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**Abstract**

The poorly differentiated carcinoma with t(15;19)(q13, p13.1) is characterized by its highly aggressive, invariably lethal clinical course. The chromosome 19 translocation breakpoint targets the BRD4 double bromodomain-containing gene, which functions in regulation of cell cycle progression. Herein we demonstrate that BRD4 is fused with nearly the entire transcript of the novel 15q13 gene, NUT (nuclear protein in testis), forming a 6.4-kb fusion oncogene, BRD4-NUT. NUT, like BRD4, is predicted to encode a nuclear protein but, unlike the ubiquitous BRD4 transcript, is expressed only in testis. These findings establish a model to elucidate the oncogenic consequences of unscheduled NUT expression and altered BRD4 function. Very few fusion oncogenes have been identified in epithelial tumors, and BRD4-NUT is the first fusion oncogene mechanism identified in a highly lethal form of carcinoma.

**Introduction**

Translocation t(15;19)(q13, p13.1) characterizes a rare, aggressive, and lethal carcinoma arising in midline organs of young people. The translocation results in a heretofore uncharacterized fusion oncogene. As described previously (1), we have determined that the chromosome 19 translocation breakpoint targets the BRD4 bromodomain gene, whereas the chromosome 15 breakpoint involves a 9-kb region on chromosome band 15q13. BRD4 is expressed normally as two alternative transcripts with identical 5′ ends; but the coding sequence of the longer BRD4 transcript is unperturbed.

In vitro (2, 3) and in vivo (4) studies of the murine BRD4 have revealed a critical role in the regulation of cell cycle progression and cellular proliferation. BRD4 associates with chromatin (3) and binds replication factor C (RFC; Ref. 2). Notably, whereas BRD4 expression regulates G2-M transition, BRD4 overexpression inhibits G1-S phase transition. In addition, in vitro studies (4) suggest a pivotal role for BRD4 in cellular proliferation during embryogenesis.

To fully characterize the molecular mechanism of oncogenesis in t(15;19)-associated carcinomas, we undertook mapping and cloning of the chromosome 15 translocation target. Herein, we demonstrate that the chromosome 15 translocation rearranges the novel gene, NUT, resulting in a BRD4-NUT fusion oncogene. We also report expression profiles for NUT and BRD4 in normal tissues. These studies reveal the first known fusion oncogene in a highly malignant form of epithelial neoplasia.

**Materials and Methods**

*Electronic Sequence Analysis.* Human ESTs in the 15q13 translocation breakpoint (Nop10p region) were identified using the Human Genome Browser (UCSC). Sequence analyses were performed using BLAST [National Center for Biotechnology Information (NCBI)].

*Cell Lines.* We established two rapidly growing, immortal cell lines from t(15;19) carcinomas, both of which have been reported previously (5, 1). The cell lines have been cytogenetically stable, with persistence of the t(15;19) translocation in all cells after more than 20 passages.

*RNA Isolation and RT-PCR.* Polyadenylate-enriched RNA was isolated using Micro FastTrack kit (Invitrogen Corporation, Carlsbad, CA). RT-PCR was performed using the SMART RACE Kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer’s instructions, with first-strand synthesis using SMART adapter primers. BRD4-NUT fusion cDNA was then amplified by nested PCR using ExTag (Takara Bio Inc., Otsu, Shiga, Japan). Primers were chosen using the Whitehead Genome Center Primer3 software, amplified by nested PCR using ExTaq (Takara Bio Inc., Otsu, Shiga, Japan).

*Northern Blot Analysis.* Polyadenylate-enriched RNA was isolated from cultured cells using Micro FastTrack kit (Invitrogen), separated by electrophoresis through a formaldehyde-containing gel, and then transferred to a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). The blot was hybridized with a 400-bp NUT cDNA probe. Details of the oligonucleotide primers are available by request.4

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3 The abbreviations used are: EST, expressed sequence tag; MTN, multiple tissue Northern (blot); UCSC, University of California at Santa Cruz; BLAST, basic local alignment search tool; RT-PCR, reverse transcription-PCR; ET, extraterminal; CBP, Creb binding protein.

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Results

Identification of an EST Flanking the Chromosome 15q13 Breakpoint. Our previously reported Southern blotting analyses (1) narrowed the chromosome band 15q13 breakpoint to a <9-kb region. We searched for genes in this region using the Human Genome Browser (Human Genome Project Working Draft, UCSC). Thereby, we identified a spliced EST (GenBank accession no. AL040312) whose sequence appeared to span the <9-kb translocation breakpoint region. BLAST analysis revealed that this EST overlapped a 2134-bp cDNA clone (GenBank accession No. AL133071) that, as ascertained by 6-frame translation Baylor College of Medicine (BCM) Search Launcher, comprised the 3' coding sequence of a novel gene. Because the eight known ESTs for this gene (GenBank) were from testis cDNA libraries and were also based on Northern blot evidence for testis-restricted expression (see below) and for the presence of typical nuclear localization signals (see below), we have preliminarily named this gene NUT (Nuclear Protein in Testis).

Identification of the BRD4-NUT Fusion Transcript. RT-PCR was performed empirically using NUT reverse primers and BRD4 forward primers (exon 10) predicted, based on our genomic localizations, to be 5' to the BRD4 translocation breakpoint. A 1-kb BRD4-NUT fusion product was amplified readily and reproducibly from each of two t(15;19)-positive cancers, whereas BRD4-NUT RT-PCR products were not obtained from t(15;19)-negative control cDNAs (Fig. 1A). Sequencing revealed that the BRD4-NUT RT-PCR products in both of the t(15;19)-positive tumors were identical, containing in-frame fusion transcripts in which BRD4 exon 10 is fused to NUT exon 2.

RT-PCR using numerous nested forward and reverse primer pairs failed to amplify a NUT-BRD4 product from either of the t(15;19)-positive carcinoma cell lines.

BRD4-NUT, BRD4, and NUT Gene Structures. The t(15;19) translocation breakpoint bisects the BRD4 longer transcript into components encoding amino acids 1–720 and 721-1372. The NH₂-terminal component (Fig. 1B) contains the BRD4 bromodomains and, therefore, might contribute chromatin-binding and potential coactivation functions (6) to the BRD4-NUT fusion oncoprotein. Other BRD4 domains in BRD4-NUT are less well characterized. These include a potential kinase domain (3), an ET protein-protein interaction domain (7), and a serine-rich potential transactivation or corepressor domain (Ref. 8; Fig. 1B).

The chromosome 15 translocation breakpoint separates NUT exon 1 (potentially encoding amino acids 1–5) from exons 2–7 (encoding amino acids 6–1127). Hence, almost the entire NUT coding sequence is contained in the BRD4-NUT fusion transcript. NUT (GenBank accession no. AF482429) is predicted to encode a Mr 120,000 nuclear protein with 64% homology to a novel Mr 66,800 protein encoded by a locus on chromosome 10.

Expression of BRD4-NUT. NUT expression was evaluated by Northern blot analysis in a t(15;19)-positive carcinoma cell line, normal human testis, and in cell lines established from other malignant tumors (Fig. 2A). A 6.4-kb putative BRD4-NUT transcript was expressed in the t(15;19)-positive carcinoma cell line, and a 3.6-kb wild-type NUT transcript was expressed in normal testis. NUT expression was evaluated by Northern blot analysis in a t(15;19)-positive carcinoma cell line, normal human testis, and in cell lines established from other malignant tumors (Fig. 2A). A 6.4-kb putative BRD4-NUT transcript was expressed in the t(15;19)-positive carcinoma cell line, and a 3.6-kb wild-type NUT transcript was expressed in normal testis. NUT expression was evaluated by Northern blot analysis in a t(15;19)-positive carcinoma cell line, normal human testis, and in cell lines established from other malignant tumors (Fig. 2A). A 6.4-kb putative BRD4-NUT transcript was expressed in the t(15;19)-positive carcinoma cell line, and a 3.6-kb wild-type NUT transcript was expressed in normal testis. NUT expression was evaluated by Northern blot analysis in a t(15;19)-positive carcinoma cell line, normal human testis, and in cell lines established from other malignant tumors (Fig. 2A). A 6.4-kb putative BRD4-NUT transcript was expressed in the t(15;19)-positive carcinoma cell line, and a 3.6-kb wild-type NUT transcript was expressed in normal testis.
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Fig. 2. Northern blot analysis of NUT (A), BRD4 (B), and β-actin (C) expression in TY82 t(15;19)-positive cell line, normal testis, and three t(15;19)-negative cancer cell lines. A, a 6.4-kb aberrant NUT transcript is seen in TY82, and a normal 3.6-kb NUT transcript is demonstrated only in testis. B, BRD4 probe hybridizes to the 6.4-kb aberrant transcript in TY82, corroborating a BRD4-NUT fusion transcript. 4.4- and 6.0-kb normal BRD4 transcripts are expressed variably in all of the samples.

Expression of BRD4 and NUT in Normal Tissues. BRD4 and NUT expression were evaluated by MTN II and III (Clontech Laboratories, Inc.). BRD4 4.4- and 6.0-kb transcripts, which likely encode the short and long BRD4 isoforms, respectively, were expressed ubiquitously (Fig. 3A). By contrast, NUT expression was highly restricted. A 3.6-kb NUT transcript was confirmed in normal testis, whereas all other tissues lacked demonstrable NUT expression (Fig. 3B).

Discussion

The studies reported herein demonstrate that the t(15;19) translocation in poorly differentiated carcinoma results in fusion of the BRD4 and NUT genes. BRD4-NUT transcripts were demonstrated by RT-PCR in two t(15;19)-positive cell lines, whereas NUT-BRD4 reciprocal transcripts were not demonstrable in either cell line. Northern blotting confirmed these findings. Therefore, we conclude that BRD4-NUT is the functional fusion oncogene associated with the t(15;19) translocation. Although the oncogenic mechanisms of BRD4-NUT have not yet been determined, the t(15;19) is highly characteristic of a clinically distinctive cancer, and it is likely that BRD4-NUT plays a pivotal pathogenetic role in this extremely lethal form of carcinoma.

Several aspects of these findings are unique and intriguing. The t(15;19)-positive poorly differentiated carcinoma is one of very few epithelial neoplasms that harbor defining translocations (9). Among this limited group, the t(15;19)-positive carcinoma is by far the most clinically aggressive. Most patients die within 3 months (1, 10). Therefore, this cancer provides one of the few models with which to study (a) the role of fusion oncogene mechanisms in epithelial neoplasia, and (b) oncogenic mechanisms of clinically aggressive neoplasia. In particular, the transforming mechanisms of bromodomain oncoproteins, although studied recently in hematological neoplasia (11), have not been evaluated in solid tumors.

Although the clinical ramifications of BRD4-NUT have not been determined in systematic studies, all of the known t(15;19)-positive carcinomas were rapidly metastasizing and extremely lethal. Therefore, we expect that diagnostic recognition of BRD4-NUT will be useful in identifying this clinically aggressive subset of carcinomas. Presumably, individuals with t(15;19)-positive carcinoma require immediate treatment with intensive systemic therapies to attempt ablation of their rapidly progressive disease. Such therapeutic efforts will be enabled by prompt molecular diagnosis of the BRD4-NUT mechanism. The clinical diagnosis of BRD4-NUT oncogenes can be accomplished by several methods. The translocation t(15;19) can be demonstrated in fresh clinical specimens by conventional karyotyping, as has been reported by several groups (10, 12–15). BRD4-NUT genomic rearrangement can

Fig. 3. Evaluation of BRD4 and NUT expression in normal human tissues. Clontech MTNs (MTN II and III) were hybridized with cDNA probes to 5′ BRD4 (A), NUT (B), and β-actin (C). BRD4 4.4 and 6.0 kb transcripts are ubiquitously expressed (A), whereas NUT is expressed exclusively in testis (B).
also be demonstrated, including in frozen or formalin-fixed specimens, by fluorescence in situ hybridization (1). Although not yet evaluated in clinical specimens, we have shown herein that the BRD4-NUT genomic rearrangement can be determined at the transcript level by RT-PCR. Furthermore, it is conceivable that NUT immunohistochemistry might provide a simple alternative to molecular detection, given that NUT expression has been identified thus far only in normal testis and t(15;19)-positive carcinoma.

The COOH-terminal end of BRD4-NUT incorporates almost the entire NUT sequence. Given that NUT expression is restricted to the testis, it is likely that unscheduled NUT expression in the t(15;19)-positive carcinomas results from oncogenic juxtaposition to BRD4, with expression then being regulated by the BRD4 promoter elements. NUT oncogenic function likely results both from ectopic expression in an epithelial cell lineage and from the structural consequences of fusion to BRD4. Expression of NUT protein, therefore, may be a specific marker of the t(15;19) carcinoma, as well as a potential therapeutic target.

BRD4 represents the first known oncogene from the BET family of bromodomain genes, which are defined by the presence of NH₂-terminal bromodomain(s) and an ET domain (7). The intact bromodomain regions and the ET domain are contained in the BRD4 component of the BRD4-NUT fusion oncprotein. The second bromodomain of BRD4 has been shown to directly bind replication factor C, a multi-subunit complex essential for DNA replication, and through this interaction, inhibits G₁-S phase transition when overexpressed (2).

Given the known functions of the longer BRD4 isoform, and the ubiquitous presence of the biologically uncharacterized short isoform, the oncogenic BRD4 mechanisms are likely to be complex in the t(15;19) carcinoma. One scenario that warrants evaluation is that the BRD4 short and long isoforms might have qualitatively different, and even opposing, functions in cell cycle regulation. Such a mechanism could be affected in the t(15;19) carcinomas, in which the translocation transacts the coding sequence of the BRD4 long isoform, without affecting that of the short isoform. These considerations suggest that the t(15;19) translocation breakpoint might abrogate BRD4 long isoform function, and it is significant that the BRD4 component in the BRD4-NUT fusion protein comprises virtually all of the predicted BRD4 short isoform. Therefore, the BRD4-NUT oncoprotein could function as a dominant negative in relationship to the BRD4 long isoform, and might also contribute a gain-of-function equivalent of the BRD4 short isoform. Notably, the BRD4 short and long isoforms are expected to compete for binding to chromatin, given that their bromodomain-containing NH₂-terminal regions are identical. Consequences of BRD4-NUT fusion that might contribute to transforming function could, therefore, include: (a) reduced expression of BRD4 long isoform; (b) increased avidity of BRD4-NUT in chromatin binding, resulting in functional inhibition of the non-fusion BRD4 isoforms; and (c) other altered BRD4 functions, e.g., perturbed interactions with corepressors/coactivators, possibly resulting from the NUT component of the fusion protein.

Although BRD4-NUT is the first example of a bromodomain-containing oncogene in a solid tumor, bromodomain-containing fusion oncoproteins have been characterized in several types of leukemia. For example, the CBF bromodomain-containing protein is rearranged in acute myelogenous leukemia with t(8;16) translocation (11), resulting in a fusion oncogene. MOZ is a transactivating component of the MOZ-CBP complex (16), which is thought to regulate transcription of genes that influence myeloid differentiation programs. In contrast, the MOZ-CBP fusion oncoprotein inhibits AML1-mediated transcription and thereby creates a leukemogenic myeloid differentiation block through dominant-negative effects on AML1 function (16). By analogy, the BRD4-NUT fusion oncoprotein might function in part via perturbed interactions between NUT and NUT-binding proteins. This possibility seems particularly likely in that nearly the entire NUT sequence is included in BRD4-NUT. However, the model of BRD4-NUT in t(15;19) carcinoma differs from that of MOZ-CBP in leukemia, because MOZ-CBP fusion alters the function of proteins that are normally expressed in the transformed cell lineage. In the case of BRD4-NUT, there are presently no clues (other than nuclear localization) as to the normal function of NUT, and there is no evidence that non-oncogenic NUT plays important functional roles, or is even expressed, in epithelial cell lines.

References


