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Determination of the prognostic significance of cyclin B1 overexpression in patients with esophageal squamous cell carcinoma

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Abstract Recent studies have identified a family of proteins referred to as cyclins, which control the cell cycle. Cyclin B1 activates cdc2, which regulates cell progression through the G2 and M phases. The main aim of this study was to examine the relationships between the cyclin B1 expression in human esophageal squamous cell carcinoma (SCC) and clinicopathological factors and prognosis of the patients. Eighty-seven cases of primary human SCC consecutively obtained at esophagectomy were immunohistochemically studied using an anti-human cyclin B1 protein antibody (2H1-H6). The relationship between cyclin B1 expression and clinicopathological factors, including prognosis, were also statistically assessed. Positive immunostaining of cancer cells, mainly in the cytoplasm, was detected in 72.4% (63/87): heterogeneous pattern in 37.9 % (33/87) and homogeneous pattern in 34.5% (30/87). The prevalence of cyclin B1 expression was significantly higher in cases with invasion deeper than the muscularis propria ($P<0.005$) and with venous invasion ($P<0.01$) than in other cases. Patients whose SCCs expressed high levels of cyclin B1 protein had a significantly poorer prognosis than did the other patients ($P<0.05$). Multivariate analysis demonstrated that cyclin B1 status was an important factor affecting survival ($P<0.05$). These findings demonstrated that overexpression of cyclin B1 protein is associated with tumor behavior and prognosis for patients with human esophageal SCC.

Key words Cyclin B1 · Esophageal squamous cell carcinoma · Immunohistochemistry

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Introduction

Several molecules controlling the cell cycle have recently been identified [4, 6, 25]. Cyclin-dependent kinases (cdks) modulate the cell cycle by phosphorylating various cellular proteins [3, 22, 27, 28]. Cyclins fill a key regulatory role in this process by activating each partner and targeting it to its respective protein substrate. Several cyclins are expressed discontinuously during the cell cycle, and their synthesis and degradation occur at sharp and well-defined timepoints in the cycle [4]. There are at least 11 distinct cyclin genes in the human genome, which fall into three categories: G1-phase cyclins (C, D1-3, E, G, and H) [21, 24, 30, 34, 36], S-phase cyclins (A and F) [4], and G2/M-phase cyclins (A and B1–2) [12, 32]. Cyclins C, D1-3, and E reach peaks of synthesis and activity during G1 and appear to regulate the phase transition from G1 to S [3, 11]. On the other hand, cyclins A and B1-2 reach maximum levels later in the cell cycle during S phase and G2 phase [3, 11, 29]. Diverse patterns of redundant expression of particular cyclins, including cyclin A, cyclin D1 and cyclin E, in different tumors have previously been reported as they relate to oncogenesis [1, 2, 9, 10, 14, 15, 17–20, 23, 34, 35]. We have recently reported overexpression of cyclins D1 and A in human esophageal squamous cell carcinoma (SCC) and have shown that cyclin D1 and/or A overexpression is associated with tumor behaviors and patient prognosis [7, 8, 13].

Cyclin B1 is an important mitotic cyclin in the G2 and M phases of the cell cycle [30, 31]. To our knowledge, there are few reports concerning cyclin B1 overexpression in human primary cancer [5, 16, 17, 33]. According to the status of immunoreaction in each cell, immunohistochemical studies of cyclin B1 protein have revealed only cytoplasmic staining in human breast lesions including normal glands [5]. In addition, Kawamoto et al. have revealed cytoplasmic staining in the nonmitotic phase and cellular staining in the mitotic phase in immunohistochemical staining of cyclin B1 protein in nonmalignant and cancerous human breast lesions [16]. Fur-

thermore, it has also been demonstrated that there are close correlations between cyclin B1 positivity and proliferative indices in human breast cancers [5, 16] and human colorectal cancers [33]. Using Northern blot analysis and Western blot analysis in human breast cancer, cyclin B1 overexpression was found in 90% of tumor cell lines examined [17]. It has also been found that the majority of colorectal cancers (88%) have much higher expression of cyclin B1 [33]. However, precise data on the expression of cyclin B1 protein in the progression of esophageal cancer or on how it relates to prognosis have not yet been determined.

In this study, we examined the expression of cyclin B1 protein in esophageal SCC immunohistochemically in an attempt to obtain evidence supporting the hypothesis that cyclin B1 is a useful prognostic marker of this type of tumor.

Materials and methods

Patients and tumor samples

Eighty-seven cases of primary human esophageal SCC obtained from consecutive esophagectomies in the Department of Surgery II of Kochi Medical School between 1982 and 1997 were studied. Patients who underwent esophagectomy in our medical school had received chemotherapy with 150 mg bleomycin p.o. (30 mg/day for 5 days) but no radiation therapy before surgery. After discharge from our hospital they were followed up, and most received chemotherapy when a recurrence was detected. Of the patients, 77 (88.5%) were men and 10 (11.5%) were women. The mean age was 62.2 years (range 41–86). Clinical staging and histopathological classification were based on the "Japanese Guide Lines for Clinical and Pathological Studies on Carcinoma of Esophagus." There were 29 (33.3%) patients with stage 0 disease (involving mucosa or submucosa with neither lymph node nor organ metastasis), 3 (3.4%) with stage I (involving the muscularis propria with neither lymph node nor organ metastasis), 15 (17.2%) with stage II (invasion reaching to the adventitia with or without lymph node metastasis but with no organ metastasis), 23 (26.4%) with stage III (definite invasion to the adventitia with or without lymph node metastasis but with no organ metastasis), and 17 (19.5%) with stage IV (invasion into the neighboring structure with either lymph node or organ metastasis). The number of deaths within 5 years after operation for each disease stage is as follows: stage 0, 4; stage I, 3; stage II, 10; stage III, 17; stage IV, 14. Tumor specimens were fixed in 10% phosphate-buffered formalin solution, processed routinely and embedded in paraffin. In each case, all available hematoxylin- and eosin-stained sections were reviewed, and a representative block was chosen for further studies.

Immunohistochemistry with antibody to cyclin B1 protein

Sections 4 μm thick from archival formalin-fixed paraffin-embedded tissue were placed on poly-L-lysine-coated slides (Sigma Chemical, St. Louis, Mo.) for immunohistochemistry (IHC). Cyclin B1 protein expression was assessed by IHC examination using an anti-human cyclin B1 monoclonal antibody (2H1-H6, dilution 1:50, Calbiochem, Newcastle, UK). After blockade of endogenous peroxidase activity, sections were pretreated in 10 mM citrate buffer (heated at 124°C) for 20 min in an autoclave oven. Then, the sections were further treated with normal goat serum for 30 min and incubated with cyclin B1 antibody at 4°C overnight. IHC staining for cyclin B1 protein was then performed using the avidin–biotin complex procedure with a streptavidin–biotin complex peroxidase kit (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). The sections were briefly counterstained with hematoxylin before mounting. Previously, using the same antibody as in the present study, Kawamoto et al. demonstrated the IHC staining of cyclin B1 protein, in non-malignant and malignant human breast lesions, revealing positive cytoplasmic staining in the nonmitotic phase [16]. Based on their IHC status, we used breast cancer tissues positive for this antibody as a positive control.

The degree of cyclin B1 staining was scored for the percentage of cells exhibiting cyclin B1 cytoplasmic staining. For cyclin B1 protein expression, cytoplasmic staining was considered positive if the chromogen was detected in more than 5% of the cell population, and the positive cases were further classified by degree of cytoplasmic staining into two groups: the first group (group I, n=30), in which cytoplasmic staining was homogeneous (over 50% positive reaction of cancer cells) was ranked as 2+, while the second group (group II, n=33), in which cytoplasmic staining was heterogeneous (5–50% positive reaction of cancer cells) was ranked as 1+. In addition, a third group (group III, n=24) was ranked as negative, meaning from nil up to less than 5% positive.

Statistical analyses

Statistical association between cyclin B1 expression and various clinicopathological factors was determined using the Chi-square test (P<0.05) for categorical variables. Survival analyses were performed using survival curves. The cumulative survival rates were calculated using the Kaplan–Meier methods, and the statistical significance of differences was determined using the log-rank test (P<0.05) (with time to death as the end-point). The simultaneous effects of more than one prognostic factor were estimated by means of the Cox proportional hazards model. These statistical analyses were performed with SPSS statistical software (SPSS, Chicago).

Association between cyclin B1 and patients' prognosis

Survival was calculated from operation to the date of death or of the last follow-up (either a clinical visit or a discussion with physicians referring each patient). Median follow-up duration was 20.5 months (in 0–60 months range). At the last follow-up, 44.8% of the patients were alive and 7 patients were clinically followed-up for more than 5 years after operation.

Results

IHC with antibody to cyclin B1 protein

Of the 87 tumors examined, 63 (72.4%) exhibited a positive immunoreaction with cyclin B1 antibody, including 30 (34.5%) cases of 2+ and 33 (37.9%), of 1+. Positive immunostaining with anti-cyclin B1 antibody was predominantly observed in both the cytoplasm and the nuclear membrane of each cancer cell, homogeneously (Fig. 1a) or heterogeneously (Fig. 1b), and diffusely in the cell during the mitotic phase (Fig. 1a, arrowheads). In normal mucosa adjacent to cyclin B1-positive or -negative tumors, it was predominantly the nuclei of the basal cell layer that were stained (Fig. 1c, arrowheads), and diffuse weak staining was also observed in the perinuclear region of the prickle cells (Fig. 1c, arrows). Peripheral regions of cancer nests were not usually stained more intensely than the center regions. Generally, no difference was observed in the degree of staining between invasive and noninvasive portions of cancers. Twenty-four tumors

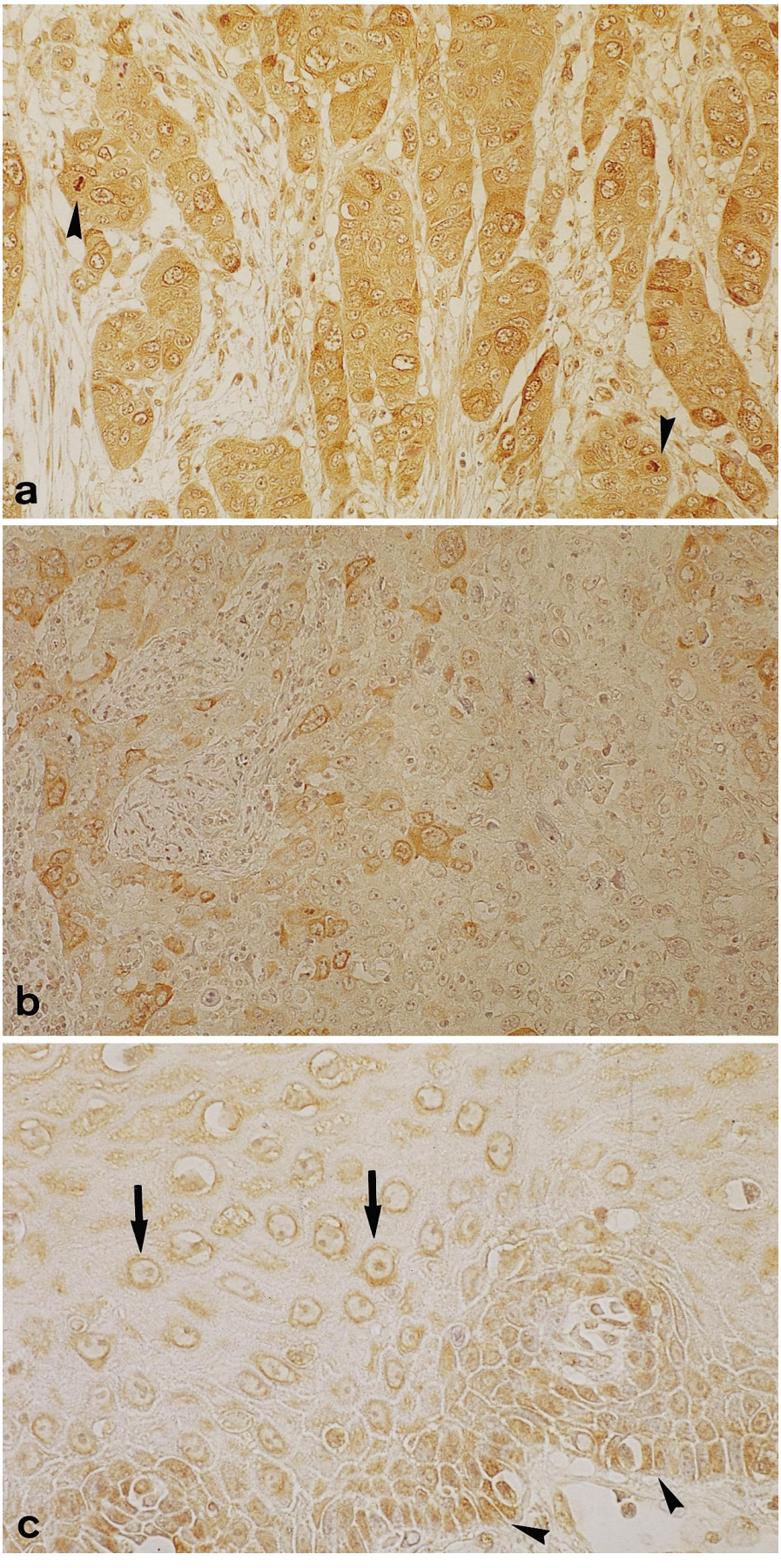

Fig. 1a–c Cyclin B1 staining patterns in human squamous cell carcinoma (SCC), as revealed by immunohistochemistry. The sections were counterstained with hematoxylin. **a** Homogeneous positive staining was observed in almost all of the cytoplasm and the nuclear membrane of cancer cells besides diffuse positivity of mitotic cells (*arrowheads*). ABC, ×200. **b** Heterogeneous positive staining was also observed in the cytoplasm and the nuclear membrane of cancer cells, showing considerable cell-to-cell variation in intensity. ABC, ×200. **c** In normal esophageal mucosa, it was predominantly the nuclei of the basal cell layer that were stained (*arrowheads*), and diffuse weak staining was often observed in the perinuclear region of the prickle cells (*arrows*). ABC, ×250

Table 1 Interrelationship between the expression of cyclin B1 protein and clinicopathological characterizations of patients with esophageal squamous cell carcinoma (*m* limited to the mucosa, *sm* involving submucosa, *mp* involving muscularis propria, *N.S.* not significant))

	Cyclin B1		*P*-value
	Positive (*n*=63)	Negative (*n*=24)	
Age			
<60	25	11	N.S.
60–70	26	6	
>70	12	7	
Sex			
Male	56	21	N.S.
Female	7	3	
Histological type			
Well	19	4	N.S.
Moderately	38	14	
Poor	6	6	
Depth[α]			
m	3	6	*P*<0.005
sm, mp	23	11	
beyond	37	7	
Nodal metastasis			
+	32	9	N.S
–	31	15	
Lymphatic invasion			
+	49	14	N.S
–	14	10	
Venous invasion			
+	34	5	*P*<0.01
–	29	19	
Histopathological stage[a]			
0	16	13	N.S.
I	3	0	
II	14	1	
III	17	6	
IV	13	4	

[a] Classification of the Japanese Society for Esophageal Disease (1992)

were negative for staining. The stroma adjacent to cancer cell nests was negative except for some inflammatory cells.

Statistical analyses

Table 1 shows the relationships between expression of cyclin B1 and clinicopathological factors. The relationships of cyclin B1-positivity with both depth of invasion ($P<0.005$) and presence of venous invasion ($P<0.01$) were significant. In sharp contrast, no significant relationship was found between cyclin B1 expression and other clinicopathological parameters, including age, sex, histological type, lymph node metastasis, lymphatic invasion and histopathological stage.

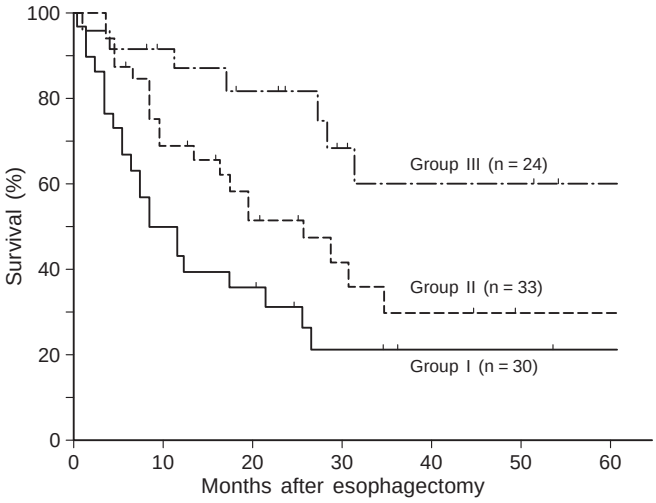

Fig. 2 The curative survival curves for patients with esophageal SCCs divided by the degree of cyclin B1 immunopositivity: group I (*n*=30), 2+ positive; group II (*n*=33), 1+ positive; group III (*n*=24), negative. There were statistically significant differences between group I and group III and between group II and group III (group I vs group III: $P<0.0005$; group II vs group III: $P<0.05$)

Table 2 Summary of multivariate Cox regression analysis with survival as the end point

Factor	Hazard ratio	95% confident limit (Cl)	*P*-value
Cyclin B1 status	2.6352	1.1692–5.9391	0.0194
Histological grade	1.0128	0.6366–1.6112	0.9572
Nodal metastasis	1.4944	0.5925–3.7693	0.3947
Venous invasion	0.9485	0.4799–1.8747	0.8792
Lymphatic invasion	1.1126	0.4595–2.6937	0.8130
Stage	1.7377	1.2574–2.4013	0.0008

Relationship between cyclin B1 expression and prognosis

Figure 2 shows a comparison of the Kaplan–Meier survival curves in the three groups. In all 87 patients tested, the postoperative 5-year survival rate in group I (*n*=30) was 20.5%, whereas that in group II (*n*=33) was 29.3%. There was a significant difference in prognosis between group II and group III ($P<0.05$). In addition, group I had a significantly poorer prognosis than group III ($P<0.0005$), but no difference was found in prognosis between groups I and II. Furthermore, when stage 0 cases were excluded, group I had a significantly poorer prognosis than group III ($P<0.05$), and there was a significant difference in prognosis between group II and group III ($P<0.05$). However, no difference was found in prognosis between groups I and II (not shown).

To determine the most informative combination of independent factors, correlations were determined among the various prognostic factors. Multivariate analysis clearly demonstrated that histopathological stage was an important factor for survival ($P=0.0008$), as was cyclin B1 status ($P=0.0194$; Table 2).

Discussion

We studied the aberrant expression of cyclin B1 protein in 87 patients with esophageal SCC and assessed relationships between cyclin B1 expression and various clinicopathological features of these patients. IHC investigation using antibody to cyclin B1 allowed precise determination of cyclin B1 expression and patterns of expression in individual tumor cells, and this procedure appears suitable for screening of cyclin B1 abnormalities in tissues and cells.

Overall, cyclin B1 immunoreactivity was detected in 72.4% (63 of 87) of esophageal SCCs, being observed predominantly in the cytoplasm and the nuclear membrane of each cancer cell and diffusely in the cells in mitotic phase. Our stainability results in cancer cells showed almost fair correlation with the previous study on breast cancer [16]. In normal esophageal mucosa, we demonstrated staining of the nuclei of the basal cell layer. Although no information is available on cyclin B1 immunostaining of the basal cells in normal esophageal mucosa, several studies have demonstrated frequent overexpression of cyclin B1 protein during the proliferative status of the cell [5, 16, 17, 33]. Therefore, our observation of nuclear immunopositivity of the basal cells with cyclin B1 antibody was also reasonable. Recently, overexpression of cyclin D1 was reported to occur in 38.2% [26] or 31.25% [13] and that of cyclin A in 39.5% [7] of esophageal SCCs. Thus, in esophageal SCC, overexpression of cyclin B1 as presented is much higher than that of cyclin D1 and that of cyclin A. Furthermore, the present study showed that overexpression of cyclin B1 protein in tumors statistically increased along with depth of invasion ($P<0.005$) and presence of venous invasion ($P<0.01$). Thus, altered expression of cyclin B1 is a frequent abnormality and may be an important event in the development of esophageal SCC.

The present study also showed that evaluation of the expression of cyclin B1 protein is particularly useful in the search for novel prognostic markers of esophageal SCC. The redundant overexpression of cyclin A [7] or cyclin D1 [13, 26] in esophageal SCC has previously been reported to be related to patient prognosis, but the relationship of redundant overexpression of cyclin B1 to patient prognosis has not yet been reported. Our univariate and multivariate analyses also demonstrated that cyclin B1 overexpression in esophageal SCC was significantly correlated with prognosis ($P<0.05$) and that cyclin B1 staining was useful to assess the patients' prognosis even if stage 0 cases were excluded. In addition, the combination of cyclin B1-positivity and prognosis was not affected by the other clinicopathological factors examined.

How overexpression of cyclin B1 participates in tumor progression remains unknown. The presence of this abnormal condition in tumor cells may indicate either that proteolytic degradation of the protein is impaired or that synthesis of the protein is not limited to a particular phase of the cycle. Under normal conditions, these processes run smoothly and cyclins undergo degradation correctly at the end of each functional phase [3, 11]. On the other hand, levels of cell cycle-specific kinases combined with their respective cyclins through particular stages of the cell cycle remain invariable throughout the cell cycle. We therefore tentatively suggest that overexpression of cyclin B1 presents redundantly in cancer cells. However, excess cyclin proteins may also form complexes with their respective kinases in a pattern in which elevated levels would be a trigger for each passage through all checkpoints of the cell cycle, resulting in uncontrolled cell division. Cell cycle regulation of the overexpressed cyclins is also perturbed. Under abnormal conditions, such overexpression of cyclin B1 may have significant consequences for the regulation of cell cycle progression. The oncogenic roles of cyclins [11] might, therefore, be related to their unscheduled expression.

Although the molecular basis for positive immunostaining of cyclin B1 is still under investigation, the present findings suggest for the first time in the literature that detection of cyclin B1 in esophageal SCC may be of prognostic significance. Detection of cyclin B1 overexpression should aid in the choice of a postoperative follow-up schedule of treatment, including radiation and chemotherapy. Since cyclin B1 can easily be demonstrated by IHC in formalin-fixed, paraffin-embedded materials, the degree of biological malignancy can easily be evaluated in each patient with esophageal SCC together with abnormal expression of other cyclins. More comprehensive studies involving greater numbers of tumors and including measurement of DNA and/or RNA levels will be required to confirm the present findings.

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