Polo-like kinase 1 (PLK1) is overexpressed in primary colorectal cancers

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PLK (polo-like kinase), the human counterpart of *polo* in *Drosophila melanogaster* and of *CDC5* in *Saccharomyces cerevisiae*, belongs to a family of serine/threonine kinases. It is intimately involved in spindle formation and chromosome segregation during mitosis. The purpose of this study was to determine whether PLK1 is overexpressed in primary colorectal cancer specimens as compared with normal colon mucosa and to assess its relation to other kinases as a potential new tumor marker. In the present study, immunohistochemical analyses were performed of PLK1 expression in 78 primary colorectal cancers as well as 15 normal colorectal specimens. Furthermore, we examined the relationship between other kinases, Aurora-A and Aurora-C, and PLK1 expression. In normal colon mucosa, some crypt cells showed weakly positive staining for PLK1 in 13 out of 15 cases, the remaining cases being negative. Elevated expression of PLK1 was observed in 57 (73.1%) of the colorectal cancers, statistically significant associations being evident with pT (primary tumor invasion) (P=0.0006, Mann-Whitney U test), pN (regional lymph nodes) (P=0.008, χ^2 test) and the Dukes' classification (P=0.0005, Mann-Whitney U test). Mean proliferating cell nuclear antigen-labeling index was 52.3%, with a range of 24.1% to 77.3%. Values for lesions with high and low PLK1 expression were 54.7±10.3% (mean±SD) and 45.9±11.9% (P=0.002, Student's t test). PLK1 was significantly associated with Aurora-A, but PLK1 staining was more diffuse and extensive than for Aurora-A or Aurora-C. Interestingly, PLK1 overexpression was significantly associated with p53 accumulation in colorectal cancers. Our results suggest overexpression of PLK1 might be of pathogenic, prognostic and proliferative importance, so that this kinase might have potential as a new tumor marker for colorectal cancers. (Cancer Sci 2003; 94: 148–152)

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)]

We earlier reported frequent impairment of the mitotic checkpoint and a molecular analysis of the mitotic checkpoint genes, *hsMAD2*, *hMAD1*, and *p55CDC*.[4, 5)] The centrosome is believed to play a crucial role in maintaining genomic stability by establishing bipolar spindles during cell division, and 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. Centrosome abnormalities are often observed in cancer cells, where they are thought to cause chromosome missegregation, important for progression of malignancy.[6–8)] Loss or dysfunction of members of the *Aurora* family of kinases leads to a failure of centrosomes to separate and form bipolar spindles.[9)] The nomenclature of Aurora/Ipl1-related kinases has been approved by many scientists working in the aurora field as a unifying classification. Similarity in N-terminal domains indicates the existence of three groups: (a) Aurora-A, HsAIRK1/Aik1/BTAK/aurora-2/ARK1/STK-15; (b) Aurora-B, HsAIRK2/Aik2/aurora-1/ARK2/STK-12/AIM-1; and (c) Aurora-C, HsAIRK3/Aik3/aurora-3/AIE2/STK-13.[10)] Recently, we revealed by immunohistochemical analysis that Aurora-A and Aurora-C proteins are frequently overexpressed in primary human colorectal cancers, with a link to progression from normal colonic epithelium to colorectal adenoma, and then to primary colorectal cancer.[11)]

The polo-like kinases (PLKs), homologous to the *Drosophila polo* kinase, are another conserved family of enzymes that play a variety of roles in the passage of cells through the M phase. PLK1 is one of these serine threonine kinases, and has been shown to be required for the precise regulation of mitosis by activating the CDC25C-CDK1 amplification loop and late mitotic events, primarily ubiquitin-dependent proteolysis.[12–15)] PLK1 has been found to be overexpressed in several tumor types, high levels of mRNA and protein expression correlating with mitotic activity and an adverse clinical outcome.[16–18)]

We have studied tumorigenesis by molecular analysis of primary colorectal cancers and provided evidence that abnormalities of mitotic kinases active in cell division and its checkpoints (the Cdk family, the Polo family, the Aurora family, the NIMA family, and the Mitotic checkpoint family) are important.[10, 11)]

In the present study, we performed a detailed analysis of PLK1 expression in primary colorectal cancers in comparison with normal colorectal tissues. Included was an examination of the relationship with other mitotic kinases, Aurora-A and Aurora-C.

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. Clinicopathological characteristics of the 78 primary colorectal cancer cases were as follows: mean age 63 years (range 21–87); 30 males, 48 females; 46 well differentiated, 28 moderately differentiated and 4 poorly differentiated. The Dukes' classification[19)] was applied in addition to the D-stage for patients with distant metastasis. Pathological TNM staging was performed according to the criteria of the American Joint Committee on Cancer.[20)] The carcinoma locations were 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. The specimens used for the immunohistochemical analysis were the same as previously described for Aurora-A and Aurora-C.[11)]

Immunohistochemistry. Immunohistochemistry was performed using the labeled streptavidin-biotin immunoperoxidase technique to determine the expression of PLK1. Four-micrometer-thick sections of formalin-fixed and paraffin-embedded samples

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were mounted on silane-coated glass slides, deparaffinized in xylene and rehydrated through a graded series of ethanols. They were microwaved in 10 m*M* citrate-phosphate buffer (pH 6.0) for antigen retrieval for 15 min for PLK1, 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. Next, the sections were incubated for 2 h with mouse anti-human PLK1 monoclonal antibody (Transduction Laboratories, Lexington, KY), diluted 100-fold in Tris-buffered saline pH 7.6 (TBS) with 1% BSA, in a humidified chamber at room temperature. 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 applied as the chromogen, and sections were counterstained with Mayer's hematoxylin and examined under a light microscope. A negative control was performed with serial sections, omitting incubation with the primary antibody.

Proliferative activity of primary colorectal cancers was determined by assessing the proliferating cell nuclear antigen-labeling index (PCNA-LI). Briefly, serial sections were immunostained with the anti-PCNA monoclonal antibody PC10 (DAKO A/S, Glostrup, Denmark) as previously described.[11] Sections were also immunostained with the anti-p53 monoclonal antibody DO-7 (DAKO A/S), and for Aurora-A and Aurora-C on serial sections.[11]

Evaluation of immunostaining. A semiquantitative evaluation was performed by two independent observers (T.T. and B.S.), who were blinded to the clinical and pathological stage of the patients, on two separate occasions. The intensity of specimen staining was scored as follows: ++, strong; +, moderate; ±, weak; and −, not detectable.[21] 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 area. In cancerous specimens, the intensity of positive staining for PLK1 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 in a minimum of 1000 tumor cells counted. Five randomly chosen microscopic fields were examined at high magnification (×200) under a light microscope for this purpose, as previously described.[11] p53 expression was scored as positive when staining was visible in more than 10% of nuclei within a specimen.[11]

Statistical analyses. The χ^2 and Fisher's exact probability tests were used to examine associations between PLK1 expression and various other parameters including clinicopathological characteristics, except for the 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 protein expression. The χ^2 test was employed to examine the correlation between PLK1 expression, and Aurora-A or Aurora-C. Statistical significance was assumed with a P value <0.05.

Results

PLK1 expression in normal colon tissue. In normal colon mucosa, some crypt cells showed weakly positive staining (±) in 13 out of 15 cases (Fig. 1A), the remaining cases being negative (Table 1). Staining was cytoplasmic in most cells. Stromal cells such as fibroblasts, smooth muscle cells and vascular endothe-

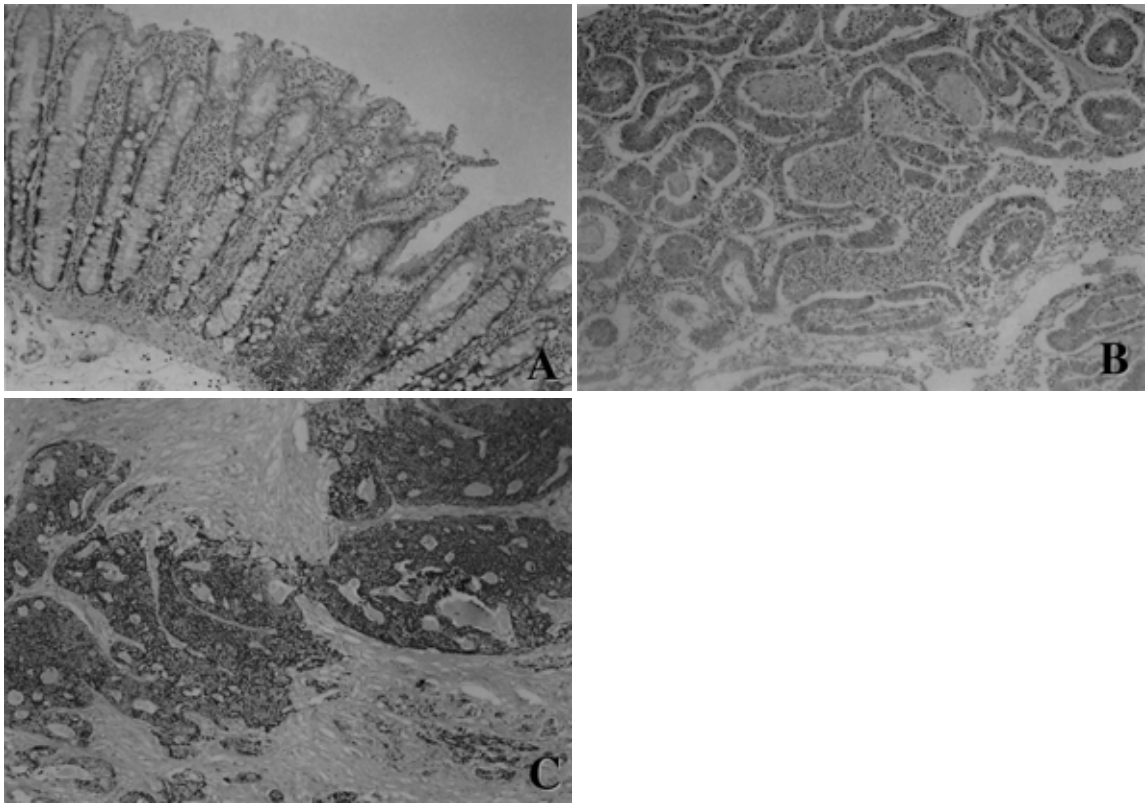

Fig. 1. Representative results of immunohistochemical staining for PLK1 in normal colon tissue and colorectal cancer. In normal colon mucosa, some crypt cells show weakly positive staining (±) for PLK1 (A). Colorectal cancer specimens frequently show strongly (++) (C) or moderately positive (+) reactions (B) in the cytoplasm of the epithelial cells for PLK1.

Table 1. PLK1 expression in human colorectal tissues

Intensity / Percentage (area)	Total	−	±			+			++			Cases with high expression[1)]
		0	<30%	30–60%	>60%	<30%	30–60%	>60%	<30%	30–60%	>60%	
Normal colon mucosa	15	2	7	5	1	0	0	0	0	0	0	0
Colorectal cancers	78	0	6	8	1	6	10	5	1	5	36	57 (73.1%)

1) High expression is defined as the total of '++' intensity and >30% positive with '+' intensity of PLK1.

lial cells were negative. Localization of immunostaining with the anti-PLK1 antibody was similar to those with anti-Aurora-A and anti-Aurora-C.

PLK1 expression in colorectal cancers. Based on the results obtained for normal colon epithelial cells, we classified tumors showing '++' intensity or >30% positive cells with '+' intensity as highly expressing PLK1. Colorectal cancer specimens frequently showed strongly (++) or moderately positive (+) reactions in the cytoplasm of the epithelial cells (Fig. 1, C and B). PLK1 was not detected in stromal cells within cancerous lesions. High expression of PLK1 was detected in 57 (73.1%) of 78 colorectal cancer specimens (Table 1).

Relationship between expression of PLK1 and clinicopathological characteristics of colorectal cancers. Significant associations were not found between PLK1 expression and clinicopathological characteristics such as sex, age, histological differentiation and tumor location (Table 2). There was a statistically significant association with pT (Table 2, P=0.0006, Mann-Whitney U test), pN (regional lymph nodes) (Table 2, P=0.008, χ^2 test), and the Dukes' classification (Table 2, P=0.0005, Mann-Whitney U test). However, high expression of PLK1 was not significantly linked with distant metastasis (M) (Table 2).

Relationship between expression of PLK1, and p53 status and PCNA-LI in colorectal cancers. p53 accumulation was detected in 44 of 78 cases (56.4%), being significantly associated with PLK1 (Table 2, P=0.048, χ^2 test). All of the examined cancer specimens showed definite, positive nuclear staining for PCNA. The mean PCNA-LI was 52.3%, with a range of 24.1% to 77.3%. Values for lesions with high and low PLK1 expression were 54.7±10.3% (mean±SD) and 45.9±11.9%, the intergroup difference being statistically significant (Table 3, P=0.002, Student's t test). PLK1-positive cells were more frequent than PCNA-positive cells.

Relationship and comparison between expression of PLK1, and Aurora-A, and Aurora-C in colorectal cancers. Elevated expression of Aurora-A was observed in 53 (67.9%) of the 78 colorectal cancers, and of Aurora-C in 40 (51.3%), as previously described.[11)] In our study, 44 of 78 colorectal cancers showed high expression of both PLK1 and Aurora-A, while 12 showed low expression of both (Table 4, P=0.004, χ^2 test). However, PLK1 expression is not statistically significantly associated with Aurora-C (Table 4, P=0.054, χ^2 test). Immunostaining of PLK1 was more diffuse and extensive than for Aurora-A or Aurora-C, while in normal mucosa the levels were similar. There was no statistically significant correlation between expression of Aurora-A or Aurora-C and PCNA-LI.[11)]

Table 2. Relationships between PLK1 expression and clinical characteristics of colorectal cancers

Clinical characteristics	Total	PLK1		
		High	Low	P value
All cases	78	57	21	
Sex				
Male	30	21	9	0.628
Female	48	36	12	
Age				
≤63 years	35	24	11	0.418
>63 years	43	33	10	
Histological differentiation				
Well	46	31	15	0.127
Moderate	28	24	4	
Poor	4	2	2	
Tumor location				
Proximal colon	23	15	8	0.104
Distal colon	25	16	9	
Rectum	30	26	4	
Dukes' classification				
A	24	11	13	0.0005
B	24	19	5	
C	19	17	2	
D	11	10	1	
pT (primary tumor invasion)				
pTis, pT1	14	4	10	0.0006
pT2	12	9	3	
pT3	35	29	6	
pT4	17	15	2	
pN (lymph node metastasis)				
pN0	47	29	18	0.008
pN1, pN2	31	28	3	
M (distant metastasis)				
M0	67	47	20	0.271
M1	11	10	1	
p53 status				
Positive	44	36	8	0.048
Negative	34	21	13	

Discussion

Polo-like kinases are a family of protein kinases known to regulate cell cycle events, particularly during mitosis. PLK, a cell cycle-regulated, cyclin-independent serine/threonine protein kinase, has been shown in recent reports to play a critical role during tumorigenesis.[12, 14, 22, 23)] Smith *et al.* earlier reported that overexpression of PLK1 causes oncogenic focus formation with NIH3T3 cells, and that the resultant cells are capable of growth to tumors in nude mice.[24)] The present study showed PLK1 to

Table 3. Relationship between PLK1 expression and PCNA-LI in colorectal cancers

		PCNA-LI[1)]	P value
PLK1	High	54.7±10.3[2)]	0.002
	Low	45.9±11.9	

1) PCNA-LI: proliferating cell nuclear antigen-labeling index.
2) Mean±SD (%).

Table 4. Relationship between expression of PLK1, and Aurora-A and Aurora-C in colorectal cancers

		Aurora-A[1)]			Aurora-C[1)]		
		High	Low	P value	High	Low	P value
PLK1	High	44	13	0.004	33	24	0.054
	Low	9	12		7	14	

1) From Takahashi *et al.* (2000).

be more overexpressed in colorectal cancers than Aurora-A or Aurora-C, with a link to the PCNA-LI. An antisense strategy against PLK1 mRNA was developed to inhibit cell proliferation of cancer cells in cell culture and in the nude-mouse tumor model.[25] Holtrich *et al.* demonstrated that PLK1 mRNA expression was clearly elevated in proliferating cells such as various cancer cell lines and tumors of different origin.[12] Moreover, Wolf *et al.* established a correlation between PLK1 mRNA expression and the prognosis of lung cancer patient.[16]

Overexpression of PLK1 in murine fibroblasts leads to an increased appearance of large cells with multiple or fragmented nuclei, which display a transformed phenotype in culture and form tumors when injected into nude mice.[24] The chromosomal instability (CIN) phenotype, which accounts for 85% of sporadic colorectal cancers, is caused by disruption of mitotic checkpoint and centrosome abnormalities. PLK1 might be a potentially attractive CIN targets because PLK1 was overexpressed in CIN colon cancer cell lines.[26] These data suggest that altered expression of PLK1 could be instrumental in cancer initiation or progression in colorectal cancers. PLK1 expression has been analyzed in seven types of primary cancers: non-small-cell lung cancer (n=111),[16] squamous cell carcinoma of the head and neck (n=89),[17] esophageal cancer (n=49), gastric cancer (n=75),[27] ovarian cancer (n=17),[28] endometrial cancer (n=20),[29] and colon cancer (n=74),[30] but generally only at the mRNA level. Immunohistochemistry was previously only applied by Takai *et al*.[28, 29] Our findings are in line with those observed for PLK1 in a series of non-small-cell lung cancers, head and neck squamous cell cancers, esophageal cancers, and ovarian cancers. The gastrointestinal mucosa is a rapidly renewing tissue, and so it has a high proliferative rate, and PLK1 was here found to be weakly expressed in the normal colon mucosa. However, a marked increase was noted in the cancers.

The most important functions of a clinical staging and a histological grading system are to estimate the prognosis accurately and to guide treatment decision-making. In tumor diagnosis, the proliferation rate is an important parameter for tumor progression, and many approaches have been adopted for its assessment, one being the PCNA-LI, which is significantly associated with PLK1 expression. Yuan *et al.* have suggested that PLK1 level is generally increased in rapidly proliferating cells,[18] and in the present study, PLK1 was significantly associated with tumor stage, particularly tumor progression to invasion and lymph node metastasis, but not with distant metastasis (M). Several reports have revealed PLK1 overexpression to be significantly correlated with poor survival, and Takai *et al.* found a link with histological grade and clinical stage in ovarian cancers.[28] Evaluation of PLK1 mRNA expression in tumors of patients belonging to post-surgical stage I or II provides an early parameter to judge their prognosis.[16] A combination of nodal stage and PLK1 expression allowed discrimination of patients with a better prognosis in the pN0/1 and pN2/3 groups. Kneisel *et al.* reported that malignant melanomas with metastases expressed PLK1 at markedly elevated levels compared to melanomas without metastases.[31] Because PLK1 is associated with tumor stage, PLK1 overexpression may be significantly correlated with poor survival in colorectal cancer cases as well as non-small lung cancers, HNSCCs, and esophageal cancers.[16, 17, 27] The combined evaluation of molecular-biological markers, such as PLK1, PCNA, and Ki-67 may have advantages. Interestingly, PLK1 expression is linked with Aurora-A expression, although PLK1-positive cells are more diffuse and extensive than with Aurora-A or Aurora-C.[11] Interestingly, high PLK1 expression was significantly associated with immunohistochemical status of p53. Therefore, PLK1 might be better as a novel tumor marker than other mitotic kinases, Aurora-A or Aurora-C, in colorectal cancer.

In conclusion, we found that progression to primary colorectal cancer is associated with increase in expression levels of PLK1, and we speculate that overexpression of PLK1 may be of pathogenic, prognostic and proliferative importance. Apart from the potential diagnostic value, modulation of PLK1 activity in tumors by chemotherapeutic agents or gene therapy may prove to be of clinical value.

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