Determination of the Prognostic Significance of Unscheduled Cyclin A Overexpression in Patients with Esophageal Squamous Cell Carcinoma

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ABSTRACT
The expression of cyclin A protein was retrospectively investigated in 124 patients with human esophageal squamous cell carcinoma with immunohistochemical techniques using antibody to cyclin A protein (BF683). Of 124 tumors, 49 (39.5%) exhibited positive staining in cancer cells with this antibody. Staining was observed predominantly but not exclusively in the nucleus. A significantly higher degree of association of immunoreactivity with this antibody was detected for advanced types of tumors (stages I, II, III, and IV) than for early tumors (stage 0; P < 0.05). Patients with cyclin A immunopositivity had a significantly poorer survival rate than did other patients (P < 0.01). These findings provide the first evidence for frequent and unscheduled overexpression of cyclin A protein in human esophageal cancer and suggest the possibility that alteration of cyclin A overexpression is associated with tumor progression and patient prognosis for esophageal cancers.

INTRODUCTION
Cyclins are prime cell cycle regulators and play a central role in the control of major checkpoints in eukaryotic cells. In humans, several types of cyclin, including these of three categories, A-type, B-type, and G1-type (C-, D-, and E-types), have now been isolated (1-5). Cyclins C, D1-3, and E reach peaks of expression in the G1/S-phase and G2 (6-8). With the discovery of cyclins and cyclin-dependent kinases, it is now possible to specifically probe the information in the management of individual patients or its independence from known prognostic factors in esophageal carcinomas.

On the basis of the findings of the above studies, we have used immunohistochemical techniques to analyze the unscheduled overexpression of cyclin A protein in a wide range of human esophageal SCCs to elucidate possible roles in tumor development and to test the hypothesis that cyclin A is a prognostic factor for this tumor. The relationship to various clinical and pathological features was then determined.

MATERIALS AND METHODS
Patients and Tissue Specimens. A total of 124 human esophageal SCCs removed by total esophagectomy between 1981 and 1996 at the Kochi Medical School were studied. Patients with these cancers included 108 men and 16 women, ages between 42 and 86 years. In all cases, histological or clinical classification was made using the Guidelines for Clinical and Pathological Studies on Carcinoma of the Esophagus established by the Japanese Society for Esophageal Disease (1992). All specimens extirpated were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. For particular cyclins, including cyclins D and E, in different tumors and cell lines have previously been reported by many authors in relation to tumorigenesis (9-24). Integration of a fragment of the hepatitis B virus genome into an intron of cyclin A was found in a hepatocellular carcinoma in association with abnormality of the cyclin A gene (2, 25). Interaction between human cyclin A and adenovirus E1A has also been reported in adenovirus-transformed cells (26, 27). Keyomarsi and Pardee (15) have recently detected the untimely appearances of cyclins A and B in G1, in breast cancer cell lines. Although little is known concerning the role of cyclin A in the pathogenesis of human tumors, these studies suggest that redundant overexpression of cyclin A gene and/or protein may be an important step in the tumorigenesis of certain tumors. However, these observations are limited in number, and how extensively each of these components is involved in various types of tumors is a matter of intense debate.

In human cancers, various amplified and activated endogenous proto-oncogenes have been demonstrated to play roles in neoplastic transformation and other biological behaviors of neoplastic cells. Previous studies of esophageal SCCs have also provided evidence for the clinical utility of analysis of several new biomarkers in the evaluation of this neoplasm (10, 28-31). However, no consensus has been reached regarding the role of the information in the management of individual patients or its independence from known prognostic factors in esophageal carcinomas.

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The abbreviation used is: SCC, squamous cell carcinoma.
Fig. 1  Cyclin A staining patterns in human esophageal SCC. A, the cyclin A-positive tumor showing diffuse invasion. B, higher magnification of the invasive lesion. Note the strong uniform staining limited to the nuclei of cancer cells. No staining is detected in the stroma adjacent to the carcinoma. C, focal and weak immunostaining are consistently detected in the basal cell layer of noncancerous squamous epithelium (arrowheads) around cyclin A-positive tumor nests (7). Negative immunostaining is observed in most of the remaining epithelial cells. A, ×100; B, ×300; and C, ×250.

Immunohistochemical Staining. The immunohistochemical assay using paraffin-embedded tissue sections has been described in detail elsewhere (29, 32). In this study, we used the monoclonal IgG1 antibody, cyclin A (dilution 1:30, CYCLIN A, BF683; Oncogene Science, Inc., Cambridge, MA). In immunoprecipitation and immunoblotting experiments, this antihuman cyclin A protein antibody (BF683) was shown to be specific for cyclin A (27). In an initial feasibility study, aimed at detecting cyclin A protein using immunohistochemistry, we compared a variety of different protocols. Microwave pretreatment was found to be more reliable than the other methods tested. Preliminary also showed that, as an alternative, microwave treatment can be used to expose the antigenic determinant in routine, formalin-fixed material while retaining satisfactory morphological preservation (29, 32). Therefore, deparaffinized tissue sections were heated at 95 ± 5°C in deionized water in a microwave oven for 5 min. After blockage of endogenous peroxidase activity with methanol containing 0.3% H₂O₂ for 30 min, the sections were incubated at 4°C overnight with the monoclonal antibody to cyclin A protein, which allows reliable identification of cyclin A overexpression even in paraffin-embedded material. After washing with 0.1 M PBS (pH 7.4), the streptavidin-biotin complex procedure was performed using a streptavidin-biotin complex peroxidase kit (DAKO LSAB kit;
Dakopatts, Kyoto, Japan) and following the directions in the kit manual. Finally, slides were counterstained with methyl green. Positive or negative controls included in each experiment were run in parallel; these included replacement of the specific or nonspecific mouse IgG1 antibodies with PBS. The experiment was repeated, yielding essentially identical patterns of cyclin A distribution in each instance in each tumor specimen.

**Statistical Analysis.** The correlations between the expression of cyclin A protein and the various clinicopathological factors considered were determined using the $\chi^2$ test at the 5% level. Survival was calculated from esophagectomy to the date of death or the date of the last follow-up (either a clinical visit or a discussion with the patient’s referring physician). Analysis of survival data was performed using survival curves and the Kaplan-Meier method and log rank test. In addition, the Cox proportional hazards model was used to calculate and estimate the postoperative survival rate and to determine the significance of each prognostic factor used in histological or clinical classification. For multivariate analysis, variables were selected on the condition that they were statistically significant and were only poorly correlated with each other.

**RESULTS**

**Immunoperoxidase Staining for Cyclin A Antibody.** Overall, 39.5% (49/124) of the surgically resected tumors had large numbers of cancer cells positive for cyclin A. Staining was predominantly but not exclusively observed in the nucleus of such tumor cells (Fig. 1, A and B). Mitotic figures were frequently observed in cyclin A-positive cancer cell nests. Although the proportion of positive cells and the degree of staining intensity varied between tumors, it was relatively straightforward to rank the degree of staining based on the overall appearance of the sections. Focal and very weak staining was consistently observed in normal mucosa adjacent to cyclin A-positive or -negative tumors and was always restricted to the basal cell layer of noncancerous squamous cell epithelium (Fig. 1 C), whereas no staining was detected in the stroma adjacent to the carcinomas (Fig. 1 B). These observations concern the expression of cyclin A provide additional evidence of the specificity of detection of this antibody used.

**Statistical Analysis.** Table I shows the relationships between the rate of detection of overexpressed cyclin A in primary tumors and clinical and pathological features. Cyclin A overexpression was significantly associated with stage I, II, III, and IV tumors, which included 95.9% (47/49) of all positive tumors. In sharp contrast, there was very little association between cyclin A positivity and stage 0 classification for tumors ($P < 0.05$). Only 13.3% (2/15) of stage 0 tumors displayed a detectable level of this protein compared to 38.1, 34.5, 37.9, and 60.0% of stage I, II, III and IV tumors, respectively. No significant association was detected between cyclin A positivity and other clinical or pathological parameters including patient sex, age, location of tumor, histological level of tumor differentiation, and performance of radiotherapy.

**Association between Cyclin A Expression and Prognosis.** The cumulative survival curves for the patients with esophageal carcinoma are shown in Fig. 2. The 50% survival period was 11 months for those with cyclin A expression ($n = 49$) and 28 months for those without cyclin A expression ($n = 75$). The cumulative survival rate of patients with cyclin A expression was significantly lower at every point of the curve than that of patients without cyclin A expression. A significant difference was detected between the patients with cyclin A-positive tumors and those with negative tumors in both 1-year ($P < 0.01$) and 2-year survival ($P < 0.01$) rates.

A multivariate analysis using the Cox stepwise proportional hazard model was used to calculate the effect of patient sex, age, location of tumor, histological level of tumor differentiation, performance of radiotherapy, histopathological stage, and cyclin A overexpression on cumulative survival rate. Of these factors, histopathological stage and cyclin A had significant affects on survival rate. A stepwise selection of these factors was made based on the relative magnitude of their contribution to survival. Analysis demonstrated that cyclin A overexpression was the important factor affecting survival ($P < 0.001$), followed by histopathological stage ($P < 0.001$).

**DISCUSSION**

We studied the overexpression of cyclin A protein in 124 patients with esophageal SCC and assessed the relationship of positivity for cyclin A protein to various clinical and pathological features of these patients. Immunohistochemistry using antibody to cyclin A made possible detailed measurement of cyclin A expression rates and patterns in individual tumor cells.

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**Table 1** Interrelationship between the expression of cyclin A protein and clinicopathological characterizations of patients with esophageal SCC

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$^a$ According to the classification of the Japanese Society for Esophageal Disease (1992).

$^b$ Ce, cervical esophagus; I, upper and middle intrathoracic esophagus; E, lower intrathoracic and abdominal esophagus.
and may be a suitable method of screening for cyclin A abnormality. A total of 39.5% (49/124) of the tumor samples exhibited diffusely increased expression of cyclin A protein, which was observed predominantly in the nuclei of cancer cells. Interestingly, cyclin A overexpression frequently occurred in tumors in stages I, II, III, and IV, and rarely in those in stage 0. According to our guidelines for esophageal carcinoma, carcinoma in situ and tumors invading the lamina propria or submucosa, which have no lymph node or distant metastases, are called "early carcinoma of the esophagus" and defined as stage 0. Our findings indicated that immunopositive reaction was more frequent in advanced carcinomas with local or distal invasion and/or lymph node metastasis, and therefore with advanced tumor stage, than in other tumors. Thus, altered and unscheduled expression of cyclin A is a frequent abnormality and may be an important event in the development of esophageal cancer. However, in the present study, precursor lesions of SCC including dysplasia were not immunohistochemically examined. Additional studies with tumors at various stages will be needed to determine the tumor stage in which cyclin A alteration is most common and the role played by this alteration in the pathogenesis of esophageal SCC.

The present study also showed that the evaluation of the expression of cyclin A protein is particularly useful in the search for novel prognostic markers in esophageal SCC. The redundant expression of cyclin D1 or E in different tumors has previously been reported to be related to tumor progression and prognosis (18, 20, 21). Our univariate and multivariate analyses also showed that cyclin A overexpression in tumors was significantly correlated with clinical outcome. Patients with overexpression of cyclin A had shorter survival than did those without it. These findings suggest that demonstration of cyclin A overexpression is a valuable prognostic indicator. In addition, our findings appear to support further the hypothesis of a relationship between unscheduled cyclin A expression and oncogenic activity in neoplastic cells.

How unscheduled overexpression of cyclin A may participate in tumor progression remains unknown. The presence of this anomalous 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, cyclins undergo degradation at the end of each functional phase (6, 7). On the other hand, levels of cell cycle-specific kinases combined with the respective cyclins through a particular stage of the cell cycle remain invariable throughout the cell cycle. We therefore tentatively suggest that overexpressed cyclin A functions redundantly in cancer cells. Excess cyclin proteins may also form complexes with their respective partner kinases in an unscheduled pattern in which the 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 unscheduled expression of cyclin A may have significant consequences for regulation of cell cycle progression. The oncogenic role of cyclins (6) might, therefore, be related to their unscheduled expression.

Although the molecular basis for positive immunostaining of cyclin A remains under investigation, this is the first report of frequent cyclin A protein overexpression in a large series of primary esophageal tumors, and is a part of ongoing investigations suggesting tumor progression. The present findings suggest that detection of cyclin A in esophageal SCC might be of prognostic significance. More comprehensive studies involving greater numbers of tumors including measurement of DNA and/or RNA levels will be necessary to determine whether increased cyclin A expression alone or in combination with other genes contributes to progression of esophageal cancer.

Fig. 2 The cumulative survival curves for patients with esophageal carcinoma divided by cyclin A immunopositivity. There was a statistical significance between the positive and negative groups ($P < 0.01$).
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Determination of the prognostic significance of unscheduled cyclin A overexpression in patients with esophageal squamous cell carcinoma.

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