Recurrence Rearrangement of the Ewing’s Sarcoma Gene, EWSR1, or Its Homologue, TAF15, with the Transcription Factor CIZ/NMP4 in Acute Leukemia

Alessandra Martini, Roberta La Starza, Hilde Janssen, Chrystèle Bilhou-Nabera, Anniek Corveleyen, Riet Somers, Ana Aventin, Robin Foà, Anna Hagemeijer, Cristina Mecucci, Peter Marynen

Center for Human Genetics, University of Leuven, Campus Gasthuisberg O&N06, B-3000 Leuven, Belgium [A.M., H.J., A.C., R.S., P.M.]; Department of Hematology, University of Perugia, Policlinico Monteluce, I-06100 Perugia, Italy [R.L.S., C.M.]; Hematology, University Victor Segalen Bordeaux, Pessac 33 604, Bordeaux, France [C.B-N.]; Hospital de Sant Pau, 08025 Barcelona, Spain [A.A.]; and University “La Sapienza,” 00161 Rome, Italy [R.F.]

Abstract

Fusions of the TET-proteins (TLS/FUS, EWSR1, and TAF15/RBP56) to different transcription factors are involved in various malignancies including Ewing’s sarcoma, primitive neuroectodermal tumors, and acute myeloid leukemia. These are thought to arise through transcriptional deregulation, with the transcription factor defining the tumor phenotype. We show that, as result of a t(12;17)(p13;q11) or its variant t(12;22)(p13; q12), the transcription factor gene CIZ/NMP4 is recurrently involved in acute leukemia through fusion with either EWSR1 or TAF15. The fusions possess transforming properties in NIH3T3 cells but do not affect the expression of CIZ target genes, suggesting a contribution to oncogenesis that is independent of the transactivating properties of the fusion protein. These results also extend the involvement of TET-protein fusions to acute lymphoblastic leukemia and suggest a role for CIZ/NMP4 in lymphoid and myeloid development.

Introduction

Oncogenic fusions involving the RNA-binding proteins of the TET family have been identified in both solid tumors and acute myeloid leukemia and are characterized by a common structure in which the COOH-terminal RNA-binding domain of the TET proteins is replaced by a transcription factor or its DNA-binding domain. The systematic and interchangeable presence of the NH2-terminal transactivation domain of the different TET proteins suggests a common oncogenic mechanism. The tumor phenotype, however, is defined by the transcription factor. Fusions to Ets factors are found in Ewing’s sarcoma. ERG fusions are found in both Ewing’s sarcoma and acute myeloid leukemia. The C/EBP factor CHOP fusions are responsible for myxoid liposarcoma, and fusions with WT1 are found in desmoplastic round cell tumors, ATF1 in clear cell sarcomas, and TEC/NOTCH1 in extraskeletal myxoid chondrosarcoma (1). The molecular mechanisms of transformation by TET fusions are not yet resolved. The TET fusions were reported to bind to the same DNA sequences as the wild-type transcription factors and to act as transcriptional activators of reporter constructs carrying those sequences, a property that is dependent on the presence of the TET-transactivating domain (2, 3). EWSR1-FLI1 induces expression of manic fringe (4) and represses expression of TGF-β type II receptor (5), and both events are linked to transformation. Other target genes of EWSR1-FLI1 include MMP3, EAT2, and CK15 (6). However, evidence was presented that also DNA binding domain-independent pathways are involved in EWSR1-FLI1-mediated oncogenesis (7, 8). More recently, it was reported that TET proteins are involved in pre-mRNA splicing, a process that is affected by oncogenic TET fusions (9–11).

We have collected seven acute leukemia cases with a novel translocation t(12;17)(p13;q11) or its variant t(12;22)(p13;q12) and show that the rearrangements affect the CIZ/NMP4 transcription factor gene on chromosome 12 and the EWSR1 or TAF15 genes on chromosome 17 and 22, respectively. The recurrent TAF15-CIZ and EWSR1-CIZ fusions described here expand the oncogenic properties of TET fusions toward lymphoid malignancies, a phenotype that must be defined by CIZ/NMP4, a transcription factor not yet implicated in hematopoiesis.

Materials and Methods

Patient Material. Seven patients presented with a diagnosis of acute leukemia: case 1, acute undifferentiated leukemia; case 2, acute myeloid leukemia, FAB-M1; cases 3–6, acute lymphoblastic leukemia with pro-B phenotype (CD10+; CD10–); and case 7, a common B-ALL. Their bone marrow karyotype showed either a t(12;17)(p13;q11) in cases 2–6 or a t(12;22)(p13; q12) in cases 1 and 7 in the majority of the metaphases. The patients were diagnosed in Bordeaux, France (case 1); Nantes, France (cases 2); Italy (cases 3–6); and Leuven, Belgium (case 7).

5’ RACE and RT-PCR. 5’-RACE and RT-PCR experiments were performed according to established protocols. mRNA was reverse transcribed with the CIZ-Ex4R1 (5’-GGTCACTGCTGCTCATTGGA) primer. The specific primers used for the 5’-RACE were CIZ-Ex4R2 (5’-TCTGGCAAACAGCTGATCCTC) and CIZ-Ex3R (5’-GAAGCCAGAGTACCGGTGTAGA). The TAF15-CIZ fusion was confirmed by heminested RT-PCR on patient cDNA using primers CIZ-Ex4R1 and CIZ-Ex4R2, in combination with primer TAF2N-Ex3F (5’-GGTATGGACAAGCATTACA). The EWSR1-CIZ fusion transcript was detected with CIZ-Ex3R1 and CIZ-Ex4R2 in combination with EWSR1bF (5’-CCCAGACTGATCCTACAGC). Plasmid Constructs. CIZ was amplified from human bone marrow cDNA with the primers CIZF1F (5’-GAGATCTAATGCGCCAGATAATGGAAGAATCCTACCT) and CIZbR1 (5’-AGGGCCAGACTGCGCAGCTGCTC)–the NH2-terminal region of TAF15 was amplified with TAF2N-F1 (5’-GAGATCTCACCACCATGGCGTCTTCAGTCTGTAGTAATCT) and TAF2N-R1 (5’-AGCCGCTGATCCTGATGCTGAGATTCTGGAAGT). The EWSR1-CIZ fusion transcript was detected with primers EWSR1F1 (5’-GGTATGGACAAGCATTACA). All PCR products were cloned in pGEM-Teasy, and...
both strands were sequenced. Expression constructs for CIZ, EWSR1, and TAF15 proteins. Both TET-proteins have a Ser-Tyr-Gln-Gly-rich (SYQG) transactivating domain at their NH2 terminus, an RNA-binding domain composed of three Arg-Gly-Gly-rich (RGG) regions and an RNA recognition motif (RRM) at the COOH terminal. A zinc finger (ZF) is present, flanked by two of the RGG boxes. CIZ contains leucine-rich (LZ), serine-rich (SR), and proline-rich (PR) domains at the NH2 terminus, followed by a nuclear localization signal (NLS), eight Krüppel-type C2H2 zinc finger domains, and a Gln-Ala repeat (QA) at the COOH terminus. Exon numbers and boundaries are marked below the protein structures. Arrowheads, breakpoints observed in the t(12;17) and t(12;22) leukemia cases. B, structure of the fusion proteins. The fusions are named after the TET-protein present (T or E), followed by the last exon number of the TET gene and the first exon of CIZ present in the fusion. The double arrows show the point of fusion; part of the amino acid sequence of the fusion proteins is shown underneath (TET-protein sequences are in italic, and CIZ sequences are roman). The leukemia cases in which a particular fusion was found are indicated on the right. The dashed lines for the T9/3 fusion show alternative splice forms detected for this fusion in the leukemic cells. C, RT-PCR detection of TAF15-CIZ and EWSR1-CIZ fusion cDNAs. Two rounds of RT-PCR were performed with primers located in the relevant exons of TAF15, EWSR1, and CIZ genes. In case 3, several bands are amplified because of the alternative splicing of TAF15. Product lengths are indicated to the right.

**EMSA.** HEK293T cells were transiently transfected with the pMSCV plasmids using the Fugene 6 Transfection Reagent (Roche) and lysed after 24 h as described (12). DNA probes were prepared by annealing the oligonucleotides 5'-CGCGTCACCTTTTCTAAAAAGACCAG and 5'-CTACGTGCTTCTTGTGAAAAGTTGGA with the 320 to 305 region of the human MMP1 promoter and filling in the 5'-overhangs with Klenow polymerase and [α-32P]dCTP. Mutant probe was prepared by annealing the oligonucleotides 5'-GCTGTCAGAGAGA and 5'-TCTCTGACACAGG.

**Promoter Reporter Assays.** The -296HMAT-Luc reporter construct with the human MMP7 promoter in pGL2Basic was a gift from L. Matrisian (Vanderbilt University, Nashville, TN; Ref. 13). HEK293T cells at 35% confluence were cotransfected with the reporter construct (150 ng), together with each of the expression vectors (or empty pMSCV-puro) and 15 ng of a β-galactosidase expression plasmid, using FuGene-6 Reagent (Roche). After 36 h, cells were lysed in passive lysis buffer (Promega) and assayed for luciferase activity using a commercial assay (Promega). All values were normalized for β-galactosidase activity. Experiments were performed three times, each point in triplicate.

**Transformation Assays.** Viral supernatants were produced in HEK293T cells cotransfected with the pMSCV-puro constructs and the pR6.1MCV packaging vector (a gift from G. Gilliland, Harvard University). NIH3T3 cells were seeded at 2 × 10^5 cells/well of a 6-well plate 24 h before adding the viral supernatants containing 8 µg/ml Polybrene for 4 h. Twenty-four h later, selection with puromycin (2 µg/ml) was started. For the focus formation assay,
the transduced NIH3T3 cells were seeded at a density of $10^6$ cells per 9-cm culture dish in DMEM/10% FCS and cultured for 3 weeks. Medium was replaced every 3.5 days. Soft agar assays were performed as described (7).

RT-PCR Analysis of Gene Expression. RNA was isolated from NIH3T3 or Ba/F3 cells expressing the different fusion genes and treated with DNase (Invitrogen). RT-PCR was performed with oligonucleotides 5'-GAGGAGCTAGCAGGTTATCTCAA and 5'-AGCTACAGTGTTGGAATCAT for Mmp3, 5'-AAGGAGATGATGAGACAGCTT and 5'-GATGCTCGCAACTTCATTTACC for Mmp7, 5'-CCCTCAATGCTGAGCTGTC and 5'-TGTCAGCCTTCTCTGTCA for manic fringe, and 5'-CTGACCAAGCGAGAGGTCTGA and 5'-TCCCTCTTTGGCATAGGTTG for East2. Thirty-five cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C, and extension for 40 s at 72°C were performed. The EWSR1-FLI1 expression plasmid was obtained from C. Denny (UCLA, Los Angeles, CA).

Results and Discussion

Cloning of t(12;17) and t(12;22). Metaphases of an acute myeloid leukemia with t(12;17)(p13;q11) and an acute undifferentiated leukemia with t(12;22)(p13;q12) as the sole anomaly were analyzed by fluorescence in situ hybridization using bacterial artificial chromosome clones mapped to chromosome region 12p13. Clone 433J6 (accession number AC079387) showed split signals in both cases. The 12p breakpoint was narrowed by Southern blot experiments, which, combined with the sequence from 433J6, indicated that a gene highly similar to rat Ciz (p130CAS interacting zinc finger, rCiz; accession number AB019281) was disrupted by both translocations. rCiz was identified as a p130CAS interacting protein (14) and is identical to the NMP4 (15). Next, we obtained two cDNAs of 1.55 and 1.74 kb, identified as a p130CAS interacting protein (14) and is identical to the NMP4 (15). Our data indicate that the transformation properties of the fusions are

leukemia. TET fusions were not described previously in acute lymphoblastic malignancies, and this novel phenotype must be defined by the COOH-terminal partner, Ciz. Bone metabolism is the only physiological process in which Ciz was implicated to date, despite its rather ubiquitous expression (14, 15). Our data indicate that Ciz is also implicated in normal and aberrant lymphoid and myeloid development.

Transforming Properties of the TET-Ciz Fusions. The oncogenic properties of the TET-Ciz fusions were analyzed by overexpression in NIH3T3 and Ba/F3 cell lines. NIH3T3 cells stably expressing TAF15-Ciz or EWSR1-Ciz displayed a clearly transformed phenotype, compared with cells transduced with empty pMSCV-puro vector or cells overexpressing Ciz (Fig. 2). The cells are smaller, grow to a higher density, and form multiple foci. Two distinct populations were observed: cells with a small round cell morphology and spindle-like cells with a "herring-bone" appearance, reminiscent of fibrosarcoma. Interestingly, a similar round cell phenotype was described for EWSR1-FLI1 and EWSR1-ETV1 (16). Controls with constructs expressing exclusively the EWSR1 or TAF15 part of the fusion, or wild-type Ciz, resulted in a normal phenotype (not shown), demonstrating that the transformation properties of the fusions are...
Expression of the fusion constructs in the interleukin 3-dependent murine pre-B lymphoid cell line Ba/F3 failed to induce growth factor independence or increased resistance to apoptosis (not shown).

Transactivating Properties of the TET-CIZ Fusions. CIZ has the properties of a transcription factor; it localizes in focal adhesions and in the nucleus of adherent cell lines, it binds specifically to the (G/C)AAAAA(A) sequence, and overexpression of CIZ was shown to activate MMP1, MMP3, and MMP7 promoters that contain the CIZ consensus sequence (14). To investigate the transactivating properties of TET-CIZ fusions, EMSAs were performed with extracts of HEK293T cells transiently expressing myc-tagged TAF15-CIZ or EWSR1-CIZ. These showed specific binding of the chimeric proteins to DNA with the CIZ consensus binding sequence; a retarded labeled band is observed that can be supershifted with antibodies against the myc-tag and disappears upon addition of unlabeled probe (Fig. 3).

Next, reporter assays were performed. Overexpressed CIZ in HEK293T cells had a weak but reproducible transactivating activity.
It is puzzling that the TET-CIZ fusions do not transactivate reporter constructs carrying CIZ binding sites, although they do bind to the CIZ DNA recognition sequence in EMSA experiments. It is possible that HEK293T cells lack some CIZ- or EWSR1/TAF15-interacting factors, preventing the full transactivating activity of the TET-CIZ proteins. However, it should be noted that HEK293T cells do express factors, preventing the full transactivating activity of the TET-CIZ fusions. Nonetheless, HEK293T cells lack some CIZ- or EWSR1/TAF15-interacting factors, preventing the full transactivating activity of the TET-CIZ fusions.

The transactivation properties of the TET-CIZ fusions were thus further explored, and the transcriptions of the CIZ target mmp7 and of the EWSR1-FLI1 targets mmp3, eat2 (6), and manic fringe (4) were analyzed in NIH3T3 and BA/F3 cells by RT-PCR (Fig. 4). No transcription of any target was seen in NIH3T3 cells expressing either TET-CIZ fusion, whereas a strong induction of mmp3, eat2, and manic fringe transcription was observed in the EWSR1-FLI1-expressing controls. Similarly, no induction of mmp3 or mmp7 was visible in the Ba/F3 cells expressing the TET-CIZ fusions. Interestingly, eat2 and manic fringe were found to be constitutively expressed in this cell line. The semiquantitative character of the RT-PCR did not allow us to determine whether the transcription of these genes was increased. Taken together, these results suggest that the CIZ fusions do not act as transcriptional activators (or repressors) for the Mmp3 or Mmp7 promoters, although they do bind the CIZ consensus sequence, or that MMPs are not the true targets of CIZ. On the other hand, it has been shown that some of the transforming activities of EWSR1-FLI1 fusions are not dependent on DNA binding and transactivation (7, 8). In this regard, the observation that TET proteins do affect pre-mRNA splicing (11, 18), a process that is affected by EWSR1-FLI1 (11, 19), TLS-ERG (9), and EWSR1-NOR1 (10), is of particular interest. Alternative splicing of particular genes can lead to modified functional properties of the gene products, e.g., dominant-negative isoforms of ikaros and a decrease of ikaros activity is associated with blast crisis in chronic myeloid leukemia (20). If transcriptional activation is not the major mechanism of action of TET-CIZ fusions, these could provide an interesting model to investigate alternative oncogenic pathways for TET fusions, such as the effect on pre-mRNA splicing.

Acknowledgments

We thank Jan Cools for thoughtful comments and Drs. G. Specchia, E. Miraglia, J. Reiffers, P. Cony-Makhoul, and A. Uyttebroeck for patient samples.

References

Recurrent Rearrangement of the Ewing's Sarcoma Gene, EWSR1, or Its Homologue, TAF15, with the Transcription Factor CIZ/NMP4 in Acute Leukemia

Alessandra Martini, Roberta La Starza, Hilde Janssen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/19/5408

Cited articles
This article cites 20 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/19/5408.full#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/19/5408.full#related-urls

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, use this link:
http://cancerres.aacrjournals.org/content/62/19/5408.
Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.