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Overexpression of Cyclin D1 in Rat Esophageal Carcinogenesis Model

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Overexpression of cyclin D1 in human esophageal carcinomas has been well documented. The aim of the present study was to assess the expression of cyclin D1 in different types of esophageal epithelial lesions induced by *N*-nitrosomethylbenzylamine (NMBA) in rats. A total of 30 rats received s.c.-injections, five times/week, of 1.0 mg/kg NMBA for a period of 5 weeks followed by the same dose once per week for another 10 weeks. An additional 15 rats were given saline and used as controls to provide normal epithelium. The tumor incidence was 100% at the termination point of 21 weeks. Seventeen rats (57%) showed nuclear staining for cyclin D1, with a great variation in the intensity, as demonstrated by using an immunohistochemical technique. The cyclin D1 positive indices were in the range of 0% to 60% of the individual cells. Negligible staining was observed for normal esophageal epithelium, with a minimal increase in hyperplastic and dysplastic lesions. A significant elevation of cyclin D1 levels was observed in tumors. However, no significant differences were found between papillomas and carcinomas. The immunohistochemical results were confirmed by western blotting analysis. Tumors, papillomas and carcinomas overexpressing cyclin D1 had elevated proliferating cell nuclear antigen (PCNA) indices ($P<0.05$). The correlation coefficient of overexpressions of PCNA and cyclin D1 was $r=0.7$ for papillomas, but only $r=0.3$ for carcinomas. The study thus provides strong evidence of relatively early overexpression of cyclin D1 during tumorigenesis in the present rat esophageal model. Cyclin D1 expression is not simply a direct consequence of increase cell proliferation.

Key words: Cyclin D1 — Rat — Esophageal carcinogenesis

Experimental animal models provide good tools for understanding the mechanisms of development underlying carcinogenesis. A number of asymmetric nitrosamines can induce rat esophageal tumors, as first shown by Druckrey *et al.*,[1,2] NMBA, a highly potent carcinogen,[1,3] is thought to be activated preferentially by P450 isozymes in the esophagus to form benzaldehyde and electrophilic metabolites in the form of methyl carbonium ions. These in turn can methylate DNA, producing O^6-methylguanine,[4,5] which has been associated with tumor induction.[6] Formation of this adduct is thought to be responsible for the activation of the Ha-*ras* oncogene that has been observed in rat papillomas induced by NMBA.[7] Although O^6-methylguanine is also formed in other sites, such as the nasal cavity, trachea, tracheal gland and lung, NMBA-induced tumorigenesis is limited to the esophagus. The actual cause of this specificity is still unknown, although van Benthem *et al.*[8] suggested that it could be due to the high rate of cell turnover in esophageal epithelial tissues. Since the establishment of the NMBA esophageal tumor model, a number of studies have been focused on histopathological aspects.[4,5,8] Recently, this model has been used to investigate the mechanisms of action of several chemopreventive agents and dietary factors in esophageal tumorigenesis.[9]

In general, cyclins are labile proteins, whose expression is limited to specific points during the cell cycle. Several studies have indicated that cyclin D1 in particular plays an important role as one of the main regulatory proteins controlling the normal progression of cells through G1.[10] Microinjection of anti-cyclin D1 antibodies or antisense cyclin D1 plasmids prevents both human and mouse fibroblasts from entering S phase.[11] Cyclin D1 thus qualifies as the prototype of a new class of cell cycle-regulatory proto-oncogenes[12] and appears to be the most likely oncogene candidate in the 11q13 amplicon.[13,14] Gene rearrangement, overexpression and amplification have been reported in a number of human tumors, including esophageal carcinomas.[15–17] Recent studies have focused on expression of this protein in animal models of carcinogenesis in mouse skin and rat mammary glands.[18–24]

Our purpose in the present work was to study cyclin D1 regulation by employing an NMBA-induced rat esophageal cancer model which allows us to analyze stage-specific events in multistage carcinogenesis *in vivo*. This was done by examining the protein expression levels in the different rat esophageal lesions.

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Abbreviations used: NMBA, *N*-nitrosomethylbenzylamine; UBI, upstate biotechnology; PCNA, proliferating cell nuclear antigen.

MATERIALS AND METHODS

Chemical NMBA with a purity >98% was purchased from Ash Stevens Inc. (Detroit, MI).

Animals Forty-five 6-week-old male F344 rats (Charles River, Kanagawa) were used in this study. The rats were housed 3 per cage in an animal room with a 12-hour light–12-hour dark cycle at a temperature of 22±2°C and 55±5% humidity. The animals were observed daily to assess their general health and provided with basal diet purchased from Oriental MF (Oriental Yeast Co., Tokyo). Body weight was measured once a week for the first 10 weeks, then daily until the end of the experiment.

Experimental design After a one-week acclimation period the rats were divided into two groups: the tumor-induction group of 30 rats treated with 1 mg/kg body weight, s.c.-injection 5 times per week of NMBA in saline for the first 5 weeks.[25] The same dose was then administered once a week for the following 10 weeks. The other group comprised 15 rats given the same amounts of saline (vehicle) alone as a control (Table I). The dosing volume was 1 ml/kg body weight. The treatment was temporarily stopped for any rat exhibiting a loss of body weight of more than 10 g/week. Animals were scheduled to be killed at the beginning of the 21st week but were killed earlier if they lost about 25% of their maximum body weight or 30–40 g/week.

Pathological analysis Rats were killed under ether anesthesia and the esophagus of each rat was excised and opened longitudinally on a white index card. Tumors ≧1 mm in diameter were counted. The approximate volume of each tumor was determined by measuring the three dimensions (height, length and width) with a millimeter ruler. Tissues were fixed in freshly prepared 10% neutral buffered formalin for one to two days before paraffin embedding. Sections of paraffin blocks were made so that the entire length of the esophagus could be analyzed on one slide. Serial sections were prepared for routine HE staining and immunohistochemistry as described below. Some tissue samples, including tumors, papillomas and carcinomas, were rapidly frozen in liquid nitrogen and stored at −80°C for western blotting analysis as described below.

Immunohistochemical reactions

Cyclin D1: Deparaffinization and blocking of endogenous peroxidase and endogenous biotin were performed as described previously.[26] The antigenicity of the formalin-fixed tissue sections was regenerated by immersing the sections in a target unmasking fluid (PharMingen, San Diego, CA, cat. no. 70001 T) for 5–20 min at 80–90°C. A microwave oven was used to achieve the desired temperature. Boiling was avoided, as this may affect the quality of the unmasking fluid. Three cyclin D1 antibodies were assessed in this study: rabbit polyclonal anti-human cyclin D1 with cross reactivity to rat protein[27] from UBI, Lake Placid, NY (cat. no. 06–137), mouse monoclonal IGg1 from Santa Cruz, CA (cat. no. SC 246), and the same antibody was kindly provided by Dr. R. Michalides of the Netherlands Cancer Institute.[28] Antibody concentrations were determined by titer. The optimum concentration of 10 μg/ml was achieved by using antibody diluent (Dako, cat. no. S 0809). Incubation was done for 2 h at 37°C or overnight at 4°C, with comparable results. The second antibody was biotinylated horse anti-mouse or anti-rat immunoglobulin linking antibody at a 1/200 dilution (Vectastain, Vector Labs, Burlingame, CA). For the avidin-biotin-peroxidase complex technique, commercially available kits (Elite Vectastain, Vector Labs) were used.[29] Labeled streptavidin-biotin with an LSAB-2 kit (Dako, Santa Barbara, CA) gave comparable results. Visualization of antibody binding was performed with Dako Chromagen tablets (cat. no. S 3000). Rat thyroid lesions known to overexpress cyclin D1 were used as a positive control. As a negative control, the primary antibody was omitted.

PCNA: Deparaffinization, microwave therapy and blocking of both endogenous peroxidase and biotin were performed as described previously.[26] Sections were then

Table I. Incidence of Epithelial Lesions and Mean Positive Indices of Cyclin D1 and PCNA in the Esophagus of 15 Normal and 30 NMBA-treated Rats

Variable	Incidence of lesions (%)	% of mean positive index (range)[a]	
		cyclin D1	PCNA
Normal epithelium	0 (0)	1 (0–2)	25 (10–31)
Hyperplasia	27 (90)	3 (3–6)	29 (10–34)
Dysplasia	23 (77)	3 (3–6)	40 (20–47)
Papilloma	25 (83)	30 (3–55)[b]	57 (15–68)
Carcinoma	12 (40)	28 (3–60)[b]	71 (17–76)

a) Rats with an undetectable levels of cyclin D1 protein among the NMBA-treated group (43%) were not included.

b) Significant increase in comparison to the values of both hyperplastic and dysplastic lesions ($P<0.01$).

reacted with mouse monoclonal antibody against PCNA (PC 10, Dako, Denmark) diluted 1/200, followed by the avidin-biotin-peroxidase complex as detailed above. As positive controls, rat bladder tumors with high PCNA expression were used.

Evaluation of staining results Cells were considered positive for cyclin D1 when clear nuclear staining could be identified. Samples with negligible or undetectable levels of cyclin D1 were considered negative. Interpretation of the PCNA binding data was in line with the description by Siglin *et al.*[25] Fields were randomly selected and individual positive cells were counted. Positive indices for both cyclin D1 and PCNA were calculated as percentage values taking the total number of examined cells into account. At least 2,000 cells were counted throughout the entire esophagus of each animal. A PCNA positive index of 30% was used as the cutoff point for two groups of rats (Table II) with a different proliferation status. This value was the lowest allowing discrimination with a significant probability (P) value.

Table II. Relation of the PCNA Positive Index to the Cyclin D1 Expression Status in NMBA-treated Rats

Variable[a]	No. of rats	Cyclin D1 expression			P-value[b]
		negative ($n=13$)	positive ($n=17$)	% of positive	
Low PCNA tumors	12	8	4	33	<0.05
High PCNA tumors	18	5	13	72	

a) A PCNA positive index value of 30% was used as a cutoff value between low and high PCNA tumors.
b) χ^2 test.

Western blotting analysis Tissue samples of rat esophageal tumors, either fresh or frozen, were used for western blotting analysis after separation of the mucosa from muscle and connective tissue. Crude extracts were prepared according to Klinge's criteria.[30] Tissues were homogenized with a Polytron in 4 volumes of buffer A: 50 m*M* Tris-HCl (pH 8.0), 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 25% glycerol, and 1 m*M* phenylmethylsulfonyl fluoride. Homogenization was accomplished with eight 5-s bursts at intervals of 10 s. The resulting homogenate was centrifuged at 40,000*g* for 45 min. The protein concentration was calculated by the Bradford method,[31] then equal quantities of proteins were fractionated on 10% sodium dodecyl sulfate polyacrylamide gels. These gels were either stained with Coomassie blue to control for balanced loading or blotted, using a semi-dry method,[32] onto nitrocellulose filters, which were probed with rabbit polyclonal anti-human cyclin D1 (UBI) using a 3T3 cell lysate as a control (UBI, NY, cat. no. 12–305) according to the manufacturer's instructions.

Statistical methods The significance of differences in incidence between groups was assessed with the χ^2 test. That of all other differences was assessed with the Mann-Whitney test. Data are given as means with the corresponding ranges. Correlation coefficients (r) were calculated using the StatView program on a personal computer. The criterion of statistical significance was $P<0.05$.

RESULTS

A decrease in average body weight was observed from week 12 with the onset of tumor development. The mean values of initial body weight for the NMBA-treated and

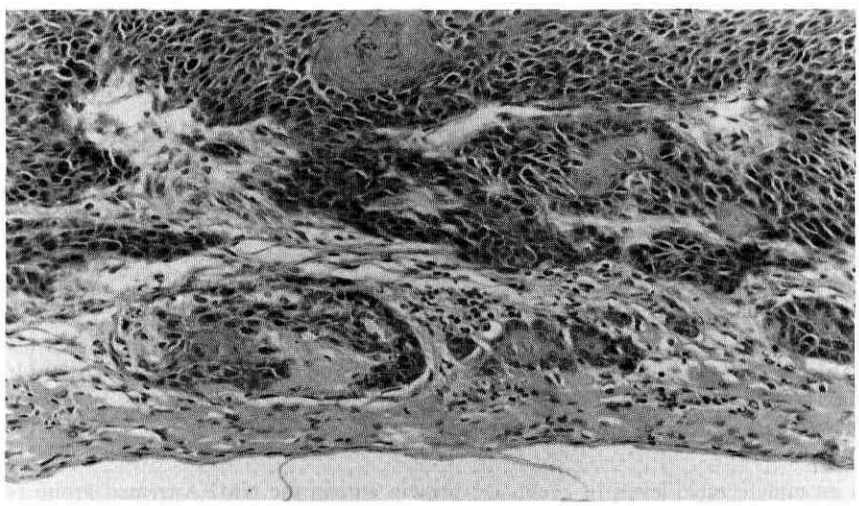

Fig. 1. HE staining of a moderately differentiated squamous cell carcinoma of rat esophagus, induced by s.c.-injections of NMBA, showing muscle layer invasion of cancer cells. (magnification ×200).

control groups were 156 and 152 g, respectively. The mean maximum body weight of 267 g was reached at the 12th week in the NMBA-treated group, while the mean final body weight of the control group at the end of the experiment (21st week) was 350 g. In the NMBA-treated group (30 rats), 20 rats were killed before the scheduled time, with a mean survival time of 16 weeks, owing to excessive weight loss. Most of the control group were killed at the end of the experiment.

The macroscopic examination of the NMBA-treated group revealed a tumor incidence of 100%. The tumors were regularly distributed throughout the esophagus with no site of significant predominance. The majority were exophytic polyps projecting into the lumen, with a mean number of 7.3 ± 1.8 (range: 1 to 14 tumors/rat). The mean tumor volume was 34.7 mm^3. One tumor had a volume of 612 mm^3 ($4 \times 17 \times 9$). This animal was killed at the 18th week as a result of rapid, progressive loss of body weight. Grossly visible tumors were not observed in the vehicle control group (15 rats).

Microscopically, the esophageal epithelial lesions were classified according to Wang's criteria[9] into four main categories: epithelial hyperplasia, dysplasia, papilloma and carcinoma. Twelve rats (40%) developed esophageal carcinomas (Table I). Seven of these lesions were found together with papilloma lesions, but carcinoma in papilloma was not observed. All carcinomas demonstrated invasion, either into the submucosal layer or the muscle layer (Fig. 1) and most were of moderately differentiated type. The total number of rats with papillomas was twenty-five (83%). All the tumors obtained were of squamous cell type. The incidences of hyperplasia and dysplasia were 90% and 77%, respectively. There was no statistically significant difference in development period between papillomas (18.3 ± 2.0 weeks) and carcinomas (18.0 ± 1.3 weeks). Both the control and the normal esophageal epithelium portions of the NMBA-treated groups showed normal histology.

Table I also summarizes immunohistochemical findings for both cyclin D1 and PCNA in terms of mean positive indices and the corresponding ranges. The amount of cyclin D1 was found to be negligible in normal rat esophageal epithelium, with a minimal increase in the expression levels in hyperplastic and dysplastic lesions. Esophageal papillomas and carcinomas, in contrast, each showed overexpression of cyclin D1 compared with hyperplastic and dysplastic lesions ($P < 0.01$). One case each of papilloma and carcinoma had a positive cyclin

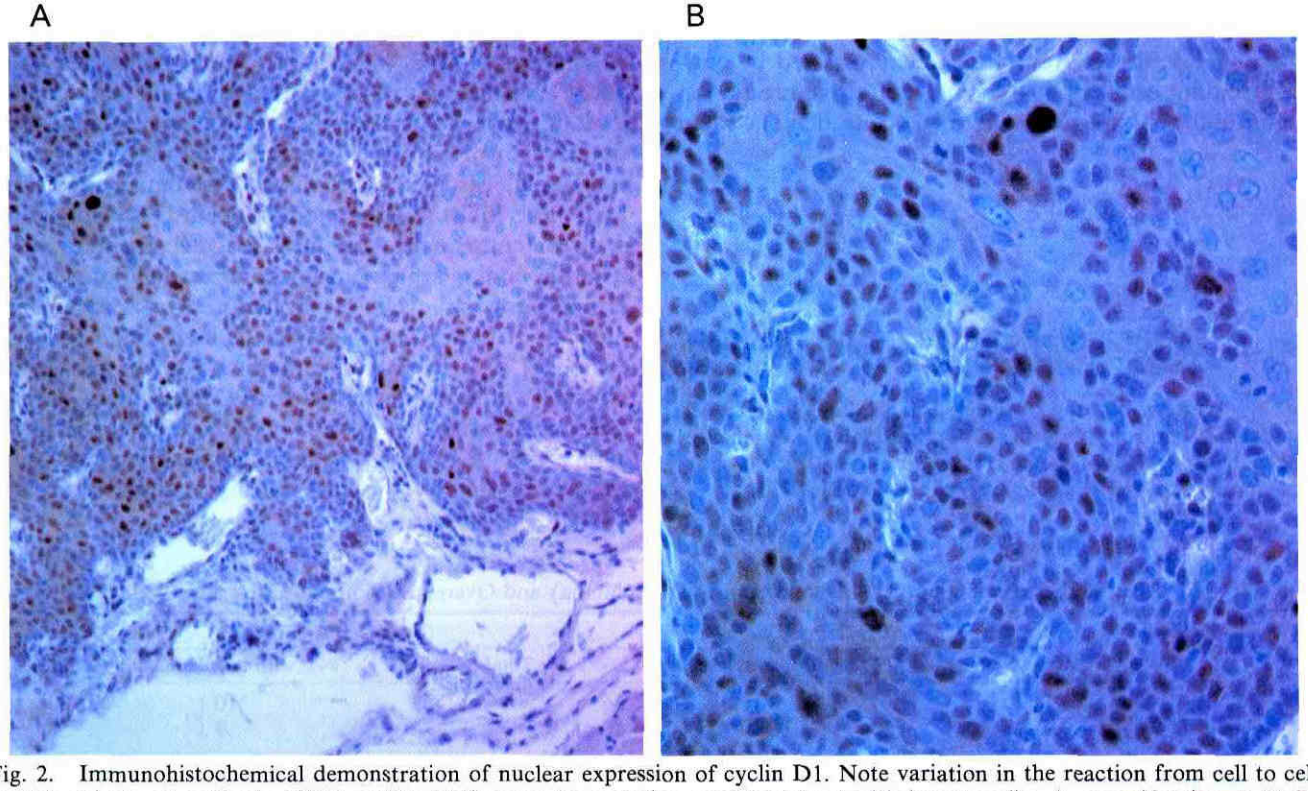

Fig. 2. Immunohistochemical demonstration of nuclear expression of cyclin D1. Note variation in the reaction from cell to cell (rabbit polyclonal antibody, UBI), avidin-biotin-peroxide complex, counterstained with hematoxylin. A, magnification $\times 40$. B, magnification $\times 200$.

D1 index below 10% (about 3%). Such cases may be considered as being intermediate regarding cyclin D1 expression status (above the normal expression level, but below the tumor category). On the other hand, no significant difference in positive indices was found between papillomas and carcinomas overexpressing cyclin D1. In the NMBA-treated group, seventeen rats (57%) showed a positive cyclin D1 nuclear reaction in individual tumors (Table II) with a great variation in the intensity (range of 3% to 60%), as demonstrated by immunohistochemistry. There were undetectable levels throughout the entire esophagus (and the tumors) of 13 rats (43%) in the NMBA-treated group. The rabbit polyclonal antibody from UBI gave the best reactions with the least background in both immunohistochemistry (Fig. 2) and western blotting studies (Fig. 3). On the other hand, PCNA staining showed a gradual increase in the mean positive index from dysplasias through papillomas to carcinomas (Table I). In normal rat esophageal epithelium the basal cells were counted for the interpretation of PCNA staining results.[25] The amount of normal esophageal epithelium in the NMBA-treated rats was very small and showed no significant immunohistochemical staining in comparison with the staining pattern of the normal esophageal epithelium of the control group.

Using a 30% PCNA positive index as the cutoff value, low and high proliferation groups were generated (see Table II). In the first group, 8 rats showed no expression of cyclin D1 in their tumors, as demonstrated by antibody binding, while only 4 rats had a positive reaction in their tumors. Conversely, 13 of 18 rats demonstrated appreciable cyclin D1 expression in their tumors in the high PCNA group, the difference being significant ($P<0.05$). Eight rats showed both PCNA and cyclin D1 overexpression in the papilloma group, while 5 did so in the carcinoma group. Thus, the correlation of PCNA and cyclin D1 overexpressions (Table III) was better in the papilloma group ($r=0.7$) than in the carcinoma case ($r=0.3$). On the other hand, there was no correlation between the expression levels of cyclin D1 and PCNA proteins and the size or number of the individual tumors.

Western blotting analysis was performed on the tissue samples of the NMBA-treated and control groups. Fig. 3 shows the results for rat esophageal tumors and normal rat esophageal tissues tested. Normal tissues express cyclin D1 protein very weakly. On the other hand, there was a great variation in the level of cyclin D1 expression in tumor tissues, comparable results being obtained with the immunohistochemical reactions and western blotting of the corresponding tumor samples. All cyclin D1-positive samples exhibited a 36 kD cyclin D1 band.

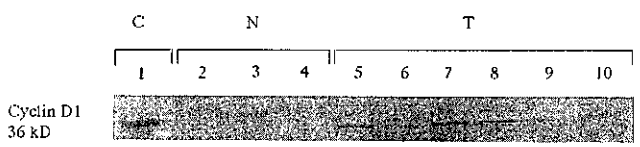

Fig. 3. Western blotting of cyclin D1. C (lane 1), 3T3 cell lysate as a control; N (lanes 2–4), normal rat esophageal mucosa with negligible cyclin D1; T (lanes 5–10), rat esophageal tumors showing variable expression of cyclin D1.

DISCUSSION

The expression level of PCNA has been widely accepted as a good indicator of cell proliferation.[23, 25] While there was a good correlation between the PCNA proliferation status and cyclin D1 overexpression in the present series of papillomas, this was not the case for the carcinomas. This is in line with the study by Kanda *et al.*,[33] in which no correlation was found between the level of cyclin D1 expression and PCNA protein expression level or the number of nucleolar organizer regions in esophageal carcinoma cell lines. The data support the hypothesis that the expression of cyclin D1 is not simply a direct consequence of increased cell proliferation.[23] Another finding arguing against a direct relation between proliferation and cyclin D1 overexpression was the large number of rats (43%) showing undetectable levels of cyclin D1 protein in their individual tumors (Table II). No such variation was noted for PCNA staining results, the contrast with the cyclin D1 case being clear from the comparison in Table I. The immunohistochemical results were confirmed by western blotting analysis to exclude

Table III. Relation between Tumor Type (Papilloma and Carcinoma) and Overexpression of PCNA and Cyclin D1

Variable	Papilloma			Carcinoma		
	Total no. of rats	No. with overexpression[a]	%	Total no. of rats	No. with overexpression[a]	%
PCNA	25	9	36	12	10	83
Cyclin D1	25	10[b]	40	12	7[b]	58

a) Overexpression is defined as cases with a positive index >30% for PCNA or a positive reaction in the cyclin D1 case.
b) Eight rats have both PCNA and cyclin D1 overexpression in the papilloma group ($r=0.7$), and 5 in the carcinoma group ($r=0.3$).

the possibility of epitope masking. Seventeen rats (57%) showed overexpression of cyclin D1 in their individual tumors. This expression percentage is in line with previous *in vivo*[15, 17] and *in vitro*[16] studies. This also excludes the hypothesis that levels of cyclin D1 expression might simply reflect differences in the relative amounts of epithelial and stromal cells between normal tissues and esophageal tumor tissues.[23]

Three findings are noteworthy. The first is the early overexpression of cyclin D1 in papillomatous lesions. This overexpression is characterized by a great variation in the intensity. The second is the absence of any significant difference in cyclin D1 expression levels between papillomas and carcinomas. The third is the loss of the significant correlation with the degree of proliferation in association with malignant progression (Table III). These findings indicate that cyclin D1 overexpression may be important for the development of tumors from epithelial lesions, such as hyperplasia and dysplasia. It seems that once the tumor formation process has been established (in papillomas), there is no need for further expression of this protein for transformation to malignancy to occur. This is supported by comparison of the expression levels of PCNA and cyclin D1. This is in line with a recent study by Naitoh *et al.*,[17] who found cyclin D1 to be already present in the early stages of human esophageal cancer development, with no variation in the degree of staining between superficial and advanced lesions. Furthermore, the finding of cyclin D1 overexpression in other benign lesions such as parathyroid adenomas[13] supports the hypothesis of an early role within the multistep process of tumorigenesis. Lukas *et al.*[32] pointed out that variation in the intensity of cyclin D1 staining, found in a number of investigations[11, 16, 32] as well as the present study, is consistent with its function in controlling G1 phase, with accumulation to a peak late in this phase, and subsequent decay before the onset of DNA synthesis.[32] This distribution of the intensity of staining from hyperplastic and dysplastic lesions to tumors is in line with the report by Wang *et al.*[34] of cyclin D1 overexpression in human esophageal carcinogenesis, but not in line with their conclusion that the lack of any significant increase during the genesis of dysplasia from hyperplastic lesions suggests no gene alteration in the very early stages.[34] However, an understanding of the crucial steps in esophageal carcinogenesis would require study of the histological changes from hyperplastic and dysplastic lesions, passing through benign tumors, to carcinomas. These broad categories are usually unavailable in studies of human esophageal carcinomas. Benign tumors such as papillomas are rare pathological findings,[35] illustrating the need for an appropriate esophageal animal model.

To our knowledge, there have been only a few reports of cyclin D1 expression in animal models, most of which were concerned with mouse skin carcinogenesis[18–21] or rat and mouse mammary carcinogenesis.[22–24] Mammary hyperplasia was demonstrated to be associated with overexpression of cyclin D1 in cyclin D1 transgenic mice, which develop a low incidence of mammary tumors after a long latent period.[22] Said *et al.*[24] reported from their study on mouse mammary cell lines and tumors that increase in the cyclin D1 protein pool was correlated with tumor progression. Sgambato *et al.*[23] detected a 10- to 15-fold increase in the level of cyclin D1 in 7 out of 9 rat mammary tumors when compared with normal glands. No data were supplied to determine how early this expression occurs in the rat mammary model.[23] Recently, however, Robles and Conti[19] demonstrated early overexpression of this protein in advanced mouse papillomas obtained after 35–50 weeks of promotion. In this regard, the rat esophageal carcinogenesis model has the advantage of a shorter development period.[4, 5] Craddock and Driver[36] reported that rat esophageal papillomas may occur as early as four weeks after treatment with NMBA and carcinomas after nine weeks.

Our data from the present study thus support the hypothesis that cyclin D1 may have proto-oncogenic properties.[12] The exact mechanisms by which it might help transform cells remain to be elucidated. However, it should be mentioned in this context that its overexpression in rodent fibroblasts can enhance cell transformation either alone[37] or in combination with an activated *ras* oncogene.[12] Filmus *et al.*[38] also reported that activated *ras* induces significant overexpression of cyclin D1 in epithelial cells derived from normal rat intestine and mouse mammary gland.

In conclusion, while there is an increase in the expression of PCNA during progression from normal epithelium to hyperplasia to dysplasia to papilloma to carcinoma, cyclin D1 expression is increased in papillomas with no further increase during progression to carcinomas. Our results support the hypothesis that the expression of cyclin D1 is not simply a direct consequence of increased cell proliferation. Furthermore, the study provides strong evidence of relatively early overexpression of cyclin D1 during tumorigenesis in the present rat esophageal model.

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