

Centrosomal Kinases, HsAIRK1 and HsAIRK3, are Overexpressed in Primary Colorectal Cancers

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Members of the recently identified family of *Homo sapiens* Aurora/Ipl1-related kinases (HsAIRKs), homologous to chromosome segregation kinases, fly *Aurora* and yeast *Ipl1*, are highly expressed during M phase, and have been suggested to regulate centrosome function, chromosome segregation, and cytokinesis. In the present study, immunohistochemical analyses were performed of HsAIRK1 and HsAIRK3 expression in 78 primary colorectal cancers and 36 colorectal adenomas as well as 15 normal colorectal specimens. In normal colon mucosa, some crypt cells showed weak positive staining in 10 and 12 out of 15 cases for HsAIRK1 and HsAIRK3, respectively, the remaining cases being negative. Elevated expression of HsAIRK1 was observed in 53 (67.9%) of the colorectal cancers, and of HsAIRK3 in 40 (51.3%). Furthermore, colorectal adenomas showed high expression of HsAIRK1 and HsAIRK3 in 11 (30.6%) and 7 (19.4%) cases, respectively, thus being intermediate between colorectal cancers and normal colorectal mucosa. Interestingly, HsAIRK1 overexpression was significantly associated with pT (primary tumor invasion) and p53 accumulation in colorectal cancers. There was no significant correlation between proliferating cell nuclear antigen-labeling index (PCNA-LI) and the levels of these proteins. The results suggest that overexpression of HsAIRK1 and HsAIRK3 might be involved in tumorigenesis and/or progression of colorectal cancers.

Key words: HsAIRK1 — HsAIRK3 — Colorectal cancer — Colorectal adenoma — PCNA-LI

Cancer is widely considered to be a genetic disease resulting from an accumulation of various genetic abnormalities.¹ A multistep genetic model of tumorigenesis has been proposed for neoplasms such as colon cancers.^{2,3} Loss of chromosomal integrity as well as genomic instability is considered to act as a driving force during the processes of tumorigenesis and tumor progression.^{4,5} During the evolution of normal cells into cancer cells, the occurrence of multiple mutations results in genetic instability. A variety of chromosomal aberrations, such as abnormal ploidy, are common in cancer cells.^{6,7} The centrosome is believed to play a crucial role in maintaining genomic stability by establishing bipolar spindles during cell division. Equal segregation of duplicated chromosomes into two daughter cells is thereby ensured. Centrosome abnormalities are often observed in cancer cells, where they are thought to cause chromosome missegregation, important for progression of malignancy.^{8–12}

Fly *Aurora* and yeast *Ipl1* gene products are known to be involved in normal chromosome segregation.^{13,14} Loss or dysfunction of *Aurora* leads to a failure of the centrosome to separate and form a bipolar spindle.¹⁴ Recent investigations revealed the presence of various members of the Aurora/Ipl1-related kinase family (AIRKs) from

yeast to human, and Giet and Prigent¹⁵ proposed a generic name, AIRKs with a species prefix and a number. Although all the members have highly homologous C-terminal kinase domains, similarity in N-terminal domains has indicated that they fall into three subgroups in mammals: (a) HsAIRK1 for human; AIK1/BTAK/aurora2/ARK1/STK15/STK6^{16–20} and MmAIRK1 for mouse; Ayk1/IAK1/ARK1,^{19,21,22} (b) HsAIRK2 for human; ARK2/AIK2/aurora1/AIM-1,^{18,19,23,24} MmAIRK2 for mouse; STK-1/ARK2^{19,25} and RnAIRK2 for rat; AIM-1,²⁶ (c) HsAIRK3 for human; AIK3/STK13/AIE2^{27–29} and MmAIRK3 for mouse; AIE1.²⁹

Previous investigations^{30–33} revealed chromosomal aberrations at chromosome 20q13, where the *HsAIRK1* gene has been mapped,^{17,34} in cancer tissues of several organs. In addition, the *HsAIRK1* gene is amplified, and its mRNA level is upregulated in breast and colorectal cancers.^{17,18,20} Recently, our group has revealed by immunohistochemistry that the HsAIRK1 protein is frequently overexpressed in primary human breast cancers.³⁵ HsAIRK1 is a human homologue of Aurora and Ipl1, known to play roles in chromosome segregation, and it has been shown to localize to the spindle pole during mitosis, especially from prophase through anaphase, suggesting a possible involvement in centrosomal functions.¹⁶ It is thus conceivable that loss of its regulation might cause an alteration in chromosomal numbers. In fact, recent studies

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revealed that overexpression of HsAIRK1 in rodent fibroblasts induced centrosome amplification, aneuploidy, and transformation, indicating that HsAIRK1 is a novel member of the oncogene group.²⁰⁾ Furthermore, the *HsAIRK3* gene is located on human chromosome 19q13.43,^{27, 28)} a region rearranged or deleted in several cancer cells.^{36–38)} HsAIRK3 is expressed cell cycle-dependently and localized in centrosomes from anaphase to cytokinesis, with overexpression reported for some cancer cell lines.²⁷⁾

In the present study, we performed detailed immunohistochemical analyses of HsAIRK1 and HsAIRK3 expression in primary colorectal cancers and colorectal adenomas as well as in normal colorectal tissues. The results demonstrated significant increases of both proteins more frequently in colorectal cancers than in colorectal adenomas.

MATERIALS AND METHODS

Patients and tissue samples Seventy-eight primary colorectal cancer specimens and 15 matched normal colon tissues from distant sites were obtained by routine surgical procedures at the Second Department of Surgery, Gifu University School of Medicine. In addition, 36 colorectal adenomas obtained by endoscopic polypectomy were also examined. Clinicopathological characteristics of the 78 primary colorectal cancer patients were as follows: mean age 63 years (range 21–87); 30 males, 48 females; 46 well differentiated, 28 moderately differentiated and 4 poorly differentiated adenocarcinomas. Those of 36 colorectal adenoma patients were: mean age 64 years (range 31–83); 21 males, 15 females; 1 adenoma with mild dysplasia, 18 with moderate dysplasia, and 17 with severe dysplasia.³⁹⁾ Colorectal cancer patients were classified according to Dukes' classification,⁴⁰⁾ in addition to D-stage for patients with distant metastasis. Pathological TNM staging was performed according to the criteria of the American Joint Committee on Cancer.⁴¹⁾ The carcinoma locations were grouped into 3 categories: 23 in the proximal colon (from the cecum to the splenic flexure), 25 in the distal colon (from the descending to the sigmoid colon), and 30 in the rectum.

Production of polyclonal antibodies against HsAIRK1 and HsAIRK3 Antibodies against HsAIRK1 and HsAIRK3 were raised and affinity-purified as previously described.^{16, 27)} Antiserum was tested for specificity by western blotting using whole cell extracts from HeLa cells.^{16, 27)}

Immunohistochemistry The immunohistochemical studies were performed using the labeled streptavidin-biotin immunoperoxidase technique to determine the expression of HsAIRK1 and HsAIRK3. Four-micrometer-thick sections of formalin-fixed and paraffin-embedded tissue samples were mounted on silane-coated glass slides. Sections were deparaffinized in xylene and rehydrated through a graded series of ethanols. They were microwaved in 10

mM citrate-phosphate buffer (pH 6.0) for antigen retrieval for 15 min for HsAIRK1, or for 5 min for HsAIRK3, then incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, followed by bovine serum albumin (BSA) for 10 min to block non-specific binding before the addition of the antibody. Next, the sections were incubated overnight with the primary antibody (the HsAIRK1 antibody was diluted 100-fold in Tris-buffered saline pH 7.6 (TBS) with 1% BSA and the HsAIRK3 antibody was diluted 300-fold in TBS with 1% BSA) in a humidified chamber at 4°C. All slides were incubated in sequence with secondary biotinylated antibody for 10 min and peroxidase-labeled streptavidin for 10 min using an LSAB kit (DAKO Corp., Carpinteria, CA). Finally, 3,3'-diaminobenzidine (DAB, DAKO Corp.) was used as the chromogen, and sections were counterstained with Mayer's hematoxyline and examined under a light microscope. For each case, two negative controls were run with serial sections. For one control section, the primary antibody was replaced with pre-immune rabbit serum, and for the other, incubation with the primary antibody was omitted. As positive controls for HsAIRK1 immunostaining, sections of primary human invasive ductal carcinoma of the breast were immunostained. HeLa cells that overexpressed HsAIRK1 and HsAIRK3 as revealed by western blotting were found to overexpress both proteins in the cytoplasm by using immunohistochemistry (data not shown). Specificity was confirmed by using antibodies preabsorbed with the HsAIRK1 and HsAIRK3 proteins before staining.

The proliferative activities of primary colorectal cancers were determined by assessing proliferating cell nuclear antigen-labeling index (PCNA-LI). Briefly, sections were immunostained with the anti-PCNA monoclonal antibody PC10 (DAKO A/S, Glostrup, Denmark) as a primary antibody using the labeled streptavidin-biotin immunoperoxidase technique, as for HsAIRK1 and HsAIRK3 staining. PC10 was diluted 100-fold and reacted with tissue specimens at room temperature for 1 h. Sections were also immunostained with the anti-p53 monoclonal antibody DO-7 (DAKO A/S) using the same protocol. DO-7 was diluted 100-fold and reacted overnight at 4°C. Immunohistochemistry of PCNA, p53, HsAIRK1 and HsAIRK3 was performed on serial sections.

Evaluation of immunostaining A semiquantitative evaluation was performed by two independent observers (T.T. and N.Y.), who were blinded to the clinical and pathological stages of the patients, on two separate occasions. The intensity of positivity of every stained specimen was scored as follows: ++, strong expression; +, medium expression; ±, weak expression; and –, expression not detectable.³⁵⁾ The extent of positive staining was further categorized into four groups (0, <30%, 30–60%, >60%), on the basis of the proportion of the positively stained

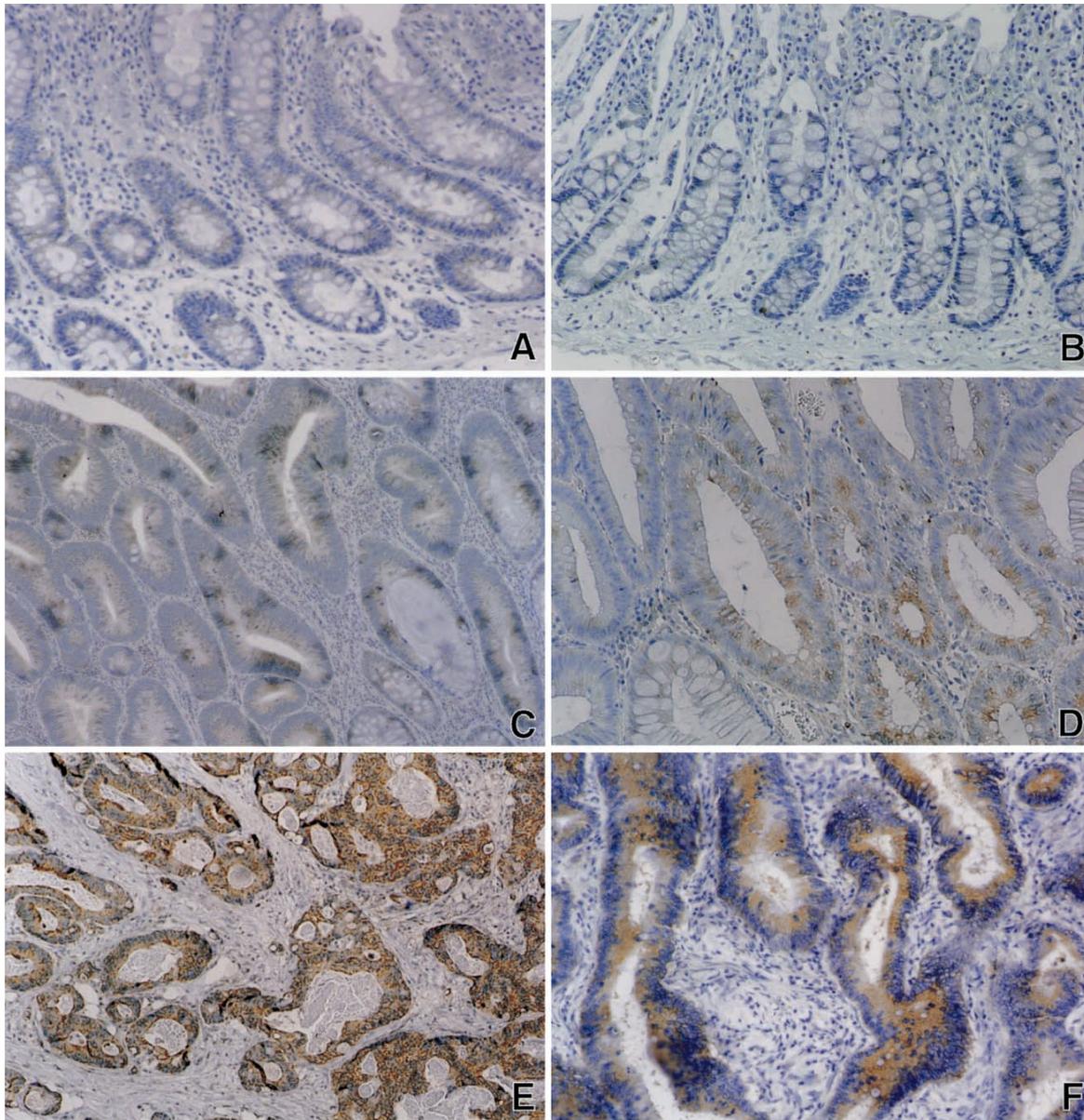


Fig. 1. Representative results of immunohistochemical staining for HsAIRK1 and HsAIRK3 in normal colon tissue, colorectal adenoma, and colorectal cancer. A, C, E: tissues immunostained with anti-HsAIRK1 polyclonal antibody, and B, D, F: tissues immunostained with anti-HsAIRK3 polyclonal antibody. In normal colon mucosa, some crypt cells show weak positive staining (\pm) for HsAIRK1 (A) or HsAIRK3 (B). The adenoma shows occasional staining of medium intensity (+) for HsAIRK1 (C) or HsAIRK3 (D). Intense and diffuse immunostaining (++) for HsAIRK1 (E) or HsAIRK3 (F) is apparent in cancer cells.

area. In cancerous specimens, the intensity of positive staining for HsAIRK1 and HsAIRK3 was evaluated as compared with adjacent normal epithelial cells in the same section as an internal control. When the intensity of positive staining of tumor cells was stronger than that of adjacent normal epithelial cells, the expression of tumor cells was evaluated as '+' or '++.'

The PCNA-LI was defined as the percentage of stained cells per a minimum of 1000 tumor cells. Five randomly chosen microscopic fields were examined at high magnification ($\times 200$) under a light microscope for this purpose. p53 expression was scored as positive when staining was visible in more than 10% of nuclei within a specimen.

Statistical analyses The χ^2 test or Fisher's exact proba-

bility test was used to examine the association between HsAIRK1 and HsAIRK3 expression and the various other parameters including clinicopathological characteristics, except for Dukes' classification and pT (primary tumor invasion), when the Mann-Whitney *U* test was applied. Student's *t* test was performed to examine the correlation between PCNA-LI and expression of both proteins. Statistical significance was assumed when the *P* value was <0.05.

RESULTS

HsAIRK1 and HsAIRK3 expression in normal colon tissue In normal colon mucosa, some crypt cells only showed weak positive staining (±) in 10 and 12 out of 15 cases for HsAIRK1 and HsAIRK3 (Fig. 1, A and B), respectively, the remaining cases being negative (Table I). Staining was cytoplasmic in most cells. Stromal cells such as fibroblasts, smooth muscle cells and vascular endothelial cells were negative. Control staining with non-immune rabbit serum or without HsAIRK1 and HsAIRK3 antibody resulted in no staining (data not shown).

HsAIRK1 expression in colorectal cancers and adenomas Based on the results obtained for normal colon epithelial cells, we classified tumors showing ‘++’ intensity and >30% positive for ‘+’ intensity as having high expression of HsAIRK1 and HsAIRK3. Colorectal cancer specimens showed frequently strong (++) or medium (+) positive reactions in the cytoplasm of the epithelial cells (Fig. 1E), that were markedly diminished with preabsorbed antibody (data not shown). HsAIRK1 was not detected in stromal cells within cancerous lesions. High expression of HsAIRK1 was detected in 53 (67.9%) of 78 colorectal cancer specimens (Table I). The figure for colorectal adenomas was only 11 (30.6%) of 36 cases (Table I). The staining localization of HsAIRK1 in adenoma specimens was similar to that observed in cancer specimens, albeit with weaker intensity and in more limited

areas (Fig. 1C). Cancer specimens showed significantly higher expression of HsAIRK1 than adenoma specimens (Table I, *P*=0.0002, χ^2 test). Incidences of high HsAIRK1 expression in adenomas with mild, moderate, and severe dysplasia were 0 of 1 (0%), 4 of 18 (22.2%), and 7 of 17 cases (41.2%), respectively. This trend towards higher HsAIRK1 expression in advanced grade of dysplasia was not statistically significant.

HsAIRK3 expression in colorectal cancers and adenomas As with HsAIRK1, HsAIRK3 immunohistochemistry showed frequent diffuse and intense staining in the cytoplasm of cancerous epithelium cells (Fig. 1F). The specificity of the HsAIRK3 antibody was also confirmed by staining after preincubation with the purified antigen. High expression of HsAIRK3 was detected in 40 (51.3%) of 78 colorectal cancer specimens and only 7 (19.4%) of 36 adenomas (Table I). The intensity and extent of positive staining in adenoma specimens were lower than in cancers (Fig. 1D). Cancer specimens showed significantly higher expression of HsAIRK3 than adenoma specimens (Table I, *P*=0.0013, χ^2 test). Incidences of high HsAIRK3 expression in mild, moderate, and severe dysplasia were 0 of 1 (0%), 1 of 18 (5.6%), and 6 of 17 cases (35.3%), respectively. Severe dysplasia showed a statistically significant increase in expression as compared with moderate dysplasia (*P*=0.041, χ^2 test).

Relationship between expression of HsAIRK1 or HsAIRK3 and clinicopathological characteristics in colorectal cancers Significant associations were not found between HsAIRK1 or HsAIRK3 expression and clinicopathological characteristics such as sex, age, histological differentiation and tumor location (Table II). There was a statistically significant association between HsAIRK1 expression and pT (Table II, *P*=0.027, Mann-Whitney *U* test), but not Dukes' classification. In particular, tumors invading beyond the submucosa showed significantly high expression of HsAIRK1 (pT; Tis, T1 vs. T2, T3 and T4, *P*=0.0091, χ^2 test). No such association was

Table I. HsAIRK1 and HsAIRK3 Expression in Human Colorectal Tissues

	Intensity (highest) Percentage (area)	Total	-				±			+			++			Cases with high expression ^{a)}
			0	<30%	30-60%	>60%	<30%	30-60%	>60%	<30%	30-60%	>60%	<30%	30-60%	>60%	
HsAIRK1	Normal colon mucosa	15	5	5	2	3	0	0	0	0	0	0	0	0	0	0
	Colorectal adenomas	36	3	5	10	2	5	7	4	0	0	0	0	0	0	11 (30.6%)
	Colorectal cancers	78	4	2	8	10	1	14	28	0	2	9	53 (67.9%) ^{b)}			
HsAIRK3	Normal colon mucosa	15	3	4	5	3	0	0	0	0	0	0	0	0	0	
	Colorectal adenomas	36	5	8	10	0	6	5	2	0	0	0	0	0	7 (19.4%)	
	Colorectal cancers	78	9	4	9	12	4	14	17	0	2	7	40 (51.3%) ^{c)}			

a) High expression is defined as expression showing “++” intensity and >30% positive with “+” intensity of HsAIRK1 and HsAIRK3.

b) Comparison of colorectal cancers and adenomas for HsAIRK1; *P* =0.0002, χ^2 test.

c) Comparison of colorectal cancers and adenomas for HsAIRK3; *P* =0.0013, χ^2 test.

Table II. Relationship between HsAIRK1 and HsAIRK3 Expression and Clinical Characteristics of Colorectal Cancers

Clinical characteristics	Total	HsAIRK1			HsAIRK3		
		High	Low	<i>P</i> value	High	Low	<i>P</i> value
All cases	78	53	25		40	38	
Sex							
Male	30	21	9	0.759	15	15	0.858
Female	48	32	16		25	23	
Age							
≤ 63 years	35	25	10	0.552	17	18	0.666
> 63 years	43	28	15		23	20	
Histological differentiation							
Well	46	29	17	0.538	23	23	0.467
Moderate	28	21	7		16	12	
Poor	4	3	1		1	3	
Tumor location							
Proximal colon	23	14	9	0.066	9	14	0.381
Distal colon	25	14	11		14	11	
Rectum	30	25	5		17	13	
Dukes' classification							
A	24	12	12	0.073	11	13	0.424
B	24	18	6		13	11	
C	19	15	4		8	11	
D	11	8	3		8	3	
pT (primary tumor invasion)							
pTis, pT1	14	5	9	0.027	6	8	0.196
pT2	12	8	4		5	7	
pT3	35	27	8		18	17	
pT4	17	13	4		11	6	
pN (lymph node metastasis)							
pN0	47	29	18	0.146	24	23	0.962
pN1, pN2	31	24	7		16	15	
M (distant metastasis)							
M0	67	45	22	0.999	32	35	0.194
M1	11	8	3		8	3	
p53 status							
Positive	44	34	10	0.045	19	25	0.102
Negative	34	19	15		21	13	

evident for HsAIRK3 expression. Furthermore, high expression of HsAIRK1 or HsAIRK3 did not show a significant association with either metastasis to regional lymph nodes (pN) or distant metastasis (M) (Table II).

Relationship between expression of HsAIRK1 or HsAIRK3, and p53 status and PCNA-LI in colorectal cancers p53 accumulation was detected in 44 of 78 cases (56.4%), being significantly associated with HsAIRK1 (Table II, $P=0.045$, χ^2 test), but not HsAIRK3 expression. All of the examined cancer specimens showed definite, positive nuclear staining for PCNA. Mean PCNA-LI was 52.3%, with a range of 24.1 to 77.3%. Values for lesions with high and low HsAIRK1 expression were $53.2\pm 10.4\%$ (mean \pm SD) and $50.5\pm 13.3\%$, respectively, and those for

Table III. Relationship between HsAIRK1 and HsAIRK3 Expression and PCNA-LI in Colorectal Cancers

Expression	HsAIRK1		HsAIRK3	
	PCNA-LI ^{a)}	<i>P</i> value	PCNA-LI	<i>P</i> value
High	$53.2\pm 10.4^b)$	0.338	54.0 ± 11.6	0.192
Low	50.5 ± 13.3		50.6 ± 11.0	

a) PCNA-LI: proliferating cell nuclear antigen-labeling index.

b) Mean \pm SD (%).

high and low HsAIRK3 expression were $54.0\pm 11.6\%$ and $50.6\pm 11.0\%$, respectively, without any statistically significant intergroup difference (Table III).

DISCUSSION

Chromosome abnormalities such as aneuploidy are frequent in human cancers.^{6,7)} Recently, it was also reported that centrosome abnormalities are often observed in cancer cells.^{8,9)} Therefore, it is appealing to hypothesize that genes involved in chromosome segregation might contribute to this phenotype. Although the precise mechanisms by which duplicated chromosomes are equally segregated during mitosis have not been clarified, the centrosome is believed to play an important role in the formation of bipolar spindles. Mutations in fly *Aurora* and yeast *Ipl1* are responsible for chromosome segregation defects, and their products encode putative serine/threonine kinases.^{13,14)} Homologous human kinases, HsAIRK1 and HsAIRK3, are also suggested to play roles in chromosome segregation and tumorigenesis. Of particular note, the facts that overexpression of HsAIRK1 in colorectal and breast cancers and its mapping on chromosome 20q13 were the first clues of an involvement in tumorigenesis.^{17,18,20,42)} Gene amplification at 20q13 is common to a variety of human malignancies, especially in the colon and breast.³⁰⁻³²⁾ Recently, it was also found that overexpression of HsAIRK1 can amplify centrosomes and transform rodent fibroblasts.²⁰⁾ These findings suggest that HsAIRK1 might be a potential oncogene for breast and colon cancers, and possibly other solid malignancies. However, a detailed analysis of HsAIRK1 expression by immunohistochemistry has not previously been performed for colorectal cancers, adenomas, and normal colon mucosa. In addition, this is the first immunohistochemical analysis of HsAIRK3 to our knowledge.

The purpose of this study was first to determine whether HsAIRK1 and HsAIRK3 are overexpressed in primary colorectal cancer specimens compared with normal colon mucosa. This was indeed found to be the case. The *HsAIRK1* gene has been shown to be amplified in 52% and its mRNA level was increased in 54% of a series of primary human colorectal cancers.¹⁸⁾ Northern and western blot analyses confirmed high expression levels of HsAIRK1 and HsAIRK2 in 12 primary colorectal cancers.⁴³⁾ In the present study, colorectal adenomas also showed high expression in an appreciable number of cases, albeit at lower levels than in colorectal cancers, with a link at least for HsAIRK3 expression with the degree of dysplastic change. Thus, premalignant lesions, which may have already reached a state of neoplastic transformation, were also positive.

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With regard to the correlation between HsAIRK1 and HsAIRK3 expression and clinical characteristics, only pT demonstrated a significant link, specific to HsAIRK1, when cancers invaded beyond the submucosa (\geq pT2). Katayama *et al.* recently reported that HsAIRK2 but not HsAIRK1 expression levels increased as a function of Dukes' stage.⁴³⁾

There was no statistically significant correlation between expression of HsAIRK1 or HsAIRK3 and PCNA-LI, so that a direct relation to cell proliferation is unlikely. We earlier showed that positive staining with HsAIRK1 is not an indication of cancer cell proliferation in primary breast cancers.³⁵⁾ Interestingly, high HsAIRK1 expression was significantly associated with immunohistochemical status of p53. It was recently reported that loss of this tumor suppressor protein can result in centrosome hyperamplification, leading to aberrant mitosis and chromosomal instability.^{44,45)} In our study, 31 of 78 colorectal cancers showed high expressions of both HsAIRK1 and HsAIRK3, while 16 showed low expressions of both. The remaining 31 cancers showed either high HsAIRK1/low HsAIRK3 expression or low HsAIRK1/high HsAIRK3 expression. Since HsAIRK1 and HsAIRK3 patterns of expression did not appear necessarily linked, the functions of these proteins might differ from each other.

In conclusion, we found that the progression from normal colonic epithelium, to colorectal adenoma, and then primary colorectal cancer is associated with increased expression levels of HsAIRK1 and HsAIRK3. Alterations of proteins forming a centrosome-associated kinase cascade may lead to chromosome segregation defects and genomic instability. Thus, it is tempting to speculate that overexpression of HsAIRK1 and HsAIRK3 may be of pathogenic and/or prognostic importance for colorectal cancers. Additional studies are currently being undertaken to examine the correlation between HsAIRKs overexpression and aneuploidy/centrosome abnormalities and to gain further insight into the biological roles of HsAIRKs.

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