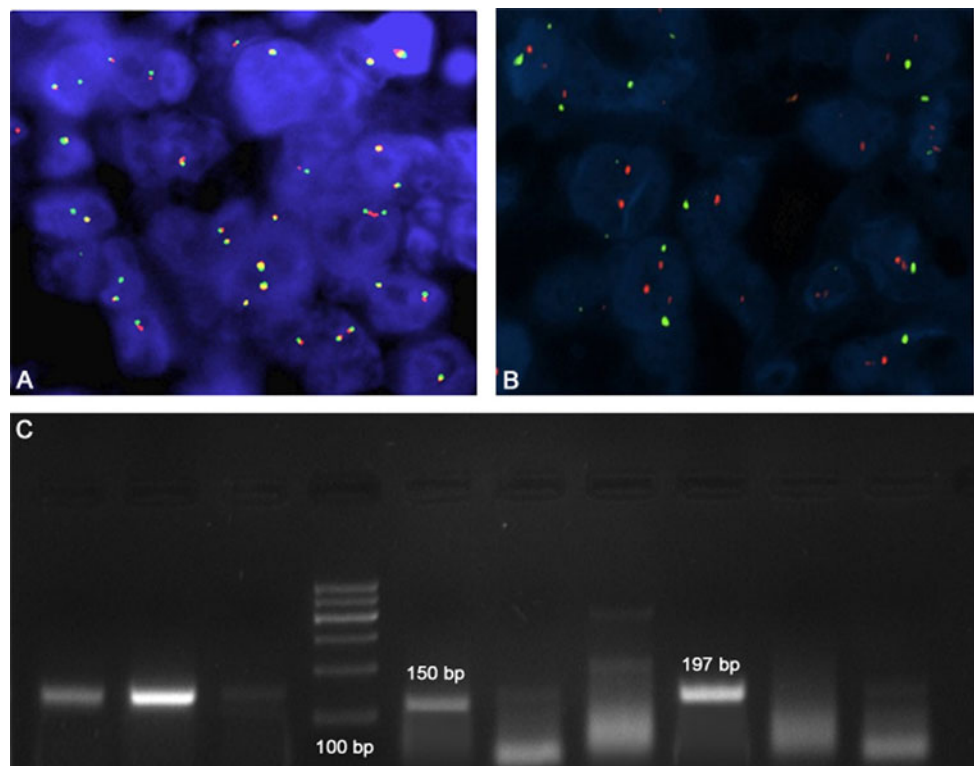
SP3 is a transcription factor belonging to the Sp/XKLF family able to recognize GC-rich DNA motifs, found in many promoters and enhancers of housekeeping genes. Moreover, sp3 is involved in cell-cycle regulation and hormone induction. Two different breakpoints have been identified for production of EWSR1/SP3 transcripts, involving EWS exon 7 with SP3 exon 6 and EWS exon 8 with SP3 exon 6. The fusion transcripts have been detected in undifferentiated small round cell sarcomas [78].

*EWSR1/SMARCA5 t(4;22)(q31;q12)*

SMARCA5 encodes for a Helicase that possesses intrinsic ATP-dependent nucleosome-remodeling activity. The protein is mainly involved in chromatin remodeling and regulation of transcription. Its deregulation has been described in gastric cancer [79]. More recently the possibility of SMARCA5 to produce a fusion transcript in extraskeletal Ewing sarcoma/primitive neuroectodermal tumor has been described. The breakpoint involved EWS exon 7 and SMARCA5 exon 8 [80].

**Molecular techniques for EWSR1 chimeric transcripts detection****FISH**

Fluorescence in situ hybridization (FISH) is a rapid diagnostic test using molecular cytogenetic techniques. FISH technique supplements conventional cytogenetics and in some cases provides additional information, which is not detected by karyotyping. In the case of EWSR1 gene, several commercial probes have been developed by different companies (Vysis (Abbott Molecular); ARUP LABORATORIES; KREATECH DIAGNOSTIC; CytoCELL Ltd.) that allow to specifically identify the breaking point in the 22q12 region of the gene (Fig. 6). However, difficulties of developing probes able to detect specific fusion partners. Since the detection of specific fusion transcripts is becoming important for prognosis and for the establishment of new therapeutic strategies, new FISH probes specific for the fusion partners of EWS have been developed. For instance, a commercial probe is able to detect the breaking of the chromosome region 11q24 related to FLI1 gene (Creative Bioarray, Abnova). However, many non-



**Fig. 6** Upward, a FISH assay showing the EWS locus rearrangement with a break-apart probe: **a** absence of translocation and **b** EWS rearrangement-positive tumor cells showing one fusion, one orange, and one green signal pattern. Arrows shows rearrangement signals (original magnification  $\times 60$ ). Under, c a Polymerase chain reaction analysis of EWS-FLI1 translocation using EWS and FLI1 primers.

Reactions were subjected to electrophoresis on a 2 % agarose gel: Lane 4 shows the DNA size marker (100 bp); Lane 1–3: Beta actin controls; Lane 5: Positive sample DNA (EWS/FLI1 type I-150 bp); Lane 8: Positive sample DNA (EWS/FLI1 type II-197 bp); Lane 6, 7, 9 and 10: Negative samples DNA

commercial probes have been developed “in-house,” by YAC and cosmid vectors, in order to detect other fusion partners of EWS, especially CREB1, FEV, WT1, ATF1. Cocktails of commercial and non-commercial probes allow to simultaneously identify translocations. Rossi et al. have developed a four-color FISH, with CREB1 proximal probe RP11-167C7 and ATF1 proximal probe RP11-189H16 labeled by nick translation with Cy5, and CREB1 distal probes G248P81788B12 and G248P89268A6 and ATF1 distal probe RP11-407N8 labeled with Cy3.

Recently, other commercial probes have been developed for detecting EWSR1/FLI1 translocation, made of a mixture of two FISH DNA probes. One of these, the Vysis LSI EWSR1 (22q12) Dual Color, Break-Apart Rearrangement Probe consists of a mixture of a ~500-kb probe labeled in SpectrumOrange, flanks the 5' side of the EWSR1 gene and extends inward into intron 4 and a second ~1,100-kb probe labeled in SpectrumGreen, flanks the 3' side of the EWSR1 gene (Abbott). Another FLI1/EWSR1 probe mix consists of green probes flanking the breakpoint region at the EWSR1 gene locus and red probes flanking the breakpoint region at the FLI1 locus (Cytocell Ltd.).

#### Qualitative PCR

The technique more widely used for the detection of fusion transcripts is represented by RT-PCR. Many studies are reported in literature, which design specific primers for the fusion partners of EWS, able to detect all fusion transcripts. The most numerous are those associated with the detection of EWSR1/FLI1 [23, 81–87] (Fig. 6) For ERG and ATF1, translocation partners have been also described several primers for its detection through specific RT-PCR [88–91], CREB1 [65, 66], NTFcA2 [77], WT1 [92, 93], ETV4 [94, 95] and CHOP [72, 96–98].

Finally, one of the most common problems associated with this detection technique is represented by the quality of archival biological sample, in fact, RNA fragmentation is more frequent, thus better pre-analytical procedures of sampling and storage must be developed [99]. Furthermore, the possibility of using biological material from the biobanks of fresh cryopreserved tissues has substantially resolved the problem of nucleic acids degradation [100].

#### Quantitative PCR

In recent years with the advent of quantitative PCR (qPCR), many molecular diagnostic tests were oriented toward this innovative system that could provide additional quantitative information. In this technique, PCR is usually performed using primers labeled with fluorescent chromophores and a suppressor molecule. Recently, this technique has been used for the detection and quantization of

the fusion transcripts, and in many cases, it is more sensitive and reliable than qualitative PCR [101, 102]. Recent reports suggest that RNA extracted from FFPE (formalin-fixed paraffin-embedded) archival materials can be successfully quantified by qRT-PCR assays, because the technique can use very small amplicons. Even in this case, the design of the probes is the main aim, because RT-PCR probes must have different characteristics and predict a particular marking which also makes them more expensive than those used for the qualitative PCR.

Different systems based on q RT-PCR have been developed for the detection of fusion transcripts in soft tumors, in particular for synovial sarcoma with Syt translocation [103–105]. A real-time RT-PCR assays specific for EWS–FLI1, EWS–ERG, EWS–ETV1, EWS–ETV4 and EWS–FEV have been recently developed [95]. However for EWSR1 translocations, only few studies have been presented in the literature.

#### Western blot

There are few information about the use of western blotting for the detection of translocations in soft tissue tumors. Wang et al. [106] applied western blotting, using an antibody against the carboxyl terminal of the FLI1 protein, for the detection of the 68-kDa EWS/FLI1 fusion protein in cultured Ewing's sarcoma cells and in four surgical biopsies of Ewing's sarcoma. Authors stressed how this method is not dependent on the quality of mRNA in the sample and involves no risk of contamination, being a powerful complement to the reverse-transcription polymerase chain reaction (RT-PCR).

#### Sequencing

Currently, sequencing represents, actually, the system for molecular investigating safer also for the detection of fusion transcripts. In fact, in most of the studies that have been carried out on the detection of EWSR1 fusion transcripts in soft tissues tumors, the sequencing of the transcript produced by PCR amplification, not only confirmed the efficiency of the investigation system used, but also the diagnosis.

#### Targeting fusion transcripts

The identification of specific fusion transcripts, including several variants of splicing of EWSR1 gene could help in establishing new biotechnological systems to block their aberrant activity in cancer cells. The most modern technologies in recent years have shown the effectiveness of “silencing systems” that could be used also to establish

specific target therapies for soft tissue tumors. First of all, there is an extensive literature concerning the use of siRNA in blocking fusion transcripts in several human malignancies. The use of this blocking system has also been described for numerous fusion transcripts, primarily associated with inhibition of production of protein transcripts Syt/SSX [107–110].

Even for the main fusion transcript of EWSR/FLI1, several studies have been carried out showing the effectiveness of this technique both *in vitro* and *in vivo* [111–113]. More recently, it was also described the possibility of reducing the Myc oncogene expression in Ewing sarcoma cell by siRNA targeting of EWS/FLI1 [114]. Several other systems have been developed to inhibit fusion transcript gene expression as antisense oligonucleotide nanocapsules (ODNs), whose main advantage consists in the rapid degradation, by nucleases, of ODNs. The suppression of EWS/FLI1 with nanocapsules is able to inhibit tumor growth in mice [115]. In addition to the possibility of interfering with fusion transcripts gene expression, other strategies are taking place, particularly those targeted to the construction of specific peptides against chimeric protein. It was described that EWS/FLI1 is able to bind RNA Helicase A (RHA). This link is fundamental for its oncogenic function in Ewing sarcoma. A specific small molecule, YK-4\_279, can block RHA binding to EWS/FLI1, inducing apoptosis in Ewing sarcoma cells and reducing the growth of tumor cell in mice [116]. It was also described a novel peptide, defined ESAP1, able to bind EWS/FLI1 chimeric protein, with high affinity, altering cell-cycle process in Ewing sarcoma cells [117]. Finally, other alternative strategies which interfere with the activity of these transcripts are related to downstream oncogenic pathways associated with them. Regarding EWS/FLI1, it was described that this transcript can bind IGFBP3 promoter, upregulating IGF1. For this reason, therapeutic strategies targeted to inhibit IGF1 receptor pathway, as monoclonal antibody figitumumab (CP-751,871), seems to be very effective in Ewing's sarcoma patients [118]. It was recently described that protein kinase PKC-B can be directly regulated by EWSR1/FLI1 chimeric protein. The loss of PKC-B causes apoptosis in Ewing sarcoma cells and reduces tumor growth in animal model [119]. Finally, another fusion transcript, EWSR1/CREB1, has been implicated in upregulation of MET oncogene in clear-cell sarcoma. The use of MET inhibitor ARQ197 already represents a good therapeutic strategy in clear-cell sarcoma [120].

## Conclusions

There are many different strategies to investigate EWS gene status. For diagnostic purpose, the choice of a

technique relies mainly on the technological support and the “know-how” available to the pathologist. In a not too distant future, blocking strategies may be implemented at all levels, from pre-transcriptional to post-translational stages, interfering with aberrant activity of chimeric transcripts and allowing to establish new and more focused therapies for soft tissue tumors.

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