Journal of Cellular Biochemistry Supplements 28/29:159–165 (1997)

Esophageal and Gastric Cardia Epithelial Cell Proliferation in Northern Chinese Subjects Living in a High-Incidence Area

Li Dong Wang,[1]* Qi Zhou,[2] and Chung S. Yang[2]

[1]Laboratory for Cancer Research, Henan Medical University, Zhengzhou, China
[2]Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, New Jersey

Abstract Linxian and the nearby county Huixian, in the Henan province in Northern China, have a very high incidence of esophageal squamous cell carcinoma (SCC). Previous studies from these counties have suggested that increased proliferation of esophageal epithelial cells, morphologically manifested as basal cell hyperplasia (BCH) and dysplasia (DYS), is an early indicator of abnormality in persons predisposed to SCC. A high incidence of gastric cardia adenocarcinoma (AC) was also found in these areas. To determine proliferation patterns of esophageal and gastric cardia epithelia with normal and different severities of precancerous lesions, we measured proliferating cell nuclear antigen (PCNA), Ki-67, and bromodeoxyuridine (BrdU) incorporation and compared the results. Esophageal biopsies (175) and gastric cardia biopsies (41) were collected from symptom-free subjects in Huixian. Of these, 23 esophageal biopsies were incubated with BrdU. The avidin-biotin-peroxidase complex (ABC) method was used to detect PCNA, Ki-67, and BrdU. The number of immunostain-positive cells was counted manually. Intense immunostaining for PCNA, Ki-67, and BrdU was observed in the cell nuclei of tissues with normal and different severities of precancerous lesions. With esophageal biopsies, both PCNA and Ki-67 increased significantly as the epithelia progressed from normal to BCH and to DYS. The number of PCNA- and Ki-67-positive cells was three times higher than that of BrdU incorporation in the same category of BCH. With cardia biopsies, the number of Ki-67 positive cells was lower in normal tissue and increased significantly from chronic superficial gastritis to chronic atrophic gastritis to DYS. Staining patterns for PCNA and Ki-67 were correlated with the histopathology of the esophagus. The correlation was not as clear with gastric cardia. BrdU studies appear to be more complicated. The PCNA and Ki-67 methods may be useful for screening high-risk esophageal and gastric cardia cancer subjects, and for monitoring chemoprevention effects. J. Cell. Biochem. Suppls. 28/29:159–165.

Key words: esophagus; gastric cardia; precancerous lesions; cell proliferation

Esophageal cancer (EC), a widely occurring disease, is a leading cause of cancer-related deaths in Linxian and Huixian of the northern Henan Province, People's Republic of China (PRC) [1,2]. Because this disease has poor prognosis, early diagnosis and prevention are of great importance. An early indicator of abnormality in persons predisposed to EC is the increased proliferation of esophageal epithelial cells, morphologically manifested as basal cell hyperplasia (BCH) and dysplasia (DYS) [3–6]. Adenocarcinoma (AC) appears to occur together with EC in many high-incidence areas in China and other countries [7,8]. AC of the gastric cardia is an under-studied topic, and cell proliferation in the pathogenesis of this disease has not been well characterized. There is evidence, however, that cardia AC differs from cancer of the rest of the stomach in terms of time, trend, risk factors, and histopathogenesis [9–11].

Cell proliferation measurements are increasingly used to assess the effects of cancer prevention trials [12–15]. Tritiated thymidine labeling of S-phase cells is an established method for identifying proliferating patterns in tissue sections. Using this labeling technique, we found esophageal BCH and DYS had higher labeling indices than normal epithelium; these precancerous lesions also showed expansion of proliferating zones toward the esophageal surface [3]. Recently, immunohistochemical identification

*Correspondence to: Li Dong Wang, MD, Laboratory for Cancer Research, Henan Medical University Zhengzhou, Henan Province 450052, People's Republic of China.
Received 1 May 1997; Accepted 10 November 1997

of cell cycle–related proteins has been used to assess cellular proliferation. The markers used include PCNA, Ki-67, and BrdU incorporation. PCNA is a 36 kd nuclear protein identified as an auxiliary protein of DNA polymerase δ. Its synthesis sharply increased in late G1 phase and also increased during S phase, but declined throughout G2 and M phases [16]. Ki-67, a nuclear antigen, is present in G1, S, and G2 phases of the cell cycle [17]. BrdU, a pyrimidine analogue of thymidine, is incorporated into DNA during cell replication in S phase and can be detected by anti-BrdU antibody [18].

In the present study, we used PCNA, Ki-67, and BrdU labeling to characterize the cell proliferation patterns of esophageal and gastric cardia epithelia with differing lesion severity. The results from these three different methods are compared.

MATERIALS AND METHODS

Tissue Collection and Processing

Esophageal biopsies (175) and gastric cardia biopsies (41) were collected from symptom-free subjects in Huixian, Henan Province, PRC. Of the 175 esophageal biopsies, 23 were immediately incubated with BrdU, 1.5 mg/100 ml (Sigma Chemical, St. Louis, MO) in a 95% basal medium with 10% fetal calf serum in a shaking incubator at 37°C for 1 h. All tissues were fixed with 80% alcohol, embedded with paraffin, and serially sectioned at 5 μm. The sections were mounted onto histostick-coated slides. Three or 4 adjacent ribbons were collected for histopathological analysis (hematoxylin and eosin stain) and immunohistochemical staining.

Histopathologic Analysis

Histopathological diagnosis for esophageal epithelia were made according to the previously established criteria [7]. Normal esophageal epithelium contained one to three basal cell layers; the papillae were confined to the lower half of the epithelium. In BCH, the number of proliferating basal cells was increased to more than three cell layers. DYS was characterized by partial loss of cell polarity and by nuclear atypia. Histopathologic classifications for gastric cardia epithelia included chronic superficial gastritis (CSG), an inflammation manifested by mild lymphocyte and plasma cell infiltration; chronic atrophic gastritis (CAG), mucosal glandular morphology eradicated partially or completely by connective tissues with interglandular space infiltrated mainly by plasma cells and lymphocytes; and DYS, characterized by nuclear atypia with or without architectural abnormalities in the gastric epithelium, but without invasion [19].

Immunohistochemical Staining

The avidin-biotin-peroxidase complex (ABC) method was used for PCNA, Ki-67, and BrdU antigen immunostaining. In brief, after dewaxing, inactivating endogenous peroxidase activity, and blocking cross-reactivity with normal

TABLE I. Cell Proliferation of Esophageal and Gastric Cardia Epithelia Measured by PCNA, Ki-67, and BrdU in Symptom-Free Subjects From Huixian†

	PCNA		Ki-67		BrdU	
Histological types	No. of biopsies examined	Positive cells/mm^2 (M ± SE)	No. of biopsies examined	Positive cells/mm^2 (M ± SE)	No. of biopsies examined	Positive cells/mm^2 (M ± SE)
Esophagus						
Normal	18	144 ± 16	16	145 ± 20	3	112 ± 56
BCH	136	338 ± 21*	96	308 ± 25*	19	103 ± 9
DYS	21	928 ± 157*	15	773 ± 229*	1	425
Gastric cardia						
Normal	9	290 ± 43	6	180 ± 41	—	—
CSG	17	356 ± 78	12	195 ± 32	—	—
CAG	11	382 ± 85	6	376 ± 134**	—	—
DYS	4	633 ± 111	2	620 ± 143**	—	—

†—, not applied for BrdU incorporation.
*Significantly different from lower-grade lesions; $P < 0.001$ by ANOVA test.
**Significantly different from lower-grade lesions; $P < 0.05$ by ANOVA test.

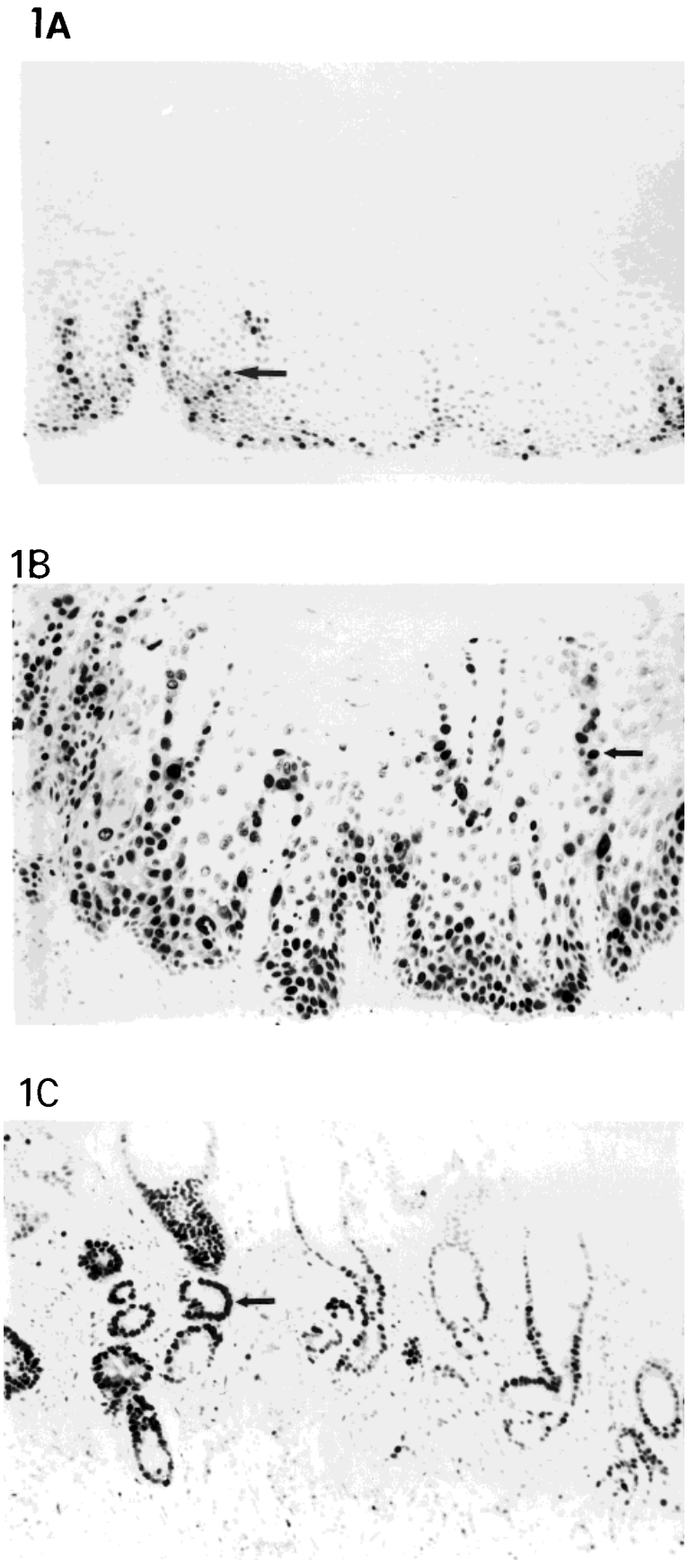
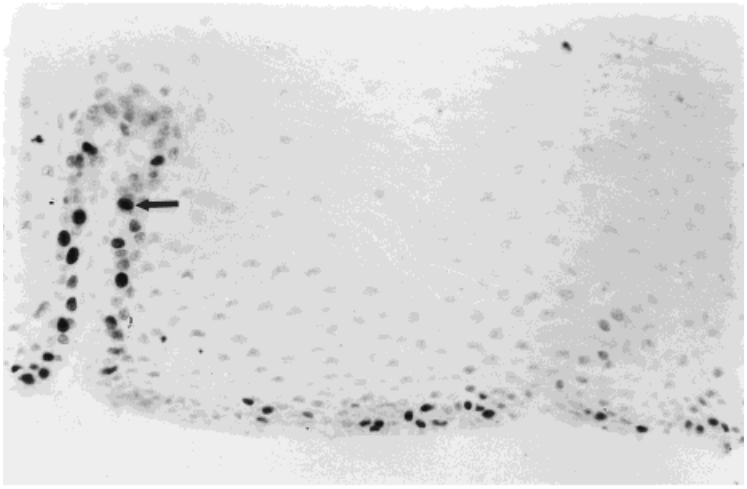
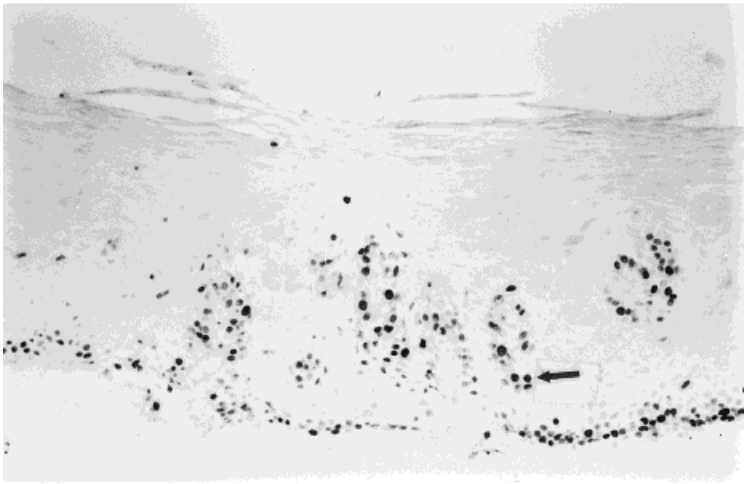


Fig. 1. Immunostaining of PCNA in biopsy samples of esophageal and gastric cardia epithelia (X100). Immunoreactivity is located in the nuclei of basal cells in the papillary regions of the normal epithelia of esophagus (**A,** arrow) and the positive cells expanded upwards in DYS (**B,** arrow). PCNA immunoreactivity is located in the gastric epithelium with CSG (**C,** arrow).

2A



2B



2C

Fig. 2. Immunostaining of Ki-67 in biopsy samples of esophageal and gastric cardia epithelia (×100). Immunoreactivity is located in the nuclei of basal cells in the papillary regions of the normal epithelia of esophagus (**A,** arrow) and the positive cells expanded upwards in DYS (**B,** arrow). Immunoreactivity of Ki-67 is located in the nuclei of cells at the base of the crypts and at the deep glands of gastric cardia epithelium with CSG (**C,** arrow).

serum, the sections were incubated overnight at 4°C with a dilute solution of the primary antibodies (1:200 for PCNA and Ki-67, 1:30 for BrdU). Primary antibodies were located by applying a biotinylated anti-primary antibody, ABC (conjugated to horseradish peroxidase) and diaminobenzidine (Bectastain Elite Kit). Normal serum blocking and omission of the primary antibody were used as negative controls. Counterstaining with H&E was used only for BrdU. The deparafinized tissue sections were heated in a microwave oven before the Ki-67 immunohistochemical staining procedure.

Quantitative Analysis of Immunostaining Results

Quantitative analysis of immunostaining results was recorded as the number of positive cells per mm^2 of biopsy epithelium as described previously [20]. This was done by counting all positive stained cells in the whole piece of biopsy tissue under ×400 magnification. Usually 24 fields were counted and the results were expressed as the number of positive cells per mm^2 of biopsy epithelium. Similar measurements were made for the gastric cardia biopsies.

Statistical Analysis

The mean ± SE of the PCNA, Ki-67, and BrdU immunostained cells (cell number/mm^2) in the esophageal and gastric cardia biopsy samples in each histologic category were calculated using univariate analysis. The ANOVA test followed by the Fisher PLSD test were used to assess the significance of differences ($P < 0.05$) among values of different histologic categories.

RESULTS

Among the esophageal biopsy samples, 78% had BCH and 12% had DYS (Table I). Most of the gastric cardia samples also had lesions of CSG and CAG. Clear nuclear immunostaining was observed for PCNA and Ki-67 in the normal and abnormal esophageal and gastric cardia epithelia (Figs. 1 and 2). In normal esophageal epithelim, PCNA immunoreactivity was observed mostly in the basal cells at the first and second layers, as well as at the papillary regions (Fig. 1A). As the esophageal tissue progressed from normal to BCH to DYS, cells stained positive for PCNA increased significantly in number ($P < 0.001$) and expanded into the upper epithelium (Table I, Fig. 1B).

A similar pattern of immunoreactivity was observed in Ki-67 (Fig. 2). In each category of lesions, similar numbers of Ki-67 and PCNA-positive cells were observed. BrdU immunoreactivity was also located in the cell nuclei of the basal cells (Fig. 3). The number of BrdU-labeled cells was similar to that of PCNA and Ki-67 in the normal epithelium, but less than PCNA and Ki-67 immunostained cells in the BCH category

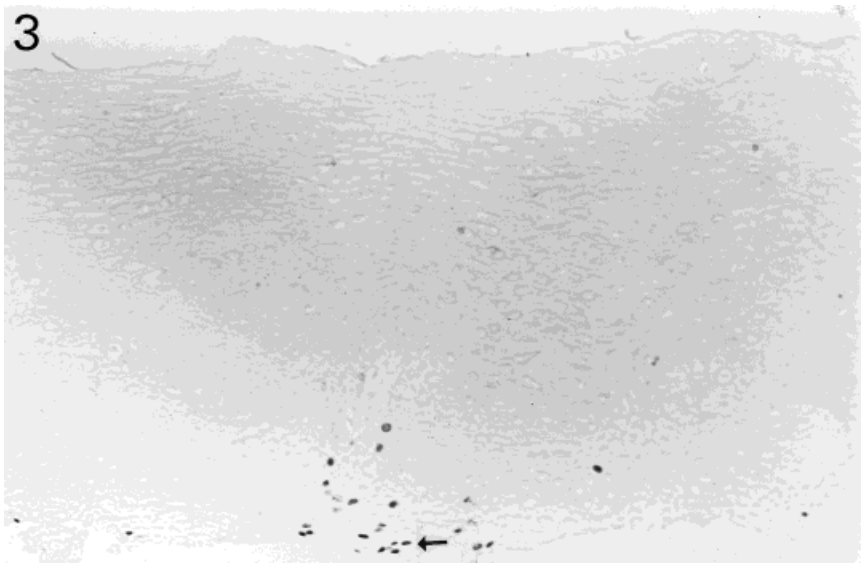


Fig. 3. Immunohistochemical studies of BrdU-labeled cells in the esophageal epithelium with BCH with ethyl green counterstain. Immunoreactivity is located in the cell nuclei of the basal cells (×200, arrow).

(Table I). In the gastric cardia, PCNA immunoreactivity was observed in basal cells of the crypt (Fig. 1C). The number of PCNA-positive cells appeared to increase slightly as the epithelia progressed from normal to CSG to CAG to DYS (Table I), but the difference was not statistically significant. In normal cardia epithelium, Ki-67 immunoreactivity was observed mostly at the basal cells of the crypt (Fig. 2). As the gastric cardia tissue progressed from normal to CAG to DYS, the Ki-67 immunostaining positive cells increased significantly in number ($P < 0.05$) and expanded to the upper part of the crypt (Fig. 2).

DISCUSSION

Consistent with previous results [4], a large number of individuals in Huixian were found to have abnormal histopathology of the esophagus. This is also true for the gastric cardia (Table I). In the present study, the immunostaining patterns for PCNA and Ki-67 were similar. Immunoreactivity for both antigens was observed in the nuclei of proliferating cells. The number of immunostain-positive cells for PCNA and Ki-67 was comparable; they increased as the proliferation zone expanded in esophageal BCH and DYS. The proliferation pattern of esophageal epithelia measured by PCNA and Ki-67 was similar to that measured previously by tritiated thymidine incorporation [3]. Technically, the PCNA method appears simpler than the Ki-67 method because PCNA is accessible by the antibody and needs no antigen unmasking step. BrdU incorporation is a reliable laboratory procedure to study DNA synthesis. The BrdU labeling index was only about a third of that obtained by PCNA or Ki-67 in esophageal samples with BCH; a difference between BCH and normal epithelium was not observed (Table I). PCNA and Ki-67 immunostaining patterns also appear to reflect the proliferation pattern, although a clear distinction between normal, CSG, and CAG was not observed with PCNA staining. Studies with larger numbers of samples may be needed to clarify this issue. Because of the relative ease of analyzing a large number of samples, PCNA and Ki-67 immunohistochemical methods may be an effective way to generate quantitative cell proliferation data in interventions with populations at high risk for esophageal and gastric cardia cancers.

REFERENCES

1. Yang CS (1980): Research on esophageal cancer in China: A review. Cancer Res 40:2633–2644.
2. Lu JB, Yang WX, Zu SK, Chang QL, Sun XB, Lu WQ, Quan PL, Qui YM (1988): Cancer mortality and mortality trends in Henan, China, 1974–1985. Cancer Detect Prev 13:167–173.
3. Wang LD, Lipkin M, Qiu SL, Yang GR, Yang CS, Newmark HL (1990): Labeling index and labeling distribution of cells in esophageal epithelium of individuals at increased risk for esophageal cancer in Huixian, China. Cancer Res 50:2651–2653.
4. Qiu SL, Yang GR (1988): Precursor lesions of esophageal cancer in high-risk populations in Henan Province, China. Cancer (Phila) 62:551–557.
5. Munoz N, Lipkin M, Crespi M, Wahrendof J, Grassi A, Shihsein L (1985): Proliferative abnormalities of the esophageal epithelium of Chinese populations at high and low risk for esophageal cancer. Int J Cancer 36:187–189.
6. Correa P (1982): Precursors of gastric and esophageal cancer. Cancer Phil 50:2554–2565.
7. Li JY, Ershow AG, Chen ZJ, Wacholder S, Li GY, Guo W, Li B, Blot WJ (1989): A case-control study of cancer of the esophagus and gastric cardia in Linxsian. Int J Cancer 43:755–761.
8. Victor T, Toit RD, Jordan AM, Bester AJ, Van Helden PD (1990): No evidence for point mutations in codons 12, 13, and 61 of the *ras* gene in high incidence area for esophageal and gastric cancer. Cancer Res 50:4911–4914.
9. MacDonald WG (1972): Clinical and pathologic features of adenocarcinoma of the esophagus. Cancer 29: 724–732.
10. MacDonald WG, MacDonald JB (1987): Adenocarcinoma of the esophagus and/or gastric cardia. Cancer 60:1094–1098.
11. Wang HH, Antonioli D, Goldman H (1986): Comparative features of esophageal and gastric adenocarcinomas. Hum Pathol 17:482–487.
12. Wang LD, Qiu SL, Yang GR, Lipkin M, Newmark HL, Yang CS (1993): A randomized double-blind intervention study on the effect of calcium supplementation on esophageal precancerous lesions in a high risk population in China. Cancer Epidemiol Biomarkers Prev 2:71–78.
13. Munoz N, Wahredorf J, Lu JB, Crespi M, Thurnham DI, Day NE, Zheng HJ, Grassi A, Li WY, Liu GL, Lang YQ, Zhang CY, Zhen SF, Li JY, Correa P, O'Conor GT, Gosch X (1985): No effect of riboflavine, retinol, and zinc on prevalence of precancerous lesions of oesophagus. Randomized double-blind intervention study in high-risk population of China. Lancet 1:111–114.
14. Rao M, Liu FS, Dawsey SM, Yang K, Lipkin M, Li JY, Taylor PR, Li B, Blot WJ, Wang GQ, Lewin KJ, Yu Y, Yang CS (1994): Effects of vitamin/mineral supplementation on the proliferation of esophageal squamous epithelium in Linxian, China. Cancer Epidemiol Biomarkers Prev 3:277–279.
15. Dawsey SM, Wang GQ, Taylor PR, Li JY, Blot WJ, Li B, Lewin KJ, Liu FS, Mark SD, Yu Y, Yang CS (1994): Effects of vitamin/mineral supplementation on the

prevalence of histologic dysplasia and early cancer of the esophagus and stomach: Results from the dysplasia trial in Linxian, China. Cancer Epidemiol Biomarkers Prev 3:167–172.

16. Celis JE, Madsen P, Celis A, Nielsen HV, Gesser B (1987): Cyclin (PCNA, auxiliary protein of DNA polymerase) is a central component of the pathway(s) leading to DNA replication and cell division. FEBS Lett 220:1–7.
17. Gerdes J, Schwab U, Lemke H, Stein H (1983): Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13–17.
18. Gratzner HG (1982): Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science 218:474–475.
19. You WG, Blot WJ, Li JY, Chang YS, Jin ML, Kneller R, Zhang L, Han ZX, Zeng XR, Liu WD, Correa P, Fraumeni JF, Xu GW (1993): Precancerous gastric lesions in a population at high risk of stomach cancer. Cancer Res 53:1317–1321.
20. Wang LD, Shi ST, Zhou Q, Goldstein S, Hong JY, Shao P, Qiu SL, Yang CS (1994): Changes in p53 and cyclin D1 protein levels and cell proliferation in different stages of human esophageal and gastric-cardia carcinogenesis. Int J Cancer 59:514–519.