Molecular Heterogeneity and Function of EWS-WT1 Fusion Transcripts in Desmoplastic Small Round Cell Tumors

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ABSTRACT

Desmoplastic small round cell tumor (DSRCT) is a primitive sarcoma with a consistent cytogenetic abnormality, t(11;22)(p13;q12). This chromosomal translocation generates a chimeric transcript that is formed by fusion of the 5’ region of the Ewing’s sarcoma gene, EWS, with the 3’ DNA-binding segment of WT1, the Wilms’ tumor suppressor gene. We collected 14 DSRCT tumor samples and examined the hybrid transcripts. We identified: (a) combinatorial heterogeneity of EWS exons fused to WT1 including use of EWS exons 7, 8, and 9; (b) subpopulations of variant transcripts in 6 of 14 tumors characterized by aberrant splicing resulting in loss of EWS exon 6 or WT1 exon 9; (c) multiple cDNA products with large internal deletions; and (d) insertion of small stretches of heterologous DNA at the fusion site or exon splice region in transcripts from two tumors. Most of the splice variants were in-frame, and small stretches of heterologous DNA at the fusion site or exon splice region in transcripts from two tumors. Most of the splice variants were in-frame, and in vitro translated fusion proteins with intact DNA-binding motifs formed complexes with a WT1 response element in gel mobility assays. Each of the chimeric proteins retains the ability to bind to the GC and TC elements of the early transcription factor EGR-1 as well as WT1 consensus sequences. We present evidence that various EWS-WT1 proteins up-regulated EGR-1 promoter activity and that this up-regulation is specifically dependent upon the absence of the exon 9 KTS domain of WT1. The molecular diversity and functionality exhibited by these fusion transcripts may have significant biological implications for their transactivating and tumorigenic potential.

INTRODUCTION

DSRCT is a primitive sarcoma with distinctive histopathological features that suggest a multilineage origin (1–3). The tumor develops primarily in pediatric or young adult patients and typically, although not exclusively, arises at intra-abdominal sites (4–6). Cytogenetic and molecular characterization of DSRCT has identified a unique chromosomal rearrangement, t(11;22)(p13;q12), associated with this tumor (7–9). This translocation juxtaposes two genes that have been implicated previously in the development of human neoplasia, the EWS gene on chromosome 22 and WT1, the Wilm’s tumor suppressor gene at 11p13. The resulting chimeric gene generates a cDNA fusion transcript that encodes an aberrant transcriptional regulatory factor comprised of the NH2-terminal region of EWS and the COOH-terminal segment of WT1 (10–12).

The hybrid transcript expressed in DSRCT is similar to the tumor-specific fusions of the EWS gene described in other human sarcomas. The prototype rearrangement is the joining of EWS with ETS family gene members (FLI-1, ERG, or ETV1) in two closely related tumors, Ewing’s sarcoma and peripheral neuroepithelioma (13–15). Other tumors with novel translocations involving EWS include malignant melanoma of soft parts with an EWS-ATF-1 fusion (16), myxoid chondrosarcomas with rearrangement of EWS and the CHN gene (17), and the EWS-CHOP gene fusion in myxoid liposarcomas (18). Several common features of the fusion transcripts generated in these malignant neoplasms have emerged. In all instances, the transcripts are comprised of the NH2-terminal effector region of EWS, whereas the RNA binding the COOH terminus is replaced by the fusion partner (19, 20). The chimeric product, which has been shown in many cases to be transforming, presumably functions through this novel combination of effector and binding regions to dysregulate a critical group of target genes (21, 22).

WT1 is a tumor suppressor gene that encodes a protein with four tandem Cys3-His2 zinc finger motifs located in the COOH terminus (23–25). Given the high level of homology between WT1 zinc finger domains 2–4 and the zinc finger region of EGR genes, WT1 recognizes the same response motifs that are bound by the EGR genes (11, 26). A WT1 splice variant (+KTS) that inserts nine nucleotides between the third and fourth zinc fingers will alter WT1 binding to EGR recognition motives (27). EGR-1 was identified initially as a member of the immediate-early response genes, which are expressed rapidly, transiently, and independently of protein synthesis during progression of quiescent cells from G0 to G1 phase of the cell cycle (11, 28–30).

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3 The abbreviations used are: DSRCT, desmoplastic small round cell tumor; EGR, early growth response; EBS, EGR binding sequence; RT-PCR, reverse transcription-PCR; EMSA, electrophoretic mobility shift assay.
EGR-1 can be induced by many factors including serum, growth factors, hormones, and neurotransmitters which, in turn, influence the transcription of other genes responsible for or involved in cell proliferation, differentiation, and development (31). A potential role of EGR-1 in the pathogenesis of some malignant tumors has also been suggested (32, 33). WTI1 binds to the consensus sequence GCCG(G/T)GGCGG (11) of the EGR-1 promoter and suppresses the transcription of EGR-1. In fact, under most circumstances WTI1 represses transcription from this site, whereas EGR-1 activates transcription through the element (26, 34). This common recognition element also exists in the promoter regions of a number of growth-related genes (11), and it plays a significant role in transcriptional regulation. It is, therefore, of interest to know whether the EWS-WTI1 hybrid proteins from DSRCT remain functionally active through interaction with the G+C-rich sequence as well as the later identified second T+C-rich motif (35).

In the present study, we collected more than a dozen samples of this rare tumor, assessed the fusion transcripts expressed in tumor cells, and have identified extensive structural heterogeneity in many of these chimeric gene products. Our studies show that the naturally occurring hybrid EWS-WTI1 products from DSRCT bind to the common recognition element 5'-GGCGGGGGC-3' in vitro and also serve as a functional protein in transactivating transcription in transfected cells.

**MATERIALS AND METHODS**

**Tumor Samples.** The protocol for specimen collection and molecular genetic analysis of DSRCT was approved by the NIH Office of Human Subject Research. DSRCT samples were obtained from consenting patients undergoing debulking surgery or from the Pediatric Oncology Group/Cooperative Human Tissue Network (Columbus, OH). Information regarding age, sex, and chemotherapy treatment prior to resection of the tumor is summarized in Table 1 for the 14 patients used in this analysis.

**RT-PCR Assay.** Total RNA was extracted from primary tumor tissues and nonneoplastic control samples by the acid guanidinium thiocyanate-phenol-chloroform method (RNA isolation kit; Stratagene). A Superscript II kit (Life Technologies, Inc.) was used to generate cDNA by reverse transcription of 5 μg of total RNA using an antisense primer at the WTI1 3' untranslated region downstream of exon 10 (5'-ACAGTG-GTGAACGTGCCTCAAGT-3'). Two-μl aliquots of the reverse transcription product were used for sequential nested PCR reactions. The hybrid WTI1 cDNA products were amplified with the respective primer sets P1 and P2 and primer sets P3 and P4; P1, 5'-ACGTTGAGAACGAGGAGGA-3' (EWS exon 1, sense); P2, 5'-GACACTGAACGGTCCCCGAGG-3' (WTI 3' untranslated region, antisense); P3, 5'-GAATGCGCTC-CACGGATTAC-3' (EWS exon 1, sense); and P4, 5'-TCAAGGCGGGACTGAGGT-3' (WTI exon 10, antisense). PCR products obtained after 30 cycles of amplification (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) were then directionally subcloned into the CloneAmp vector (Life Technologies, Inc.). The cloned PCR fragment was then sequenced using the dideoxy DNA sequencing kit from United States Biochemicals Corp.

**Genomic DNA Isolation and PCR Amplification.** High molecular weight genomic DNA was prepared as described previously (36). Nested PCR amplification of EWS exon 6 and WTI exon 9 was performed in 50-μl reaction mixtures containing 0.5--2.0 μg of DNA in the presence of 0.5 unit of Taq polymerase (Amplitaq; Perkin-Elmer) using a Perkin-Elmer 480 DNA thermocycler. The amplification procedure consisted of 2 min of denaturation at 94°C, followed by 35 cycles at 94°C (1 min), 55°C (2 min), and 72°C (3 min), ending with a 7-min extension at 72°C. The primary reaction product was diluted 1:100, and 2 μl were used as substrate for the secondary PCR reaction. For amplification of EWS exon 6 and surrounding intronic regions, the primer sets P5 and P6 (primary) and the primer sets P7 and P8 (secondary) were designated as follows: P5, 5'-GTATTTATTTCAGCTCAA-3' (EWS exon 6, 5' intron, sense); P6, 5'-TATCACTGGCGACAGTCGAG-3' (EWS exon 6, 3' intron, antisense); P7, 5'-AACATAAATGCTTATGGTATGG-3'; and P8, 5'-AAACAATACCTTACGCGAGG-3' (P7 and P8 are the internal primers of P5 and P6). Amplification of WTI1 exon 9 and adjacent 5' and 3' introns was performed with two primer sets, P9 and P10 (primary) and primer sets P11 and P12 (secondary) as follows: P9, 5'-GTCTCACTGGCAGGTCTAAA-3' (EWS exon 9, 5' intron, sense); P10, 5'-CAGTGAGCTTCTTCATCTT-3' (WTI exon 9, 3' intron, antisense); P11, 5'-GGGCTACGTGCCCACAT-3'; and P12, 5'-TCATTCTCTTCTCTCTCTG-3' (P11 and P12 are the internal primers of P9 and P10). The genomic PCR products were then subcloned into CloneAmp plasmids.

**cDNA Cloning and Western Immunoblotting.** Plasmids were constructed by inserting the full-length EGR-1 cDNA coding region (248–1879 bp) or EWS-WTI1 chimeric cDNA products (variably designated 9/8, 8/8, 7/8, 7/8α, 7/8β, and 7/8γ) into the pcDNA3 (Invitrogen) or pcDNA3/neo (Promega Corp., Madison, WI) expression vector. Plasmids were sequentially transcribed and translated in vitro using the TNT-coupled reticulocyte lysate system (Promega) under the control of the T7 promoter. Parallel in vitro translation reactions were performed in the absence and presence of [35S]methionine. The molecular weight and quality of the in vitro translation products were verified by SDS-PAGE and Western immunoblotting.

**EMSA.** The EBS response element bound by EGR proteins and WTI1 has been described previously (11). Double
stranded probe with three tandem G+C-rich EBS binding sites was constructed by annealing the sense/antisense oligonucleotides of (GCGGGGGCG)3 in annealing buffer. The binding site oligonucleotide was 5’-end labeled by phosphorylation with T4 polynucleotide kinase (Life Technologies, Inc.) in the presence of [γ-32P]ATP. A total of 10,000 cpm of oligonucleotide probe was used for each EMSA reaction.

Protein concentrations of the various chimeric gene products were equalized by measuring [35S]methionine incorporation corrected for the methionine content, and equivalent amounts were used in the gel shift assay.

In standard EMSA reactions, in vitro-translated proteins (2.5–5 μl) were preincubated in a total volume of 15 μl for 15 min on ice with 1 μg of poly(deoxyinosinic-deoxycytidylic acid) (Sigma) in binding buffer [20 mM HEPES (pH 7.9), 70 mM KCl, 5 mM MgCl2, 10 μM ZnCl2, 12% glycerol, and 2 mM DTT] before addition of 32P-radiolabeled probe. Reactions were then allowed to proceed to equilibrium for 20 min at room temperature. The mixtures were electrophoresed for ~2 h at 250 V on nondenaturing polyacrylamide gels [5% (60:1 acryl-bisacylamide), 0.045 M Tris-borate, 0.001 M EDTA] at room temperature. Gels were dried and subjected to autoradiography.

DNA Transfections and Luciferase Assays. The human osteosarcoma cell line, Saos-2, was maintained in RPMI 1640 with 10% FCS. Cells were plated at a density of 5 × 104 cells/well in a six-well plate 24 h prior to transfection. Transfections were carried out according to the lipofectin transfection protocol provided by Life Technologies, Inc. In addition to 4 μg of cDNA constructs and 0.5 μg of reporter plasmid, cells were cotransfected with 1 μg of β-galactosidase expression plasmid (Promega) to serve as an internal control for transfection efficiency. Forty-eight h after transfection, cell extracts were prepared, aliquots were normalized for transfection efficiency by assay of β-galactosidase activity, and luciferase activity was determined by following the protocol in the Luciferase Assay System kit (Promega).

For in vivo transactivation assay, EGR-1 promoters (~602 to 12 bp and ~552 to 12 bp; Ref. 37) were cloned into the pGL-2 luciferase reporter vector at the BglII site. The plasmid containing EGR/WT1 binding element alone was also constructed by inserting three-tandem G+C EBS element repeats into the pGL-2 vector driven by SV40 promoter.

RESULTS

Heterogeneity of EWS/WT1 Fusion Transcripts. Full-length coding region isolates of the EWS/WT1 fusion transcript were generated by RT-PCR assay using total cellular RNA obtained from tumor material of 14 patients with the histopathological diagnosis of DSRCT (Table 1). Obvious differences in size between the PCR-amplified fragments isolated from several tumors were noted after agarose gel separation of the reaction products. Further heterogeneity was identified when multiple clones of the fusion product from each tumor were assessed by sequence analysis. A schematic representation of the fully sequenced fusion transcripts cloned from the 14 tumors is shown in Fig. 1. The prototypical EWS/WT1 fusion transcript most frequently isolated from the productively rearranged der 22 allele was formed by fusion of EWS exons 1–7 and WT1 exons 8–10 (designated 7/8) and was ~1.1 kb in size. This transcript was identified in 12 of the DSRCT samples. In addition, alternatively sized fusion transcripts larger and/or smaller than 1.1 kb were amplified from 8 of 14 tumors. Sequence analysis of the fusion transcripts determined that the size variation was usually, although not exclusively, attributable to the extent of EWS sequence present. For example, fusion transcripts isolated from tumors 13 and 14 were larger than the prototype 7/8 fusion because these RNA products used EWS exon 8 (8/8) or EWS exon 9 (9/8), respectively, to join with WT1 (Fig. 1). Several additional tumor transcripts were isolated with large internal deletions of EWS sequence; these transcripts remained in-frame despite loss of part or all of EWS exons 5, 6, 7, and 8. The shortest hybrid transcript was a 600-bp fragment expressed in tumor 3. This chimera, formed by EWS exons 1–5 with WT1 exon 10, was frame-shifted at the fusion site and terminated prematurely by a stop codon.

Further examples of structural heterogeneity between the chimeric transcripts were revealed by sequence analysis of multiple cDNA products. A minimum of four clones was examined from each tumor. Two recurrent isoforms, characterized by
deletion of EWS exon 6 or deletion of WT1 exon 9, were identified as fusion transcript subpopulations in 6 of the 14 DSRCTs. Two tumors had transcripts with deletion of exon 6, one tumor had exon 9-deleted transcripts, whereas three tumors expressed both subpopulations with an exon 6 or exon 9 deletion. No clones were identified that encoded a fusion protein with deletion of both exons.

Two additional levels of molecular diversity were characterized in these variant fusion transcripts. The first involved the naturally occurring KTS alternative splice. The KTS splice alteration is located in WT1 exon 9, and the hybrid transcripts with deleted WT1 exon 9 a priori did not express this splice isoform. However, +KTS and −KTS isoforms of all fusion transcripts with an intact WT1 exon 9 were identified.

A second aberrant structural feature of the DSRCT cDNA products, noted in two fusion transcripts, was the presence of short stretches of heterologous DNA inserted at a splice site or fusion breakpoint. One of four 7/8 cDNA transcripts from tumor 12 contained a 15-bp insertion (AAGGACCAGTAGACG) between EWS exons 4 and 5 that maintained an in-frame protein with the introduction of five amino acids (E-G-T-S-T). This 15-bp addition did not originate from known EWS intronic sequence. All of the 8/8 transcripts isolated from tumor 13 contained a 6-bp (CCAACC) in-frame insertion at the EWS-WT1 fusion site.

Quantitative PCR Analysis of EWS and WT1 Genomic Loci. The high percentage of DSRCTs expressing fusion transcripts with deleted EWS exon 6 or WT1 exon 9 suggested that this was a nonrandom alteration that may have resulted from a common structural defect in these tumors. We wished to quantify the levels of expression of these deleted transcripts and identify whether there was a common structural defect responsible for this alternative splicing. RT-PCR analysis using radiolabeled primers that flanked these respective exons was performed to establish the ratio of alternatively spliced forms and full-length fusion transcripts. This analysis included total RNA from DSRCT, with and without variant transcripts. In each tumor with EWS exon 6 or WT1 exon 9 deletions, an appropriately sized deletion fragment was evident below the major gene product. Quantitative analysis of these bands by Phosphorimager suggested that the full-length:variant transcript ratio was approximately 100:1 (data not shown). This result was in contrast with the recovery of these variant transcripts from plasmid subclones, suggesting that the variant transcripts were present at levels of at least 10–20% of the full-length transcript.

We then assessed the EWS exon 6 and WT1 exon 9 genomic regions of several DSRCTs expressing alternatively spliced transcripts. Our objective was to identify possible structural alterations of these areas, particularly of splice recognition sites at exon-intron junctions, that would explain the genesis of these variants. By using nested PCR with intronic primers flanking the respective exons, these genomic regions were amplified from four tumors, and the entire exon as well as the 5’ and 3’ exon/intron junctions of multiple clones was sequenced. In each tumor examined, there was no evidence of somatic alterations in the genomic DNA sequence.

DNA Binding Analysis of Variant Transcripts. Most EWS-WT1 fusion proteins retains WT1 zinc finger motifs 2, 3, and 4, which have a high level of homology to the corresponding zinc finger domains of the EGR family of transcription factors. To determine whether the multiple DSRCT fusion transcripts exhibited similar DNA binding specificity for response elements recognized by EGR-1 and WT1, we performed a gel mobility shift assay with a radiolabeled GC-rich consensus trimer designated EBS. EWS-WT1 chimeric proteins were expressed by in vitro translation of cloned cDNAs in the pcDNA neo expression vector using the TNT T7-Coupled Reticulocyte Lysate System (Promega). EGR-1 cDNA (nucleotides 248-1879; Ref. 38) was also subcloned into pcDNA3 (Invitrogen) and in vitro translated. The in vitro-translated proteins were assessed by Western immunoblot analysis using an antisem specific for the COOH-terminal end of WT1 protein. The fusion clones produced proteins of various sizes (estimated between Mₙ, 23,000 and 52,000), appropriate for the insert length, and all were recognized by the WT1 antisemur, confirming that the cDNA transcripts isolated from DSRCTs encoded immunoreactive proteins (Fig. 2).

As seen in Fig. 3, the EWS-WT1 chimeric proteins showed a similar binding specificity to the response elements recognized by WT1 and the EGR protein. However, as expected, fusion transcripts lacking exon 9, which encodes the third WT1 zinc finger motif, did not bind nor did the proteins containing the naturally occurring three amino acid (+KTS) splice isoform in exon 9, which disrupts the third WT1 zinc finger region. The fusion transcripts encoded by tumors 13 and 14 that joined EWS exons 8 and 9, respectively, with WT1 exon 8 also recognized the response element, suggesting that the presence of these alternative exons adjacent to the zinc finger motif did not inhibit binding. These (−KTS) fusion proteins also bind with similar
DSRCT Molecular Heterogeneity

The transactivation through the EBS site is increased 4–5-fold when cotransfected with EGR-1 (Fig. 4). Unlike the three tandem EBS repeats in reporter plasmid, WT1 lacks its NH₂-terminal transactivation domain (39, 40). This finding is significant because WT1 has been shown largely to suppress the transcription. Thus, the NH₂ terminus of EWS is responsible for transactivation. The transactivation domain(s) may reside in the EWS exons 1–5 because transfection of naturally occurring deletion of EWS exons 6, 7, 8, and 9 individually or collectively did not result in any significant changes in the regulatory ability of the fusion protein (Fig. 4).

To directly investigate the effect of the EWS-WT1 fusion proteins on EGR-1 promoter activity, the EGR-1 promoter fragment (−602 to 12) was cloned into the pGL2 reporter vector and cotransfected with various EWS-WT1 constructs into Saos-2 cells. This EGR-1 5‘ upstream untranslated region was shown to be critical and sufficient for regulating EGR-1 transcription (37). Our studies indicate that EGR-1 promoter activity was significantly increased 4–5-fold when cotransfected with the (-KTS) fusion constructs as compared with the control +KTS forms (Fig. 5). Unlike the three tandem EBS repeats in reporter plasmid, there is only one GC-rich EBS element in the EGR-1 promoter. Because transactivation through the EBS site is increased with the number of EBS repeats present (data not shown), it is conceivable that some additional element(s) may be present in the EGR-1 promoter region that could also be transactivated, to a certain degree, by the EWS-WT1 proteins. Indeed, we observed only 2.5–3-fold increase in EGR-1 promoter activ-
gence of both fusion partners, all in-frame DSRCT chimeras are joined to WT1 exon 8.

A second level of DSRCT fusion transcript heterogeneity is the precise deletion of a single EWS exon 6 or WT1 exon 9 in the protein-coding region of the transcript. Quantitative RT-PCR analysis indicated that these alternatively spliced subpopulations represent only a small percentage of the total level of fusion transcript expressed in DSRCT cells. However, the frequent occurrence of these deletion variants (one or both deleted transcripts were present in 6 of 14 tumors) suggests a potential functional role. These two alternatively spliced transcripts were subcloned from tumor 4, which had been obtained prior to any therapeutic interventions (Table 1). This result suggests that the genotoxic effects of radiation or chemotherapeutic drugs were unlikely to be implicated in the altered splicing. We examined the exon-intron borders of EWS exon 6 and WT1 exon 9 using genomic DNA from four of the six tumors and found no evidence of mutation or deletion at these sites. These variations appear to be examples of exon skipping without genomic mutation. We also excluded the possibility that loss of EWS exon 6 or WT1 exon 9 might represent a physiological splice selection used in the generation of normal EWS or WT1 transcripts, because we were unable to identify any examples lacking EWS exon 6 or WT1 exon 9 from several normal tissues. Exon 9 encodes the third WT1 zinc finger motif, and loss of this region in the EWS-WT1 chimera disrupts the binding activity of this transcription factor. EWS exon 6 is located in a region of the encoded protein with transactivating potential, although the impact of its deletion on these functions of EWS cannot currently be predicted.

Two DSRCTs (tumors 12 and 13) showed heterologous stretches of DNA in the fusion transcript. A 15-bp segment of heterologous DNA was inserted in a tumor cDNA message to create a variant in-frame transcript. The origin of this DNA stretch was not evident but may represent a mini-exon that is created by alternative splicing in a subfraction of fusion transcripts in this tumor. The DSRCT hybrid transcript from tumor 13 had a unique insertion/deletion event that contributed to formation of the chi-
meric fusion site. This region contained a 6-bp DNA segment (CCAAATT) that was inserted at the breakpoint site between EWS exon 8 and WT1 exon 8 sequences, whereas the first 4 bp of WT1 exon 8 were eliminated (44). This alternative splice site originated from a LINE-1-related element that integrated in the genomic breakpoint region, possibly during the recombination event (45). Both of these alterations may reflect a high level of splicing abnormalities detected in many malignancies.

The function of the EWS gene is presently unclear, although the COOH-terminal region contains an RNA-binding domain (46), and the NH2-terminal region is homologous to eukaryotic RNA polymerase II (13, 15, 19). However, the occurrence of fusion products containing EWS and other transcription factors implicates the NH2 terminus of EWS as a transactivating or regulatory domain. The replacement of the NH2 terminal transcriptional activation domain of transcription factor, FLI-1, with the NH2 terminal portion of EWS in Ewing’s sarcoma and peripheral neuroectodermal tumor results in similar or stronger transcriptional activation at promoters responsive to native FLI-1 (47, 48). Another example involves the fusion of EWS with AFF-1, a member of ATF/cyclic AMP-responsive element binding protein transcription factor family in clear cell sarcoma. In this case, the EWS-fusion domain functions as an efficient regulatory domain for the transcriptional activation properties of the EWS-AFF-1 chimeric protein (16). Recently, an artificially fused EWS-WT1 (~KTS) fusion protein has been reported to increase IGF-1 promoter activity by ~300%, whereas the fusion protein containing KTS has no effect (49). Our observations of transactivation of the EGR-1 promoter by various endogenous EWS-WT1 fusion proteins extend this picture. It is yet unclear whether IGF-1 and/or EGR-1 are the only target genes of the EWS-WT1 fusion proteins.

In summary, chimeric EWS-WT1 RNA messages encoded in DSRCTs exhibit significant molecular diversity. EWS combinatorial variability, internal deletion, exon skipping of coding region sequence, and random nucleotide insertions contribute to the heterogeneity of these tumor-specific gene products. Structural differences in EWS-FLI1 transcripts may correlate with survival benefit in some Ewing’s sarcoma patients (50). Whether the different DSRCT chimeric proteins are associated with different clinical outcomes remains to be determined. These findings have implications, however, for the design of therapeutic strategies targeting the fusion transcript. These approaches will need to be considered for DSRCT, a disease that is generally refractory to conventional treatment modalities (51, 52). As an immediate-early gene, EGR-1, plays a critical role in the cascade of sequential gene induction that links extracellular signals for growth and differentiation to the complement of distal genes that comprise the specific response to exogenous stimuli. It is possible that transcriptional activation of EGR-1 and perhaps other genes by the combinatorially heterogeneous EWS-WT1 fusion proteins may constitute a possible mechanism for the malignant transformation and progression of DSRCTs.

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