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Overexpression of cyclin B1 in human colorectal cancers

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Abstract The expression of the human cyclin B1 gene was investigated with Western blot analysis in human colorectal carcinomas and in adjacent non-neoplastic colorectal mucosas. Out of 41 cancers, 36 (88% of patients) showed much higher expression of cyclin B1 than did the non-neoplastic mucosa. Proliferating-cell nuclear antigen (PCNA) immunohistochemistry revealed that the labeling indexes of these cancer tissues were $47.3 \pm 11.3\%$ while those of the mucosa were $15.6 \pm 5.5\%$. Only 5 cancers (12% patients) demonstrated the same expression level of cyclin B1 as the mucosa; however, the PCNA labeling indexes were $42.3 \pm 11\%$ for the cancer tissue, compared to $12.6 \pm 2.4\%$ for the mucosas. Southern blot analysis showed that there was no change of the cyclin B1 gene at the somatic DNA level in spite of its high expression at the protein level. These results proved that majority of colorectal cancers express high levels of cyclin B1, consistent with a high rate of cell proliferation, whereas a small fraction of these cancers lose control of cyclin B1 expression, diverging from their fast cell proliferation.

Key words Cyclin B1 · Western blot · Southern blot · PCNA · Colorectal cancer

Abbreviation *PCNA* proliferating-cell nuclear antigen

Introduction

Unconstrained cell division is the most important characteristic of cancer cells. It is due to the gain or loss of function of proteins that constitute the cell-cycle machinery (Clurman and Roberts 1995). In the core of the cell cycle are cyclins and cyclin-dependent kinases (Pines 1993). There is increasing evidence to prove that, in cancer cells, cyclins and cyclin-dependent kinases are out of normal control. The cyclin A gene is highly expressed in human hematological malignancies and in some breast carcinomas (Keyomarsi and Pardee 1993; Paterlini et al. 1993). The cyclin D1 gene is amplified and overexpressed in some human esophageal cancers and hepatocellular carcinomas (Jiang et al. 1992; Nishida et al. 1994). Cyclin E is also reported to be amplified and overexpressed in human gastric cancers (Akama et al. 1995). Recently we reported the expression of cyclins A, D1 and E in human primary colorectal cancers (Wang et al. 1996). Cyclin E was overexpressed in a majority of the cancers and only a small fraction of the cancer expressed higher levels of cyclin A and cyclin D1. Some colorectal cancers even showed a reduced expression of cyclin A and cyclin D1.

To our knowledge, there are no reports concerning cyclin B in human primary cancer. Cyclin B, is an important mitotic cyclin which, with $p34^{cdc2}$, functions in the G2 and M phases of the cell cycle. Since colorectal cancer cells move through their cell cycle very quickly, the cyclin B gene is supposed to be very active in this type of cancer. However, on the basis of our previous research, cyclin A, the other mitotic cyclin, which acts in S, G2 and M phases, turned out to be under-expressed in most colorectal cancer tissues. These reasons prompted us further to do further experiments. In the present study, the expression of the human cyclin B1 gene was investigated with Western blot analysis in human colorectal carcinomas and in adjacent non-neoplastic colorectal mucosas. The cyclin B1 gene at the somatic DNA level was also checked with Southern blot analysis. The activity of cell proliferation was evaluated by proliferating-cell nuclear antigen (PCNA) immunohistochemistry. All results were

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compared with clinico-pathological parameters of the cancer patients.

Materials and methods

Patients

Surgical tissues of colorectal cancers from 41 patients (23 men and 18 women), 44–84 years old, at the Tokai Central Hospital and Hashima Municipal Hospital, Gifu Prefecture, Japan, were used. They were 37 adenocarcinomas, 2 mucinous cancers, 1 undifferentiated cancer and 1 adenosquamous carcinoma. Of the 37 adenocarcinomas, 19 were well differentiated, 17 were moderately differentiated and one was poorly differentiated. All diagnoses were checked by two of the authors.

Samples

The whole surgical samples were washed clean with cold saline after removal at operation. The colorectal cancer tissues without necrosis and the tissues of colorectal mucosa of normal appearance, more than 5 cm away from the cancer site, were cut, immersed in liquid nitrogen and kept at −80°C until used for protein and DNA extraction. A piece of cancer tissue and a piece of adjacent mucosal tissue from each case were fixed in buffered formalin and embedded in paraffin for PCNA immunohistochemical staining and staining with hematoxylin/eosin (Hall et al. 1990; Wang et al. 1996). It was microscopically confirmed that none of the cancer tissues contained any necrotic tissue and the adjacent mucosa did not contain cancer tissue.

Western blotting analysis

The cancer tissue and the apparently normal colorectal mucosa were homogenized and sonicated in buffer, containing 50 mM TRIS/HCl pH 7.4, 120 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 mM NaF, 200 μM sodium orthovanadate, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 0.5% Nonidet P-40, and then centrifuged at 10 000 *g* for 30 min at 4°C (Kosaka et al. 1993). The supernatant protein was quantified with the BCA protein assay kit (Rockford, Ill.). A 100-μg sample of this total protein from each preparation was applied to 12% of sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidine difluoride membrane (Millipore Corporation, Bedford, Mass.) (Sambrook et al. 1989a). This membrane was probed with anti-(human cyclin B1) monoclonal antibody (Upstate Biotec. Inc., New York), followed by horseradish-peroxidase-linked second-antibody incubation and detection using the enhanced chemoluminescence. Western blotting detection system (Amersham, Buckinghamshire, UK). All the results were confirmed again using another piece of tissues with the same procedures.

Southern blot analysis

Probe

Total RNA was extracted from human colon mucosa tissue using RNAZol (Biotex, Houston, Tex.). A 1-μg sample of the total RNA was used to synthesize cDNA with oligo(T) nucleotides. The cyclin B1 cDNA was amplified by the polymerase chain reaction (PCR): one step at 94°C for 3 min and then 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min with the primers 5′-CAA GTT ACA CCT TTG CCA CAG-3′ and 5′-GTT CTG CTT CTC CCC GCT GA-3′ (Pines and Hunter 1989). This 1351-base pair PCR product, which covered all the coding sequence of human cyclin B1, was cloned into plasmid pCR II by TA cloning (Invitrogen, San Diego, Calif.). The sequence of cloned cyclin B1 was confirmed by partially sequencing with a sequenase kit, version 2.0 (Amersham, USB, Ohio). The inserted cyclin B1 cDNA, which served as probe, was cut from plasmids and labeled with [α-^{32}P] dCTP (Amersham, UK), using a random-primer labeling kit (Takara, Japan).

Electrophoresis and hybridization

Genomic DNA was extracted pure from the frozen colorectal cancer tissues and adjacent mucosal tissues by the phenol/chloroform method (Blin and Safford 1976). Samples of 10 μg DNA were completely digested with *Eco*RI, electrophoresed in 0.8% agarose (Sambrook et al. 1989b) and then transferred onto Hybond-N$^+$ nylon membrane (Amersham). After prehybridization, the membrane was hybridized overnight with the labeled cyclin B1 probe and then washed and exposed on Kodak film for 48 h.

Results

Western blot analysis

The expression of cyclin B1 was much higher in cancer tissues than that in mucosa in 36 cases (Table 1, Fig. 1A). As shown in Fig. 1A, the results were very clear and the difference between the expression level of cancers and that of their mucosas was unequivocal. We quantified the Western blot bands by computerized densitometry; the densities of the bands from cancer tissues were 2- to 100-fold those of adjacent mucosas (data not shown). Only 5 cancers demonstrated the same expression level as their mucosas. Although the Western blot bands of mucosas and the cancer from case number 9 (the case number used in our previous paper) were invisible, feeble bands were confirmed if exposed for a little longer (Fig. 1A) (Wang et al. 1996). PCNA labeling indexes are also presented in Table 1. The mean PCNA labeling index of 41 cancer tissues was $46.6 \pm 11.1\%$ and that of mucosas was $15.2 \pm 5.2\%$. Although the expression of cyclin B1 did not differ between the cancer and the mucosa in 5 cases,

Table 1 Summary of cyclin B1 expression and proliferating-cell nuclear antigen (*PCNA*) labeling indexes in the cancer tissue (*T*) and adjacent mucosa (*M*)

B1 expression	No. cases	PCNA (%)	
		T	M
Total	41	46.6 ± 11.1*	15.2 ± 5.2
T > M	36	47.3 ± 11.3*	15.6 ± 5.5
T = M	5	42.3 ± 11.0*	12.6 ± 2.4
T < M	0		

* $P < 0.01$ compared with mucosa, by Student's *t*-test

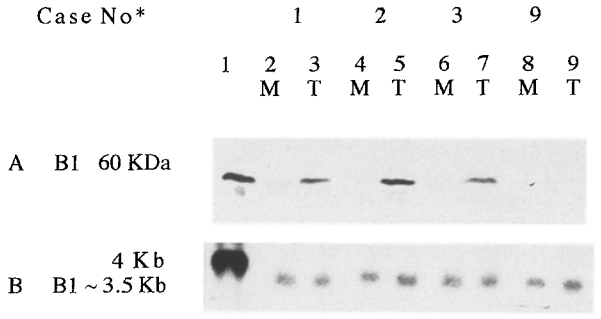

Fig. 1A,B The case numbers are those used in Table 1 of the previous paper. The representative bands of Western blots (**A**) and Southern blots (**B**) of 4 cases are shown. *T* cancer, *M* adjacent mucosa. **A** *Lane 1* the Western blotting band of a colonic adenocarcinoma cell line. Three cancer tissues showed higher expression of cyclin B1 (*lanes 2–7*). The fourth case (*lanes 8, 9*) showed no change in expression. If the film was exposed for a little longer to the samples, feeble bands could be seen. **B** *Lane 1* DNA ladder marker. No change was found between the cancer and mucosa

the mean of PCNA labeling index of the cancer tissues was 42.3 ± 11.0% and that of their mucosas was 12.6 ± 2.4%. We compared the expression level of cyclin B1 in cancer tissues to other clinico-pathological parameters, and no significant correlation was found.

Southern blot analysis

As Fig. 1B reveals, the Southern blot bands of 41 cancer tissues were equivalent to those of the adjacent mucosas. We carefully repeated Southern blot analysis with another piece of tissue and reconfirmed the results.

Discussion

The G2/M transition, one of the restriction points of the cell cycle, is basically controlled by cyclins A and B and the cdc2 kinase. In cycling cells, the level of cdc2 kinase is constant while cyclins must accumulate to some threshold level to start mitosis (Murray and Kirschner 1989). In a previous study we found that the *cdc2* gene was highly expressed in all colorectal cancers examined. In the present research, the majority of colorectal cancers (88%) had a much higher expression of cyclin B1, in keeping with the high rate of cell proliferation. High expression of cyclin B1 may be another reason for the rapid proliferation in this kind of cancer. The data indicate that the cyclin B1 gene would be positively involved in colorectal tumorigenesis. Whether its expression is related to progression of the cancer is a fascinating topic and a study of the expression of the B1 gene in adenoma and in metastatic lesions of the cancer is underway in our laboratories.

Equivalent expression of the cyclin B1 between cancer and mucosa was found in 5 patients although we

Table 2 Summary of cyclin A, B1, D1 and E expression in the 5 cancers with an expression level of cyclin B1 equivalent to that of the mucosa. The number in parentheses is the one used in our previous paper (Wang et al. 1996). ↑ The expression level of the cyclin in cancer is higher than that in mucosa; = level is the same in the cancer and mucosa; ↓ the expression level of the cyclin in cancer is lower than that in mucosa. These 5 cancers were all adenocarcinoma. Cancers 1, 3 and 5 penetrated the muscularis externa and involved serosa. Cancers 2 and 4 infiltrated the muscularis externa. Cancers 1 and 5 metastasized to local lymph nodes

Cyclin	Cyclin expression in cases 1–5				
	1(9)	2(18)	3(19)	4(24)	5(33)
A	=	↓	=	↑	=
B1	=	=	=	=	=
D1	↓	=	↑	=	↓
E	↑	↑	↑	↑	=

carefully repeated Western blot analysis in these 5 cases, using different parts of cancer and mucosa tissues. We have summarized the results for cyclins A, D1, B and E in these 5 cases (Table 2). The cyclin A gene was also under-expressed or expressed at an equivalent level to that of adjacent mucosa in 4 cases. Only 1 cancer showed higher expression of cyclin A. Since cyclins A and B1 are indispensable for the G2/M transition of the cell cycle. It remains unclear why these 5 cancer tissues could retain a high proliferation as their PCNA labeling indexes showed without increase of cyclin B1 or cyclin A expression. Nevertheless, these data suggest that some other constituent in these cancer tissues may compensate for the function of cyclin A and cyclin B1 to maintain their fast cell division. In fact, Lu and Hunter recently demonstrated that, besides the mitotic cyclins/cdc2 kinase pathway, there might be a NIMA (never in mitosis A) pathway, which does not depend on cyclins and cdc2 kinase for entry into mitosis in vertebrate cells (Lu and Hunter 1995).

Cyclin B has a subfamily consisting of cyclins B1, B2 and B3 (Gallant and Nigg 1994; Jackman et al. 1995). Although cyclins B1 and B2 share a variety of common features (they increase in S and G2 phases, peak at mitosis, and both associate with cdc2 kinase), they are distributed in different organelles and may have different biological functions. Chicken cyclin B3 was cloned and characterized. It possesses the features of cyclin B and as well as those of cyclin A. It is tempting to detect the expression of cyclin B3 in the cancer tissues with no expression change of cyclin A and cyclin B1, though human cyclin B3 has not yet been discovered.

Overexpression of some cyclins such as D1 and E was coordinated to their gene amplification (Akama et al. 1995; Jiang et al. 1992; Nishida et al. 1994). The high expression of the B1 gene in the cancer drove us to check this gene at the DNA level. However, no change was found on the basis of Southern blot analysis in the cancer regardless of its expression level. Therefore, cyclin B1 gene may be transcriptionally and

post-transcriptionally regulated in normal and neoplastic colorectal tissues, in agreement with the result in Hela cells (Pines and Hunter 1989).

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