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# *hCDC4* Gene Mutations in Endometrial Cancer[1]

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## Abstract

**Cyclin-dependent kinase 2 activated by cyclin E is involved in the initiation of DNA replication and other S phase functions. Consistent with this role, cyclin E protein accumulates at the $G_1$-S phase transition and declines during early S phase. This profile of expression is the result of periodic transcription and ubiquitin-mediated proteolysis directed by $SCF^{hCdc4}$. However, in many types of human tumors cyclin E protein is elevated and deregulated relative to the cell cycle by an unknown mechanism. Here, we show that the F-box protein hCdc4 that targets cyclin E to the SCF (Skp1-Cull-F-box) protein ubiquitin ligase is mutated in at least 16% of human endometrial tumors. Mutations were found either in the substrate-binding domain of the protein or at the amino terminus, suggesting a critical role for the region of hCdc4 upstream of the F-box. *hCDC4* gene mutations were accompanied by loss of heterozygosity and correlated with aggressive disease. The *hCDC4* gene is localized to chromosome region 4q32, which is deleted in over 30% of human tumors. Our results show that the *hCDC4* gene is mutated in primary human tumors and suggest that it may function as a tumor suppressor in the genesis of many human cancers.**

## Introduction

Cyclin E accumulates at the $G_1$-S phase transition and is involved in the initiation of DNA replication and other S phase programs (1–4). Its degradation during early S phase is triggered by phosphorylation on residues Thr 62 and/or Thr 380 and is dependent on SCF[3] ubiquitin ligase activity (5–7). SCF ubiquitin ligases are composed of a core complex of Cul1, Rbx1, and Skp1 linked to a variable component known as an F-box protein, which provides substrate specificity. We (6) and others (8, 9) have recently identified hCdc4 (also designated Fbw7 and Ago) as the F-box protein that targets appropriately phosphorylated cyclin E for SCF-mediated ubiquitination. Although cyclin E protein levels are tightly controlled during the cell cycle, in many types of human tumors the level is often elevated and deregulated relative to the cell cycle (10–12), and this phenotype has been associated with poor patient prognosis (13–16). Furthermore, deregulated cyclin E expression can generate tumors in a mouse model (17). Although the basis for cyclin E-mediated tumorigenesis is not known, the fact that constitutive cyclin E expression causes genomic instability (18) suggests a possible mechanism whereby elevated levels of chromosome loss may accelerate LOH. On the other hand, the molecular mechanism(s) by which cyclin E protein becomes deregulated in tumors has remained elusive. We have observed that elevated cyclin E protein levels in tumor-derived cell lines often occurs without a coordinate increase in cyclin E mRNA, suggesting the involvement of a posttranscriptional process (data not shown). We, therefore, first characterized the human *CDC4* gene and then determined whether *hCDC4* gene mutation could be responsible for the cyclin E phenotype observed in human tumors.

## Materials and Methods

**hCDC4 Genomic Organization.** The *hCDC4* genomic locus was identified using the high throughput genomic sequence database. The *hCDC4* gene was found to be contained within BAC clones RP11–555K12 (200147 bp, GenBank accession no. AC023424) and RP11–461L13 (208580 bp, GenBank accession no. AC080078). The internet tool NIX (Nucleotide Identify X[4], United Kingdom HGMP Resource Centre) was used to aid the identification of several untranslated 5′ exons embedded within a predicted CpG island. RT-PCR was used to confirm all exon predictions.

**Cell Cycle Analysis.** HeLa cells were synchronized by thymidine-nocodazole double block procedure. Treatment with 2 mM thymidine was for 20 h, followed by release for 3 h, then incubation with nocodazole (75 ng/ml) for 12 h. Cells were released from nocodazole, and samples were taken every 1.5 h for 21 h. Cyclin E and PSTAIRE (CDK1 + CDK2) were detected by Western blot using monoclonal antibody HE12 and an anti-PSTAIRE antibody, respectively. hCdc4 protein was detected by immunoprecipitating 500 $\mu$g of lysate with an anti-hCdc4 antibody, followed by Western blotting. Cell cycle progression was monitored by fluorescence-activated cell-sorting analysis.

**Tumor Analysis.** DNA, RNA, and protein were isolated from 51 fresh frozen endometrial adenocarcinomas. Tumors were graded and staged according to International Federation of Gynecologists and Obstetricians guidelines. Protein extracts were prepared in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin-pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM vanadate, and 10 mM NaF]. Phosphorylated cyclin E was detected by subjecting 5–100 $\mu$g of lysate (normalized for cyclin E protein levels) to Western blot analysis (7.5% SDS polyacrylamide gels) using the monoclonal antibody HE12. PCR primers used for SSCP analysis are available upon request. All samples displaying aberrant SSCP banding patterns were confirmed by an independent analysis, followed by DNA sequencing. Corresponding formalin-fixed tumor-free tissues were used as controls. LOH was determined for microsatellite markers D4S1554, D4S1572, D4S1586, D4S1607, D4S1615, D4S171, and D4S2915. The PCR primers and conditions used were as described by the manufacturer (Research Genetics). Quantitative RT-PCR for hCdc4 was performed on 100 ng of poly-A+ RNA as a template and primers 5′-ATGGGCCCTGCTCTTCACTTCATGTCC-3′ and 5′-CACTGTGCGTTGTATGCATC-3′ in a 20-cycle PCR reaction ($T_{an}$ = 55°C). Primers specific for human protein phosphatase 1 were used as a control (Stratagene, La Jolla, CA).

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[3] The abbreviations used are: SCF, Skp1-Cull-F-box; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.

[4] Internet address: http:menu.hgmp.mrc.ac.uk/menu-bin/Nix/Nix.pl.

## Results

**The *hCDC4* Gene Locus.** We searched various genome and expressed sequence tag databases to determine the organization of the *hCDC4* gene (see "Materials and Methods"). The *hCDC4* gene locus maps to chromosome region 4q32, which is frequently deleted in a broad spectrum of human tumor types (19) and is composed of 4 untranslated and 13 coding exons spanning approximately 210 kb of the human genome (Fig. 1*a*). RT-PCR was used to confirm exon order using HeLa cell mRNA as a template (data not shown). Search of the expressed sequence tag databases combined with published reports (6, 8, 9, 20, 21) revealed the existence of three primary splice variants (designated $\alpha$, $\beta$, and $\gamma$). Ten common 3′ exons are alternatively spliced to three different 5′ coding exons. RT-PCR demonstrated that all three variants are expressed in HeLa cells, although the $\gamma$-form was difficult to amplify, suggesting a low abundance (data not shown). Northern blot analysis using probes specific for the various 5′ exons demonstrated that the $\alpha$-splice variant of *hCDC4* is expressed as a 5.5-kb mRNA, whereas the $\beta$- and $\gamma$-forms both are expressed as 4-kb mRNAs (data not shown). A CpG island is present 123 kb upstream of the first coding exon of the $\alpha$-form of hCdc4, and four small noncoding exons are differentially spliced to the $\alpha$-coding exon (data not shown). The $\alpha$-form of hCdc4 was also found to be expressed in all human tissues analyzed, whereas the 4-kb mRNA representing the $\beta$- and/or $\gamma$-forms was present at lower levels, except in skeletal muscle, brain, and to a lesser degree, heart (Fig. 1*b*). Previously, we showed that the $\alpha$-form is also the predominant *hCDC4* mRNA expressed in tumor-derived cell lines (6), although the $\beta$-form could be detected at lower levels. hCdc4 protein levels were found to not vary significantly during the cell cycle in HeLa cells (Fig. 1*c*).

***hCDC4* Gene Analysis in Endometrial Adenocarcinomas.** We next determined whether the *hCDC4* gene is altered in human tumors. Endometrial adenocarcinomas were used in our analysis because previous reports have shown that elevated cyclin E levels occur frequently in these tumors (22) and sufficient tissue was available for protein, RNA, and genomic DNA analysis. Western blot analysis on 51 frozen tumor specimens showed that 8 specimens contained elevated levels of cyclin E protein, and 2 of these tumors showed the phenotype was likely because of genomic amplification of the cyclin E locus (data not shown). Interestingly, we observed an accumulation of phosphorylated cyclin E in seven tumors (Fig. 2*a*). This assessment was based on low mobility on SDS-PAGE and confirmed by phosphatase treatment (Fig. 2*b*). Surprisingly, five of these seven tumors contained only low to moderate levels of cyclin E protein (Table 1).

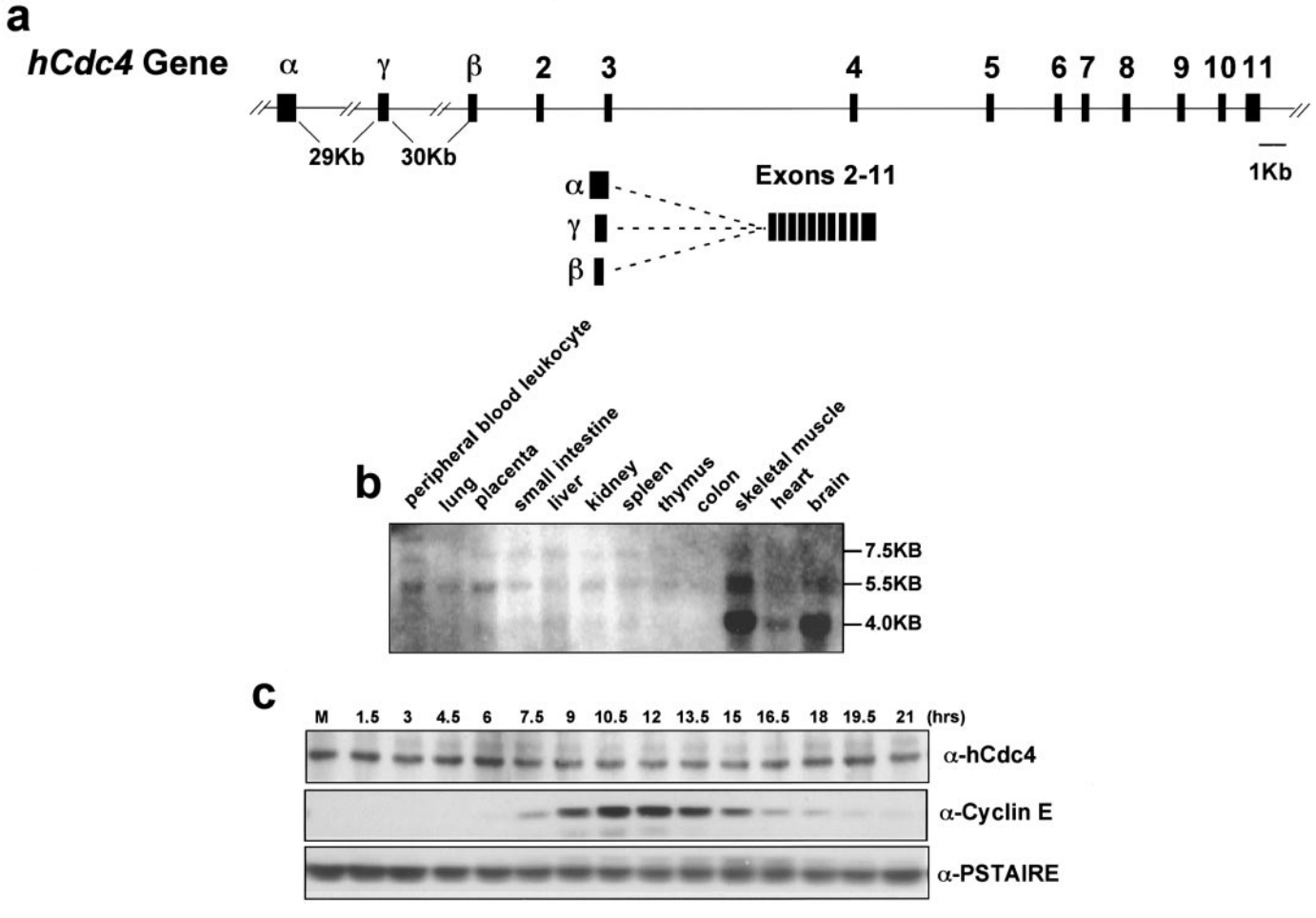


Fig. 1. Genomic organization of the *hCDC4* gene and expression in human tissues and cycling cells. *a*, genomic organization of the *hCDC4* locus localized to chromosome region 4q32. Alternative splicing joins exons 2–11, containing the F-box motif and seven WD40 protein-binding motifs, to one of three different 5′ exons. *b*, tissue blot of hCdc4 in human tissues. A multi-tissue Northern blot was probed with exons 2–11 of the hCdc4 cDNA. The $\alpha$-form of hCdc4 corresponds to the 5.5-kb band, and the $\beta$- and $\gamma$-forms correspond to the 4.0-kb band. *c*, *hCdc4* expression during cell cycle progression. HeLa cells were released from M phase arrest, and samples were taken at the indicated time points. hCdc4 protein was detected by immunoprecipitation/Western blotting. Cells enter S phase of the cell cycle at approximately 10.5 h.

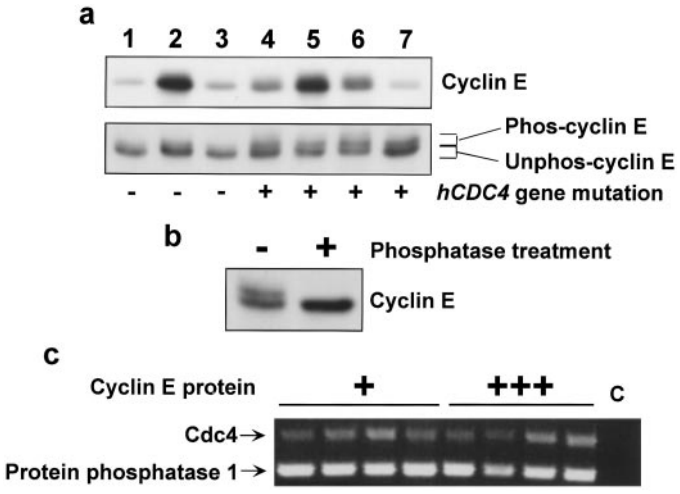


Fig. 2. Analysis of cyclin E and *hCDC4* in endometrial adenocarcinomas. *a*, cyclin E protein levels and phosphorylation status in endometrial adenocarcinomas. Cyclin E protein levels in tumors were determined by Western blotting with anticyclin E antibody (*top*). Cyclin E levels were normalized, and lysates were run on 7.5% SDS-PAGE gels and probed with anti-cyclin E antibody (*bottom*). A slower migrating band is observed in tumors that contained inactivating mutations of hCdc4 (*Lanes 4–7*). *b*, phosphatase treatment of lysate displaying altered mobility of cyclin E. Lysate from tumor in *Lane 6* of *a* was treated with λ-phosphatase for 60 min on ice, run on a 7.5% SDS-PAGE gel, and subjected to Western blot analysis with anti-cyclin E antibody. *c*, RT-PCR analysis of *hCdc4* expression in endometrial adenocarcinomas. RT-PCR reactions were performed on poly-A+ RNA isolated from tumors to determine level of hCdc4 expression as compared with control protein phosphatase 1.

Northern blotting and RT-PCR analysis revealed that *hCDC4* mRNA was expressed over a range of 2–4-fold in all tumors analyzed, and the level of expression did not correlate with cyclin E protein level or phosphorylation status (Fig. 2*c* and data not shown). We performed SSCP analysis of the *hCDC4* gene from tumors that exhibited either elevated cyclin E levels or phosphorylated cyclin E or both (13 of 51). In addition, *hCDC4* SSCP analysis was performed on 15 control tumors that had neither elevated cyclin E levels nor phosphorylated cyclin E. Aberrant SSCP banding patterns, indicative of mutations, were observed in eight tumors (Fig. 3 and Tables 1–3). Interestingly, six of eight tumors with *hCDC4* gene mutations also accumulated phosphorylated cyclin E, and only two of these tumors contained elevated cyclin E protein levels (Table 1 and Fig. 2). A wild-type SSCP banding pattern was observed for 14 of 15 control tumors (with neither elevated nor phosphorylated cyclin E). DNA sequencing demonstrated that six of eight mutations occurred within the seven WD40 repeat domains of hCdc4 that are proposed to be involved in substrate recognition (Table 3). Four mutations introduced a stop codon within the WD40 repeat region. Previously, we have shown that truncations within the hCdc4 protein that delete WD40 repeats eliminate cyclin E binding (6). Furthermore, two missense mutations occurred at Arg residues that are conserved in the Cdc4 homologues of *Saccharomyces cerevisiae*, *Drosophila*, *Caenorhabditis elegans*, and human (6, 9). On the basis of the putative structure of the Cdc4 protein, one of these Arg residues (codon 465) is located on the surface of the β-propeller structure that is proposed to be involved in phosphorylation-dependent recognition of cyclin E (9). Mutation of this residue has previously been shown to abolish cyclin E binding *in vitro* (9). One mutation (Glu→Tyr, codon 124) occurred outside the WD40 repeat regions, in the 5′ exon of the α-form of h*CDC4*. Another mutation was localized to the 5′ exon of the β-form (GTT→ATT, codon 23). This mutation was obtained from one of the "control" tumors that had neither elevated nor phosphorylated cyclin E. Interestingly, of the eight *hCDC4* gene mutations detected, the six that were localized to the WD40 repeat region and, therefore, presumably prevented substrate binding occurred in tumors with an accumulation of phosphorylated cyclin E. In contrast, the two tumors that contained *hCDC4* gene mutations that were localized to the aminoterminal region of hCdc4 and predicted not to affect substrate binding did not accumulate phosphorylated cyclin E (Tables 1 and 3). Thus, the accumulation of phosphorylated cyclin E may depend on the inability of hCdc4 to bind substrate. In addition, because not all of the tumors were subjected to SSCP analysis (only those showing elevated cyclin E levels or increased cyclin E phosphorylation and 15 controls), it is conceivable that additional *hCDC4* mutations were missed. Thus, eight *hCDC4* mutations in 51 tumors is likely a minimal estimate. No mutations were detected in paired normal tissue DNA corresponding to any tumor, confirming that all of the *hCDC4* gene mutations in tumors were of somatic origin (Fig. 3).

The SSCP data suggested that most of the tumors that contained *hCDC4* gene mutations did not retain a wild-type allele of *hCDC4*, as indicated by the absence of a wild-type banding pattern (Fig. 3 and data not shown). We examined these tumors further for LOH of several markers surrounding the *hCDC4* gene on chromosome region 4q32. Evidence of LOH was observed in seven cases where informative heterozygosities were apparent in matched nontumor samples, confirming the loss of the remaining *hCDC4* allele (Fig. 3). A single tumor was noninformative at all loci analyzed.

## Discussion

Our results are the first to demonstrate *hCDC4* gene mutations in primary human tumors and suggest that the F-box protein hCdc4 may function as a tumor suppressor in the genesis of endometrial carcinoma. In most cases, *hCDC4* gene alterations were accompanied by a coordinate LOH of the remaining wild-type allele consistent with Knudson's (23) "two-hit" hypothesis of tumor suppressor genes. *hCDC4* gene mutations were significantly correlated with high-grade (G3) tumors ($P = 0.05$, $\chi^2$ test) and trended toward high-stage tumors ($P = 0.104$). Furthermore, within a subset of patients ($n = 19$) analyzed for evidence of pelvine lymph node involvement, there was a significant correlation with hCDC4 mutations [*i.e.*, 100% (3 of 3) with tumors having *hCDC4* mutations had positive lymph nodes compared with 25% (4 of 16) without mutations ($P = 0.036$, Fisher's exact test)]. Although the data are limited, they suggest that hCdc4 gene mutations may correlate with particularly aggressive disease.

*hCDC4* may also be involved in the genesis of many other tumor types because deletion of chromosome region 4q32 has been

Table 1 *Analysis of endometrial adenocarcinomas*

| No. | Grade/stage | ↑ Cyclin E | Phos-cyclin E | *hCDC4* mutation | Lymph node[a] |
|---|---|---|---|---|---|
| 1 | G2/1.3 | + | − | − | n/a |
| 2 | G3/3.1 | + | − | − | − |
| 3 | G3/1.3 | + | − | − | − |
| 4 | G3/4.2 | + | − | − | n/a |
| 5 | G3/2.2 | + | − | − | n/a |
| 6 | G3/3.3 | + | − | + | + |
| 7 | G3/3.3 | + | + | + | + |
| 8 | G3/1.3 | + | + | + | n/a |
| 9 | G2/3.3 | − | + | + | + |
| 10 | G3/1.3 | − | + | + | n/a |
| 11 | G3/1.3 | − | + | + | n/a |
| 12 | G3/n/a | − | + | + | n/a |
| 13 | G3/2.1 | − | + | − | − |
| 14–51 | Average grade, 1.4<br>Average stage, 2.4 | − | − | 1/15 | 4/13 |

[a] Positive pelvine lymph nodes. n/a, not available.

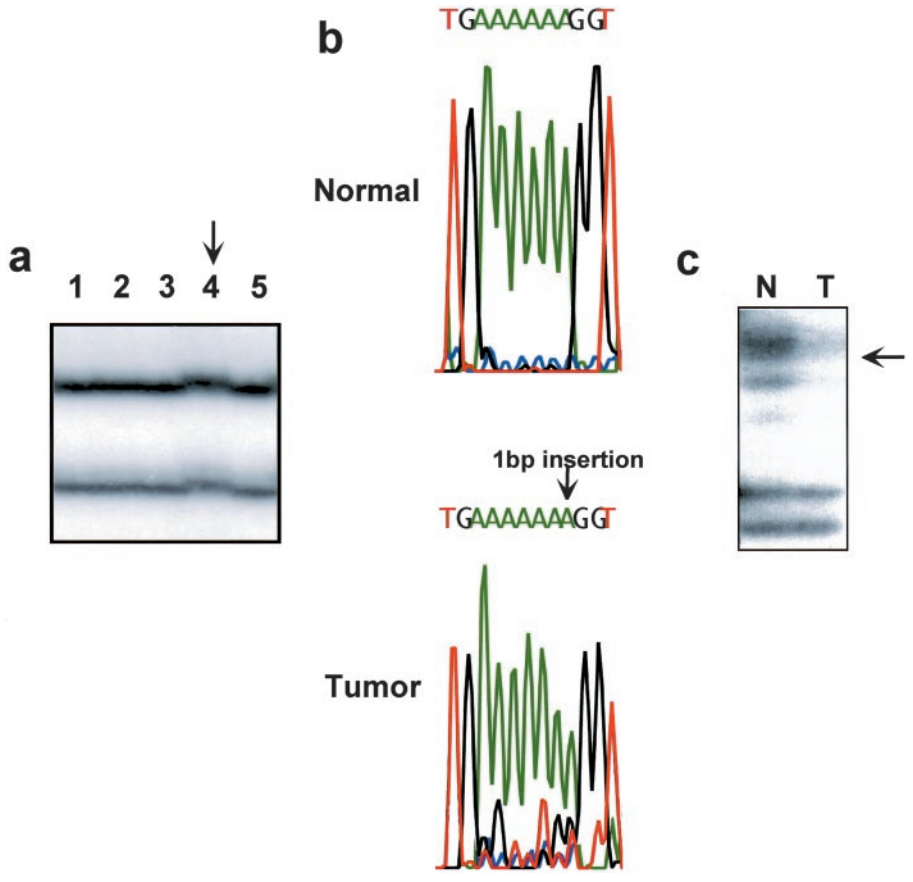


Fig. 3. Genetic analysis of *hCDC4* gene in endometrial adenocarcinoma. *a*, SSCP gel. An altered banding pattern and absence of a normal banding pattern is observed in tumor from *Lane 4* (corresponding to tumor 6 in Table 1). *b*, sequencing chromatogram of tumor displaying altered SSCP banding pattern. A single base (A) insertion at codon 472 in exon 8 introduces a stop codon at codon 476, resulting in a truncated protein. *c*, LOH analysis. PCR-based analysis of microsatellite locus D4S1586 near chromosome region 4q32 demonstrates loss of upper allele and retention of a single mutant allele of *hCDC4*.

reported in 31% of all neoplasms, including 67% of lung cancers, 63% of head and neck cancers, 41% of testicular cancers, and 27% of breast cancers (19). Remarkably, the 16% frequency of *hCDC4* gene alterations detected in this study is comparable with the frequency (17%) of 4q32 deletion reported for endometrial adenocarcinomas (19). Further study is necessary to determine whether the frequency of 4q32 LOH in these other tumor types corresponds to the involvement of *hCDC4* or a yet to be identified tumor suppressor gene.

It is currently unclear how alteration of hCdc4 function contributes to tumorigenesis. Tumors containing *hCDC4* gene mutations within the WD40 binding domains accumulated phosphorylated cyclin E, but four of six of these tumors expressed a low to moderate level of cyclin E protein. These results are unexpected, in that impairment of cyclin E degradation processes would be expected to result in an increase in the steady-state level of cyclin E protein in its phosphorylated form. This was confirmed by experiments where an F-box-deleted and, thus, presumably dominant negative form of hCdc4 was introduced into wild-type cells by adenoviral transduction (6). We have found that cyclin E mRNA levels are not down-regulated in tumors containing *hCDC4* gene mutations (data not shown), eliminating a potential role for transcriptional regulation. Alternatively, other proteolytic mechanisms of cyclin E, possibly the Cul3 pathway (24), could be up-regulated in tumors with *hCDC4* gene mutations, but this hypothesis awaits further investigation.

Normally, cyclin E accumulation is limited to a narrow window at the $G_1$-S phase boundary of the cell cycle (25). Deregulated cyclin E-associated kinase activity relative to the cell cycle and not elevated cyclin E protein *per se* may be the critical link between *hCDC4*

Table 2 *Summary of analysis*

| | Tumor grade (G3/G2) | *hCDC4* gene mutation |
|---|---|---|
| ↑ Cyclin E protein (8) | 7/1 | 3/8[a] |
| Phos-cyclin E (7) | 6/1 | 6/7 |
| Neither (38) | 16/22 | 1/15 |

[a] Two of these tumors also contained phos-cyclin E.

Table 3 *hCDC4 gene mutations in endometrial carcinomas*

| No. | Mutation | Codon[a] | Result | LOH 4q32 |
|---|---|---|---|---|
| 6 | GAG→TAT | 124 | Glu→Tyr | + |
| 7 | AAA→(TC)A (2-bp insertion) | 371 | Lys→Ser, term codon 376 | + |
| 8 | CGA→TGA | 367 | Arg→Ter | + |
| 9 | CGT→CAT | 465 | Arg→His | ni[b] |
| 10 | CGA→CAA | 479 | Arg→Gln | + |
| 11 | CGA→TGA | 658 | Arg→Ter | + |
| 12 | AGA→A(A)G (1-bp insertion) | 472 | Arg→Lys, term codon 476 | + |
| 14 | GTT→ATT | 23[c] | Val→Ile | + |

[a] Codon number based on α-form of *hCDC4*.
[b] ni, noninformative at all loci analyzed.
[c] Mutation in 5′-exon of β-form of *hCDC4*.

mutation and tumorigenesis. In support of this hypothesis, we have found that cyclin E protein is deregulated in a breast tumor-derived cell line containing a *hCDC4* gene mutation.[5] Additional studies are currently underway to determine whether cyclin E expression is deregulated in tumors containing inactivating mutations of *hCDC4*.

One surprising finding of this study is that mutations in the unique domains of either of two alternatively spliced variants of *hCDC4* are associated with tumorigenesis. $\alpha$-Variant-specific mutation is consistent with the observation that this form is predominant in tumors and tumor-derived cell lines, but $\beta$-variant-specific mutation is difficult to rationalize in this context. These data, however, suggest that the functions of these two alternatively spliced variants are not redundant.

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