Molecular Heterogeneity and Function of *EWS-WT1* Fusion Transcripts in Desmoplastic Small Round Cell Tumors[1]

Jun Liu,[2] Marion M. Nau, Jimmy C. Yeh, Carmen J. Allegra, Edward Chu, and John J. Wright

Department of Medicine and Pharmacology, Yale Cancer Center, Yale University School of Medicine and Veterans Affairs Connecticut Healthcare System, New Haven, Connecticut 06520 [J. L., E. C.], and Medicine Branch, Division of Clinical Science, National Cancer Institute, NIH, Bethesda, Maryland 20889 [M. M. N., J. C. Y., C. J. A., J. J. W.]

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 *in vitro* translated fusion proteins with intact DNA-binding motifs formed complexes with a *WT 1* 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[3] 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 NH_2-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* gene family 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 NH_2-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 Cys_2-His_2 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 G_0 to G_1 phase of the cell cycle (11, 28–30).

Received 3/27/00; revised 6/21/00; accepted 6/26/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by Grants CA 16359 and CA 75712 from the National Cancer Institute (to E. C.) and the Veterans Affairs Merit Review from the Department of Veterans Affairs (to E. C.).
[2] To whom requests for reprints should be addressed, at Veterans Affairs Connecticut Healthcare System, Cancer Center-111D, 950 Campbell Avenue, West Haven, CT 06516. Phone: (203) 932-5711, extension 4033; Fax: (203) 937-4869.

[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.

Table 1 Clinical features of the 14 study patients with intra-abdominal DSRCTs

Case no.	Sex	Age	Previous chemotherapy
1	M	14	Yes
2	M	36	Yes
3	M	30	No
4	M	17	No
5	M	24	Yes
6	M	20	No
7	M	34	Yes
8	M	15	No
9	M	17	No
10	M	13	Yes
11	M	13	Yes
12	F	20	Yes
13	F	10	Yes
14	M	37	Yes

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 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). *WT1* binds to the consensus sequence GCGC(G/T)GGGCG (11) of the *EGR-1* promoter and suppresses the transcription of *EGR-1*. In fact, under most circumstances *WT1* 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-WT1* 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-WT1* products from DSRCTs bind to the common recognition element 5′-GCGGGGGCG-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 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 *WT1* 3′ untranslated region downstream of exon 10 (5′- ACAGTGTGTGAACTGCTTTCAAGT-3′). Two-μl aliquots of the reverse transcription product were used for sequential nested PCR reactions. The hybrid *EWS-WT1* cDNA products were amplified with the respective primer sets P1 and P2 and primer sets P3 and P4: P1, 5′-ACGTTGAGAGAACGAGGAGGA-3′ (EWS exon 1, sense); P2, 5′-GACACTGAACGGTCCCCGAGG-3′ (WT1 3′ untranslated region, antisense); P3, 5′-AAAATGGCGTCCACGGATTAC-3′ (EWS exon 1, sense); and P4, 5′-TCAAAGCGCCAGCTGGAGTTT-3′ (WT1 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 *WT1* 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′-GTATTTATTTCCAGGCTTAA-3′ (*EWS* exon 6, 5′ intron, sense); P6, 5′-TATCACTGCGACAGTCAGAT-3′ (*EWS* exon 6, 3′ intron, antisense); P7, 5′-AATCATAACATTGCTTATTG-3′; and P8, 5′-ATAACATACCTTAGCTGAAG-3′ (P7 and P8 are the internal primers of P5 and P6). Amplification of *WT1* 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′-CCTCCAGCTGCCGGAAGTCAG-3′ (*WT1* exon 9, 5′ intron, sense); P10, 5′-CAGTGAGCATCTTTCCATCTT-3′ (*WT1* exon 9, 3′ intron, antisense); P11, 5′-TGGGCCTCACTGTGCCCACAT-3′; and P12, 5′-TCCATCTTCTCCTTCTCTGTA-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-WT1* chimeric cDNA products (variously designated 9/8, 8/8, 7/8, 7/8Δ6, 7/8wΔ9, and others) into the pcDNA3 (Invitrogen) or pCI/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 [^{35}S]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 *WT1* 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-^{32}P]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 [^{35}S]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 $MgCl_2$, 10 μM $ZnCl_2$, 12% glycerol, and 2 mM DTT] before addition of ^{32}P-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-bisacrylamide), 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 5×10^5 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 *Bgl*II 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.

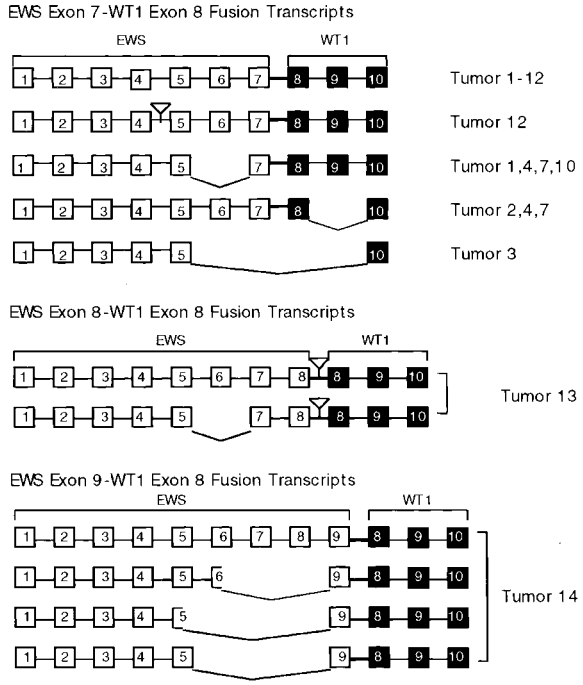

Fig. 1 Schematic illustration of *EWS-WT1* fusion transcripts isolated from DSRCTs. Three major types of chimeric *EWS-WT1* transcripts were identified resulting from different combinations of *EWS* exons 7, 8, or 9 and *WT1* exon 8. Variant transcripts isolated from the same tumors are depicted *below* the full-length representations. Precise deletion of exons is indicated by *lines* joining intact *boxes*. Internal deletion involving portions of exons 5 and 6 noted in tumor 14 are designated with *open exon boxes*. Heterologous DNA insertions (tumor 12 and all tumor 13 transcripts) are denoted by *closed* and *open triangles*, respectively. The *EWS-WT1* chimeric protein joins the NH_2 terminus 264 amino acids (exons 1–7), 325 amino acids (exons 1–8), or 337 amino acids (exons 1–9) of *EWS* to 93 (−KTS) or 102 (+KTS) COOH terminus amino acids of *WT1* exons 8–10. Nucleotide and predicted amino acid sequences of *EWS* and *WT1* have been reported previously (53, 54).

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 (AAGGGACCAGTACAG) 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 quantitate 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 immunoblotting analysis using an antiserum specific for the COOH-terminal end of *WT1* protein. The fusion clones produced proteins of various sizes (estimated between M_r 23,000 and 52,000), appropriate for the insert length, and all were recognized by the *WT1* antiserum, confirming that the cDNA transcripts isolated from DSRCTs encoded immunoreactive proteins (Fig. 2).

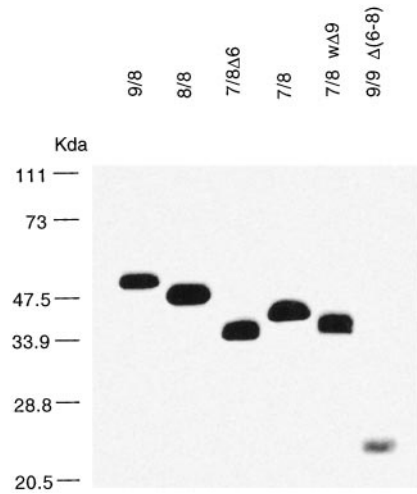

Fig. 2 Western immunoblot analysis of *in vitro*-translated *EWS-WT1* fusion products. RT-PCR products were directionally subcloned into expression plasmid pCI neo (Promega) and expressed in a coupled transcription-translation system using the T7 RNA polymerase promoter (Promega). After resolution on a SDS-8% polyacrylamide gel, proteins were identified with polyclonal antisera (Santa Cruz) directed against the *WT1* COOH terminus. 9/8, 8/8, and 7/8 designate full-length hybrid proteins with alternative EWS fusion exons. 7/8Δ6 indicates an *EWS* exon 6-deleted protein. 7/8wΔ9 denotes a *WT1* exon 9-deleted protein, and 9/8Δ (6−8) refers to the tumor 14 fusion protein with an *EWS* exon 6−8 internal deletion. *Left*, position of prestained protein standards (in thousands).

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

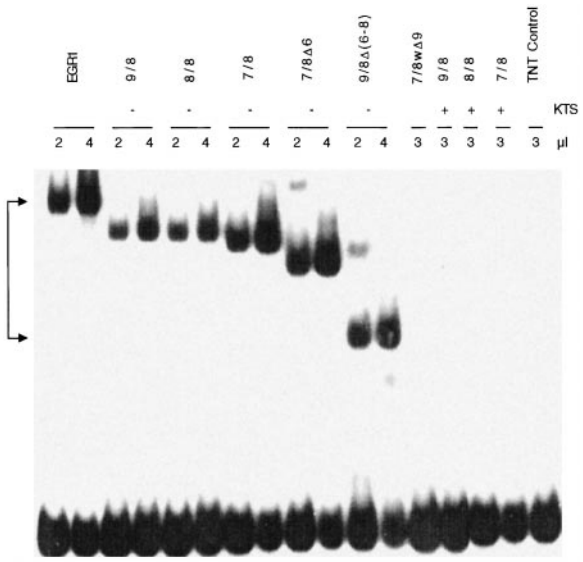

Fig. 3 EMSA of the *EWS-WT1* fusion proteins and target sequences: (GCGGGGGCG). *EWS-WT1* (−KTS) fusion proteins bind to EBS, and the binding is KTS dependent. *EWS-WT1* (−KTS) cDNAs, 9/8, 8/8, 7/8, 7/8Δ6, 7/8wΔ9, and 9/8Δ (6–8) were subcloned from pAMP1 into the pCI-neo expression vector (Promega). *EGR-1* cDNA (nucleotides 249-1879; Ref. 38) was subcloned into pcDNA3 (Invitrogen). Constructs were *in vitro* translated using a TNT Coupled Reticulocyte Lysate system (Promega). Specific amounts in μl (indicated on the figure) of translated protein or the TNT lysate were preincubated on ice in a buffer containing 1 μg poly(deoxyinosinic-deoxycytidylic acid):poly(deoxyinosinic-deoxycytidylic acid), 20 mM HEPES (pH 7.9), 70 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.01 mM $ZnCl_2$ and 12% glycerol. An oligonucleotide containing three EBS copies end-labeled with [γ^{32}P]ATP (Amersham) using T4 polynucleotide kinase (~4–7 × 10^5 cpm/ng) was added to the reaction mixture and incubated for 30 min at room temperature. The DNA-protein complexes were resolved on a 5% native polyacrylamide gel (60:1, acrylamide:*N*,*N*′-methylene-bis-acrylamide) at 200–300 V in 0.5 × TBE buffer. Different size bands reflect the size of different *in vitro*-translated proteins, as indicated by the *side bracket*.

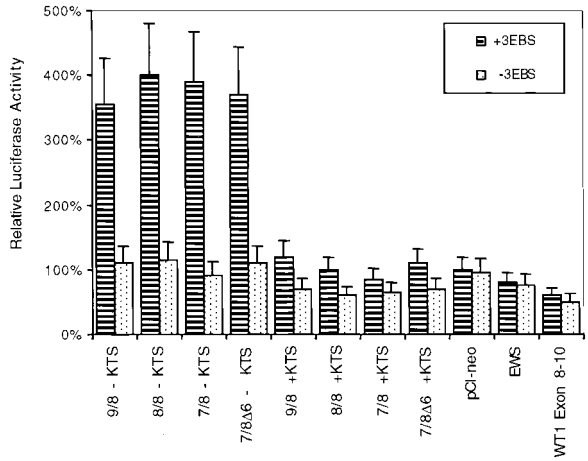

Fig. 4 EWS-WT1 (−KTS) fusion proteins stimulate luciferase reporter activity through EBS. ▤ and ▨, transfection results obtained using the pGL2 Luciferase Reporter Vector (Promega) with or without three EBS repeats, respectively. The following were also cloned into the pCI-neo Mammalian Expression Vector (Promega) as different controls: full-length *EWS-WT1* fusion cDNAs; the full-length EWS coding region; and the exon 8–10 coding region of *WT1*, beginning with an artificially created ATG. The cloned cDNAs were verified by sequence analyses. Transfections were performed in duplicate with 5 × 10^5 Saos-2 cells in six-well plates. Four μg of various *EWS-WT1* and control constructs, 0.5 μg of either plus or minus EBS reporter vectors, and 1 μg of internal control β-galactosidase vector were mixed with 15 μl of lipofectin according to protocol (Life Technologies) for 10 min at room temperature. The mixtures were then added to the cells in 1 ml of 5% FCS containing RPMI 1640. An additional 1 ml of 15% FCS RPMI 1640 was added 14 h after transfection, and the cells were harvested 48 h later for the luciferase activity assay. Transfections were carried out as described in "Materials and Methods."

affinity to another recently defined *WT1/EGR-1* DNA binding motif, 5′-TCCTCCTCCTCCTCTCC-3′ (Ref. 35; data not shown).

Transcription Activation of *EGR-1* Promoter through *EGR-1/WT1* Binding Site by Fusion EWS-WT1 Proteins from DSRCTs. WT1 has been shown to suppress transcription through its binding to EBS (26, 34). To test whether these EWS/WT1 fusion proteins also regulate transcription through this target sequence, a DNA fragment containing three tandem EBS repeats was inserted into the pGL2 luciferase reporter plasmid driven by the SV40 promoter. This reporter construct was then cotransfected into the human osteosarcoma Saos-2 cell line with *EWS/WT1* cDNAs constructs in the pCI/neo expression vector (Promega). As shown in Fig. 4, cotransfection of the (−KTS) *EWS/WT1* expression vectors increased the activity of the EBS-containing reporter promoter 4–5- fold when compared with the promoter without the EBS repeats. The *WT1* 3′ terminus containing the remaining three zinc fingers was essential because *EWS* full-length protein did not transactivate the reporter. In contrast, all *EWS-WT1* constructs with the KTS insert were unable to stimulate promoter activity. The control *WT1* exon 8–10 did not show any regulatory activity because this truncated *WT1* lacks its NH_2-terminal transactivation domain (39, 40). This finding is significant because *WT1* has been shown largely to suppress the transcription. Thus, the NH_2 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-

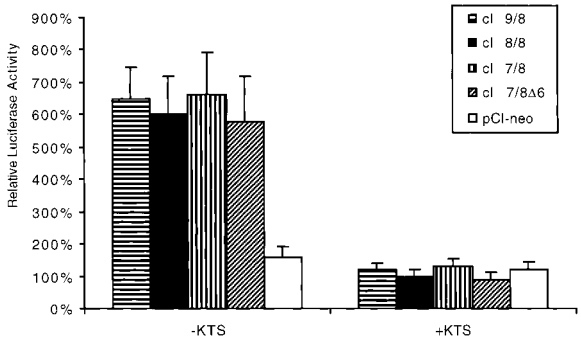

Fig. 5 Up-regulation of *EGR-1* promoter activity by −KTS fusion proteins. ▤, clone 9/8; ■, clone 8/8; ▥, 7/8; ▨, clone 7/8Δ6; □, pCI-neo vector alone. The various −KTS constructs were mixed with the pcDNA3 plasmid containing *EGR-1* promoter region at a ratio of 1:8 (0.5 μg of *EGR-1* promoter plasmid:4 μg of −KTS construct) along with 1 μg of internal control plasmid, β-galactosidase. *Bars*, SD.

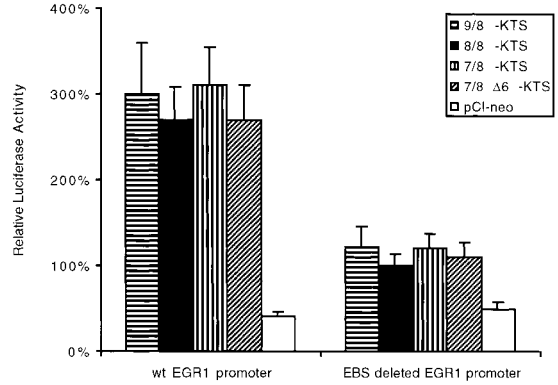

Fig. 6 EWS-WT1 (−KTS) fusion proteins stimulate the promoter activity of *EGR-1* through the EBS. Transfections were carried out using the *EWS-WT1* expression constructs (described in the Fig. 4 legend) and either construct containing the *EGR-1* promoter region corresponding to the transcriptional start site with the EBS (nucleotides −602 to 12), or construct without the EBS (nucleotides −552 to 12), cloned into pcDNA3. Duplicate samples were transfected into Saos-2 cells at a ratio of 1:8 (0.5 μg *EGR-1* construct:4 μg of *EWS-WT1* construct). *Bars*, SD.

ity when the full-length promoter was used compared with one in which only the region containing the EBS site was deleted (Fig. 6). This result confirmed that various *EWS-WT1* (−KTS) chimeric proteins are able to act as transcription activators in regulating *EGR-1* promoter activity. The classic *EGR-1* binding element plays a critical role as the target of the transactivation. In addition to the GC-rich EBS element, there may be other as yet unidentified sequences that can be bound and regulated by the *EWS-WT1* fusion proteins.

DISCUSSION

This report describes the first comprehensive analysis of *EWS-WT1* chimeric cDNA transcripts isolated from 14 adolescent and adult patients diagnosed with DSRCT. There have been sporadic reports of alternatively spliced fusion *EWS-WT1* transcripts from DSRCT patients (4, 10, 41–43). A sufficient large number of tumor samples were collected that appear to represent a wide range of heterogeneity resulting from the chromosomal translocation of DSRCT. In this study, we provide evidence for a remarkable level of molecular diversity that characterizes these tumor-specific fusion transcripts. The fusion transcript heterogeneity was present at several levels. Specifically, at least three types of chimeric *EWS-WT1* RNA transcripts were generated by different combinations of *EWS* exons with *WT1*. The essential structural elements of the chimeric protein included an *EWS* region of variable length combined with three of the four *WT1* DNA-binding zinc finger domains located in exons 8, 9, and 10. The prototypical *EWS-WT1* fusion transcript present in the majority of DSRCTs results from a fusion of the first seven *EWS* exons and *WT1* exons 8–10. This transcript was isolated in 12 of the 14 tumors examined. Fusion transcripts combining *EWS* exons 1–8 or *EWS* exons 1–9 with the 3′ *WT1* region were also identified in one tumor each. The combinatorial variability of *EWS* exons in these DSRCT fusion transcripts was consistent with the extensive *EWS* heterogeneity in *EWS-FLI1* chimeras expressed in Ewing's sarcomas and primitive neuroectodermal tumors. In these tumors, fusions formed by *EWS* exons 6, 7, 9, and 10 have been noted. In contrast to our findings, *EWS* exon 8 has never been joined in-frame with *FLI1* to form a functional protein. Unlike the *EWS-FLI1* fusions, which exhibit heterogeneity 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 (CCAATT) 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 NH_2-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 NH_2 terminus of *EWS* as a transactivation or regulatory domain. The replacement of the NH_2 terminal transcriptional activation domain of transcription factor, *FLI-1*, with the NH_2 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 *ATF-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-ATF-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.

ACKNOWLEDGMENTS

We are grateful to Anna Hughes of the Cooperative Human Tissue Network and Drs. P. Sugarbaker, P. Thomas, J. Porter, C. Roberts, I. Somani, C. Scheurch, M. Zielenska, and M. Kanning for providing tissue samples. We thank Dr. W. Laskin for pathological review of tissues and Dr. J. Powell for computer analysis, discussions, and review of the manuscript.

REFERENCES

1. Ordonez, N. G., Zirkin, R., and Bloom, R. E. Malignant small-cell epithelial tumor of the peritoneum coexpressing mesenchymal-type intermediate filaments. Am. J. Surg. Pathol., *13:* 413–421, 1989.

2. Gonzalez-Crussi, F., Crawford, S. E., and Sun, C-J. Intraabdominal demoplastic small-cell tumors with divergent differentiation. Am. J. Surg. Pathol., *14:* 633–642, 1990.

3. Gerald, W. L., Miller, H. K., Battifora, H., Mirttinen, M., Silva, E. G., and Rosai, J. Intraabdominal demoplastic small round-cell tumor. Am. J. Surg. Pathol., *15:* 499–513, 1991.

4. Gerald, W. L., Rosai, J., and Ladanyi, M. Characterization of the genomic breakpoint and chimeric transcripts in the *EWS-WT1* gene fusion of desmoplastic small round cell tumor. Proc. Natl. Acad. Sci. USA, *92:* 1028–1032, 1995.

5. Parkash, V., Gerald, W. L., Parma, A., Miettinen, M., and Rosai, J. Desmoplastic small round cell tumor of the pleura. Am. J. Surg. Pathol., *19:* 659–665, 1995.

6. Tison, V., Cerasoli, S., Morigi, F., Ladanyi, M., Gerald, W. L., and Rosai, J. Intracranial desmoplastic small-cell tumor. Report of a case. Am. J. Surg. Pathol., *20:* 112–117, 1996.

7. Sawer, J. R., Tryka, A. F., and Lewis, J. M. A novel reciprocal chromosome translocation t(11;22)(p13;q12) in an intraabdominal desmoplastic small round-cell tumor. Am. J. Surg. Pathol., *16:* 411–416, 1992.

8. Biegel, J. A., Conard, K., and Brooks, J. J. Translocation (11;22)(p13;q12): primary change in intra-abdominal desmoplastic small round cell tumor. Genes Chromosomes Cancer, *7:* 119–121, 1993.

9. Rodriguez, E., Sreekantaiah, C., Gerald, W., Reuter, V. E., Motzer, R. J., and Chaganti, R. S. K. A recurring translocation, t(11;22)(p13;q11.2), characterizes intra-abdominal desmoplastic small round-cell tumors. Cancer Genet. Cytogenet., *69:* 17–21, 1993.

10. Ladanyi, M., and Gerald, W. Fusion of the *EWS* and *WT1* genes in the desmoplastic small round cell tumor. Cancer Res., *54:* 2837–2840, 1994.

11. Rauscher, F. J. I., Morris, J. F., Tournay, O. E., Cook, D. M., and Curran, T. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science (Washington DC), *250:* 1259–1262, 1990.

12. Brodie, S. G., Stocker, S. J., Wardlaw, J. C., Duncan, M. H., McConnell, T. S., Feddersen, R. M., and Williams, T. M. *EWS* and *WT-1* gene fusion in desmoplastic small round cell tumor of the abdomen. Hum. Pathol., *26:* 1370–1374, 1995.

13. Delattre, O., Zucman, J., Plougastel, B., Desmaza, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., Aurias, A., and Thomas, G. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumors. Nature (Lond.), *359:* 162–165, 1992.

14. Giovannini, M., Biegel, J. A., Serra, M., Wang, J. Y., Wei, Y. H., Nycum, L., Emanuel, B. S., and Evans, G. A. EWS-erg and EWS-Fli1 fusion transcripts in Ewing's sarcoma and primitive neuroectodermal tumors with variant translocations. J. Clin. Investig., *94:* 489–496, 1994.

15. Sorensen, P. H., Lessnick, S. L., Lopez-Terrada, D., Liu, X. F., Triche, T. J., and Denny, C. T. A second Ewing's sarcoma translocation, t(21;22), fuses the *EWS* gene to another ETS-family transcription factor, ERG. Nat. Genet., *6:* 146–151, 1994.

16. Fujimura, Y., Ohno, T., Siddique, H., Lee, L., Rao, V. N., and Reddy, E. S. The *EWS-ATF-1* gene involved in malignant melanoma of soft parts with t(12;22) chromosome translocation, encodes a constitutive transcriptional activator. Oncogene, *12:* 159–167, 1996.

17. Clark, J., Benjamin, H., Gill, S., Sidhar, S., Goodwin, G., Crew, J., Gusterson, B. A., Shipley, J., and Cooper, C. S. Fusion of the *EWS* gene to CHN, a member of the steroid/thyroid receptor gene superfamily, in a human myxoid chondrosarcoma. Oncogene, *12:* 229–235, 1996.

18. Panagopoulos, I., Hoglund, M., Mertens, F., Mandahl, N., Mitelman, F., and Aman, P. Fusion of the *EWS* and *CHOP* genes in myxoid liposarcoma. Oncogene, *12:* 489–494, 1996.

19. Zucman, J., Melot, T., Desmaze, C., Ghysdael, J., Plougastel, B., Peter, M., Zucker, J. M., Triche, T. J., Sheer, D., Turc-Carel, C., *et al.* Combinatorial generation of variable fusion proteins in the Ewing family of tumours. EMBO J., *12:* 4481–4487, 1993.

20. Ladanyi, M. The emerging molecular genetics of sarcoma translocations. Diagn. Mol. Pathol., *4:* 162–173, 1995.

21. May, W. A., Gishizky, M. L., Lessnick, S. L., Lunsford, L. B., Lewis, B. C., Delattre, O., Zucman, J., Thomas, G., and Denny, C. T. Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. Proc. Natl. Acad. Sci. USA, *90:* 5752–5756, 1993.

22. Lessnick, S. L., Braun, B. S., Denny, C. T., and May, W. A. Multiple domains mediate transformation by the Ewing's sarcoma *EWS/FLI-1* fusion gene. Oncogene, *10:* 423–431, 1995.

23. Bruening, W., Winnett, E., and Pelletier, J. Wilms' tumor: a paradigm for insights into development and cancer. Cancer Investig., *13:* 431–443, 1995.

24. Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., *et al.* Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell, *60:* 509–520, 1990.

25. Gessler, M., Poutska, A., and Cavenee, W. Homozygous deletion in Wilms' tumors of a zinc-finger gene identified by chromosome jumping. Nature (Lond.)., *343:* 774–778, 1990.

26. Madden, S. L., Cook, D. M., Morris, J. F., Gashler, A., Sukhatme, V. P., and Rauscher, F. J., III. Transcriptional repression mediated by the WT1 Wilms' tumor gene product. Science (Washington DC), *253:* 1550–1553, 1991.

27. Haber, D. A., Sohn, R. L., Buckler, A. J., Pelletier, J., Call, K. M., and Housman, D. E. Alternative splicing and genomic structure of the Wilms' tumor gene *WT1*. Proc. Natl. Acad. Sci. USA, *88:* 9618–9622, 1991.

28. Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., *et al.* A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell, *53:* 37–43, 1988.

29. Christy, B. A., Lau, L. F., and Nathans, D. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA, *85:* 7857–7861, 1988.

30. Varnum, B. C., Lim, R. W., Kujubu, D. A., Luner, S. J., Kaufman, S. E., Greenberger, J. S., Gasson, J. C., and Herschman, H. R. Granulocyte-macrophage colony-stimulating factor and tetradecanoyl phorbol acetate induce a distinct, restricted subset of primary-response *TIS* genes in both proliferating and terminally differentiated myeloid cells. Mol. Cell. Biol., *9:* 3580–3583, 1989.

31. Khachigian, L. M. C., Collins, T. Early growth response factor 1: a pleiotropic mediator of inducible gene expression. J. Mol. Med., *76:* 613–616, 1998.

32. Le Beau, M. M., Espinosa, R. D., Neuman, W. L., Stock, W., Roulston, D., Larson, R. A., Keinanen, M., and Westbrook, C. A. Cytogenetic and molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases. Proc. Natl. Acad. Sci. USA, *90:* 5484–5488, 1993.

33. Huang, R. P., Darland, T., Okamura, D., Mercola, D., and Adamson, E. D. Suppression of v-sis-dependent transformation by the transcription factor, Egr-1. Oncogene, *9:* 1367–1377, 1994.

34. Crosby, S. D., Puetz, J. J., Simburger, K. S., Fahrner, T. J., and Milbrandt, J. The early response gene *NGFI-C* encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. Mol. Cell. Biol., *11:* 3835–3841, 1991.

35. Wang, Z. Y., Qiu, Q. Q., Enger, K. T., and Deuel, T. F. A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. Proc. Natl. Acad. Sci. USA, *90:* 8896–8900, 1993.

36. Wright, J. J., Gunter, K. C., Mitsuya, H., Irving, S. G., Kelly, K., and Siebenlist, U. Expression of a zinc finger gene in HTLV-1 and HTLV-II transformed cells. Science (Washington DC), *248:* 588–591, 1990.

37. Sakamoto, K. M., Bardeleben, C., Yates, K. E., Raines, M. A., Golde, D. W., and Gasson, J. C. 5′ upstream sequence and genomic structure of the human primary response gene *EGR-1/TIS8*. Oncogene, *6:* 867–871, 1991.

38. Suggs, S. V., Katzowitz, J. L., Tsai-Morris, C., and Sukhatme, V. P. cDNA sequence of the human cellular early growth response gene *Egr-1*. Nucleic Acids Res., *18:* 4283, 1990.

39. Madden, S. L., Cook, D. M., and Rauscher, F. J., III. A structure-function analysis of transcriptional repression mediated by the WT1 Wilms' tumor suppressor protein. Oncogene, *8:* 1713–1720, 1993.

40. Rauscher, F. J., III. The WT1 Wilms tumor gene product: a developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. FASEB J., *7:* 896–903, 1993.

41. Chan, A. S., MacNeill, S., Thorner, P., Squire, J., and Zielenska, M. Variant EWS-WT1 chimeric product in the desmoplastic small round cell tumor. Pediatr. Dev. Pathol., *2:* 188–192, 1999.

42. Shimizu, Y., Mitsui, T., Kawakami, T., Ikegami, T., Kanazawa, C., Katsuura, M., Obata, K., Yamagiwa, I., and Hayasaka, K. Novel breakpoints of the *EWS* gene and the *WT1* gene in a desmoplastic small round cell tumor. Cancer Genet. Cytogenet., *106:* 156–158, 1998.

43. Antonescu, C. R., Gerald, W. L., Magid, M. S., and Ladanyi, M. Molecular variants of the *EWS-WT1* gene fusion in desmoplastic small round cell tumor. Diagn. Mol. Pathol., *7:* 24–28, 1998.

44. de Alava, E., Ladanyi, M., Rosai, J., and Gerald, W. L. Detection of chimeric transcripts in desmoplastic small round cell tumor and related developmental tumors by reverse transcriptase polymerase chain reaction. A specific diagnostic assay. Am. J. Pathol., *147:* 1584–1591, 1995.

45. Liu, J., Nau, M. M., Zucman-Rossi, J., Powell, J. I., Allegra, C. J., and Wright, J. J. LINE-I element insertion at the t(11;22) translocation breakpoint of a desmoplastic small round cell tumor. Genes Chromosomes Cancer, *18:* 232–239, 1997.

46. Burd, C. G., and Dreyfuss, G. Conserved structures and diversity of functions of RNA-binding proteins. Science (Washington DC), *265:* 615–621, 1994.

47. Ohno, T., Rao, V. N., and Reddy, E. S. EWS/Fli-1 chimeric protein is a transcriptional activator. Cancer Res., *53:* 5859–5863, 1993.

48. Bailly, R. A., Bosselut, R., Zucman, J., Cormier, F., Delattre, O., Roussel, M., Thomas, G., and Ghysdael, J. DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. Mol. Cell. Biol., *14:* 3230–3241, 1994.

49. Karnieli, E., Werner, H., Rauscher, F. J., III, Benjamin, L. E., and LeRoith, D. The IGF-I receptor gene promoter is a molecular target for the Ewing's sarcoma-Wilms' tumor 1 fusion protein. J. Biol. Chem., *271:* 19304–19309, 1996.

50. Zoubek, A., Dockhorn-Dworniczak, B., Delattre, O., Christiansen, H., Niggli, F., Gatterer-Menz, I., Smith, T. L., Jurgens, H., Gadner, H., and Kovar, H. Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients? J. Clin. Oncol., *14:* 1245–1251, 1996.

51. Farhat, F., Culine, S., Lhomme, C., Duvillard, P., Soulie, P., Michel, G., Terrier-Lacombe, M. J., Theodore, C., Schreinerova, M., and Droz, J. P. Desmoplastic small round cell tumors: results of a four-drug chemotherapy regimen in five adult patients. Cancer (Phila.), *77:* 1363–1366, 1996.

52. Kushner, B. H., LaQuaglia, M. P., Wollner, N., Meyers, P. A., Lindsley, K. L., Ghavimi, F., Merchant, T. E., Boulad, F., Cheung, N. K., Bonilla, M. A., Crouch, G., Kelleher, J. F., Jr., Steinherz, P. G., and Gerald, W. L. Desmoplastic small round-cell tumor: prolonged progression-free survival with aggressive multimodality therapy. J. Clin. Oncol., *14:* 1526–1531, 1996.

53. Zucman, J., Delattre, D., Desmaze, C., Plougastel, B., Joubert, I., Melot, T., Peter, M., De Jong, P., Rouleau, G., Aurias, A., *et al.* Cloning and characterization of the Ewing's sarcoma and peripheral neuroepithelioma t(11;22) translocation breakpoints. Genes Chromosomes Cancer, *5:* 271–277, 1992.

54. Gessler, M., Konig, A., and Bruns, G. A. The genomic organization and expression of the *WT1* gene. Genomics, *12:* 807–813, 1992.

Clinical Cancer Research

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

Jun Liu, Marion M. Nau, Jimmy C. Yeh, et al.

Clin Cancer Res 2000;6:3522-3529.