Fusion of the NH$_2$-Terminal Domain of the Basic Helix-Loop-Helix Protein TCF12 to TEC in Extraskeletal Myxoid Chondrosarcoma with Translocation t(9;15)(q22;q21)$^1$

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Abstract

Extraskeletal myoid chondrosarcomas (EMCs) are characterized by recurrent t(9;22) or t(9;17) translocations resulting in fusions of the NH$_2$-terminal transactivation domains of EWS or TAF2N to the entire TEC protein. We report here an EMC with a novel translocation t(9;15)(q22;q21) and a third type of TEC-containing fusion gene. The chimeric transcript encodes a protein in which the first 108 amino acids of the NH$_2$-terminus of the basic helix-loop-helix (bHLH) protein TCF12 is linked to the entire TEC protein. The translocation separates the NH$_2$-terminal domain of TCF12 from the bHLH domain as well as from a potential leucine zipper domain located immediately downstream of the breakpoint. These results demonstrate that the NH$_2$-terminal transactivation domains of EWS or TAF2N are not unique in their ability to convert the TEC protein into an oncogenically active fusion protein, and that they may be replaced by a domain from a bHLH protein that presumably endows the fusion protein with similar functions.

Introduction

Previous cytogenetic studies have revealed two recurrent chromosome translocations in EMC, i.e., a t(9;22)(q22;q12) and a t(9;17)(q22;q11) (1–5). Both translocations result in fusion genes in which the TEC gene at q22 is the 3’ partner gene (3–7). TEC encodes an orphan nuclear receptor belonging to the steroid/thyroid receptor super family. In the t(9;22), TEC is fused to the EWS gene at 22q12 (6, 7). The resulting fusion protein consists of the NH$_2$-terminal transactivation domain of EWS linked to the entire TEC protein. EWS, which was originally identified as the target gene rearranged in Ewing’s sarcoma with t(11;22)(q24;q12), encodes a putative RNA binding protein containing a central RNA binding RNP-I motif (8). In the variant translocation t(9;17), the EWS-related gene TAF2N at 17q11 has been shown recently to replace EWS as a fusion partner to TEC (3–5).

The NH$_2$-terminal domain of EWS is also fused to a variety of transcription factors in several other sarcomas, including Ewing’s sarcoma, clear cell sarcoma of tendons and aponeuroses, and desmoplastic small round cell tumor (reviewed in Ref. 9). A third member of the EWS and TAF2N gene family, TLS/FUS, is also involved in a similar gene fusion in myoid liposarcoma in which the NH$_2$-terminal domain of TLS/FUS is linked to the entire coding region of the transcription factor CHOP as a result of a t(12;16)(q13;p11) (reviewed in Ref. 9). Thus, the NH$_2$-terminal transactivation domains of the EWS family of RNA binding proteins are regularly fusion partners of various transcription factors in sarcomas, suggesting that they might have important oncogenic properties. Indeed, it has been shown that EWS-FLI1, TAF2N-FLI1, TLS-CHOP, and EWS-CHOP can transform 3T3 cells and that the transforming activity is dependent on the presence of the NH$_2$-terminal parts of EWS, TAF2N, and TLS (10–12).

We report here a third type of TEC-containing fusion gene occurring in an EMC with a novel translocation t(9;15)(q22;q21). The chimeric transcript encodes a protein in which the NH$_2$-terminal domain of the bHLH protein TCF12 is linked to the entire TEC protein. These findings indicate that the NH$_2$-terminal domains of EWS or TAF2N are not unique in their ability to convert the TEC protein into an oncogenically active fusion protein.

Materials and Methods

Tumor Material and Cytogenetic Analysis. Fresh tumor tissue was obtained from a 71-year-old man who had a 2-year history of a slowly growing right thigh mass. The tumor arose in the vastus lateralis and measured 25 cm in greatest dimension on computed tomography and magnetic resonance imaging. Two additional tumor nodules, 3 and 4 cm each, were found in the pectoralis major muscles. Fine-needle aspiration of the thigh mass revealed a sarcoma. The primary tumor was radically excised, and the chest wall metastases were excised locally. The patient received no adjuvant treatment; he developed skeletal and soft tissue metastases and died 10 months after diagnosis.

Macroscopically, the primary tumor was multinodular, solid, gray-white, and partly hemorrhagic. Histologically, it was characterized by a predominately solid growth of relatively small, uniform cells with minimal cytoplasm; oval, reniform, and clefted nuclei; finely distributed chromatin; and small nucleoli (Fig. 1). Mitotic activity was low. There was abundant extracellular proteinaceous material and a scant amount of basophilic myxoid matrix. The differential diagnosis was broad. The negative panel of immunostains (epithelial, melanocytic, lymphoid, myeloid, muscular, and primitive neuroectodermal markers), strong vimentin positivity, and certain ultrastructural features (abundant mitochondria, glycogen deposits, cytoplasmic projections, irregularly clefted nuclei, and prominent nucleoli) were compatible with the diagnosis of cellular-solid variant of EMC, as described previously (13). The cytogenetic and molecular genetic findings (see below) as well as the ultrastructural detection of scattered neurosecretory granules in the tumor cells were also consistent with this diagnosis (14).

Primary cultures were established from a fresh, unfixed specimen of the primary tumor as described previously (3). Chromosome preparations were made from exponentially growing primary cultures, and these were subsequently G-banded and analyzed using standard procedures.

SKY Analysis. Three- to five-day-old slides were treated with a pepsin solution (12 µg/ml) for 4 min prior to hybridization. The SkyPaint probe used contains a cocktail of 24 differentially labeled, chromosome-specific painting probes (ASI-Applied Spectral Imaging, Ltd., Migdal Ha’Emek, Israel). The

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$^3$The abbreviations used are: EMC, extraskeletal myoid chondrosarcoma; bHLH, basic helix-loop-helix protein; RT-PCR, reverse transcription-PCR; SKY, spectral karyotyping; DAPI, 4’-6-diamidino-2’-phenylindole dihydrochloride.

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conditions for hybridization, posthybridization washes, and detection were essentially as recommended by the manufacturer. Chromosomes were counterstained with DAPI containing an antifade solution. Image acquisition was achieved with the SpectraCube system (ASI) mounted on a Zeiss Axioplan 2 Imaging microscope equipped with a custom designed optical filter cube (SKY-1; Chroma Technology, Brattleboro, VT) and a DAPI filter (15). Analysis of spectral images was performed using the SkyView software (ASI).

RNA Isolation, RT-PCR, and Nucleotide Sequence Analyses. Total RNA was extracted from frozen tumor tissue using the Trizol (Life Technologies, Inc.) method. For cDNA synthesis, 5 μg of total RNA were reverse-transcribed using the SuperScript Preamplification System according to the manufacturer’s manual (Life Technologies, Inc.). An aliquot of 0.25 μg of the resulting first-strand cDNA was amplified using the appropriate primer sets. Thirty-six cycles of PCR (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C) were performed with 1 μl of cDNA in 50 μl reaction volumes. The AmpliTaq Gold (Perkin-Elmer Applied Biosystems) DNA polymerase was used for the amplification reactions. The following EWS-, TAF2N-, TCF12-, and TEC-derived primers were used: EWS ex. 7 fwd, 5’-CCACATGTTACCCACCCCA; TAF477U24, 5’-GAGCAGTCAAATTATGATCAGCAGC; TCF12.1, 5’-GGACTCAGGAAGGCTTGAGTT (3, 6). As control for intact RNA and cDNA, an RT-PCR reaction for expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was performed on all cDNAs used. PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and subsequently sequenced with an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems). The resulting sequences were analyzed using basic local alignment search tool (BLAST) searches (National Center for Biotechnology Information).

Results and Discussion

In an effort to identify novel chromosome translocations and fusion genes in EMC, we have cytogenetically analyzed a series of new EMC cases including a tumor with a novel translocation t(9;15). The karyotype of this tumor was 48, X, −Y, t(9;15)(q22;q21), +12, +der(15)(t9;15)(q22;q21), +19 [8] (Fig. 2A). There were also six cells with a normal karyotype. To confirm the t(9;15) and to search for possible cryptic rearrangements, we also performed SKY analysis. Detailed analysis of the SKY and DAPI band images from eight metaphases corroborated the t(9;15) and the duplication of the der(15) marker as well as the presence of extra copies of chromosomes 12 and 19 (Fig. 2B). No cryptic rearrangements were detected in any of the cells analyzed. To the best of our knowledge, this is the first description of a reciprocal t(9;15)(q22;q21) translocation in EMC, raising the possibility that it represents a third variant translocation.

Interestingly, there are two other known cases of EMC with involvement of chromosomes 9 and 15; one case with a t(9;22;15)(q31; q12.2;q25) (Ref. 16), and another with a t(9;17;15)(q22.q11.2;q22) (Ref. 17). The latter case most likely represents a variant translocation of the recently described t(9;17)(q22;q12) resulting in a TAF2N-TEC fusion gene, whereas the former is probably a variant of the classical t(9;22)(q22;12), resulting in an EWS-TEC fusion gene. However, we found no evidence of rearrangements of chromosomes 17 or 22 using SKY in our case. To exclude the possibility of a cryptic EWS-TEC or TAF2N-TEC fusion, we performed RT-PCR experiments with primers specific for these transcripts. No amplification product was obtained using the TAF2N and TEC primers. However, amplification with the primer set EWS ex. 7 fwd and TEC RevA resulted in a fragment shorter than the known EWS-TEC fusion transcripts (data not shown). Nucleotide sequence analysis of this fragment revealed TEC sequences fused to sequences derived from the TCF12 gene. The TCF12 sequences were fused to TEC 2 nucleotides upstream of the ATG initiation codon. Upstream of the TCF12 sequence was a sequence that did not match any known sequences in GenBank and to which the EWS primer had reannealed. This sequence most likely corresponds to parts of an intron within the TCF12 gene. TCF12 has been mapped previously to 15q21 (18), i.e., to the same band as the breakpoint on 15q in the t(9;15)(q22;q21). Collectively, these observations indicate that TCF12 is the target gene on 15q in the t(9;15).

Fig. 2. A, partial G-banded karyotype showing the t(9;15)(q22;q21) translocation, an extra copy of the der(15) marker, loss of the Y chromosome, as well as trisomy for chromosomes 12 and 19. B, SKY karyotype visualizing each of the 24 human chromosomes in a separate color. All abnormalities detected by G-banding, including the (9;15), could be confirmed by SKY. Translocated chromosome segments are indicated.
To obtain additional evidence supporting the existence of a TCF12-TEC fusion transcript in this EMC, we designed new primers located upstream of the previously identified TCF12 sequence (primers TCF12.1 and TCF12.2) and downstream of the identified TEC sequence (TEC RevD). Amplification with primers TCF12.1 and TEC RevD generated a fragment of 545 bp, consistent with the presence of such a transcript (Fig. 3A). Similarly, amplifications with the TCF12.1 and TCF12.2 primers and the TEC primer RevC resulted in fragments of the expected size, i.e., 472 and 421 bp, respectively (Fig. 3A). In contrast, analysis of control RNA from an EMC with a t(9;22)(q22;q12) and a known EWS-TEC fusion (6) failed to show a TCF12-TEC fusion transcript (data not shown). These observations are consistent with the presence of a chimeric transcript containing 5’ sequences of TCF12 fused to the entire coding region of TEC. The identity of the putative fusion transcript was confirmed by nucleotide sequence analysis of a 421-bp fragment using the TCF12.2 and TEC RevC primers (GenBank accession no. AF289510). This fragment was shown to correspond to a chimeric transcript in which the first 325 nucleotides of the coding sequence of TCF12 were fused in-frame to the first coding exon of TEC (Ref. 19; Fig. 3f). The breakpoint in TEC corresponds to a known fusion point within intron 2 found in both EWS-TEC and TAF2N-TEC gene fusions (3–6). The genomic structure of the TCF12 gene is not known, but it is likely that the breakpoint is located within an intron also in this gene. The translocation separates the NH2-terminal domain of TCF12 from the bHLH domain as well as from a potential leucine zipper domain located immediately downstream of the breakpoint (Fig. 4). The putative TCF12-TEC fusion protein is expected to consist of the first 108 amino acids of the NH2-terminus of TCF12 linked to the entire TEC protein.

TCF12 (also known as HTF4 and HEB) encodes a bHLH transcription factor belonging to the class A family (also referred to as the E-proteins; Refs. 20–22). Other members of this family are E2A (also known as E12 and E47), TCF4 (also known as E2–2 and ITF2), and the Drosophila daughterless protein Da (reviewed in Refs. 21 and 22). The class A bHLH proteins are ubiquitously expressed transcription factors playing a key role in the regulation of cell growth and differentiation (reviewed in Refs. 23 and 24). Recent studies have shown that these proteins can act as general negative regulators of cell proliferation through mechanisms involving both enhancement of the expression of several cyclin-dependent kinase inhibitor genes and promotion of cell death through apoptosis (25). TCF12 binds specifically to oligomers of E-box motifs related to the immunoglobulin enhancer kappa-E2 and the SV40 AP4 site (20). It can also form heterodimers with other bHLH proteins of both class A and class B, including e.g., E2A, TAL1, myogenin, and MyoD (21, 22, 26, 27). TCF12 has been implicated in both myogenesis and hematopoiesis (21, 22, 27). We are unaware of any previous reports implicating TCF12 in tumorigenesis.

The TCF12-TEC fusion generated by the t(9;15) is the first example of a fusion protein in EMC lacking the NH2-terminal parts of EWS or TAF2N. This finding demonstrates that these domains are not unique in their ability to convert the TEC protein into an oncogenically active fusion protein. Interestingly, there is no obvious sequence homology between the NH2-terminal parts of TCF12 and EWS/TAF2N other than that all three domains are serine rich; TCF12 has 19 serine residues among the first 108 amino acids. There are also potential N-glycosylation, protein kinase C phosphorylation, and tyrosine kinase phosphorylation sites present in the NH2-termini of both TCF12 and TAF2N. Whether these sites are functionally significant for the fusion proteins is presently not known. However, it is likely that the NH2-terminal domain of TCF12 contributes functions to the fusion protein similar to the corresponding domains of EWS and TAF2N.

Interestingly, another member of the class A family of bHLH transcription factors is involved in gene fusions in pre-B cell acute lymphoblastic leukemias with t(1;19)(q23;p13) and t(17;19)(q22;p13) translocations (28–30). Both translocations result in fusion of the NH2-terminal transactivating domain of the bHLH protein E2A to the DNA binding domain of either the homeobox containing protein PBX1 or the leucine zipper containing protein HLF. The fusion proteins are strong transactivators of transcription that contribute to leukemogenesis by altering the expression of genes normally responsive to PBX1 and HLF (29). These findings as well as our own findings suggest that other genes encoding bHLH proteins may also be found as 5’ partners in fusion genes in EMCs and other types of sarcomas.

Our findings further emphasize the significance of TEC in the fusion proteins of EMC. The TEC protein is an orphan nuclear receptor highly homologous to two other nuclear receptors, NGFI-B and NURR1 (6, 7, 19). TEC has been implicated previously in

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Fig. 3. Detection of TCF12-TEC fusion transcripts by RT-PCR. Amplification with primer sets specific for the 5’ parts of TCF12 and TEC result in products of 545 bp (primers TCF12.1 and TEC RevD; Lane 1), 472 bp (primers TCF12.1 and TEC RevC; Lane 2), and 421 bp (primers TCF12.2 and TEC RevC; Lane 3), respectively, consistent with a fusion of the first 325 nucleotides of TCF12 to exon 3 of TEC. Lane 4, control PCR reaction with primers TCF12.2 and TEC RevC but without cDNA template. The DNA Molecular Weight Marker VIII (Boehringer Mannheim) was used as a molecular weight marker. R, nucleotide and deduced amino acid sequences of parts of the TCF12-TEC cDNA fragment amplified using the TCF12.2 and TEC RevC primer set. * ATG initiation codon in TEC; vertical line, fusion point between TCF12 and TEC. Primer sequences are underlined.

Fig. 4. Schematic representation of the TEC and TCF12 proteins as well as of the predicted TCF12-TEC fusion protein. Known functional domains are indicated: AD, activation domain; DBD, DNA binding domain; LBD, ligand binding domain; NTD, NH2-terminal domain; LZ, potential leucine zipper domain; CAS, class A-specific domain. Arrow, fusion point.
apoptosis as well as in the control of cell proliferation as an immediate-early-gene product (reviewed in Refs. 19, 31, and 32). Recent studies have shown that the transcriptional activation capacity of TEC is mostly attributable to sequences located within the COOH-terminal domain (32), and that addition of the NH2-terminal domain of EWS to the TEC protein significantly increases its transcriptional activation properties (31). Available data indicate that TEC is expressed in most tissues, with the highest expression levels found in the central nervous system (19, 32). Presumably, the TEC-containing fusion proteins exert their oncogenic activity by activating the expression of TEC target genes not normally expressed in the targeted cells of EMC (31). This is also in line with the previous observation that the unrearranged TEC allele was not expressed in two EMC cases containing EWS-TEC fusions (33).

The diagnosis in the present case was difficult because of the unusual histological appearance of the tumor; thus, the differential diagnosis was broad. Detection of the t(9;15)(q22;q21) and the resulting TCF12-TEC fusion gene strongly supported the diagnosis of EMC. As demonstrated previously for the EWS, TAF2N, and TLS/FUS series of fusion genes, there is clearly a correlation between tumor type and the 3′ partner gene (reviewed in Ref. 9). Fusion genes having TEC as the 3′ partner have thus far been found only in EMC (9). The identification of a third translocation involving TEC in EMC increases the ability to unequivocally establish the diagnosis. Techniques designed to identify these translocations and fusion genes are essential for the definitive diagnosis of morphological variants of EMC as illustrated in this case.

It is unclear whether the three different fusion genes identified in EMC are associated with particular morphological features, such as tumor cellularity and neuroendocrine differentiation. Our previous finding of solid-cellular areas in EMC with EWS-TEC and TAF2N-TEC fusions suggests that this is unlikely. Similarly, the presence of neurosecretory granules appears to be unrelated to the type of fusion gene, because we have observed them in EMC with all three types of fusions. The clinical course of EMC is quite variable, and morphological features are of little prognostic value (13). Whether any of the three fusion genes carry any clinical or prognostic significance is unclear at this time. It is, however, interesting to note that this patient, who had a rapidly progressive clinical course, had a duplication of the der(15) marker chromosome, which is likely to encode the oncogenic fusion protein (3). Further studies of additional cases with long-term follow-up are necessary to address these issues.

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