Overexpression and Mistargeting of Centromere Protein-A in Human Primary Colorectal Cancer[1]

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ABSTRACT

Aneuploidy is the hallmark of many human cancers. Recent work has strongly suggested that chromosome missegregation during mitosis is the main cause of aneuploidy and contributes to oncogenesis. Centromere protein (CENP) -A is the centromere-specific histone-H3-like variant essential for centromere structure and function. It plays a central role in the assembly of the protein complex, termed kinetochore, which is indispensable for equal chromosome segregation. In this study, we demonstrate that the kinetochore protein CENP-A was overexpressed in all of 11 primary human colorectal cancer tissues. CENP-A mRNA was also upregulated, indicating that overexpression of CENP-A occurred at the transcriptional level. Immunostaining with anti-CENP-A antibodies showed increased CENP-A signals in the tumor cells. Moreover, coimmunostaining of CENP-B, a centromere-associated DNA binding protein, with CENP-A showed mistargeting of CENP-A to noncentromeric chromatin in the tumor cells. These results suggest that overexpression of CENP-A could play an important role for aneuploidy in colorectal cancers.

INTRODUCTION

The genetic instability in human cancers appears to be widely accepted. In most cancers, this instability is observed at the chromosomal level, which is predominantly the gain or loss of entire chromosomes, also known as aneuploidy (1). Aneuploidy is found in nearly all tumor types, and develops early during tumorigenesis, as seen in carcinoma *in situ* or precancerous lesions of the colon, cervix, and esophagus (reviewed in Refs. 2, 3). In addition, *in vitro* studies using human and rodent cells have shown that aneuploidy is required for neoplastic transformation (4, 5). These results suggest that aneuploidy plays an important role in the development and progression of cancer.

Because aneuploidy represents an abnormal number of chromosomes, it could result from the missegregation of chromosomes during cell division. Potential defects in many mitotic processes could lead to the unequal segregation of chromosomes. These include chromosome condensation, sister-chromatid cohesion, kinetochore assembly, centrosome duplication, microtubule dynamics, and checkpoints for proper progression of the cell cycle. Several regulatory genes of these mitotic targets have been shown recently to be involved in the induction of aneuploidy and carcinogenesis. For example, STK15/BTAK/aurora2 and vertebrate securin are overexpressed in human tumors and have exhibited transforming activity *in vitro* (6–8). The checkpoint genes *hBUB1* and *hBUBR1* were found to be mutated in a small fraction of human colorectal cancers, and the exogenous expression of the mutant BUB1 confers an abnormal spindle checkpoint (9).

Kinetochore is a large multiprotein complex that is assembled on centromeric DNA and serves as the attachment site for spindle microtubules, which is essential for proper chromosome segregation during mitosis. Although its malfunction could be a major cause of aneuploidy, there is no direct relationship between kinetochore defects and tumorigenesis. Among the several kinetochore proteins discovered to date, CENP-A[3] is one of the first identified kinetochore components in humans (10). It is a unique histone H3-like protein that has been found only at active centromeres and believed to be a central element in the epigenetic maintenance of centromere identity (reviewed in Refs. 11, 12). CENP-A is well conserved among eukaryotes (13–15) and mutations or knockouts of CENP-A result in chromosome missegregation (16).

These observations raise important questions regarding the altered expression of CENP-A in tumor cells and whether malfunctions of CENP-A could cause aneuploidy and lead to cancer. Therefore, we examined CENP-A protein levels in colorectal cancers. We found that CENP-A was highly overexpressed in most cancer tissues when compared with adjacent normal mucosa. In addition, it partially did not colocalize with a centromere-associated DNA binding protein CENP-B in the tumor cells. Our results suggest that higher levels of CENP-A in colorectal cancer result in its association with noncentromeric regions of chromatin and partial disruption of the kinetochore complex.

MATERIALS AND METHODS

Human Tissues Samples. Tissues from 11 cases of primary colorectal cancer were surgically resected (Table 1). Written informed consent was obtained from each patient before surgery. The excised samples were obtained within 1 h after the operation from tumor tissues and corresponding nontumor tissues 5–10 cm remote from the tumor. All of the excised tissues were placed immediately in liquid nitrogen and stored at −80°C until additional analysis.

Protein Extraction and Immunoblotting. Frozen tissue samples were solubilized in lysis buffer {7 M urea, 2 M thiourea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.1 M DTT, 2% IPG buffer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and 40 mM Tris} using a Polytron homogenizer (Kinematica, Littau-Luzern, Switzerland) after centrifugation (100,000 × g) for 1 h at 4°C. The supernatant proteins were separated by electrophoresis on 7.5–15% gradient gels (Nikkyo Technos DRC, Tokyo, Japan). Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) in a tank transfer apparatus (Bio-Rad, Hercules, CA), and the membranes were blocked with 5% skim milk in PBS. Nuclear centromere autoantibody positive control (ANA serum; The Binding Site, Birmingham, United Kingdom) diluted 1:1000, antihuman CENP-A monoclonal antibody (17) diluted 1:500, rabbit anti-PCNA (FL-261) antibody (Santa Cruz, Santa Cruz, CA) diluted 1:100, and goat anti-β-actin antibody (Santa Cruz) diluted 1:500 in blocking buffer were used as primary antibodies. Goat antihuman IgG HRP conjugate (Jackson, West Grove, PA) diluted 1:10,000, goat antirabbit IgG HRP (Bio-Rad) diluted 1:3,000, and rabbit antigoat IgG HRP (Cappel, West Chester, PA) diluted 1:500 in blocking buffer were used as secondary antibodies. Antigens on the membrane were detected

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[3] The abbreviations used are: CENP, centromere protein; FISH, fluorescence *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; HRP, horseradish peroxidase; RT-PCR, reverse transcription-PCR.

Table 1 *Clinical features of patients with colorectal cancer*

	Sample no.	Age	Sex	Location[a]	Dukes' stages
1	1	82	Male	S	B
2	112	72	Male	S	C
3	6	57	Male	S	B
4	117	49	Male	C	D
5	118	85	Male	S	B
6	24	64	Female	C	C
7	27	51	Male	R	D
8	28	67	Male	R	C
9	29	51	Male	R	C
10	32	80	Male	A	B
11	35	72	Female	S	B

[a] S, sigmoid colon; C, cecum; R, rectum; A, ascending colon.

by enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech). The intensity of each band was measured by NIH Image.

Immunohistochemistry. Tissues were fixed on slide glasses with 4% paraformaldehyde in PBS for 5 min at room temperature. After washing three times with PBS, samples were permeabilized with 0.5% Triton X-100 in PBS for 5 min and refixed with 4% paraformaldehyde/PBS for 5 min. In some experiments tissues were fixed with acetone for 10 min at 4°C. Nonspecific binding of antibodies was blocked with blocking buffer (1% BSA or 10% fetal bovine serum/PBS) for 1 h. Samples were incubated for 1 h with antihuman CENP-A monoclonal antibody diluted 1:500 and/or goat antihuman CENP-B (Y-17) antibody (Santa Cruz) diluted 1:25 in blocking buffer. After washing with PBS, samples were incubated with 1:500 diluted Alexa Fluor 488 or 568-conjugated goat antimouse IgG secondary antibody and Alexa Fluor 594-conjugated donkey antigoat IgG secondary antibody (Molecular Probes, Eugene, OR) for 1 h. DNA was counterstained with DAPI III Counterstain

(Vysis, Abbott Park, IL). Samples were observed with fluorescence microscopy (Leica QFISH; Leica Microsystems, Tokyo, Japan).

RT-PCR and Real-Time Quantitative PCR. Total RNA and genomic DNA were extracted from tumor and nontumor tissues with RNeasy Mini kit and DNeasy Tissue kit (Qiagen). cDNA was synthesized from total RNA with the first-strand cDNA Synthesis kit for RT-PCR (Roche, Mannheim, Germany). Using the cDNA as a template, CENP-A cDNA was amplified with suitable primers: forward 5′-TAGGCGCTTCCTCCCATCAA-3′, reverse 5′-GCCGAGTCCCTCCTCAAG-3′. For the control, GAPDH cDNA was amplified. Serial dilutions of the template cDNA were made for PCR reactions to optimize the PCR products within the linear range. Real-time quantitative PCR of CENP-A cDNA using the LightCycler instrument (Roche) was carried out in 20 μl of reaction mixture containing LightCycler DNA Master SYBR Green I (FastStart Taq DNA polymerase, deoxynucleotide triphosphate, buffer, SYBR Green I), 3.0 mM MgCl$_2$, and 0.5 μM each of forward (5′-ACAAG-GTTGGCTAAAGGA-3′), and reverse primer (5′-ATGCTTCTGCTGC-CTCTT-3′) in a LightCycler capillary. The CENP-A genomic DNA was also quantified by real-time PCR as described above. However, the following primers were used: forward 5′-ACGCCTATCTCCTCACCTT-3′ and reverse 5′-TGGCTGAGCAGGAAAGAC-3′. LightCycler software version 3.3 (Roche) was used for analysis of quantitative PCR. Optimization of primers was performed at the Nihon Gene Research Laboratories Inc. (Sendai, Japan).

FISH Analysis. The FISH method for touch preparations described previously (18, 19) was slightly modified. Briefly, slides of touch preparations of fresh or frozen tissues were air-dried. After washing in PBS, slides were incubated in 75 mM KCl for 10 min at room temperature and then fixed in 3:1 methanol:acetic acid for 10 min. Slides were treated with 0.1 mg/ml of RNase A in 2× SSC for 30 min at 37°C, washed in 2× SSC, and incubated in 0.005% pepsin solution for 5 min at 37°C. After washing in PBS, slides were dehy-
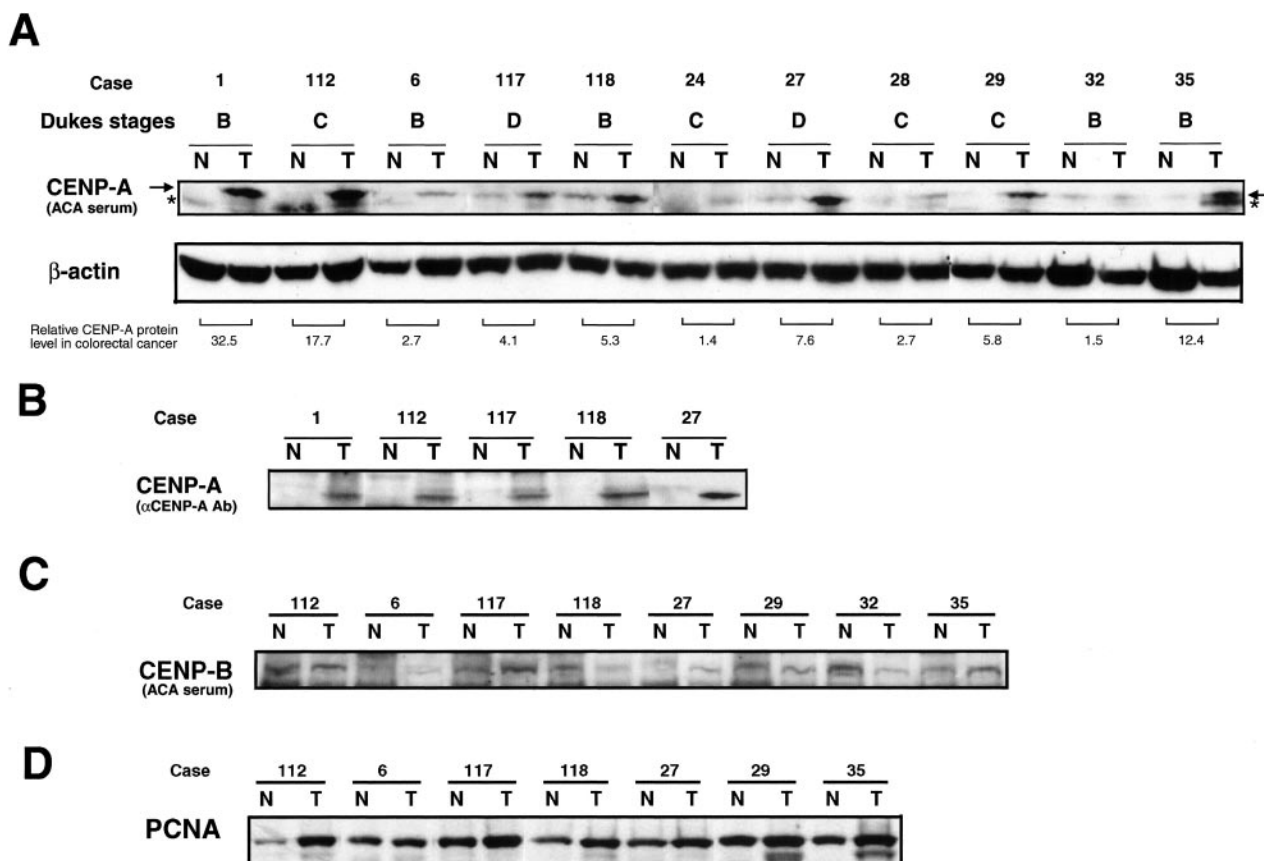

Fig. 1. CENP-A protein was increased in primary colorectal cancers. Total protein lysates were prepared from matched samples of tumor (*T*) and adjacent normal tissue (*N*). Equal amounts of protein from each pair were resolved on 7.5–15% gradient polyacrylamide gel and immunoblotted with several antibodies. *A*, CENP-A protein is indicated by the *arrow*. Immunoblotting was also performed with β-actin antibody as a loading control. Intensity of each band was measured by NIH Image, and the relative mean CENP-A protein levels between tumor and normal tissue normalized with β-actin were calculated from at least three experiments. * shows a nonspecific band. Dukes' stage of each tumor is noted. *B*, CENP-A protein levels were detected with anti-CENP-A antibody instead of ANA serum. *C*, CENP-B protein detected with ANA serum. *D*, PCNA protein levels in colorectal cancer tissues.

drated in 70%, 85%, and 100% ethanol. Slides were then incubated in 2× SSC/0.1% NP40 solution for 30 min at 37°C and dehydrated again. Target DNA was denatured for 5 min at 73°C in 70% formamide/2× SSC solution. Ten μl of probes to pericentromeric regions of chromosome 7 (CEP7 Spectrum Green), 8 (CEP8 Spectrum Orange), 12 (CEP12 Spectrum Orange), and 15 (CEP15 Spectrum Green; Vysis, Downers Grove, IL) were also denatured for 5 min at 73°C, then hybridized to the target DNA by incubating overnight at 37°C. Posthybridization washes were performed as described previously (18). Hybridization signals were observed and analyzed with Leica QFISH (Leica). At least 100 nuclei of each sample were evaluated for chromosome counts.

RESULTS

Aneuploidy that is observed in many types of cancer could result from the abnormal function of proteins involved in chromosome segregation. Thus, we examined alterations in any factors that are essential for proper chromosome segregation in human primary cancers. CENP-A, an essential protein for the kinetochore assembly, was overexpressed in all 11 of the cases of primary colorectal cancer, which was detected by Western blot using anticentromere antibody (ANA serum; Fig. 1A). The relative expression level of CENP-A protein in tumors compared with adjacent normal tissue varied from 1.5 to 32.5. CENP-A overexpression in the colorectal tumors shown above was also confirmed by Western blot using antihuman CENP-A monoclonal antibody (Fig. 1B). Because CENP-A is a component of the human kinetochore complex, other kinetochore proteins might also be overexpressed.

ANA serum contains antibodies against CENP-B and CENP-C, as well as CENP-A; thus, the expression of these proteins was also examined. As shown in Fig. 1C, expression levels of CENP-B protein were similar in tumor and adjacent normal tissues. Bands corresponding to CENP-C could not be detected with ANA serum (data not shown). This overexpression of CENP-A in colorectal cancers is not because of the higher rates of proliferation of the tumor cells, because the relative expression level of PCNA protein were comparable between tumor and normal tissues in most of the cases (Fig. 1D).

To determine whether overexpression of CENP-A is the result of increased transcription, CENP-A mRNA levels of colorectal cancer cells and normal colon epithelium were examined by RT-PCR (Fig. 2A). All of the cases, except case 32, showed higher CENP-A mRNA levels in tumor cells compared with normal cells. Also, relative mRNA levels in tumor cells correlated well with relative protein levels shown in Fig. 1A. The increased CENP-A mRNA level in colorectal cancer cells was additionally confirmed by real-time quantitative RT-PCR (Fig. 2B). CENP-A gene amplification was also tested by real-time quantitative PCR using genomic DNA from the same matched tumor/normal samples used in the above experiments. As shown in Fig. 2C, no amplification was observed in tumors. These results indicate that overexpression of CENP-A occurred at the transcription level.

The majority of cancers have been shown to lose or gain chromosomes, and some colorectal cancer cell lines are often polysomic (1). These observations have raised the question of whether overexpression of CENP-A may be the consequence of polyploidy. To address the question, FISH analysis of normal and tumor cells were performed with several centromere probes. Touch preparations from three colorectal cancers in which CENP-A was overexpressed were obtained, and the number of centromeric signals for chromosomes 7, 8, 12, and 15 were counted (Table 2). Polyploid cells were hardly observed in normal tissue (1–4%); in contrast, all of the three tumors showed polyploidy at least in one chromosome. Whereas >80% of chromosomes 7, 8, and 12 in the tumor 1 were polyploid, only chromosome 15 in tumor 2 (14.1%) and chromosome 7 in tumor 3 (49.2%) were polyploidy, suggesting that not all of the tumor cells are highly polyploid. These results indicate that overexpression of CENP-A is not merely a consequence of polyploidy in colorectal cancers.

To check whether CENP-A was overexpressed in cancer cells and not in surrounding mesenchymal cells, tissue sections of colorectal cancers and adjacent normal tissues were stained with antihuman CENP-A monoclonal antibodies (17). CENP-A existed as discrete dots in the nucleus, which is typical for centromere-kinetochore
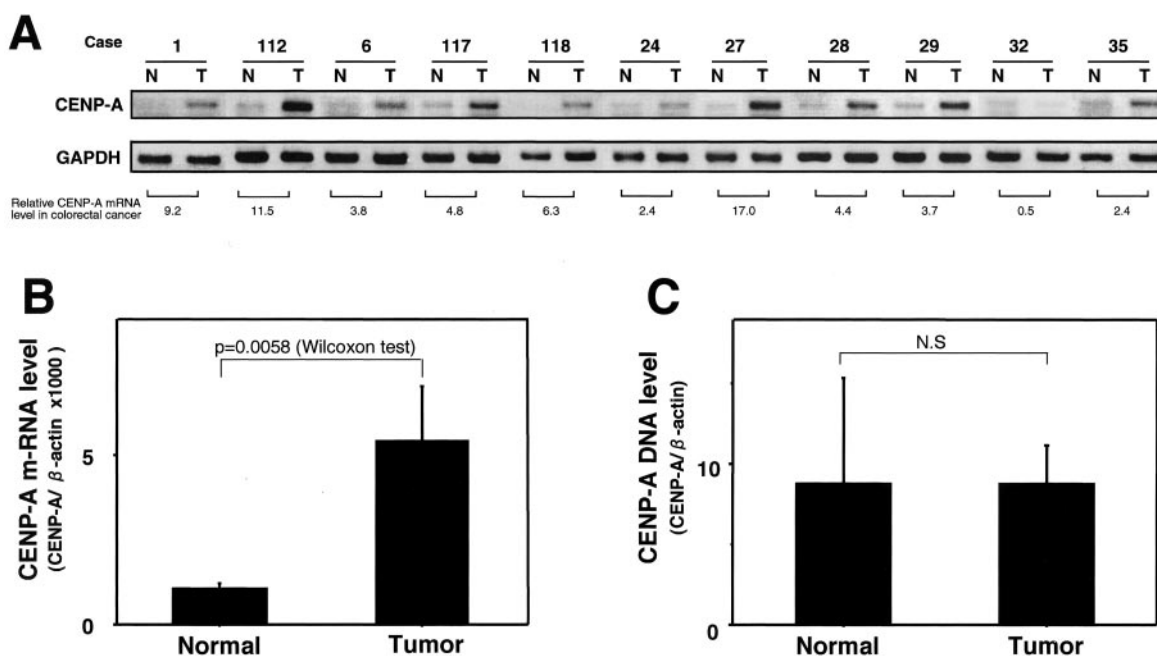

Fig. 2. CENP-A gene is overexpressed but not amplified in colorectal cancer. A, total RNAs were prepared from matched samples of tumor (T) and adjacent normal tissue (N), and RT-PCR was performed. Intensity of each band was measured by NIH Image, and the relative mean CENP-A mRNA levels between tumor and normal tissue normalized with GAPDH mRNA levels were calculated from at least three experiments. In most of the cases, CENP-A mRNA in tumors was increased when compared with in normal tissues. B, comparison of CENP-A mRNA levels between tumors and adjacent normal tissues by real-time quantitative RT-PCR. C, quantitation of CENP-A gene copy number in tumors and normal tissues by real-time quantitative PCR; bars, ±SD.

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Table 2 *FISH analysis of colorectal cancer tissues*

For each chromosome tested, percentage of chromosomal number are shown. Case 1 is highly aneuploid, whereas Case 2 and 3 are near diploid.

	Normal			Tumor 1			Tumor 2			Tumor 3		
	Chromosome number (%)			Chromosome number (%)			Chromosome number (%)			Chromosome number (%)		
	1	2	$\geqq$3	1	2	$\geqq$3	1	2	$\geqq$3	1	2	$\geqq$3
Chromosome 7	16.5	82.5	1.0	3.7	7.4	88.9	11.6	82.0	6.4	3.1	47.7	49.2
Chromosome 8	18.2	77.8	4.0	4.8	14.4	80.8	23.6	71.9	4.5	8.4	77.9	13.7
Chromosome 12	13.9	84.5	1.6	2.1	12.3	85.6	12.7	82.6	4.7	10.0	74.3	15.7
Chromosome 15	19.4	77.7	2.9	21.8	49.1	29.1	20.3	65.6	14.1	14.9	76.2	8.9

staining in both normal and tumor cells. Examination of the several tissue sections under microscope showed increased CENP-A staining in tumor cells compared with normal epithelial cells, indicating that CENP-A is indeed increased in the tumor cells (Fig. 3, *B* and *D*).

The observation shown above raised an important question of where overexpressed CENP-A localizes in tumor cells. CENP-A is known as a core component of kinetochore and recruits a subset of other kinetochore proteins. Thus, mislocalization of CENP-A could contribute to ectopic centromere formation, which would cause chromosome missegregation. To examine this hypothesis, tissue sections were coimmunostained with anti-CENP-A and anti-CENP-B antibodies, and individual cells were examined with higher magnification (Fig. 4*A*). Given that CENP-B binds to centromeric α-satellite DNA, it was used as a centromere marker. Whereas almost all of CENP-A and CENP-B signals colocalized in normal cells, >10% of CENP-A did not colocalize with CENP-B in tumor cells (Fig. 4, *A* and *B*). This result suggests not only that CENP-A associates with noncentromeric chromosomal regions, but it dissociates from native centromeres.
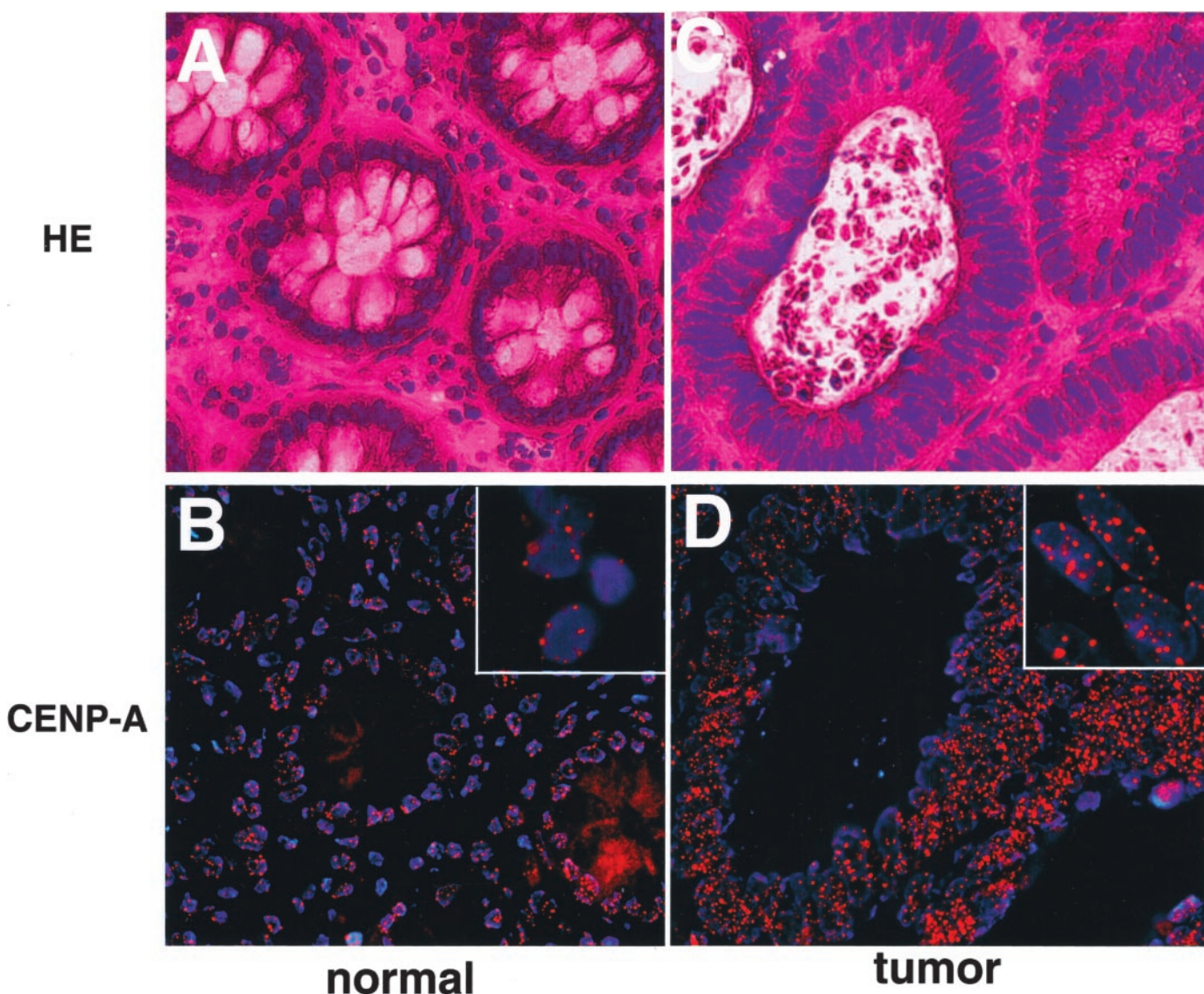

Fig. 3. Immunostaining of human colon tissue with anti-CENP-A antibody. Normal colon epithelium (*A* and *B*) and colon cancer tissue (*C* and *D*) were fixed with 4% paraformaldehyde and were stained with H&E (*A* and *C*) or with anti-CENP-A antibody (*B* and *D*). CENP-A staining was increased in the cancer cells when compared with the normal epithelial cells. Original magnification is ×40. Higher magnification view (×63) is shown in the *inset* of *B* and *D*.
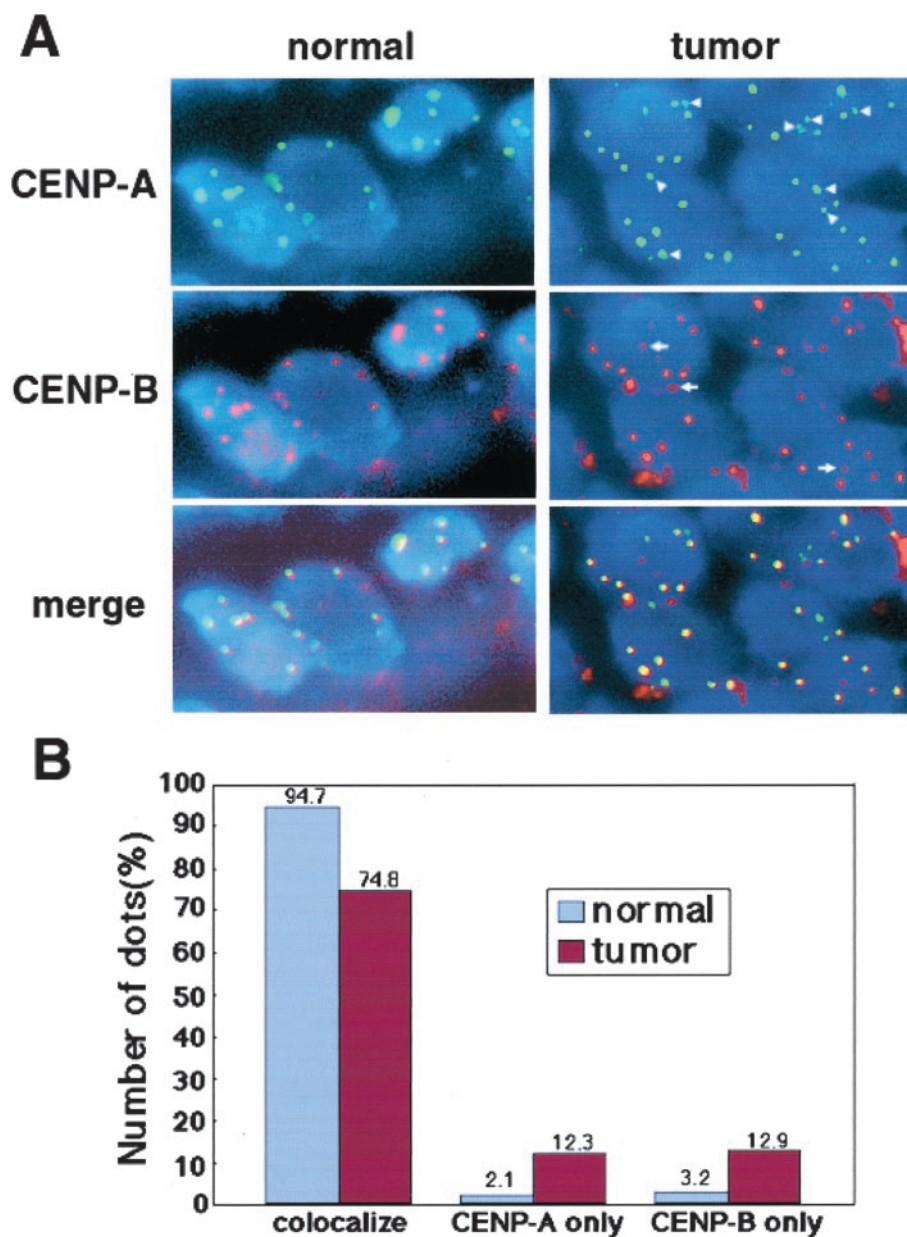
Fig. 4. Coimmunostaining of human colon tissue with anti CENP-A and anti CENP-B antibodies. *A,* normal colon epithelium and colon cancer tissue were fixed with acetone, and were stained with anti-CENP-A and anti-CENP-B antibodies. *Arrowheads* show the CENP-A that does not colocalize with CENP-B. *Arrows* show the CENP-B that does not colocalize with CENP-A. Whereas all of the CENP-A and CENP-B colocalize in normal cells, some of the CENP-A and CENP-B signals do not colocalize in tumor cells. *B,* number (%) of CENP-A and CENP-B dots that colocalize or do not in normal and tumor cells. At least 300 dots of cells were counted respectively.

DISCUSSION

In this report, we presented the first evidence that a kinetochore protein, CENP-A, is overexpressed in primary colorectal cancer. We have found that the overexpressed CENP-A in tumor cells is likely to be mistargeted to a noncentromeric region of the chromosome. In addition, CENP-A disappeared from the centromere of certain chromosomes, which suggests the disruption of native kinetochore complexes. These observations would provide a new insight for understanding chromosomal instability in colorectal cancers.

In recent years, many genes have been identified to provoke chromosome missegregation when mutated in yeast cells (20, 21). In addition to STK15/BTAK/aurora2, Bub1, Mad2, and v-securin, of which the malfunctions induce aneuploidy, defects in several processes involving chromosome condensation, sister-chromatid cohesion, and kinetochore structure could also cause aneuploidy. Some kinetochore proteins, such as CENP-F or INCENP, were up-regulated in human cancer cells (22, 23); however, the real functions of the proteins were still unclear. In contrast, CENP-A is an essential protein

for centromere function. It distinguishes the centromeric chromatin from other chromatin by replacing histone H3 in the centromere-specific nucleosome (24). Furthermore, it is required for the recruitment of other kinetochore proteins to the centromere (16). Thus, inappropriate expression of CENP-A could induce abnormal kinetochore function and chromosome missegregation.

If overexpression of CENP-A causes aneuploidy, what is the mechanism? One explanation may be ectopic centromere formation. Van Hooser *et al.* (25) demonstrated recently that overexpression of CENP-A in HeLa cells results in its incorporation into the entire length of chromosomes and recruits a subset of centromere-kinetochore components to noncentromeric regions of chromatin, which creates prekinetochore complex. The situations in colorectal cancer cells are similar. CENP-A was overexpressed in the cancer cells and was mistargeted to noncentromeric regions. Note that we could not observe the association of CENP-A with entire chromosomes, because mitotic cells were hardly seen in the tissue sections. Although overexpression of CENP-A alone in HeLa cells is not sufficient for

complete kinetochore assembly at noncentromeric chromosome regions, active neocentromere might be assembled in colorectal cancer cells by some additional mechanisms. Thus, it is important to find additional factors necessary for complete centromere activity that may be altered in colorectal cancer.

Absence of CENP-A from certain centromere is somewhat unexpected. Although the precise mechanism is unclear, overexpression of CENP-A might deplete other centromere-kinetochore components and disrupt the kinetochore complex. Another possibility is that mistargeting of CENP-A to noncentromeric regions of chromatin alters the conformation of chromosome, which might prevent normal kinetochore assembly. In fact, overexpressed CENP-A in HeLa cells was found predominantly on the euchromatic arms of chromosomes, with reduced levels of CENP-A in the pericentric heterochromatin of certain chromosomes (25). Additional investigations are needed to clarify the mechanism of mistargeting of CENP-A in colorectal cancer and how it contributes to chromosomal instability.

How is CENP-A overexpressed in colorectal cancer? We demonstrated that CENP-A mRNA was also increased, which indicates that the overexpression of CENP-A occurred at the transcriptional level. Thus, some enhancer proteins may be activated, although such transcriptional factors or enhancer elements are unknown. Investigations of the regulation of CENP-A transcription are needed. Expression of CENP-A is also known to be regulated in the cell cycle. It is synthesized in G_2 after DNA synthesis occurs, which might be important for centromeric nucleosome assembly (26). The expression of CENP-A and centromere assembly should be regulated tightly during the cell cycle, and errors in this regulation may lead to aneuploidy. It would be insightful to examine the expression level of CENP-A throughout the cell cycle in colorectal cancer cell lines.

The chromosomal instability seen in most solid tumors might provide new therapeutic targets if the precise mechanism could be fully uncovered. Genes that potentially affect chromosome segregation are the most promising candidates. If CENP-A overexpression is the driving force of tumor progression, suppression of its expression level in cancer cells might cease tumor proliferation. In fact, microinjection of antibodies to CENP-A arrests HeLa cells in interphase (27). CENP-A possesses a COOH-terminal histone-fold domain that is similar to histone H3 and a highly variable NH_2-terminal domain. This unique NH_2-terminal domain could be a useful target to design an anticancer drug.

ACKNOWLEDGMENTS

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