CDC4 Mutations Occur in a Subset of Colorectal Cancers but Are Not Predicted to Cause Loss of Function and Are Not Associated with Chromosomal Instability

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Abstract

CDC4/FBXW7 is part of a ubiquitin ligase complex which targets molecules such as cyclin E, c-myc, and c-jun for destruction. *CDC4* mutations occur in several cancer types and are best described in colorectal tumors. Knockout of *CDC4 in vitro* in colorectal cancer cells causes changes suggestive of chromosomal instability (CIN). In $p53^{+/-}$ mice, radiation-induced lymphomas show deletion or mutation of one copy of *CDC4* and knockdown of *CDC4* leads to increased aneuploidy in mouse fibroblasts. We screened 244 colorectal tumors and 40 cell lines for *CDC4* mutations and allelic loss. Six percent (18 of 284) of tumors, including near-diploid (CIN−) lesions, harbored *CDC4* mutations and there was no association between mutation and CIN (polyploidy). The *CDC4* mutation spectrum in colorectal tumors was heavily biased towards C:G>T:A changes, either missense mutations at critical arginine residues or nonsense changes in the 5′ half of the gene. The reasons for this odd mutation spectrum were unclear but C:G>T:A changes were not found more often than expected at *APC, K-ras*, or *p53* in the same tumors and we found no specific defects in DNA repair to account for the observations. No colorectal tumor was found to carry two *CDC4* mutations predicted to abolish protein function; partial loss of CDC4 function may therefore cause tumorigenesis. The *in vitro* studies, therefore, did not assess the functional effects of mutant alleles which are found *in vivo*. *CDC4* mutations may be selected primarily to drive progression through the cell cycle although CIN might be an important secondary effect in some cancers. (Cancer Res 2005; 65(24): 11361-6)

Introduction

Many cancers have aneuploid and polyploid karyotypes. Such cancers are often said to have chromosomal instability (CIN) on the basis that they have acquired a tendency to gains and losses of large genomic regions (1). While questions remain as to the cause, mechanism, and timing of CIN, it has been suggested that it results from specific, somatic mutations which cancers acquire. A recently proposed CIN gene for human cancers, particularly colorectal carcinoma, is *CDC4* (F-box and WD40 domain protein 7, *FBW7, FBXW7*, Archipelago; ref. 2).

CDC4 is an evolutionarily conserved protein which acts as a bridge between a substrate and the Skp1-Cull-F-box ubiquitin ligases. The NH_2-terminal region of the protein principally encodes the F-box and the COOH-terminal consists of eight WD40 repeats which are involved in substrate recognition and binding. A CDC4 substrate is cyclin E (CCNE; ref. 3), which is involved in the progression from G_1 to S phase in the cell cycle. Overexpression of CCNE or loss of CDC4 is expected to activate cyclin-dependent kinase 2 and target CDKN1B for degradation, leading to elimination of CDKN1B from the cell and progression of the cell cycle from G_1 to S phase. *CCNE* has been found to be amplified and/or overexpressed in several cancer types (e.g., refs. 4, 5) and mutations of *CDC4* have been found in breast and ovarian cancer cell lines (6–8), endometrial cancers (9, 10), and, recently, colorectal carcinomas and adenomas (2). Other probable CDC4 substrates include Notch4, c-myc, and c-jun.

Hubalek et al. (11) searched for *CDC4* mutations in endometrial cancers. Excluding isoform-specific changes, they found mutations in 6 of 12 aneuploid and/or polyploid cancers and in none of three diploid lesions. Whereas these data were suggestive of a link between *CDC4* change and CIN, the association was formally nonsignificant ($P = 0.19$, Fisher's exact test). Willmarth et al. (12) studied CCNE expression in a panel of breast cancer cell lines; one of which, SUM149PT, was known to harbor a *CDC4* mutation. Although SUM149PT showed evidence of CIN, there was no obvious overall association between level of CCNE expression and degree of aneuploidy/polyploidy.

In the most comprehensive study to date of *CDC4* changes and their consequences, Rajagopalan et al. (2) found *CDC4* mutations in 22 of 190 colorectal cancers and 4 of 58 adenomas. Nineteen mutations were amino acid substitutions and 10 were nonsense changes; six tumors, all cancers, had biallelic mutations, including allelic loss. The great majority of mutations in the cancers (19 of 25) occurred in exons 7 to 10. In addition to nonsense mutations which were predicted to disrupt substrate binding, most of the missense mutations occurred within the series of eight WD40 repeats (13) within the CDC4 protein. These mutations were predicted to disrupt CCNE binding to CDC4. Having identified *CDC4* mutations in colorectal cancers, Rajagopalan et al. (2) tested the possibility that the changes resulted in CIN, as had previously been proposed for CCNE (14). Knockout of

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CDC4 in vitro in near-diploid colorectal cancer cell lines showed raised levels of CCNE plus cytologic abnormalities, including increased micronuclei and nuclei of atypical morphology. Mitosis was prolonged, with abnormal chromosome alignment in metaphase and aberrant chromosome numbers. These changes were strongly suggestive of CIN.

A recent study (15) identified deletions of *CDC4* in radiation-induced lymphomas from $p53^{+/-}$ mice. Two mutations were also found, one nonsense change and one missense alteration, although neither change was typical of the mutations found in human cancers. Because biallelic changes were not found in any tumor, it was suggested that *CDC4* is a haploinsufficient tumor suppressor in mice. Knockdown of *CDC4* by small interfering RNA in p53 wild-type embryonic fibroblasts led to an increase in the proportion of aneuploid cells. The levels of CDC4 targets Notch4 and c-jun were increased in the "knockdown" cells but that of cyclin E was not.

Several questions remain about the role of *CDC4* in colorectal and other cancers in humans. Foremost among these is whether or not mutations are associated with the CIN phenotype *in vivo*. Previous studies using fresh/frozen tumors have not resolved this question. *In vitro* studies which have shown effects of *CDC4* mutation on chromosome segregation (2) inevitably relied on colorectal cancer cell lines. These have undoubtedly acquired multiple changes during their progression from normal colonocyte to malignancy, leaving open the possibility that loss of *CDC4* has effects which would not generally occur *in vivo*. It remains plausible, therefore, that *CDC4* mutations are selected primarily for their effects on cell cycle progression rather than CIN. We have screened 202 fresh-frozen colorectal carcinomas, 10 metastases, and 32 adenomas, as well as 40 colorectal cancer lines, for somatic *CDC4* mutations and compared the results with the CIN and microsatellite instability (MSI) status of the tumors.

Materials and Methods

Samples. A series of 32 fresh frozen colorectal adenomas, 202 carcinomas, and 10 metastases, all with paired normal bowel, was obtained from St. Mark's Hospital, London and the John Radcliffe Hospital, Oxford. Fixed tissue was obtained from the same tumors. All cancers contained more than 60% neoplastic cells as assessed using routine histology. DNA was extracted from each tumor sample and paired normal bowel using standard methods. Samples were studied on an anonymized basis according to Harrow and Oxford local research ethics guidelines. A panel of 40 colorectal cancer cell lines was also analyzed (C10, C125, C32, C70, C75, C80, C84, C99, CACO2, COLO205, COLO320D, COLO741, CX-1, DLD-1, GP5D, HCA46, HCA7, HCT8, HCT116, HRA19, HT29, LM1863, LOVO, LS1034, LS180, LS411, LS174T, PCJW, RKO, SCKO-1, SW1417, SW1222, SW48, SW480, SW837, SW948, T84, VACO4A, VACO4S, and VACO5).

***CDC4* mutation screening and loss of heterozygosity.** All samples were screened for somatic *CDC4* mutations using fluorescence single-strand conformational polymorphism (SSCP) analysis. Primers were designed to amplify the coding regions and exon-intron boundaries of exons 1 to 10. Oligonucleotides and reaction conditions used to amplify each fragment are available from the authors. Samples were run at 18°C and 24°C on the ABI3100 capillary sequencer. All tumors with bandshifts on fluorescence-SSCP analysis were sequenced in forward and reverse orientations for that exon using a new, unlabeled PCR product, the ABI BigDye Terminator Ready Reaction Mix (PE Applied Biosystems, Warrington, United Kingdom), and the ABI377 semiautomated sequencer. Loss of heterozygosity (LOH) analysis was done at six microsatellite markers near *CDC4* (details available from the authors) using tumor and constitutional DNA. Products were run on the ABI377 sequencer and results were analyzed using Genotyper software. Constitutionally homozygous markers were scored as noninformative. Allelic loss was considered present if the relative ratio of normal/tumor peak areas was <0.5 or >2, having corrected for the relative areas in the constitutional DNA.

Flow cytometry. For assignment of ploidy in the colorectal cancers, a 50-μm section of paraffin-embedded tissue was cut from each block. The section was placed between two sheets of 3-mm filter paper in a histopathologic cassette and dewaxed in 100% xylene (BDH, Oxford, United Kingdom) overnight. The section was rehydrated in an ethanol series (100%, 95%, 90%, 70%, 50%) and rinsed twice in water. The tissue was then removed from the slide with a scalpel and digested with pepsin solution [0.5% pepsin (Sigma, Poole, United Kingdom), 0.9% NaCl, pH 1.5] for 30 minutes at 37°C. After centrifugation for 5 minutes at room temperature, cells were washed twice in PBS before flow cytometric analysis. All tumors with evidence of an aneuploid and/or polyploid flow cytometry peak, distinct from the diploid peak and corresponding to a DNA index of >1.2, were classed as CIN+. For the cell lines, full karyotype and chromosome counts were done using standard methods.

Microsatellite instability. MSI was assessed using the mononucleotide repeats BAT25 and BAT26. Allelic bandshifts typical of MSI at either marker caused that tumor to be classified as MSI+.

Other molecular changes. Tumors were screened for *APC* mutations using fluorescence-SSCP analysis (for smaller exons) and denaturing high-performance liquid chromatography analysis (for larger exons and exon 15) so as to cover the coding sequence and intron-exon boundaries of exons and the region of exon 15 before codon 1600 (details available from authors). Any samples with bandshifts were sequenced for the appropriate fragment in forward and reverse orientations from a new PCR product. Only truncating mutations were classed as pathogenic. p53 (exons 5-8) was screened using SSCP analysis based on silver-stained midi-gels, followed by sequencing of samples with bandshifts in forward and reverse orientations from a new PCR product. Mutations at *K-ras* (codons 12, 13, and 61) were detected using direct sequencing in forward and reverse orientations as previously reported (details available from the authors).

Rasmol. Clustal X PPC was used to align human CDC4 with the equivalent mouse, *Drosophila*, and *S. cerevisiae* enzymes. Given the relatively high levels of conservation within the WD40 repeats between the *S. cerevisiae* and human protein sequences (13), we used the former as a template on which to map human mutations. Molecular figures were drawn with Rasmol V2.7.1.2 using the protein databank file 1NEX.

***TDG* and *UDG* mutation screening.** Tumors with *CDC4* mutations were screened for *TDG* and *UDG* mutations using fluorescence-SSCP analysis so as to cover the coding sequence and intron-exon boundaries of exons 1 to 10 for *TDG* and exons 1 to 6 for *UDG* (details available from authors). Any samples with bandshifts were sequenced for the appropriate fragment in forward and reverse orientations from a new PCR product.

Quantitative real-time PCR analysis. Single-stranded cDNA was synthesized from RNA isolated from colorectal cancer cell lines as recommended by the manufacturer (Amersham Pharmacia, Amersham, United Kingdom). Amplification of the full coding sequences of *CDC4, MGMT, TDG*, and *UDG* was done using standard PCR protocols (primers and details available from authors). mRNA expression levels were determined with a TaqMan PCR assay using the ABI Prism 7700 sequence detector (PE Applied Biosystems).

Western blot analysis. c-myc, c-jun, and cyclin E proteins were characterized for size and levels in colorectal cancer cell lines using standard Western blot techniques. Briefly, 5×10^6 cells were detergent lysed, denatured, and separated on a 7.5% acrylamide gel followed by charged transfer to polyvinylidene difluoride membrane (Millipore, Watford, United Kingdom). Samples were probed with mouse anti-c-myc (9E10, Cancer Research UK, London, United Kingdom)], rabbit anti-c-jun and rabbit anti–cyclin E (Santa Cruz Biotetechnology, Santa Cruz, CA) and detected using enhanced chemiluminescence (Amersham Pharmacia Biotech). Rabbit and mouse anti-β-actin (Sigma) were used as a loading control.

Results

We detected a total of 18 protein-truncating or missense *CDC4* mutations (one per tumor) in 11 primary colorectal carcinomas, 2 metastases, 3 adenomas, and 2 cancer cell lines (Table 1).

Table 1. *CDC4* mutations detected in this study (*A*) and in that of Rajagopalan et al. (ref. 2; *B*)

(*A*)

CDC4 exon	Nucleotide change	Amino acid change	Mutation type	LOH	Specimen	Sample ID	Ploidy (modal chromosome no.)	MSI	*APC* mutation	*K-ras* mutation	*p53* mutation
3	C670T	R224X	Nonsense	No	Metastasis	3822/93M	Diploid	+			
4	C832T	R278X	Nonsense	No	Adenoma	13103/93P	Diploid	−			
4	C832T	R278X	Nonsense	No	Carcinoma	452/94C	Aneuploid				
4	C832T	R278X	Nonsense	No	Cell line	C32	Hypertriploid (74)	−	776FS+LOH	None found	None found
4	C845A	S282X	Nonsense	No	Carcinoma	284A	Aneuploid	−	None found	None found	None found
6	C1099T	R367X	Nonsense	No	Carcinoma	1644a	Aneuploid	−	None found	None found	R273H
7	C1177T	R393X	Nonsense	No	Carcinoma	1350	Diploid	−	R213X(C>T); 1411FS	None found	None found
8	C1393T	R465C	Missense	No	Carcinoma	10593/90C		+			
8	C1393T	R465C	Missense	No	Adenoma	15935P*	Aneuploid	−			
8	C1393T	R465C	Missense	No	Carcinoma	307	Aneuploid	−	R1114X(C>T)	None found	None found
8	G1394A	R465H	Missense	No	Metastasis	15935/93M	Aneuploid	−			
9	G1436A	R479Q	Missense	No	Carcinoma	13854/93C	Aneuploid	−			
9	G1436A	R479Q	Missense	No	Carcinoma	12829/93C	Diploid	+			
9	G1436A	R479Q	Missense	No	Adenoma	4624/93P	Diploid	−			
9	C1513T	R505C	Missense	LOH	Carcinoma	8244/9/93C		−			
9	C1513T	R505C	Missense	No	Cell line	LOVO	Hyperdiploid (49)	+	R1114X(C>T); 1430FS	G>A c13	None found
9	G1514C	R505P	Missense	No	Carcinoma	17205/93C	Diploid	−			
10	C1787T	S596F	Missense	LOH	Carcinoma	1494	Diploid	−	1413FS	None found	None found

(*B*)

Tumor	CDC4 exon	Nucleotide change	Amino acid change	Mutation type*	LOH
1	3	C418T	Q220X	Nonsense	No
2	3	C430T	R224X	Nonsense	No
3	3	C430T	R224X	Nonsense	No
4	4	C592T	R278X	Nonsense	No
	10	C1505T	S582L	Missense	No
5	4	C592T	R278X	Nonsense	No
6	5	C667T	Q303X	Nonsense	No
7	5	A696C	R312S	Missense	No
8	7	C937T	R393X	Nonsense	No
	10	C1505T	S582L	Missense	No
9	7	C937T	R393X	Missense	No
	10	C1505T	S582L	Missense	No
10	7	G950A	G397D	Nonsense	LOH
11	8	G1028T	G423V	Missense	LOH
12	8	C1073T	S438F	Missense	No
13	8	G1098A	W446X	Nonsense	No
14	8	C1153T	R465C	Missense	No
15	8	C1153T	R465C	Missense	No
16	8	C1153T	R465C	Missense	No
17	8	G1154A	R465H	Missense	No
18	8	C1153T	R465C	Missense	No
19	9	G1196A	R479Q	Missense	No
20	9	G1196A	R479Q	Missense	No
21	9	G1196A	R479Q	Missense	No
22	9	G1196A	R479Q	Missense	No
23	9	G1217A	W486X	Nonsense	No
24	9	G1388A	R543K	Missense	No
25	9	A1394G	Y545C	Missense	LOH
26	10	C1505T	S582L	Missense	No

NOTE: Absence of data means not done.

*The types of some mutations have been corrected from those originally reported by Rajagopalan et al. (2) Supplementary Table S1.

"Two hits" at *CDC4* were found in 2 of the 18 samples and both of these tumors (8244/9/93C and 1494) harbored a missense mutation accompanied by allelic loss. *CDC4* mutations were equally common in aneuploid/polyploid (n = 8) and near-diploid (n = 8) lesions (P = 0.30, Fisher's exact test, compared with all cancers studied). There was, moreover, no association between *CDC4* mutation and MSI (P = 0.18, Fisher's exact test, compared with all cancers studied).

The location of the mutations within *CDC4* was nonrandom. All five mutations NH_2-terminal to the WD40 repeats were nonsense changes whereas 11 of 13 mutations in the WD40-repeat region (codons 364-692, exons 7-11) were missense changes (P = 0.002, Fisher's exact test). Most WD40-region missense changes affected arginine residues (R465C, R465H, R479Q, R505C, and R505P). In these respects, our data confirm the finding of Rajagopalan (Table 1; ref. 2) and supplement it with the finding of mutations at arginine 505. Arginines 465, 479, and 505 are highly conserved residues which lie at the apices of blades of the "β-propellers" produced by the WD40 repeats in CDC4. These residues are thought to be involved in determining substrate-specific binding of CDC4 to CCNE (Fig. 1).

Our other missense mutation, S596F, involved a residue in the sixth WD40 repeat of CDC4 which is conserved in five of the other repeats and which is invariant among human, mouse, yeast, and *Drosophila*. Using molecular modeling, we found that the serine may stabilize interactions between residues within the blades of the propeller. Mutation to phenylalanine would disrupt these interactions (Fig. 1).

The other missense mutations of Rajagopalan et al. (2) mostly lie within the WD40 repeats (Fig. 1) but did not clearly affect substrate binding. S582L is not at a conserved residue but lies close to a conserved lysine which sits around the substrate binding pocket. G397D affects a highly conserved amino acid and lies within the β3 domain of WD repeat 1. G423V is at a moderately conserved residue within the β6 region of WD repeat 2 and S438F affects a highly conserved site within the β 7 domain of WD repeat 2. Y545C affects a highly conserved tyrosine residue around the binding pocket which is encoded within the β17 domain of WD repeat 5. Rajagopalan et al. (2) also found a missense mutation, R312S, which is the sole missense mutation NH_2-terminal to the WD40 repeats and is conserved in the F-box domain motif. We speculate that mutation of this residue upsets the structure of the F-box domain, interrupting interaction with Skp1 and breaking down the Skp1-Cull-F-box complex.

For further analysis of the *CDC4* mutation spectrum in colorectal tumors, we combined the published data of Rajagopalan et al. (2) with our own findings because the two mutation data sets had large areas in common. The types of *CDC4* mutations seemed to be highly atypical. Simple inspection showed that almost all detected point mutations in *CDC4* (42 of 47) were C:G>T:A changes. C:G>T:A mutations are among the most common spontaneous changes and they typically account for about 60% to 70% of the point mutations in tumor suppressor genes (e.g., *p53*; ref. 16). A further striking observation was that not one of the protein-truncating *CDC4* mutations was a frameshift change. In genes such as *APC* and *p53*, frameshift mutations are usually in an excess over nonsense changes (16, 17). Almost all the nonsense mutations in *CDC4* were evidently caused by C:G>T:A changes.

One possible explanation for these observations was that *CDC4* mutations tended to occur in tumors that were deficient in a specific form of DNA repair. We tested various possible sources of excess C:G>T:A changes, first by screening for mutations in DNA repair genes which encode proteins that prevent the accumulation of C:G>T:A changes. Screening of methylguaninemethyltransferase (*MGMT*), thymidine DNA glycosylase (*TDG*), and uridine DNA glycolsylase (*UDG*) revealed no changes. We had

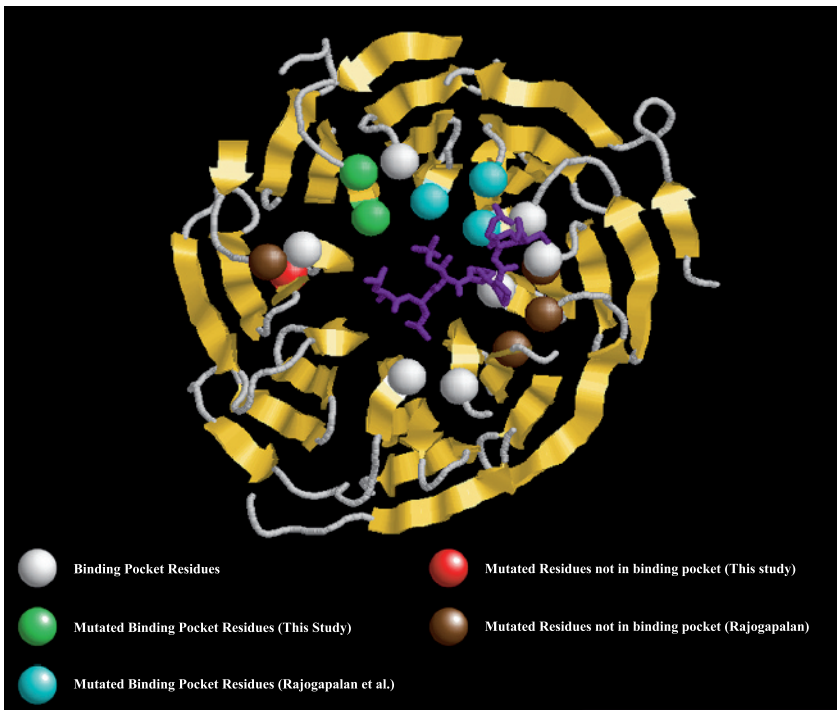

Figure 1. Highly conserved arginines at apices of β-propellors in *S. cerevisiae* CDC4 (*gold*) shown with phosphorylated cyclin E (*purple*). Residues with mutations detected *in vivo* are shown colored.

previously analyzed some of the tumors for MGMT expression using immunohistochemistry (18); 2 of 4 cancers with *CDC4* mutations showed decreased or absent MGMT expression, compared with 27 of 31 *CDC4* wild-type lesions ($P = 0.45$, Fisher's exact test). Quantitative real-time PCR analysis of *MGMT, TDG,* and *UDG* mRNA expressions in the colorectal cancer cell lines showed no reduced expression in the lines with *CDC4* mutations (details not shown). We then searched for bias towards C:G>T:A mutations in other genes (*APC, K-ras*, and *p53*) in those tumors with *CDC4* mutations, but no association was apparent (Table 1).

The mutation spectrum of *CDC4* mutations in the combined data set revealed further complexity. Only one of the 19 tumors with a "propellor" mutation of the critical arginine residues involved in CCNE binding had biallelic mutations (Table 1) but biallelic changes were found in 8 of 25 cancers with other mutations ($P = 0.02$, Fisher's exact test). In respect of the critical arginine mutations within the propellors, therefore, the data suggest that a "second hit" may not be necessary. Further consideration of the mutations which did not involve the "propellor" arginines showed that, although some cancers of these tumors did have "second hits," in no case were there two mutations which were predicted to abolish protein function. Thus, where biallelic *CDC4* mutations were found, they almost always comprised a missense mutation not involving a WD40-arginine, together with allelic loss or a nonsense change (Table 1). By contrast, the mutation spectrum of most tumor suppressors comprises biallelic mutations, both of which are predicted to negate protein function, in about 25% to 50% of cases (16, 17). Reverse transcription-PCR and Western blotting showed the presence of full-length mRNA and CDC4 protein (and no truncated protein) in each of the two colorectal cancer cell lines with *CDC4* mutations, showing that the missense mutations did not lead to absent CDC4 protein.

Western blot analysis of the CDC4 substrate cyclin E revealed variable expression levels, consistent with multiple mechanisms of activating this protein in tumors. Both of the *CDC4*-mutant colorectal cancer cell lines showed relatively high cyclin E levels (2- to 3-fold higher than the mean) compared with the other cell lines, consistent with the predicted effect of reduced CDC4 activity on cyclin E. No clear association was seen between *CDC4* mutations and c-myc expression (details not shown) and expression of c-jun was absent or very weak in all lines tested.

Discussion

The data on colorectal tumors from this study and the work of Rajagopalan et al. (2) are by far the largest surveys of *CDC4* mutations which have been undertaken. Our own data suggested a frequency of mutation of 6% (18 of 284) in colorectal tumors, and, when combined with the findings of Rajagopalan et al. (2), the mutation frequency was 8% (44 of 532). Our data agree with those of Rajagopalan et al. (2) in that *CDC4* mutations are most often missense changes that affect arginine residues in WD40 propellers; these mutations are predicted to cause reduced substrate binding. Protein-truncating *CDC4* changes are the next most common type of mutation, and these tend to occur NH_2-terminal to the WD40 repeats. *CDC4* mutations occur in adenomas as well as carcinomas.

Rajagopalan et al. (2) showed that somatic "knockout" of *CDC4* in colorectal cancer cell lines HCT116 and DLD1 led to a CIN-like phenotype *in vitro*. Small interfering RNA knockdown had similar effects (2, 15). We have shown that *in vivo CDC4* mutations are not associated with CIN+ (aneuploid/polyploid) lesions. The two sets of data can be reconciled by observation of the mutation spectrum in *CDC4*. No colorectal tumor has been reported which acquired two *CDC4* mutations predicted to abolish protein function. Of the tumors with biallelic *CDC4* mutations, all had one missense change accompanied by either allelic loss or a nonsense mutation. These findings contrast with "classic" tumor suppressor loci, at which at least a proportion of cancers harbor two detectable mutations which truncate the protein and/or abolish its function. In fact, the observed *CDC4* mutation spectrum in colorectal tumors suggests that absence of protein function might be selectively disadvantageous. Given that we have no evidence for overexpression of the mutant *CDC4* allele and that CDC4 is not predicted to act as a multimer, haploinsufficiency and/or reduced function is the most likely explanation for the observed spectrum of *CDC4* mutations. This finding is consistent with the prediction made by Mao et al. (15) from their studies of radiation-induced mouse tumors. Given that CDC4 is involved in the closely controlled temporal regulation of cell cycle entry (13, 19), we hypothesize that the mutations observed cause partial loss of function, allowing more rapid cell cycle progression without being so fast as to trigger other regulatory checkpoints.

The absence of protein-truncating *CDC4* mutations in the 220 codons 3′ to codon 486 suggests that mutations in this region do not have sufficiently profound effects on CDC4 function. The *CDC4* mutation spectrum was very unusual in other respects. All of the protein-truncating, and most of the missense, mutations found in *CDC4* in colorectal tumors have been C:G>T:A changes. We have found no evidence to ascribe these observations to specific mutations or loss of expression of DNA repair genes. Another striking feature was the absence of *CDC4* frameshift changes (even in MSI+ cancers); in genes such as *RB1, PTEN, APC*, and *p53*, frameshifts generally predominate over nonsense changes. The nonsense mutations in *CDC4* cluster between codons 220 and 393 and preferentially target arginine residues 224, 278, and 393 (10 of 17 nonsense changes). No sequence peculiarities within the *CDC4* gene can readily explain the observed bias towards nonsense mutations.

Analysis of *CDC4* mutations in other cancer types, predominantly endometrial cancers and ovarian cancer cell lines, has been on a smaller scale. The studies of other cancer types show broad agreement with the colorectal carcinoma data (6, 9, 10) although biallelic protein-inactivating mutations have been found in some of the former tumors. The unusual *CDC4* mutation spectrum may be restricted to colorectal tumors, but there may also have been important methodologic differences between studies (e.g., criteria for assessing allelic loss and methods of mutation detection).

In conclusion, our data suggest that *CDC4* mutations are not selected primarily in colorectal tumors for their effects on CIN but for some other reason, probably to drive progression through the cell cycle through overexpression of cyclin E and/or to avoid delays or apoptosis which result from G_1-S checkpoints. CIN may result as an important secondary effect of this process in some cancers although the *CDC4* mutant alleles found *in vivo* have not yet been assessed in functional assays. The *CDC4* mutation spectrum in colorectal tumors is heavily biased towards C:G>T:A changes but the reasons for this are unclear. The genetic data suggest that complete loss of CDC4 function occurs rarely *in vivo* in colorectal tumors, consistent with tumorigenesis by haploinsufficiency and/or partial loss of function.

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