Cloned fusion product from a rare t(15;19)(q13.2;p13.1) inhibit S phase in vitro


Background: Somatically acquired chromosomal translocation is a common mechanism of oncogene activation in many haematopoietic tumours and sarcomas. However, very few recurrent chromosomal translocations have been reported in more common epithelial tumours such as lung carcinomas.

Methods: We established a cell line HCC2429 from an aggressive, metastatic lung cancer arising in a young, non-smoking woman, demonstrating a t(15;19)(q13.2;p13.1). The breakpoints on chromosomes 15 and 19 were cloned using long distance inverse PCR and we determined by RT-PCR that the translocation results in a novel fusion transcript in which the 3' end Brd4 on chromosome 19p is fused to the 5' end of NUT on chromosome 15q.

Results: In total, 128 lung cancer tissues were screened using fluorescent in situ hybridisation, but none of the tumours screened demonstrated t(15;19), suggesting that this translocation is not commonly found in lung cancer. Consistent with previous literature, ectopic expression of wild type Brd4 was shown to inhibit G1 to S progression. However, we also found that the Brd4-NUT fusion augments the inhibition of progression to S phase compared with wild type Brd4.

Conclusion: Alteration in cell cycle kinetics is important in tumorigenesis, although the exact role of Brd4-NUT fusion protein in the pathogenesis of lung cancers remains unclear and need to be further elucidated.

MATERIALS AND METHODS
Cell culture, plasmids, and transfection
The t(15;19) lung cancer cell line HCC2429 was established from a 34 year old, non-smoking woman, with no family history of cancer. We also established the EBV lymphoblastoid cell line (BL2429) from the peripheral blood of the same patient. These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The cell lines 293T, 293H, and NIH3T3 were maintained in Dulbecco’s modified essential medium with 10% fetal calf serum. Brd4 long and Brd4-NUT clones were constructed using IMAGE clone 6287189, and PCR amplification of HCC2429 derived.
cDNA, with NM_058243 (Brd4) and XM_171274 (NUT) as templates. The clones were sequence verified and subcloned into a pGFP<sup>3</sup>-C1 N-terminus fusion vector (Perkin-Elmer Life Sciences, Boston, MA, USA). Plasmid DNA was prepared using standard methods.

Transfection of 293H was accomplished using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, using 12 µg DNA per 100 mm petri dish. After 48 hours, transfected cells were flow sorted for GFP expression. The cells were then harvested at 0 and at 16 hours after being cultured in the presence of complete medium, stained with propidium iodide and analysed by flow cytometry. BrdU incorporation in 3T3 was determined by incubating cells with BrdU (10 µmol/L) for 30 minutes after transfection with the control, wild type Brd4, and fusion constructs. Because of low transfection efficiency with fusion constructs, the cells were flow sorted using an antibody to GFP and fixed in cold 70% ethanol. The cells were then washed with phosphate buffered saline (PBS) and resuspended in 0.1 mol/L borax with 0.025% Tween and 0.025% BSA. The cells were incubated with primary antibody for 30 minutes, followed by two washes with 0.5% bovine serum albumin/PBS, and incubation with the secondary antibody for 30 minutes. The cells were then washed and resuspended in medium containing propidium iodide.

Pulse chase labelling experiments were carried out using 293T cells transfected with wild type Brd4 and fusion constructs. Cells were switched to RPMI medium lacking methionine and cysteine 15 min before the addition of 250 µCi of Expe<sup>35S</sup><sup>35S</sup> Protein Labelling Mix (NEN, Torrance, CA, USA) for 1 hour. The labelling pulse was terminated by replacing labelling medium with complete medium. Following the indicated chase period, the cells were lysed in RIPA buffer (20 mmol/L Tris pH 8.5, 100 mmol/L NaCl, 0.2% sodium deoxycholate, 0.2% NP-40, 0.2% Triton X-100, 0.1% sodium dodecyl sulphate, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulphonyl fluoride, 4 µg of aprotinin/ml, 2 µg of pepstatin/ml), and the lysate was clarified by centrifugation at 14 000 g for 15 minutes and immunoprecipitated with GFP mAb (Zymed, San Francisco, CA, USA) and protein G agarose (Sigma, St. Louis, MO, USA). Radiolabelled proteins found in the immunoprecipitate were resolved by reduced sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the dried gel was autoradiographed.

Long distance inverse PCR cloning of Brd4 rearrangement

High molecular weight DNA from HCC2429 was digested to completion with KpnI and ligated at low concentration to promote the formation of monomeric circles. The DNA was then purified using a Wizard column (Promega, Madison, WI, USA), and 10 ng was amplified in a nested PCR reaction using LA Taq polymerase (Takara Bio, Inc., Japan) and primers designed to anneal to the region between two KpnI sites and within exons 11 and 12 of Brd4. These primers were: external R31546-F2 (5′-GGGAGACAGTAAACAAAAGACAGACGACAGAGG-3′) and external R31546-R2 (5′-ACGTGCAGTGCCGGCCCTCAGGAGAT-3′), and nested primers internal R31546-F3 (5′-AGCTCACAATCCTGTCTTGGACGT-3′) and internal R31546-R3 (5′-GATGCTTGGGTGTTCGTGAGTG-3′). After PCR, aliquots of the reaction were run in a 0.8% agarose gel. The band was then excised from the gel, purified using Qiagen spin columns (Qiagen, Hilden, Germany), and cloned into the Eco RV-digested pBluescript SKIII (+) (Stratagene, La Jolla, CA, USA) plasmid vector. The sequences were checked against the Genbank sequences using the BLAST program from the US National Center for Biotechnology Information.

Northern blot analysis and exon specific RT-PCR

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Northern blot analysis was performed using 10 µg of total cellular RNA per sample and hybridised with a 0.8 kb Brd4 cDNA probe (Genbank accession number, NM_058243) and 0.7 kb NUT cDNA probes (Genbank accession number, AF482429) respectively. Using RT-PCR, the cDNA was synthesised using 5 µg of total RNA with SuperScript II reverse transcriptase (Invitrogen) and oligo-dT primer (Roche Applied Science, Indianapolis, IN, USA). PCR amplification was carried out to confirm the Brd4-NUT fusion transcript. After 35 rounds of PCR (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds), the PCR product was electrophoresed in a 2% agarose gel. The sequences of primers used were Brd4-ex7S (5′-GGATAGCTCCTGCGACAGTGA-3′) and LOC-AS1 (5′-CGGCACATGTTTGCATGTC-3′).

Fluorescence in situ hybridisation analysis using tissue microarrays

Tissue microarrays were prepared from paraffin blocks of 128 lung tumours (52 cases of squamous cell carcinoma, 45 cases of adenocarcinoma, 9 cases of large cell carcinoma, 1 case of small cell carcinoma, 7 cases of carcinoid tumour, and 14 cases that were classified as non-small cell lung cancers without histological subtype) and from 20 normal lung tissues. Samples were collected from surgeries performed at the Vanderbilt University Medical Center between 1989 and 2000. Haematoxylin and eosin stained sections from all tissue blocks were reviewed by a board certified pathologist, and areas to be core for array production were selected with marking ink. Cores 0.6 mm in diameter were taken in triplicate from the selected areas of each specimen and inserted into a recipient paraffin block. From the arrays, 5 µm sections were cut, mounted onto charged slides, and subjected to interphase FISH.

Interphase nuclei were probed using dual colour FISH. For the chromosome 15 probe, we used the BAC clone RP11-70A1 (Research Genetics, Huntsville, AL), which is approximately 300 kb 3′ of NUT. The chromosome 19 cosmid clone R22329, located approximately 300 kb 5′ of Brd4, was obtained from the Lawrence Livermore National Laboratory. Briefly, 2 µg of DNA were labelled by nick translation with either Spectrum Orange labelled or Spectrum Green dUTP (Vysis, Downers Grove, IL, USA). Tissue sections were deparaffinised, followed by washes in 100% ethanol and 1 mol/L sodium thiocyanate for 10 minutes. The sections were then digested with pepsin (4 mg/ml) in 0.2 mol/L HCl for 8 minutes at 37°C, and dehydrated with a graded ethanol series. Prior to hybridisation, the slides were denatured in 70% formamide/2× SSC for 5 minutes at 72°C, dehydrated in graded ethanol, and incubated with a hybridisation mixture consisting of 50% formamide, 2× SSC, 10 µg Cot-1 DNA, and 100 ng of both Spectrum Orange labelled and Spectrum Green labelled probes. After 48 hours of incubation at 37°C, the slides were washed at 45°C in 50% formamide/2× SSC for 10 minutes and counterstained with antifade solution containing DAPI. The slides were examined for fluorescent signals with the MDS system from Applied Imaging. Nuclei in which the nuclear boundaries were broken were excluded from the analysis.

RESULTS

Molecular cloning of t(15;19) breakpoint in HCC2429

We previously described the identification and mapping of a somatically acquired balanced t(15;19) translocation arising in an aggressive, metastatic lung carcinoma. We also localised the breakpoint on chromosome 19p to the 3′ 488 bp region of Brd4 on between two BamHI sites. Using

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positional cloning with fluorescence in situ hybridisation on metaphase arrested cells, the hybridisation signals from cosmid R31546, which contains nearly the entire Brd4 gene, were found on both der(19) and normal chromosome 19. In our Southern blot mapping of the breakpoint region, DNA digestion with KpnI from HCC2429 showed one rearranged band of 2.6 kb and a considerably larger, germline fragment at 4.2 kb. Based on this finding, we elected to clone the rearranged allele using LDI-PCR as previously described.

Using two pairs of nested primers within the two KpnI sites, a 2.2 kb product was amplified (fig 1A, B). Sequence analysis of the LDI-PCR product identified the precise location of the breakpoint on chromosome 19p and chromosome 15q, and the breakpoint on 19p13.1 was determined to be in the intron between exons 11 and 12 of Brd4 (fig 1C). The breakpoint on 15q13.2 is approximately 610 bp upstream of a hypothetical gene, LOC256646. Recently, this hypothetical gene was renamed as NUT (nuclear protein in testis), after it was found to be associated with a t(15;19) in two paediatric head and neck tumours. The function of this gene is unknown, but its expression is reported to be limited to the testis in adults. We also used LDI-PCR to clone the reciprocal breakpoint on der(15). Comparative analysis of the recombined and germ-line region indicated a complex reciprocal recombination involving a short insertion of chromosome 7 between chromosomes 15 and 19 (data not shown). This finding is not unusual as other neoplasia associated translocations such as t(8;14) in Burkitt’s lymphomas, t(9;22) in acute myeloid leukaemia and t(11;22) in Ewing’s sarcomas also are rarely true end to end fusions. Instead, they are often accompanied by additional genomic aberrations, such as deletions, insertions, inversions, and duplications.

Detection of the Brd4-NUT Fusion Transcript

As the breakpoint on chromosome 19 lies between exons 11 and 12 in the Brd4 gene and upstream of transcription start sites of both NUT and NOLA3, we tested whether the translocation resulted in a fusion transcript containing both Brd4 and NUT sequences. A reverse transcription (RT) PCR analysis was performed using a sense primer from Brd4 exon 7 (Brd4-ex7S) and an antisense primer from NUT (LOC-AS1). We were able to readily amplify the predicted 733 bp fragment in HCC2429 containing the t(15;19), but not in the lymphoblastoid cell line, BL2429 (fig 2A). The PCR product was subcloned, and sequence analysis revealed that nucleotide 2380 (exon 11) of Brd4 (GenBank accession no. NM_058243) was fused in frame with nucleotide 172 (exon 9) of NUT (XM_171724), resulting in a novel 5622 bp fusion transcript recently reported in two poorly differentiated carcinoma head and neck tumours with t(15;19)(q13;p13.1). Although the transcriptional direction of NOLA3 on the reciprocal translocation partner was opposite that of Brd4, we examined whether the 3’ region of Brd4 (exons 12-20) could fuse with NOLA3 upstream of NUT and centromeric to the breakpoint. As expected, no product was detected (data not shown), suggesting that the 5’-Brd4-NUT-3’ fusion transcript is the only novel product resulting from the t(15;19).

To examine the expression of the Brd4-NUT fusion transcript, we performed Northern blot analysis using a Brd4 5’-end cDNA probe on total RNA from HCC2429, BEAS-2B (a normal lung epithelial cell line), and two lung cancer cell lines NCI-H1819 and NCI-H460. A 6.4 kb putative Brd4-NUT transcript was expressed only in HCC2429 with t(15;19), whereas a 6.0 kb transcript, but not the 4.4 kb transcript, which probably encode the long and short isoform
respectively, was detected in normal and lung cancer cell lines (fig 3A). Using a NUT cDNA probe, we were able to detect the fusion transcript in HCC2429 (fig 3B) but not the non-rearranged NUT transcript. We analysed NUT expression in 20 lung cancer cell lines by RT-PCR, and no expression was found in any of these samples (data not shown). This finding is consistent with previous reports that NUT expression is confined to the testis, and we show that it is also not commonly expressed in lung cancers.

Fluorescence in situ hybridisation analysis in a lung cancer tissue microarray
To determine the frequency of t(15;19) in lung cancer, we used a paraffin based FISH assay, using cosmid probe R22329 upstream of Brd4 and RP11-70A17 downstream of the NUT gene. As a control, a paraffin embedded cell line, HCC2429, was used, which clearly demonstrated the juxtaposition of the NUT telomeric (green) signal and the Brd4 centromeric (red) signal (fig 3C, arrow). We screened a total of 128 lung cancer specimens including 41 adenocarcinomas and 52 squamous cell carcinomas, but no t(15;19) fusion signal was detected. Of note, polyploidy was frequently observed (fig 3C, lower panel).8 As translocation between chromosomes 15 and 19 appears to be a rare event in lung cancer, we also examined whether the Brd4 locus is a frequent target for rearrangement in lung cancers. We used fluorescent labelled probes telomeric and centromeric to the Brd4 locus about 400 kb apart to screen the 128 lung tumour tissue arrays. No separation of the two signals was seen (data not shown), suggesting that, like t(15;19), rearrangement of this region near Brd4 is also not a common event in lung cancer.

The Brd4-NUT fusion protein localises to the nucleus
To evaluate potential biological activity of the fusion protein, we inserted the Brd4-NUT fusion cDNA clone, and the wild type Brd4 (long isoform) open reading frame into an N-terminal GFP expression vector. Like Brd4, Brd4-NUT localised to the nucleus. The level of fusion protein product was consistently much lower than that observed with Brd4 or GFP alone (fig 4A). In vitro translation of Brd4 short isoform (a rare spliced variant), Brd4, and Brd4-NUT cDNA produced proteins of predicted sizes, as confirmed by Western blot analysis (fig 4B). We placed the fusion construct into different cloning vectors and moved GFP fusion from the C to the N terminal without any appreciable difference in the ability of 293T to express the fusion product (data not shown). Pulse chase analysis with 35S-cysteine and 35S-methionine showed that the low level of fusion product appears to be the result of low synthesis and not of decreased half-life (fig 4C).

The ectopically expressing Brd4-NUT fusion protein inhibits S phase in vitro
Brd4 belongs to the BET family of nuclear proteins that interacts with acetylated chromatin, and overexpression of Brd4 has been reported to inhibit cell cycle progression from G1 to S phase.7 Given the role of Brd4 in cycle cell progression, we examined the effect of the fusion product on cell cycle after synchronisation. Inexplicably low expression of fusion protein with transient transfection required that the transfected cells be flow sorted for GFP expression prior to cycle cell analysis. We examined cell cycle profiles immediately after sorting and 16 hours after incubation with complete medium (fig 5A). After sorting with flow cytometry, the vast majority of cells were arrested in G1. After 16 hours, an appreciable portion of cells transected with the control vector progressed through S phase and into G2/M (black arrow). Consistent with previously published data, ectopic expression of Brd4 in 293H results in the delay of progression...
from G₁ to S, in which many cells have just exited from G₁ (fig 5A, grey arrow) compared with cell lines transfected with vector control. Interestingly, the cells transfected with the Brd4-NUT protein remained in G₀/G₁, suggesting that the fusion protein product from the t(15;19) enhances normal Brd4 function. The effect of ectopic expression of fusion product on the S phase was also determined by measuring BrdU uptake. Compared with VC and wild type Brd4, BrdU incorporation was severely suppressed, suggesting that ectopic expression of fusion product inhibit S phase progression (fig 5B).

DISCUSSION

Common epithelial tumours such as lung cancer frequently exhibit marked chromosome abnormalities, which can be classified broadly into numerical (aneuploidy) and structural alterations (such as deletions, translocations, and amplifications). To date, a large number of recurrent chromosomal translocations has been identified and characterised in leukaemias, lymphomas, sarcomas, and some rare tumours. In the more common epithelial tumours, recurring reciprocal translocations have not been found. In the present study, we have succeeded in localising the precise breakpoint to chromosome 19 between exons 11 and 12 of Brd4 and have determined that exon 11 of the Brd4 gene was fused to the second exon of NUT. In addition, we have determined that the breakpoint chromosome 15 lies 610 bp upstream from the first exon of a gene recently named NUT (nuclear protein in testis). The t(15;19) is an uncommon but recurring reciprocal chromosome translocation observed in highly aggressive epithelial tumours, and to date, 10 cases, including our index case, have been published in the literature. Although Brd4 is reported to have two alternative transcripts, we were not able to detect the short transcript in either normal lung epithelium or cancer cell lines, a finding consistent with the results of Houzelstein et al. Bromodomains are evolutionarily conserved 110 amino acid motifs found in several eukaryotic transcription factors, which interact with acetyl lysine, at least in the context of short histone H3 and H4 peptides. The families of proteins containing bromodomains include most nuclear histone acetyltransferases (HATs), the SWI/SNF family of proteins, the CREB binding protein/p300, and the

Figure 4  Expression of fusion product and wild type Brd4 in transiently transfected 293T. (A) Top, bright field; bottom, dark field. Subcellular localisation of BRD4-NUT and BRD4 long isoform in 293T visualised with fluorescent and bright field microscopy. Similar to BRD4, fusion product is localised in the nucleus. Of note, the level of expression of fusion product is markedly less then that seen with BRD4. The experiment was repeated several times with similar results. In contrast, GFP expression of empty vector is seen in the cytoplasm. (B) Immunoblot using a GFP antibody demonstrates constructs (Brd4, Brd4-short isoform [SI] and Brd4-NUT) of expected size. (C) A 35S-pulse chase analysis demonstrated a much lower level of fusion protein compared with that of wild type Brd4. However, the half life of both proteins appeared to be comparable.
BET proteins. The BET proteins, including Brd4, differ significantly from other bromodomain proteins in their coding sequence and carry two tandem bromodomains with a C-terminal extraterminal domain. Using fluorescence photobleaching, Brd4 was found to interact with acetylated chromatin in living cells in a bromodomain dependent manner, and this interaction was shown to be crucial for progression through the G2/M transition. Furthermore, other investigators have shown that ectopic expression of Brd4 induces G1 arrest and inhibits S phase progression through binding with replication factor C. Thus, it is clear from our data and from the literature that perturbation of Brd4 alters cell cycle kinetics.

The fusion transcript retains both bromodomains and the BET domain in the NH2-terminus. The mechanism by which t(15;19)(q13.2;p13.1) contributes to the tumorigenesis of lung cancer remains unclear. Studies of deletion mutants have shown that the bromodomains are important for chromatin interaction. Interestingly, although the function of the C-terminal domain is relatively unknown, studies of Brd4 deletion mutants retaining both bromodomains but lacking the C-terminal domain enhance binding to chromatin in the presence of trichostatin A, a histone deacetylase inhibitor. Furthermore, the ΔC mutant enhances Brd4 interaction with GTPase activity of SAP-1 and inhibits progression to S phase. These observations suggest that the C-terminal domain negatively regulates Brd4 interactions with chromatin. The loss of the C-terminal domain by the translocation, juxtaposing the NUT transcript, may therefore enhance Brd4 activity. This hypothesis is supported by our observation that the fusion protein inhibits G1/S phase progression to a greater degree than wild type Brd4.

Virtually all known fusion products as a result of recurrent translocation have been demonstrated to have oncogenic activity. Thus, it is unclear how the inhibition of S phase progression by ectopic expression of fusion product can contribute to lung cancer tumorigenesis. Other investigators have shown that Brd4 expression is induced by growth factors and that its normal expression is important for G2 to M progression; whether this observation is part of a positive or negative feedback response is undetermined. In our study, malignant transformation was not observed using transformation assays with NIH3T3 and Rat-1A. In addition, cells transfected with the fusion construct died, or stable transfected cells did not express fusion product (data not shown).

While the low expression of fusion protein may be related to a transfection aberrance unique to this construct, it is possible that Brd4 functions as a tumour suppressor gene, owing to its role in cell cycle arrest, and that the t(15;19) translocation results in haploid insufficiency. Another possibility is that, similar to some fusion oncogenes such as BCR-ABL, further genetic insults are required for transformation Rat-1 cells. The breakpoint on chromosome 19 is 45 kb upstream of Notch3, a gene important in cancer and cell fate determination. This translocation is associated with high
expression of Notch3, a receptor from a family of proteins important in cellular differentiation and cancer. Thus, a cooperative locus effect cannot be discounted. Finally, ectopic expression of cell cycle proteins such as Brd4 in an in vitro system is a highly artificial environment and may not reflect their true function. Thus, in vivo and further confirmatory studies are needed to assess the true role of t(15;19) and Brd4-NUT in neoplastic transformation.

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