Mutation of hCDC4 Leads to Cell Cycle Deregulation of Cyclin E in Cancer

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Abstract

hCDC4, the gene that encodes the F-box protein responsible for targeting cyclin E for ubiquitin-mediated proteolysis, has been found to be mutated in a number of primary cancers and cancer-derived cell lines. We have observed that functional inactivation of hCDC4 does not necessarily correlate with elevated levels of cyclin E in tumors. Here we show, however, that hCDC4 mutation in primary tumors correlates strongly with loss of cell cycle regulation of cyclin E. Similarly, a breast carcinoma-derived cell line mutated for hCDC4 exhibits cell cycle deregulation of cyclin E, but periodic expression is restored by reintroducing hCDC4 via retroviral transduction. Conversely, small interfering RNA-mediated silencing of hCDC4 deregulates cyclin E with respect to the cell cycle. These results indicate that hCDC4 function is an absolute prerequisite for cell cycle regulation of cyclin E levels, and loss of hCDC4 function is sufficient to deregulate cyclin E.

Introduction

In dividing human somatic cells, cyclin E, an activator of cyclin-dependent kinase 2 (Cdk2), is expressed with a distinct cell cycle periodicity, with accumulation beginning late in G1 and decline occurring through S phase (1–3). These kinetics are thought to be the result of periodic transcription of the gene encoding cyclin E combined with activation-specific protein degradation determined by autophosphorylation of cyclin E-Cdk2 complexes on specific threonine residues of cyclin E (4–6). Degradation of phosphorylated cyclin E is then mediated by a protein-ubiquitin ligase of the Skp1-Cull-F-box (SCF) family containing the F-box protein specificity factor hCdc4 (Ref. 7; also known as Fbw7 or hAgo; Refs. 8 and 9). The link between overexpression and/or deregulation of cyclin E and human carcinogenesis (10–14) suggests that proper degradation of cyclin E may be important in preventing malignant transformation. Consistent with this view, mutations in the gene encoding hCdc4, required for targeting phosphorylated cyclin E for ubiquitylation, have been linked to cancer (7, 8, 15). Surprisingly, though, not all tumors mutated for hCDC4 express elevated levels of cyclin E (15). This unexpected finding is potentially explained by reports that cyclin E can be turned over via alternative pathways (16–18). Here we demonstrate, however, that cell cycle regulation of cyclin E depends on hCdc4, suggesting that deregulation of cyclin E expression relative to the cell cycle may be a more critical factor in carcinogenesis than simple overexpression and that cyclin E cell cycle deregulation may be useful as a marker of patient prognosis.

Materials and Methods

Cells and Media. Sum149PT, a breast cancer-derived cell line, was obtained from the University of Michigan Breast Cell Tissue Bank and grown in medium recommended by the supplier. ZR75–1 was obtained from the American Type Culture Collection. ZR75–1, HEK239, and 293 Phoenix cells were grown in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and l-glutamine (Invitrogen). Human telomerase reverse transcriptase-immortalized human mammary epithelial (IME) cells were a kind gift of J. W. Shay (The University of Texas Southwestern Medical Center, Dallas, TX). IME cells were grown in MCDM 131 media (Invitrogen), supplemented with 1% fetal bovine serum (Invitrogen), 10 ng/ml epidermal growth factor (Calbiochem), 30 ng/ml bovine pituitary extract (BD Bioscience), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 5 μg/ml human holo-transferrin, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and l-glutamine (Invitrogen). All cells were grown at 37°C in 5% CO2.

Antibodies. Primary antibodies used in this study are as follows: mouse monoclonal anticyclin E and cyclin A antibodies (19); rabbit polyclonal anti-Cdk2 antibodies (M2; Santa Cruz Biotechnology), and polyclonal antibodies against human hCdc4, described previously (7). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) and are as follows: FITC-conjugated donkey antirabbit IgG and antimouse IgG, Cy3-conjugated donkey antirabbit IgG and antimouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Western blot analysis and immunoprecipitations were performed as described previously (7, 15).

Immunohistochemistry, Immunoprecipitation, and Western Blot Analysis. Paraffin-embedded sections (2 μm) were deparaffinized in xylene and hydrated by a stepwise incubation in 100, 95, and 70% ethanol. Antigen retrieval was performed by boiling sections for 10 min in antigen-unmasking solution (Vector Laboratories, Inc.). Immunohistochemical staining with anticyclin E and anticyclin A antibodies was performed using the ABC kit (Vector Laboratories, Inc.). Western blot analysis and immunoprecipitations were performed as described previously (7, 15).

Immunofluorescence and Deconvolution Microscopy. For immunofluorescence analysis of cyclin E and cyclin A, cells were plated onto hemacytometer glass coverslips (Orbeco Analytical Systems, Inc., Farmingdale, NY) 24–30 h before fixation. Cells were fixed in methanol for an hour at room temperature and then processed for immunofluorescence as described previously (1). Fluorescence data were collected using a DeltaVision wide field optical sectioning microscope system based on an Olympus IX-70 inverted epifluorescence microscope (Applied Precision, Issaquah, WA). Images were captured at intervals of 0.2 μm, using a 60× 1.4 numerical aperture neofluor oil immersion lens, and all images shown were generated from a single central section of the 3-dimensional image stack (z-stack). Images were processed via a constrained iterative deconvolution algorithm.

Recombinant Retrovirus Production and Transduction. Retroviral vector-containing supernatants were obtained after transient transfection of the 293T-derived packaging cell line Phoenix-Ampho with the retroviral plasmid vector pBabe Puro, containing a hCDC4a construct. Sum149PT cells
(1 × 10^6) were transduced, and a stable cell line expressing hCdc4e protein was obtained after selection, as described previously (20).

**RNA Interference.** RNA interference experiments were done by infecting HEK293 cells with pSuperRetro (21), either targeting a region in exon 3 of hCdc4e (AAGGGCCAAACAGCGCCGCA) or enhanced green fluorescent protein (GGTTAGACGCTTCCTCGCCCAA) as a control. Cells were grown in medium containing 250 ng/ml puromycin to select for cells having integrity the construct.

**Laser Scanning Cytometry.** A CompuCyte (Cambridge, MA) laser scanning cytometer equipped with a 20-mW argon-ion air-cooled laser and a 5-mW HeNe laser equipped with a DP11 digital camera (Olympus, Vienna, Austria) were used to measure propidium iodide (PI) and FITC fluorescence. Cross-talk corrections were determined. Scanning was done using the 20× objective. PI fluorescence was used as the contouring parameter. Overlapping nuclei were automatically excluded from the counting by special statistical filters. DNA histograms were generated based on DNA content measured in these cells.

**RT-PCR.** Quantitative RT-PCR for hCdc4e was performed on 100 ng of poly-A+ RNA as a template using primers 5′-ATGGGCCCCGCTCTTCTACTTCATGTC-3′ and 5′-CAGTGTGGCCTTGTTATGCTAC-3′ in a 20-cycle PCR reaction (T_m = 55°C).

**Statistics.** To evaluate the significance of the correlation between hCDC4 mutation and cyclin E deregulation, data from Table 1 were analyzed using Fischer’s exact test. If the criterion for cyclin E deregulation was taken as regional-negative nuclear staining at >50%, the P = 0.009. If the criterion was regional-positive nuclear staining at >50%, the P = 0.003.

### Results and Discussion

Analysis of a set of endometrial tumors indicated that approximately 20% had mutations in hCDC4 with an accompanying loss of heterozygosity (15). However, many of these tumors did not exhibit elevated levels of cyclin E compared with tumors without hCDC4 mutations (Fig. 1A; Table 1; Ref. 15). We therefore sought to determine whether another aspect of cyclin E regulation might be impaired by functional inactivation of hCDC4. Cyclin E expression was analyzed by immunohistochemistry in paraffin-embedded sections of material from six tumors harboring mutations that completely eliminated hCDC4 function and was compared with expression in sections from seven randomly chosen control tumors. hCdc4 exists as three isoforms (α, β, and γ) resulting from the differential splicing of three distinct first exons to 10 common exons (15). Therefore, in addition, three tumors with splice variant isoform-specific hCDC4 mutations were analyzed, two mutated in exon1β and one mutated in exon1α. As a control for the proliferative state of cells in the respective samples, serial sections were analyzed immunohistochemically for expression of cyclin A. Typically, approximately 20% of tumor cell nuclei stained positive for cyclin A, consistent with rapid proliferation (Refs. 22 and 23; Fig. 1, B–D; Table 1). In sections obtained from all but one of the tumors not mutated for hCDC4, a similar percentage of nuclei (approximately 30%) stained positive for cyclin E (Fig. 1E; Table 1). The exception was tumor 9 in Table 1, which was quite heterogeneous for cyclin E staining but which had regions with up to 60% positive nuclei. However, for tumors bearing mutations expected to eliminate all hCdc4 function (i.e., mutations that inactivated all hCdc4 isoforms), higher frequencies of cyclin E staining cells were always observed (Fig. 1F; maximal regional levels of 70–100%; in only one case was a maximal level of 50% observed; 16 in Table 1). Neither of the β-isomorph-specific mutations (7 and 8 in Table 1) showed deregulation of cyclin E, suggesting that this isoform is not involved in cyclin E targeting. However, the α-isomorph-specific mutation (Fig. 1G; 10 in Table 1) showed strong deregulation of cyclin E, indicating that this isoform is required for cyclin E regulation. Regional heterogeneity of staining within individual sections was often observed providing the ranges given in Table 1 and could in some cases be attributed to contamination with nontumor-derived tissue. It is also conceivable that regional heterogeneity of hCDC4 mutation or loss of heterozygosity exists within some tumors. Furthermore, cyclin E could be observed in mitotic cells of hCDC4-mutated tumors, but not in nonmutated tumors (Fig. 1, I and H, respectively), clearly indicating a deregulation of cyclin E expression relative to cell cycle progression. Therefore, although hCDC4 mutation in endometrial tumors does not necessarily lead to elevated expression of cyclin E, it confers a loss of cyclin E cell cycle regulation, with the exception of β-isomorph-specific mutations. Interestingly, tumors that had elevated cyclin E levels but were not mutated for hCDC4 maintained normal cell cycle regulation of cyclin E (Fig. 1E; Table 1). On the other hand, one tumor (tumor 9), which showed some deregulation of cyclin E, had no apparent mutation in the hCDC4 gene. However, initial detection of mutations was based solely on single-strand conformational polymorphism analysis, and only exons showing variant patterns were sequenced. It is conceivable that tumor 9 (or some regions of tumor 9) contains an hCDC4 mutation that was not detected by single-strand conformational polymorphism analysis.

To determine whether mutation of hCDC4 is sufficient to account for loss of cell cycle regulation of cyclin E, we analyzed the relationship between cyclin E periodicity and hCdc4 function in a cell line that could be manipulated for hCDC4 expression. SUM149PT, a breast tumor-derived cell line mutated for hCDC4, which exhibits reduced turnover of cyclin E (7), was shown by immunofluorescence microscopy to express cyclin E in all cells (Fig. 2, A and B). In the same population, only 50% of cells stained positive for cyclin A, a typical of rapidly growing cells in culture (Refs. 22 and 23; Fig. 2A, middle column and Fig. 2B). The same cell line was transduced with a recombinant retrovirus programmed to express the M_r 110,000 (α) isoform of hCdc4, and the transduced cell population was analyzed again for cyclin E expression. Immunoblot analysis of a protein extract obtained from the transduced population (SUM149PT+hCdc4) using anti-hCdc4 antiserum confirmed the restoration of hCdc4 expression (Fig. 2C). Significantly, accumulation of hyperphosphorylated cyclin E, a hallmark of hCdc4 inactivation (15), was reversed in the SUM149PT+hCdc4 population (Fig. 2D). Immunofluorescence analysis of cyclin E in the SUM149PT+hCdc4 population revealed that cell cycle regulation of cyclin E expression was indeed restored. Only 60% of nuclei now stained positively for cyclin E and only 50% of the cyclin A-positive population now stained positively for cyclin E.
E (Fig. 2, bottom row and Fig. 2B). Because cyclin A has been shown to be expressed from the beginning of S through G2 phase (23), these data are consistent with a window of cyclin E expression extending from mid-G1 through mid-S phase (1). Interestingly, a virtually identical pattern of cyclin E and A expression is observed in a randomly chosen mammary carcinoma-derived cell line (ZR75-1) not mutated for hCDC4 (Fig. 2B). Furthermore, a similar but not identical pattern is observed in nontransformed IME cells (Fig. 2A, top row and Fig. 2B). These data collectively confirm that normal cell cycle regulation has been restored. In the case of the human mammary epithelial cells, there is less overlap between cyclin E- and cyclin A-expressing populations, a property characteristic of nontransformed cells when compared with a significant subset of tumor-derived cells (10).

To provide a quantitative assessment of the consequences of re-
technology has been shown previously to result in increased levels of cyclin E, but the effects on cell cycle regulation of cyclin E have not been reported (9). HEK293 cells were transduced with a retrovirus programmed to express a small duplex RNA targeted to the region of hCdc4 mRNA corresponding to exon 3 of the gene, common to all hCdc4 isoforms. Both hCdc4 mRNA and protein were dramatically reduced in the hCdc4-targeted cells relative to control transductions with a retrovirus that targets enhanced green fluorescent protein (Fig. 4A). Immunofluorescence analysis of cyclin E in the respective transduced cells indicates that both the frequency and intensity of cyclin E staining are dramatically increased in response to hCdc4 targeting by small interfering RNA. (Fig. 4, B and C). As in the SUM149PT cell line, knocking down hCdc4 by RNA interference in HEK293 cells results in cyclin E being expressed in every cell (Fig. 4, B and C). Interestingly, whereas telophase cells were never cyclin E-positive in the control cells (Fig. 4D, top row), they were when hCdc4 was knocked down by small interfering RNA transduction (Fig. 4D, bottom row). Thus, loss of hCdc4 function is sufficient to deregulate cyclin E expression relative to cell cycle progression and causes accumulation of cyclin E at inappropriate times.

Because many hCDC4-mutated tumors did not exhibit cyclin E overexpression (15), we sought to determine whether another parameter of cyclin E expression might be perturbed, accounting for a link between hCDC4 mutation and carcinogenesis. In all of the hCDC4-mutated tumors analyzed (except for one marginal case), cyclin E appeared to be deregulated relative to the cell cycle, in that an abnormally high percentage of nuclei stained positively for cyclin E and cells undergoing mitosis contained high levels of cyclin E. Such frequencies of cyclin E staining were rarely observed in tumors not mutated for hCDC4, even those with high levels of cyclin E. Simi-
larly, a mammary adenocarcinoma-derived cell line mutated for hCDC4 showed cell cycle deregulation of cyclin E expression (7). The deregulation of cyclin E throughout the cell cycle without overexpression suggests that temporal misregulation of cyclin E-Cdk2 kinase activity may be more important than quantitative misregulation. Indeed, recent data suggest that cyclin E-Cdk2 activity in late mitosis and early G1 can interfere with prereplication complex assembly and thus cause impairment of DNA replication. 4 Impairment of DNA replication may in turn cause genomic instability, which is likely to promote carcinogenesis. Indeed, deregulation of cyclin E has been shown to induce genomic instability in cultured cells (24). Thus, cell cycle deregulation leading to phosphorylation of regulatory proteins at inappropriate times may be the critical factor in cyclin E-mediated pathology rather than overexpression.

We have reported previously that in endometrial tumors, hCDC4 mutation is correlated with metastasis (15). Although the data were not comprehensive, all tumors with hCDC4 mutation that had been analyzed had metastasized (excluding tumors with mutations in exon 1β). Because metastasis is relatively rare in endometrial cancer (approximately 15% in the non-hCDC4-mutated samples for which analysis had been carried out), this is a significant finding. Therefore, cyclin E cell cycle deregulation, which correlates strongly with hCDC4 mutation (P < 0.01, Fischer’s exact test), may serve as a convenient prognostic marker for individualizing patient treatment.

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References


Correction

In the article by S. Ekholm Reed et al., titled “Mutation of hCDC4 Leads to Cell Cycle Deregulation of Cyclin E in Cancer,” which appeared in the February 1, 2004 issue of Cancer Research (pp. 795–800), a hyphen was omitted from the surname of one of the co-primary authors. The correct author list is as follows: Susanna Ekholm-Reed, Charles H. Spruck, Olle Sangfelt, Frank van Drogen, Elisabeth Mueller-Holzner, Martin Widschwendter, Anders Zetterberg, and Steven I. Reed.
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