

Apoptosis and its relationship with cell proliferation, p53, Waf1p21, bcl-2 and c-myc in esophageal carcinogenesis studied with a high-risk population in northern China

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Subject headings esophageal neoplasms; apoptosis; precancerous lesions; Waf1 p21 gene; p53 gene; bcl-2 gene; c-myc gene

Abstract

AIM To determine the extent of apoptosis and its possible relationship with the changes of p53, Waf1p21, bcl-2, and c-myc at different stages of esophageal carcinogenesis.

METHODS Two hundred and forty-one esophageal biopsy samples from symptom-free subjects and 38 surgically resected esophageal carcinoma tissues from a high-risk population for esophageal cancer in Henan, China were used in this study. Apoptotic cells and apoptotic bodies were identified by well-established morphological criteria. The extent of apoptosis and its possible relationship with the rate of cell proliferation (PCNA) and changes of p53, Waf1p21, bcl-2, and c-myc were analyzed in samples with esophageal precancerous and cancerous lesions.

RESULTS The apoptotic cells, identified morphologically, were located in the same proliferative compartment of hyperproliferative cell population in the esophageal epithelia as the cells immunostaining-positive for p53, bcl-2, c-myc and PCNA. The apoptotic indices (total number of apoptotic cells and apoptotic bodies per mm² of the tissue section) were low in the normal epithelia, and increased significantly as the lesions progressed from BCH to DYS and to SCC. The extent of apoptosis correlated well with the cell proliferation indices based on PCNA. The total number of positive cells for p53 stain was much higher than that of apoptotic cells. No difference in apoptotic indices was found between p53-positive and p53-negative samples. Waf1p21-positive cells resided in

cell layers were higher in number than p53 and PCNA-positive cells. The number of immunostaining positive cells for Waf1p21 increased slightly from normal to BCH, but decreased in DYS and SCC. Positive staining samples for bcl-2 and c-myc increased as the lesions progressed from BCH to DYS and to SCC. No apparent correlation between apoptosis and Waf1p21, bcl-2 or c-myc expression was observed.

CONCLUSION The extent of apoptosis was low in normal esophageal epithelium and increased as the lesions progressed. The apoptotic cells were located in the same hyperproliferative cell compartment as cells immunostaining-positive for p53, bcl-2, c-myc and PCNA, but no apparent correlation between apoptosis and these parameters was observed, possibly due to the complexities of molecular changes in esophageal carcinogenesis.

INTRODUCTION

Carcinoma of the esophagus (EC) is a widely occurring disease in Huixian and Linxian counties of Henan Province in northern China and remains a leading cause of cancer-related death^[1,2]. The development of human esophageal squamous cell carcinoma (SCC) is a multistage progressive process^[3-6]. 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), dysplasia (DYS) and carcinoma in situ (CIS), which could be considered precancerous lesions of EC. Changes in cell proliferation and cell death may be a key factor contributing to the rate of neoplastic progression and tumor growth. Although previous studies on the subjects from these high incidence areas have suggested the importance of epithelial cell hyperproliferation in human esophageal carcinogenesis, little information is available on cell death and the molecular mechanisms involved.

Recent evidence suggests that apoptosis, a genetically programmed cell death, is of fundamental importance in tumorigenesis^[7,8]. There

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Received 1998-07-28

are several genes which participate in the regulation of apoptosis, and bcl-2 has been identified as an apoptosis inhibitor^[9-11]. In spite of a lack of detectable autonomous transforming activity, bcl-2 has been shown to cooperate with c-myc in immortalizing cells^[12]. Under certain conditions, constitutive expression of c-myc induces apoptosis and it can be suppressed by bcl-2. It appears that the c-myc-cooperating oncogenic activity of bcl-2 is related to its inhibition of apoptotic pathways^[9].

The tumor suppressor gene p53, a transcription factor, has been identified as a participant in the cellular DNA damage response. Upon DNA damage, p53 up regulates Waf1p21 to cause G1 arrest, which allows time for damaged DNA to be repaired, or for triggered apoptosis to eliminate genetically damaged cells^[13-16]. The apoptosis promoting capacity of p53 is presumably due to its ability to activate bax, a gene that encodes an inhibitor of bcl-2^[15]. Bax prote in inhibits the function of bcl-2 leading to increased apoptosis. Recent reports indicate that Waf1p21 can also induce apoptosis^[17]. As the Waf1p21 is up-regulated by wild type p53 protein, the levels of Waf1p21 protein may reflect the functional status of p53.

Our previous studies indicated that accumulation of p53 protein occurred at early stages of human esophageal carcinogenesis, even in histopathologically normal epithelium^[18-19]; p53 gene mutations were observed in some of these samples^[20-22]. The results indicated that p53 protein accumulation and gene mutation could be an early event in esophageal carcinogenesis. The loss of normal p53 function, for instance due to mutations, could delay apoptosis^[15].

Quantitative analysis of both apoptosis and proliferation should provide important insights into cancer development, but such analysis has not been made in samples at different stages of esophageal carcinogenesis. In this study, we have investigated the extent of apoptosis and its relationship with cell proliferation and changes of p53, Waf1p21, bcl-2 and c-myc in human esophageal epithelia with different severities of precancerous and cancerous lesions from subjects in Henan, China.

MATERIALS AND METHODS

Tissue collection and processing

Esophageal biopsy tissues were collected from 241 symptom-free subjects, and surgically-resected EC specimens were collected from 38 patients in Huixian and Linxian, China. Of these 279 subjects, there were 160 males (20 to 71 years of age with a mean±SD of 48±14 years) and 119 females (20 to 79 years of age with a mean±SD of 47±16 years). None of these cancer patients received any treatment of chemotherapy or radiotherapy before

the operation. All specimens were fixed with 85% 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), for apoptosis analysis, and for immunohistochemical staining.

Histopathology analysis

Histopathological diagnosis for esophageal epithelia were made according to cellular morphological changes and tissue architecture using previously established criteria^[23]. In brief, the normal esophageal epithelium contained 1-3 proliferating basal cell layers; the papillae were confined to the lower half of the epithelium. In BCH, the proliferating basal cells were increased to more than 3 cell layers and less than half of the whole epithelial thickness. DYS was characterized by the partial loss of cell polarity and cell nuclear atypia. SCC was characterized by confluent and invasive sheets of cohesive, polymorphous cells with hyperchromatic nuclei.

Immunohistochemical staining for PCNA, p53, Waf1p21, bcl-2 and c-myc

The avidin-biotin-peroxidase complex (ABC) method was used for PCNA, p53, Waf1p21, bcl-2 and c-myc antigen detection (Oncogene Science, Inc., Manhasset, NY). In brief, after dewaxing, inactivating endogenous peroxidase activity, and blocking cross-reactivity with normal serum, the sections were incubated overnight at 4 °C with a diluted solution of the primary antibodies (1:200 for PCNA, 1:1 000 for p53, 1:20 for Waf1p21 and bcl-2 and 1:150 for c-myc). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain Elite Kit). Normal serum blocking and omission of the primary antibody were used as negative controls^[18,19].

Quantitative analysis of immunostaining results

Quantitative analysis of nuclear immunostaining results was recorded as the number of positive cells per mm² of the tissue section as described previously^[19]. This was done by counting all the positive stained cells in the whole piece of tissue under a microscope with magnification ×400; usually 24 fields for biopsy and 50 fields for surgically resected specimens were counted.

Analysis of apoptotic index

Apoptotic cells and apoptotic bodies were identified by well-established morphological criteria^[7,24-26]. In

general, the morphologic characteristics attributed to apoptotic cells include cell shrinkage, disconnection with neighboring cells, nuclear chromatin condensation, maintenance of cytoplasmic membrane integrity, strong eosinophilic cytoplasm and lack of an inflammatory reaction. Apoptotic bodies were defined as small, roughly spherical or ovoid cytoplasmic fragments, some of which contain nuclear fragments. Apoptosis was scored in coded slides by microscopic examination of H & E-stained sections at 400X. Quantitation of apoptotic cells and bodies was performed in the following manner^[25]. The serial sections of the tissues were first scanned under a microscope with low magnification, and the tissue slides were selected based on the following criteria: flat with good orientation, clear cell and tissue structure, and uniform staining. All identified apoptotic cells and bodies in the slide were counted. In SCC, only fields with the least amount of stroma were selected for counting. Apoptotic cells or bodies were not evaluated from the vicinity of necrotic areas. In cases in which many apoptotic bodies were found clearly in one cell, the group of apoptotic bodies was counted as one. Quantitative analyses of apoptotic changes were recorded as apoptotic cell indices (ACI, the number of apoptotic cells per mm² of the tissue), apoptotic body indices (ABI, the number of apoptotic bodies per mm² of the tissue) and apoptotic indices (AI, the combined number of apoptotic cells and bodies per mm² of the tissue).

Statistical analysis

The mean±SE of the PCNA, p53 and Waf1p21 immunostaining positive cells/mm² and the AI in the esophageal biopsy and surgically resected EC samples in each histologic category were calculated by univarious analysis, compared by the Wilcoxon rank-sum test for unpaired data and correlated by Pearson's correlation. The Chi-squared test was used for the percentage of samples with immunostaining positivity.

RESULTS

Apoptotic indices in esophageal precancerous and cancerous lesions

Both apoptotic cells and apoptotic bodies were observed in tissues with different severities of lesions (Table 1). The apoptotic cells usually had small nuclei and condensed eosinophilic cytoplasm. In normal epithelia and epithelia with BCH and DYS, apoptotic cells were located among the hyperproliferative basal cells (Figure 1). In SCC, most of the apoptotic cells were seen among the peripheral cancer cells, but not in the keratinized

areas of the tumor. The apoptotic cells were usually scattered single cells which had highly condensed nuclear chromatin and cytoplasm as well as strong eosinophilic staining of the cytoplasm. No inflammatory response was observed around these apoptotic cells. The apoptotic bodies most frequently appeared as a single structure separated from the surrounding intact cells by a clear halo (Figure 1). The AI and the frequency of apoptosis occurrence was low in normal epithelia, but increased significantly from BCH to DYS and to SCC ($P<0.01$, Table 1). The ratio of ACI and ABI reversed from 1.5:1 in normal epithelia to 1:1.7 in SCC, suggesting a higher turnover of apoptosis activity in cancers than in normal tissues.

Immunoreactivity for PCNA, p53, Waf1p21, bcl-2 and c-myc in different stages of esophageal carcinogenesis

Intense immunostaining of p53 protein and PCNA was observed in the cell nuclei of tissues with different lesions. In brief, similar immunostaining patterns were observed for PCNA and p53, but the number of PCNA-positive cells was higher than that of p53 in the same histological category (3- to 4-fold, Tables 1 and 2). As the esophageal tissue progressed from BCH to DYS and to SCC, the PCNA and p53 immunostaining positive cells increased significantly in number and expanded upwards in the epithelium. This finding is consistent with our previous report^[19].

The Waf1p21 positive cells were located at the third and forth cell layers which was 2 to 3 cell layers higher than those staining positive for PCNA and p53 in esophageal epithelia (Figure 2). Waf1p21 immunostaining was confined to the cell nucleus. The percentage of samples with Waf1p21 positive immunostaining was similar in normal and BCH esophageal epithelia, but the number of Waf1p21 immunostaining positive cells was slightly higher in BCH than in normal (Table 2). As the lesions progressed to DYS and to SCC, the percentage of samples with Waf1p21 immunoreactivity and the number of immunostaining positive cells decreased. The total number of Waf1p21 positive cells appeared to be lower than that of p53 in normal and BCH esophageal epithelia, much lower in DYS (24 fold) and markedly lower in SCC (162 fold).

Bcl-2 immunoreactivity was observed only in the cytoplasm of the cells. Bcl-2 positivity was seen in none of the 23 morphologically normal esophageal epithelial samples but was observed in 16% (5/31) of the BCH, 40% (4/10) of the DYS and 86% (6/7) of the SCC samples (Figure 3). C-myc immunoreactivity was observed in both the

nuclei and the cytoplasm of the cells. The percentage of the samples with positive immunostaining for c-myc was 13% (4/31) in normal epithelia, increased to 25% (15/61) in BCH and markedly increased to 54% (7/13) in DYS, and 59% (16/27) in SCC (Figure 3). Strong nuclear staining for c-myc was noted in esophageal glandular cells in submucosal tissues.

Apoptosis in relation to cell proliferation

Both AI and the number of PCNA positive cells increased significantly as the lesions progressed from BCH to DYS and to SCC ($P<0.01$, Table 1, Figure 4). Pearson's correlation analysis showed a good correlation between AI and the proliferation index in esophageal carcinogenesis ($r = 0.363$, $P<0.001$). In the same category of lesions, the number of PCNA positive cells was several hundred fold higher than that of AI (Table 1).

Apoptosis in relation to p53, Waf1p21, bcl-2 and c-myc

In samples with p53 positive staining, the AI was similar in the normal epithelia and BCH (Table 3). All 31 samples with DYS lesions were p53-positive. The AI for samples with p53-positive stain appeared to be slightly higher than those with p53-negative stain, but this difference was not statistically significant (Table 3). The total number of p53 positive cells per mm² was higher than that of apoptotic cells in morphologically normal esophageal epithelia, BCH and DYS, and much higher in SCC (Tables 1 and 2). In bcl-2 staining positive samples, the AI was low in BCH, but increased in DYS and SCC. The AI in BCH, DYS and SCC with bcl-2 positive stain was not significantly different from the corresponding samples with bcl-2 negative stain (Table 3). The AI in normal, BCH, DYS and SCC with c-myc positive staining were also not significantly different from those with c-myc negative stain (Table 3).

Table 1 Apoptosis and cell proliferation in esophageal epithelia with different severities of lesions

Histology	Apoptosis					PCNA immunostaining	
	Number of samples examined	Percent of samples with apoptosis	Apoptotic indexa ($\bar{x}\pm s$)			Number of samples examined	Number of positive cells/mm ² ($\bar{x}\pm s$)
			AI	ACI	ABI		
Normal	38	24	0.3±0.7	0.2±0.5	0.1±0.4	31	200±113
BCH	156	32	0.6±0.9	0.3±0.6 ^b	0.2±0.5	106	286±150 ^b
DYS	47	79 ^c	1.8±1.5 ^b	0.9±0.9 ^b	0.8±1.0 ^b	31	719±389 ^b
SCC	38	100 ^c	2.5±2.1 ^b	0.9±0.9	1.6±1.5 ^b	26	1 261±45 ^b

^aACI, the number of apoptotic cells per mm²; ABI, the number of apoptotic bodies per mm²; AI, the number of both apoptotic cells and apoptotic bodies per mm². ^bStatistically different from the adjacent low-grade lesion by Wilcoxon rank-sum test, ^c $P<0.01$.

Table 2 Changes of p53 and Waf1p21 in esophageal epithelia with different severities of lesions

Histology	p53 immunostaining		Waf1p21 immunostaining	
	% of samples with positive stain % (n/N) ^a	Number of positive cells/mm ² ($\bar{x}\pm s$)	% of samples with positive stain % (n/N) ^a	Number of positive cells/mm ² ($\bar{x}\pm s$)
Normal	74 (23/31)	53±7839	(11/28)	34±72
BCH	87 (102/117)	74±66	38 (14/37)	53±10 ^b
DYS	100 (31/31)	262±341 ^b	27 (3/11)	11±20 ^b
SCC	89 (23/26)	1 297±1110 ^b	14 (1/7)	8±22

^an, positive cases; N, total cases examined. ^bStatistically different from the adjacent low-grade lesion by Wilcoxon rank-sum test, $P<0.01$.

Table 3 Apoptotic indexes in samples with positive and negative immunoreactivity for p53, bcl-2 and c-myc in esophageal epithelia with different severities of lesions

Histology	p53 immunostaining				bcl-2 immunostaining				c-myc immunostaining			
	Positive		Negative		Positive		Negative		Positive		Negative	
	Na	AIb	N	AI	N	AI	N	AI	N	AI	N	AI
Normal	23	0.4±0.8	8	0.2±0.4	0	0	23	0.2±0.6	4	0.6±1.2	27	0.3±0.6
BCH	102	0.4±0.8	15	0.3±0.6	5	0.1±0.3	26	0.5±1.1	15	0.8±1.1	46	0.5±1.1
DYS	31	1.8±1.5	0		4	1.7±1.3	6	1.9±0.4	7	2.0±1.7	6	0.7±0.7
SCC	23	2.3±2.1	3	1.1±0.4	6	2.0±0.9	1	2.8	16	2.2±2.2	11	2.0±1.8

^aN, Number of samples examined.

^bAI, apoptotic index, i.e., the number of apoptotic cells and apoptotic bodies per mm².

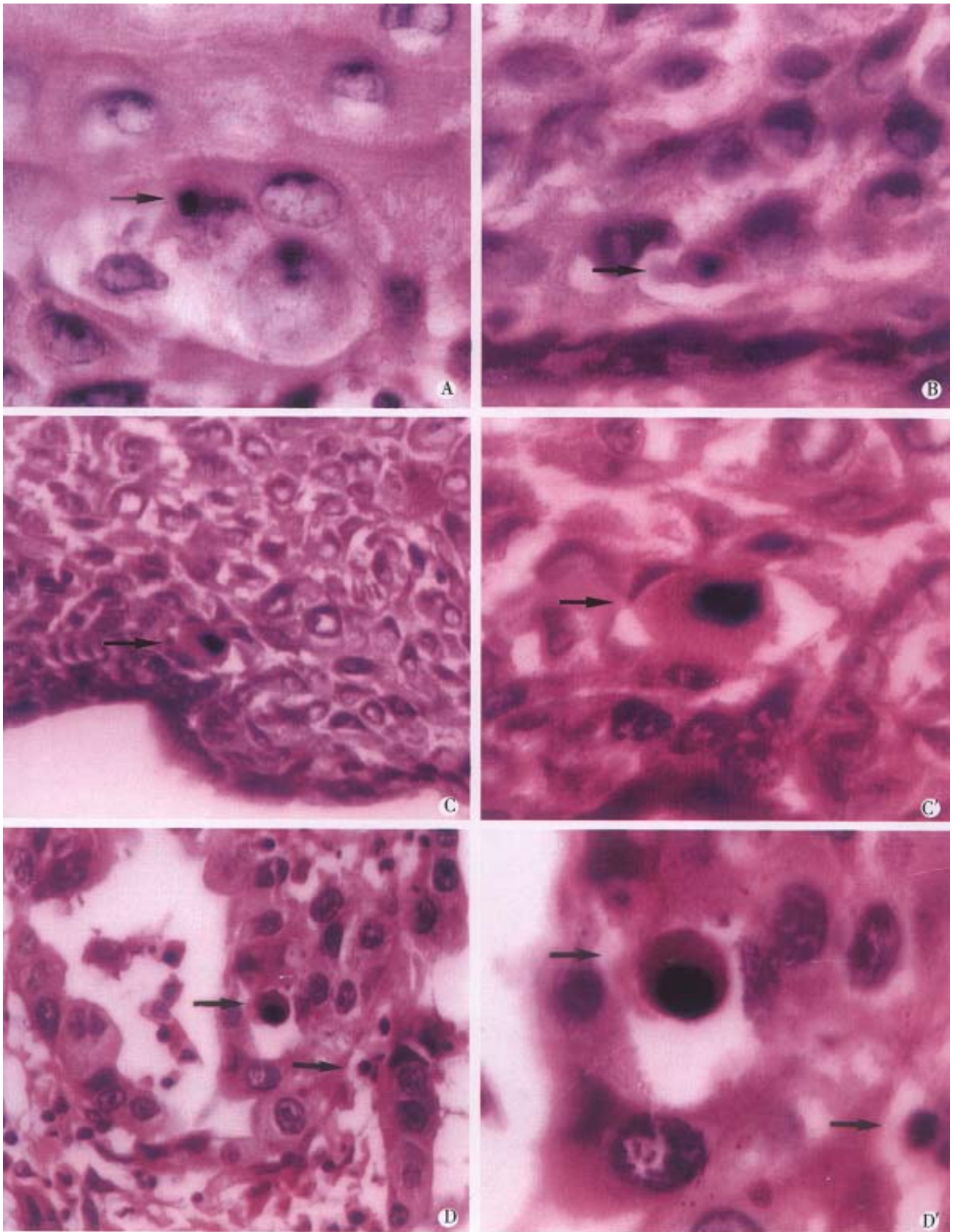


Figure 1 Apoptotic cells in human esophageal epithelia (H & E staining). Micrographs illustrate the apoptotic cells in normal tissue (A) and the tissues with lesions of BCH (B), DYS (C) and SCC (D). $\times 400$. C' and D' were the higher magnification of C and D. $\times 1\,000$. Scattered apoptotic cells (arrow) were located among the compartments of dysplastic and hyperproliferative cells. The apoptotic bodies most often appeared as a single structure separated from the surrounding intact cells by a clear halo (C).



Figure 2 Immunostaining of Waf1p21 in biopsy samples of esophageal BCH. The Waf1p21-positive immunostaining cells were located at the third and fourth cell layers of the epithelium (arrow). $\times 400$

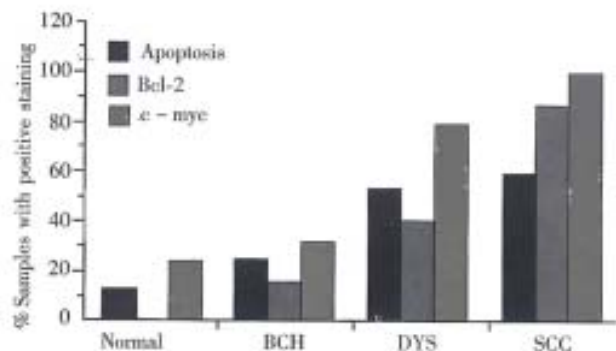


Figure 3 Changes of apoptosis and immunoreactivity of bcl-2 and c-myc in esophageal normal epithelia and epithelia with different severities of lesions.

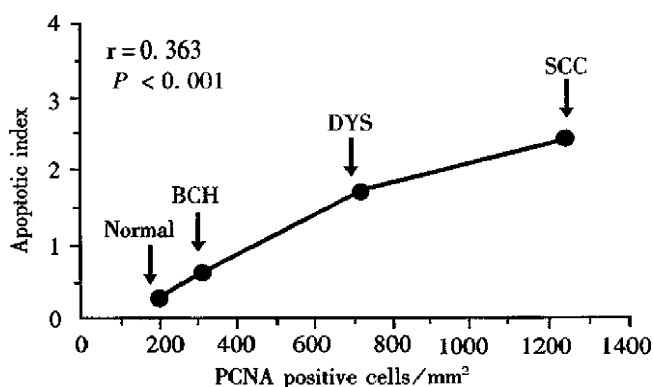


Figure 4 Changes of apoptosis and cell proliferation determined by PCNA in esophageal carcinogenesis.

DISCUSSION

The detection of apoptotic cells and apoptotic bodies by normal light microscopy is based on several well-established morphological features^[7,24-26]. These features include condensation of chromatin and cytoplasm as well as fragmentation of the cells

which leads to the appearance of membrane-bound apoptotic bodies.

An interesting observation in this study was that apoptotic cells were located in the same compartment of hyperproliferative cell population in the esophageal epithelium as the cells staining positive for p53, bcl-2, c-myc and PCNA. This finding was consistent with the recent report on human lung and breast tumors^[25,27], but not consistent with the report on apoptosis detection by terminal deoxynucleotidyl transferase mediated UTP nick end labeling (TUNEL) method^[28]. In our samples the apoptotic cells were also found at the superficial layers of the esophageal epithelium by TUNEL (data not shown). The superficial cells of the epithelium are usually at the late stage of differentiation, and the cells gradually die with DNA fragmentation which may be detected by the TUNEL method. TUNEL has been used to detect apoptotic cells in many studies^[27,29], but different opinions exist concerning the interpretation of the results^[30]. It has been reported that apoptotic cells were not detected by TUNEL, but were observed on routine H&E stained sections in colorectal carcinomas^[31,32]. The most meaningful standard of apoptotic cell death in studying carcinogenesis, we believe, is the characteristic morphological changes.

Another observation is that the number of cells undergoing apoptosis was low in esophageal epithelia with different lesions. p53 is known to act as a cell cycle check-point protein and to induce apoptosis following DNA damage^[14,33,34]. Inactivation of wild-type p53 function, frequently reflected by the accumulation of mutated p53 protein, could delay apoptosis^[35]. In the present study, no distinctive difference was noted in the level of AI between the p53 positive and p53-negative lesions. It is possible that the effect of p53 inactivation on apoptosis in these cases is masked by other dominant regulators.

Expression of bcl-2 oncoprotein has been shown to block apoptosis in various experimental systems^[36]. In the present study, we observed a low level of apoptosis and high frequency of bcl-2 and c-myc expression in DYS and SCC, which is in keeping with the role of bcl-2 in blocking apoptosis. The lack of difference in AI between the bcl-2 positive and negative groups suggest that other factors may dominate the regulation of apoptosis and override the inhibitory effects of bcl-2.

A third observation is the inverse correlation between Waf1p21 and p53 and PCNA. In this study a low number of Waf1p21-positive cells, much lower than that of p53 and PCNA, in DYS and SCC was observed. Waf1p21 has been shown to bind to Cdk2 and to be a potent Cdk inhibitor, and also has been shown to bind to PCNA and to inhibit DNA

replication *in vitro*. In esophageal DYS, p53 mutation has been identified frequently^[20-22]. The low levels of Waf1p21 at the stage of DYS may be related to the functional loss of p53. Other mechanisms may also be responsible for the lack of Waf1p21 expression in DYS and SCC^[37]. The distribution of Waf1p21-positive cells was different from that of p53 and PCNA in that Waf1p21-positive cells reside in the middle third of epithelium, a better differentiated area, and are usually located 2 to 4 cell layers higher than that of p53- and PCNA-positive cells, suggesting that the expression of Waf1p21 is related to the presence of wild type p53 or with cell differentiation.

In summary, we found that AI was at a low level in normal esophageal epithelia and increased as the lesions progressed. The low number of Waf1p21-positive cells may be due to functional loss of p53 by mutations. The relationship between Waf1p21 and bcl-2 positivity and apoptosis is not clear, possibly due to the complexities of molecular changes in esophageal carcinogenesis.

Acknowledgments We would like to thank Dr. Guang Yu Yang for his helpful discussions, Mr. Darren Seril for technical help, and Ms. Dorothy Wong for the preparation of this manuscript.

REFERENCES

- Yang CS. Research on esophageal cancer in China: a review. *Cancer Res*, 1980; 40(7):2633-2644
- Lu JB, Yang WX, Zu SK, Zhang QL, Sun XB, Lu WQ *et al.* Cancer mortality and mortality trends in Henan, China, 1974-1985. *Cancer Detect Prev*, 1988; 13(2):167-173
- Correa P. Precursors of gastric and esophageal cancer. *Cancer (Phila.)*, 1982; 50(11):2554-2565
- Munoz N, Crespi M, Grassi A, Wang GQ, Shen Q, Li ZC. Precursor lesions of oesophageal cancer in high risk population in Iran and China. *Lancet*, 1982; 1(5):876-879
- Qiu SL, Yang GR. Precursor lesions of esophageal cancer in high-risk populations in Henan Province, China. *Cancer (Phila.)*, 1988; 62(3):551-557
- Wang LD, Lipkin M, Qiu SL, Yang GR, Yang CS, Newmark HL. Labeling index and labeling distribution of cells in esophageal epithelium of individuals at increased risk for esophageal cancer in Huixian, China. *Cancer Res*, 1990; 50(9):2651-2653^a
- Kerr JFR, Winterford CM, Harmon BV. Apoptosis: its significance in cancer and cancer therapy. *Cancer*, 1994; 73(8):2013-2026
- Kastan MB, Canman CE, Leonard CJ. p53, cell cycle control and apoptosis: implications for cancer. *Cancer Metas Rev*, 1995; 14(1):3-15
- Uhlman EI, D'sa Eipper C, Subramanian T, Wagner AJ, Hay N, Chinnadurai G. Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. *Cancer Res*, 1996; 56(10):2506-2509
- Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood*, 1992; 80(5):879-886
- Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol*, 1994; 124(1):1-6
- Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature (Lond)*, 1988; 335(3):440-442
- Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci USA*, 1992; 89(16):7491-7495
- Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, 1991; 51(23):6304-6311
- Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science (Washington DC)*, 1994; 266(13):1821-1828
- Shimamura A, Fisher DE. p53 in life and death. *Clinical Cancer Res*, 1996; 2(3):435-440
- Sheikh MS, Rochefort H, Garcia M. Overexpression of p21 Waf1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. *Oncogene*, 1995; 11(9):1899-1905
- Wang LD, Hong JY, Qiu SL, Gao HK, Yang CS. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. *Cancer Res*, 1993; 53(8):1783-1787
- Wang LD, Shi ST, Zhou Q, Goldstein S, Hong JY, Shao P *et al.* Changes in p53 and cyclin D1 protein levels and cell proliferation in different stages of human esophageal and gastric cardia carcinogenesis. *Int J Cancer*, 1994; 59(4):514-519
- Gao HK, Wang LD, Zhou Q, Hong JY, Huang TY, Yang CS. p53 tumor suppressor gene mutation in early esophageal precancerous lesions and carcinoma among high risk populations in Henan, China. *Cancer Res*, 1994; 54(16):4342-4346
- Wang LD, Zhou Q, Hong JY, Qiu SL, Yang CS. p53 protein accumulation and gene mutations in multifocal esophageal precancerous lesions from symptom free subjects in a high incidence area for esophageal carcinoma in Henan, China. *Cancer*, 1996; 77(7):1244-1249
- Shi ST, Feng B, Yang GY, Wang LD, Yang CS. Immunohistochemical sequencing (IHSS) of p53 tumor suppressor gene in human oesophageal precancerous lesions. *Carcinogenesis*, 1996; 17(1):101-106
- Wang LD, Qiu SL, Yang GR, Lipkin M, Newmark HL, Yang CS. 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*, 1993; 2(1):71-78
- Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer*, 1972; 26(2):239-257
- Meyn RE, Stephens LC, Mason KA, Medina D. Radiation-induced apoptosis in normal and pre-neoplastic mammary glands *in vivo*: significance of gland differentiation and p53 status. *Int J Cancer*, 1996; 65(3):466-472
- Ei-Labban NG, Osorio-Herrera E. Apoptotic bodies and abnormally dividing epithelial cells in squamous cell carcinoma. *Histopathology*, 1986; 10(5):921-931
- Tormanen U, Eerola AK, Rainio P, Vahakangas K, Soini Y, Sormunen R *et al.* Enhanced apoptosis predicts shortened survival in non-small cell lung carcinoma. *Cancer Res*, 1995; 55(23):5595-5602
- Ohbu M, Saegusa M, Okayasu I. Apoptosis and cellular proliferation in oesophageal squamous cell carcinomas: differences between keratinizing and nonkeratinizing types. *Virchows Arch*, 1995; 427(3):271-276
- Tatebe S, Ishida M, Kasagi N, Tsujitani S, Kaibara N, Ito H. Apoptosis occurs more frequently in metastatic foci than in primary lesions of human colorectal carcinomas: analysis by terminal-deoxynucleotide-transferase-mediated dUTP-biotin nick end labeling. *Int J Cancer*, 1996; 65(2):173-177
- Mundle SD, Gao XZ, Khan S, Gregory SA, Preisler HD, Raza A. Two in situ labeling techniques reveal different patterns of DNA fragmentation during spontaneous apoptosis *in vivo* and induced apoptosis *in vitro*. *Anticancer Res*, 1995; 15(4):1895-1904
- Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM *et al.* Inhibition of apoptosis during development of colorectal cancer. *Cancer Res*, 1995; 55(5):1811-1861
- Staunton MJ, Gaffney EF. Tumor type is a determinant of susceptibility to apoptosis. *Am J Clin Pathol*, 1995; 103(3):300-307
- Marx J. Cell death studies yield cancer clues. *Science*, 1993; 259(5):760-761
- Lane DP. p53, guardian of the genome. *Nature*, 1992; 358(6381):15-16
- Bartek J, Bartkova J, Vojtesek B. Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene*, 1991; 6(9):1699-1703
- Bissonnette RP, Echeverri F, Mahboubi A, Green DR. Apoptotic cell death induced by c-mys is inhibited by bcl-2. *Nature*, 1992; 359(6395):552-554
- Barbareschi M, Caffo O, Doglioni C, Fina P, Marchetti A, Buttitta F *et al.* P21Waf1 immunohistochemical expression in breast carcinoma: correlations with clinicopathological data, oestrogen receptor status, MIB1 expression, p53 gene and protein alterations and relapse free survival. *Br J Cancer*, 1996; 74(2):208-215