Advances in Brief

Fusion of the EWS and WT1 Genes in the Desmoplastic Small Round Cell Tumor

Marc Ladanyi and William Gerald
Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Abstract

The desmoplastic small round cell tumor (DSRCT) is a recently recognized type of primitive sarcoma defined by a predilection for young males, aggressive clinical behavior, widespread abdominal serosal involvement, and a primitive histological appearance with prominent desmoplasia and striking divergent, multilineage differentiation. Previous cytogenetic case reports have identified a recurrent translocation, t(11;22) (p13;q12). We have characterized this translocation at the molecular level in a panel of five DSRCTs using a candidate gene approach. Southern blot analysis revealed recurrent rearrangement of both EWS, located at 22q12, and rearranged in other tumor-specific translocations in Ewing's sarcoma and clear cell sarcoma, and of WT1, the gene at 11p13 involved in a subset of Wilms' tumor. Consistent clonality of the translocated EWS and WT1 bands in multiple enzyme digests indicated fusion of the genomic sequences, presumably due to the translocation t(11;22) (p13;q12). Northern blotting showed aberrant EWS and WT1 transcripts of the same size, suggesting the presence of a chimeric messenger RNA. This was confirmed by reverse transcriptase polymerase chain reaction using an EWS exon 7 primer and WT1 exon 8 or 9 primers, which revealed single polymerase chain reaction products consistent with a junction of EWS exon 7 to WT1 exon 8. DSRCT thus represents the third primitive sarcoma in which the EWS gene is involved and the first instance of recurrent rearrangement of a tumor suppressor gene, WT1, in a specific tumor type. The different translocation partners of the EWS gene, all of which are putative or definite transcription factor genes, may be responsible for the biological differences between DSRCT, Ewing's sarcoma, and clear cell sarcoma.

Introduction

The DSRCT is a recently identified distinctive malignant neoplasm characterized by a predilection for young males, widespread abdominal serosal involvement not related to a particular organ system, prominent desmoplasia, and aggressive clinical behavior (1). A striking feature of this tumor is frequent divergent, multilineage differentiation, showing epithelial, neural, and myogenic immunophenotypes (1). Its relationship to other primitive tumors affecting young people, such as ES, PNET, Wilms' tumor, and embryonal rhabdomyosarcoma, is still unclear.

Karyotypes have been reported in five cases of DSRCT. In four of these five cases, a clone with a breakpoint in the 22q11.2-13 region was present, and in three of these four cases the clone was present as the translocation t(11;22) (p13;q11.2-12; Refs. 2-5). Band 22q12 is the site of the EWS gene, which is rearranged in the tumor-specific translocations, t(11;22) (q24;q12) and t(12;22) (q13;q12), of ES and CCS (also known as malignant melanoma of soft parts), respectively.

In light of the karyotypic data and the involvement of the EWS gene in two other primitive sarcomas, we examined the configuration of the EWS gene in DSRCT. At 11p13, WT1 was screened as a candidate breakpoint locus because of its involvement in Wilms' tumor, which shares some histopathological features of DSRCT, and because it codes for a transcription factor like other translocation partners of EWS (6, 7). We report evidence of consistent fusion of the EWS and WT1 genes in DSRCT, resulting in a chimeric EWS-WT1 RNA.

Materials and Methods

Frozen tissue or extracted DNA was available in five cases of histologically typical DSRCT. Routine histopathology and immunohistochemical analysis were performed at the time of initial diagnosis as described elsewhere (1). All five cases showed evidence of multiphenotypic differentiation with expression of neural, myogenic, and epithelial lineage markers. Brief clinical and pathological descriptions follow.

Case 1 was a 16-year-old male who presented with pain and an abdominal mass. He was found to have multiple tumors involving the omentum, colon, and pelvis. The tissue studied was obtained from a resection of residual tumor following chemotherapy. The histological appearance of the tumor was characteristic of DSRCT (Fig. 1), and immunohistochemical studies showed expression of neural, myogenic, and epithelial markers in the tumor. Case 2 was a 26-year-old male who presented with low back pain of 2-months duration and a left upper quadrant mass. Imaging studies revealed widespread tumor in lungs, liver, pelvis, and mesentery. The tissue studied was obtained from a diagnostic needle aspiration biopsy. Pathological examination showed a typical DSRCT with myogenic and epithelial differentiation demonstrated by immunohistochemical studies. The clinical and pathological details in Case 3 have been reported previously and illustrated (Case 2 in Ref. 5). Morphologically, this was a classic DSRCT in a 32-year-old male, and the tumor reacted with antibodies to keratin, desmin, and S100 protein. Case 4 was a 17-year-old female who presented with difficulty swallowing. She was found to have a posterior mediastinal tumor encasing the pleura, the hilum of the left lower lobe of lung, and pericardium. The tumor was biopsied, and histopathology showed isolated clusters of DSRCT within normal fibroadipose tissue. Immunohistochemical studies revealed expression of neural and myogenic antigens in the tumor cells. Case 5 was a 24-year-old male who presented with headaches, vomiting, vertigo, and hearing loss and was found to have meningioma-based intracranial mass. Imaging studies showed no evidence of intraabdominal pathology. Histopathology of the resected tumor showed a typical DSRCT with immunoreactivity for keratin and desmin.

Genomic DNA was extracted from snap-frozen tissues by a standard manual organic extraction protocol (8) or on an automated DNA extractor (Model 340A; Applied Biosystems, Foster City, CA) following the manufacturer's protocols. Total RNA was extracted by a modified Chomczynski method (9). Southern blots of genomic DNA digested with EcoRI or HindIII were hybridized overnight with probes radiolabeled to a high specific activity by random priming. The hybridized filters were washed at high stringency and filmed for 1 to 4 days. Placental DNA was used as germline, i.e., normal, control. For Northern blotting, approximately 10 µg of total RNA were electrophoresed in a 1% agarose-2.2 M formaldehyde gel (8). The gel was stained with ethidium bromide for visualization of RNA and then destained prior to capillary transfer.

For the EWS gene, a 741-base pair partial cDNA probe generated by PCR, as described previously (10), was used. This probe corresponds to nucleotides

Received 2/14/94; accepted 4/21/94.

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 Supported by American Cancer Society Clinical Oncology Career Development Award #93-38 (to M. L.).
2 To whom correspondence and requests for reprints should be addressed at Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.
3 The abbreviations used are: DSRCT, desmoplastic small round cell tumor; ES, Ewing's sarcoma; PNET, peripheral neuroectodermal tumor; CCS, clear cell sarcoma; cDNA, complementary DNA; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction.

2837
527 to 1267 of the EWS cDNA and includes exons 7 to 11, which constitute the genomic EWS breakpoint cluster region in ES/PNET (6, 11). In a previous study, this probe was shown to detect EWS rearrangement in 87% of unselected cases of ES or PNET, including all cases with a typical or complex t(11;22) (q24;q12) (10). Southern and Northern blot filters were also rehybridized with a 1.8-kilobase EcoRI fragment of WT33, a WT1 cDNA (gift of K. Call and D. Housman, M.I.T., Cambridge, MA) (12). This WT1 probe hybridizes to all 10 exons of the WT1 gene; almost all EcoRI and HindIII-digested genomic fragments of WT1 are detected, except for a portion of the relatively large fifth intron (13, 14).

RT-PCR was performed on total RNA using the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT), according to the recommended protocol, on an automated thermal cycler (Omnigene; Hybaid, Middlesex, United Kingdom). Reverse transcription was performed for 30 min at 42°C on 1 μg of total RNA using random hexamers. The reverse transcriptase is inactivated at 99°C for 5 min. The PCR reagents were then added. The final MgCl2 concentration was 1.75 mM, and the amount of each primer was 30 pmol/reaction. The cycling parameters were: 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 30 s, and followed by a final extension at 72°C for 5 min. The forward primer for EWS exon 7 was 5'-TCCFACAGCCAAGCTCCAAGTC-3' (primer 22.3 in Ref. 6). The reverse primers for WT1 exons 8 and 9 were 5'-ACCTTGGTTCACACGTCTTGTG-3' and 5'-GACCAGGAACCTTGGCTGAC-3', respectively.

Results

Nongermline bands were identified in multiple enzyme digests on Southern blots probed with EWS in Cases 1, 2, 3, and 5, but not in Case 4 (Fig. 2). Southern blot analysis of the WT1 gene was performed in four cases (cases 1, 3, 4, and 5) and revealed nongermline bands in Cases 1, 3, and 5 (Fig. 3). In all three cases, one or more of the rearranged WT1 bands comigrated with rearranged EWS bands in both enzyme digests (Fig. 3), indicating the presence of a rearrangement between the EWS and WT1 genes. Karyotypic data in Case 3 have been reported previously (see “Discussion”). Cytogenetic analysis was not performed on the other four cases.

High molecular weight RNA was obtained only in Case 3. Northern blotting in this case showed a broad EWS band, suggestive of an aberrant transcript of slightly larger size (approximately 2.5 kilobases) than the expected 2-kilobase EWS transcript (Fig. 4). Rehybridization with a WT1 probe showed a single abnormally sized transcript in Case 3, apparently comigrating with the aberrant EWS transcript. The normal 3-kilobase WT1 transcript was seen in the cell line K562. The finding in Case 3 of aberrant EWS and WT1 transcripts of the same size suggested the presence of a chimeric mRNA resulting from the putative translocation between the EWS and WT1 genes.

RT-PCR using an EWS exon 7 primer and WT1 exon 8 or 9 primers in Cases 1 and 3 revealed identical single PCR products consistent in size with a junction of EWS exon 7 to WT1 exon 8 in both cases (Fig. 5). Sequencing of the PCR product in Case 3 showed an in-frame fusion of EWS exon 7 to WT1 exon 8.5 No RNA and no tissue for RNA extraction were available in Cases 2, 4, and 5.

Discussion

The unique features of DSRCT suggest that it represents a novel, distinct neoplastic entity. Predilection for young adults, especially males, frequent serosal abdominal involvement, prominent desmo-
FUSION OF EWS AND WTI IN DESMOPLASTIC SMALL ROUND CELL TUMOR

Fig. 4. Northern blot analysis of EWS and WTI in Case 3. The positions of the 18S and 28S ribosomal RNA bands are indicated on the left. Approximately 10 μg of total RNA from acute myeloid leukemia cell line K562, cultured fibroblasts, Case 3, and B-cell lymphoma cell line SU-DHL-4 were electrophoresed. Ubiquitous expression of the 2-kilobase EWS transcript is seen in all samples. In addition, Case 3 appears to show an aberrant EWS transcript approximately 2.5-kilobases in size (>). Rehybridization of the 2-kilobase EWS transcript is seen in all samples. In addition, Case 3 appears to show an aberrant EWS transcript approximately 2.5-kilobases in size (>). The transcript in Case 3 (>) is abnormally small (approximately 2.5 kilobases) and appears to comigrate with the aberrant EWS transcript.

plastic response, and the presence of multilineage differentiation, as demonstrated by immunohistochemistry, are helpful in establishing the diagnosis (1). Its histological and clinical attributes do, however, overlap with other primitive tumor types including ES, PNET, rhabdomyosarcoma, rhabdoid tumor, and Wilms' tumor. The precise nosologic position and cell of origin of DSRCT remain controversial, and its molecular pathogenesis is completely unknown. The concept of DSRCT as an entity has recently been strengthened by the identification of a specific recurrent chromosomal abnormality. Cytogenetic data have been published in 5 cases of DSRCT (2—5). The "classic" translocation in DSRCT appears to be t(11;22) (p13;q12), seen in 3 cases (2, 4, 5). A fourth case showed a "variant" t(2;21;22) (3), and the last case (Case 3 in the present report) contained one cell with del(22q12) and one cell with del(11p13) (5).

Chromosome band 22q12 is the site of the EWS gene. This gene is involved in three sarcoma-associated translocations, t(11;22) (q24;q12) and t(21;22) (q22;q12) in ES, and t(12;22) (q13;q12) in CCS (6, 7, 15). Indeed, EWS was first identified by the cloning of the ES translocation; its normal function remains unclear, although one portion appears to encode an RNA binding domain (6). The 11q24 and 21q22 breakpoints in the ES translocations lie respectively within the FLI1 and ERG genes, members of the ETS family of transcription factors (6, 15). The 12q13 breakpoint in CCS involves another transcription factor gene, ATF-1 (7). In all three translocations, the sequence-specific DNA binding domain encoded by a transcription factor gene is juxtaposed to putative regulatory elements in the proximal portion of the EWS gene, replacing the proposed RNA binding domain of the latter (6, 7, 15).

The 22q12 breakpoints in ES/PNET are clustered in a 7-kilobase segment of genomic DNA between exons 7 and 11 of EWS (11, 16). The breakpoints in the two cases of CCS analyzed so far were located in the same region of the EWS gene (7). Using an EWS cDNA probe that spans the breakpoint cluster region, we detected EWS rearrangement in four of five cases of DSRCT. The negative result in Case 4 may have been due to insufficient tumor in the sample submitted for molecular analysis. Indeed, histopathological examination of the remainder of the resected tissue in this case showed only isolated nests of tumor.

Karyotypic information was only available in Case 3 of our five cases. The clonal karyotype was near-tetraploid and contained del(1)(p34), numerical abnormalities, and an unidentified marker chromosome (5). However, the cytogenetic analysis also detected one cell with del(22) (q12) and one cell with del(11) (p13) (5). Although none of the other cases we studied were karyotyped, we hypothesized that a (11;22) (p13;q12) may be present at least in the four rearranged cases, similar to the cytogenetic findings in three of five published cases. In Case 3, this translocation may have been masked as the marker chromosome or present in cells that did not yield analyzable metaphases.

On the assumption that the structure of this translocation involving the EWS gene is similar to the translocations in ES/PNET and CCS, transcription factor genes localized to 11p13 were considered as candidate translocation partners in DSRCT. Putative or definite transcription factor genes mapped to 11p13 include the Wilms' tumor zinc-finger gene, WTI (12), the aniridia paired box and homeobox gene, AN2/PAX6 (17), and TSG2/RHOM2, a LIM-domain gene.

Fig. 5. RT-PCR analysis of chimeric EWS/WTI mRNA in Case 3. The reactions in Lanes 1 and 3 were performed on 1 μg of total RNA from Case 3. The reactions in Lanes 2 and 4 were carried out in the absence of RNA. Lanes 1 and 2 used the EWS primer in combination with WTI exon 8 primer, and Lanes 3 and 4 used the EWS primer in combination with WTI exon 9 primer (see "Materials and Methods"). Lane M contains a DNA size marker, HaeIII-digested PhiX174 DNA (sizes of selected bands indicated in base pairs). In Case 3, a single PCR product was seen with each primer pair (Lanes 1 and 3). The sizes of the products were consistent with a junction of EWS exon 7 to WTI exon 8 (see "Results" and "Discussion"). PCR products of the same size were also obtained in Case 1 with the same primer sets, but no products were seen with RNA from acute myeloid leukemia cell line K562 (results not shown).
involved in the t(11;14) (p13;q11) of T-cell acute lymphoblastic leukemia (18, 19). Wilms' tumor is a primitive renal tumor which may in some cases show heterologous differentiation, somewhat akin to the multilineage differentiation in DSRCT. The WT1 gene is expressed in early stages of renal differentiation and in blastemal-predominant Wilms' tumor (20, 21). For this reason, WT1 was considered a prime candidate translocation partner in 11p13.

Using a WT1 cDNA probe, we detected rearranged bands comigrating with the rearranged EWS bands in multiple enzyme digests in Cases 1, 3, and 5. No WT1 rearrangement was detected in Case 4, which also lacked a detectable EWS rearrangement, possibly because of insufficient viable tumor in the sample studied (see above). The demonstration of comigration of rearranged bands provides strong molecular evidence of a classic or complex t(11;22) (p13;q12), resulting in a rearrangement between EWS and WT1. Furthermore, Northern blot analysis of Case 3 showed aberrant EWS and WT1 transcripts of the same size (2.5 kilobases), suggesting the presence of a chimeric EWS-WT1 RNA species resulting from the translocation. EWS is ubiquitously expressed (6); in contrast, WT1 expression is tissue and developmental stage specific (12). The strong expression of a transcript hybridizing with a WT1 probe in DSRCT may thus be considered significant in itself; in addition, the transcript appears smaller than the known splice variants of WT1 (13). The other translocations involving EWS result in chimeric transcripts (6, 7, 15). That the rearrangement between EWS and WT1 in DSRCT follows the same pattern was confirmed in at least two of our cases by RT-PCR using an EWS exon 7 primer and WT1 exon 8 or 9 primers, which revealed a single PCR product of the same size in both cases. Sequencing of the PCR product showed an in-frame junction of EWS exon 7 to WT1 exon 8. Thus, this chimeric RNA encodes a putative protein in which the RNA-binding domain of EWS is replaced by the three carboxy terminal zinc fingers of the WT1 DNA-binding domain. The structural consequences of this translocation therefore appear similar to the other translocations involving EWS (6, 7, 15).

The cloning of chromosomal translocation breakpoints in sarcomas has begun to provide insights into their pathogenesis and is opening new avenues for molecular diagnosis. The sarcoma translocations cloned thus far appear to involve transcription factor genes (6, 7, 15, 22, 23). The oncogenicity of chimeric transcription factors resulting from tumor-specific translocations is a common theme in hematological neoplasms (24). The promiscuous pairing of the EWS gene with other genes may thus determine the phenotype of several primitive sarcomas. The situation appears analogous to the pairing of the MLL/HRX gene with various partners in different types of acute leukemia (25, 26). The further elucidation of the pathogenesis of DSCRT, a tumor type characterized by divergent, multilineage differentiation should be particularly interesting.

Acknowledgments

We thank Gina Cavalcire and Linhui Cao for technical assistance; Amelia Panico and Kim Kong for photography; and Juan Rosai, R. S. K. Chaganti, Eduardo Rodriguez, V. Tison, and Syed Hoda for providing some of the tissue samples.

References


Fusion of the *EWS* and *WT1* Genes in the Desmoplastic Small Round Cell Tumor

Marc Ladanyi and William Gerald


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/11/2837

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.