

Flow cytometric analysis of DNA, telomerase content and multi-gene expression in esophageal epithelial dysplasia

Lian-Fu Zuo, Pei-Zhong Lin, Fing-Ying Qi, Jian-Wen Guo, Jiang-Hui Liu

Lian-Fu Zuo, Jian-Wen Guo, Jiang-Hui Liu, Hebei Provincial Tumor Institute, Shijiazhuang 050011, Hebei Province, China

Pei-Zhong Lin, Department of Pathology, Tumor Institute, Chinese Academy of Medical Sciences, Beijing 100021, China

Feng-Ying Qi, Department of Pathology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China

Supported by the National Key Technologies Research and Development Program of China during the 9th Five-year Plan Period, No.96-906-01-02

Correspondence to: Lian-Fu Zuo, Hebei Provincial Tumor Institute, Shijiazhuang 050011, Hebei Province, China. zuolianfu@sina.com
Telephone: +86-311-6033511 **Fax:** +86-311-6077634

Received: 2003-05-10 **Accepted:** 2003-06-19

Abstract

AIM: To investigate the alteration of molecular events and the early carcinogenesis mechanism of esophageal epithelial cells in the high incidence area of esophageal cancer.

METHODS: Esophageal epithelial cells of esophageal cancer patients were collected from the high incidence area in China. Content of DNA and telomerase as well as multi-gene expressions such as p⁵³, p²¹ and cyclin D₁ in esophageal precancer cells were quantitatively analysed by flow cytometry (FCM) with indirect immunofluorescence technique and DNA propidium iodide fluorescence staining.

RESULTS: FCM analysis results showed the DNA content increased significantly and the heteroploid rate was 87.9 % in occurred carcinogenesis. P⁵³ protein accumulation and ras p²¹ increase were seen in the early carcinogenesis of the esophagus. The positive rate of p⁵³ and ras p²¹ was 100 % (5/5, 4/4 respectively) in the cancer group. Telomerase and oncogene cyclin D₁ were over- expressed in all of the cancer patients.

CONCLUSION: Increased DNA content and heteroploid rate, accumulation of p53 protein, and over-expression of p21, telomerase and cyclin D₁ proteins were early molecular events during the development of esophageal cancer.

Zuo LF, Lin PZ, Qi FY, Guo JW, Liu JH. Flow cytometric analysis of DNA, telomerase content and multi-gene expression in esophageal epithelial dysplasia. *World J Gastroenterol* 2003; 9 (11): 2409-2412

<http://www.wjgnet.com/1007-9327/9/2409.asp>

INTRODUCTION

Esophageal cancer is one of the most common malignant tumors, and many studies have been made on it^[1-3]. Former studies focused primarily on precancerous lesions and carcinogenesis of esophagus. But early carcinogenesis of esophageal cancer is unclear. In the present study, we investigated the DNA, telomerase content and P⁵³, ras P²¹ and cyclin D₁ multi-gene product of exfoliated cell samples from Cixian County, a high incidence area of esophageal cancer in Hebei Province of China

with flow cytometry. It may provide a theoretic basis for understanding the carcinogenesis of esophageal epithelial cells and early diagnosis of esophageal cancer.

MATERIALS AND METHODS

Specimen collection

All samples were collected from Cixian County, a high incidence area of esophageal cancer, Hebei Province, China. One thousand nine hundred and sixteen cases of exfoliated cell specimens of esophagus were obtained with a mesh saccule. The exfoliated cell sample in each case was divided into two parts. One part was used for smear preparation on slides with papanicolaou stain for cytologic diagnosis, another part was used for preparation of a single cell suspension for flow cytometry analysis.

Specimen preparation for FCM DNA analysis

Exfoliated cells from 1916 cases for DNA analysis were obtained as follows. The exfoliated cells were washed out from the saccule with 0.9 % NaCl solution, centrifuged and fixed in 70 % ethanol. Before DNA analysis the cell samples were washed off 70 % ethanol in 0.9 % NaCl solution. Cell suspension (1×10⁶ cells/ml) was centrifuged (5 min, 1 000 r/min) and washed twice with 0.9 % NaCl solution. After centrifugation the cells were stained in one million of propidium iodide (PI) solution (PI 50 µg/ml with triton-x-100 and RNase) for 30 minutes and filtered through a 47 µm nylon mesh to remove cellular fragments and clusters. Chicken red blood cells were added to the sample before stained as an internal standard for calibration of the FCM instrument.

The antibodies for immunofluorescence detection of telomerase, P⁵³, P²¹ and cyclin D1 protein were described as below: (1) Monoclonal antibody (MoAb) P⁵³ was a mouse antibody against human P⁵³ protein (Clone PAB1801, Oncogene Science, Inc. U S. working concentration 1:100). (2) MoAb P²¹ was a mouse antibody against human pan-ras P²¹ protein (Clone F-132, Santa Cruz, US, working concentration 1:100). (3) MoAb cyclin D1 was a mouse antibody against human cyclin D1 protein (Clone DCS-6, Santa Cruz, US, working concentration 1:100). (4) Telomerase was rabbit antibody against human telomerase associated protein (TP1) polyclonal antibody (Clone C-20, Santa Cruz, US, working concentration 1:50). (5) The second antibody was used with FITC-conjugated goat anti-mouse/anti-rabbit IgG (Tackson Immunoresearch Laboratories Inc, code number 115-095-003/115095, working concentration 1:100).

Labeling method

The sample fluorescence staining was performed using indirect immunofluorescence labeling method. Samples from 100 cases were randomly selected for each antibody labeling. Each sample (10⁶ cells/ml) was washed twice with PBS and incubated in water-bath for 30-min at 37 °C with 100 µl antibody (p53, p21, cyclin D1, telomerase). The samples were then washed twice with PBS and incubated in water-bath for 30 min at 37 °C with 100 µl of the second antibody of FITC-conjugated goat anti-mouse/rabbit IgG. The cell suspension

was washed, and resuspended in 1.0 ml PBS, filtered through a 47 μ m nylon mesh, analyzed by flow cytometry, before the samples were analyzed.

Three control samples were used. One sample was used as negative control with PBS replacing the first/second antibody. Another sample was used as positive isotype control with only the first antibody incubated. The remaining sample was incubated only with the second fluorescence antibody as positive isotype control.

Flow cytometry measurement of DNA and immunofluorescence

The stained samples were analyzed in a FACS 420 flow cytometer (FACS 420 Fluorescence Activated Cell Sorting, Becton. Dickinson, Sunnyvale, California, U S A). The light source was a 2w argon ion laser using a wave-length of 488 nm. The working power was 300 mw. Single parameter was measured respectively in DNA (with a liner mode) and each protein (with a Log mode). Usually, 10 000 cells for each sample were analyzed. The analytic data were processed with a HP-300 consort 300 computer. The coefficient of variation (CV) of the instrument was adjusted within 5 % using PI staining chicken red blood cells.

Measured data analysis

DNA ploid analysis DNA content was presented as the DNA index (DI), $DI = G0/1$ peak average channel value in experimental sample DNA histogram / $G0/1$ peak average channel value in normal cell sample DNA histogram. DNA ploid was judged according to DI value. The normal epithelial cells in each sample were used as internal standard reference cell for diploid DNA value, DI in diploid cell was 1.0. A diploid DNA histogram was defined as $DI = 1.0 \pm 2$ cv. An aneuploid DNA histogram was defined as $DI \neq 1.0 \pm 2$ cv.

Cell cycle analysis DNA cell cycle was analyzed using a software of DNA histogram distribution (sum of broadened retangled model). The phases in $G0/1$.S.G2M were calculated according to DNA content distribution histogram. The proliferation index (PI) was used to present cell proliferation activity $PI(\%) = S + G2M / G0/1 + S + G2M$.

Quantitative analysis of P⁵³, rasP²¹, cyclin D1 and telomerase expression Fluorescence index (FI) was used to describe P⁵³, P²¹, cyclin D1 and telomerase expression. Following FI calculated formulation: $FI = \text{average fluorescence intensity of sample protein expression} - \text{average fluorescence intensity of isotype control} / \text{average fluorescence intensity of normal control}$. The sample was considered positive when FI was above 1.0.

Statistical analyses

Chi-square test and *t* test were used for statistical analysis of the results. The study was double blind with a group of cytologists making cytologic diagnosis, while another group performed DNA analysis.

RESULTS

Cytologic diagnosis

The cytologic observation showed that among the 1 916 cases of exfoliated cell smears, 217 were normal, 306 were mild dysplasia, 952 were moderate dysplasia, 349 were severe dysplasia and 92 were esophageal cancer.

Relationship between DI and cytologic diagnosis

The FCM DI value and corresponding cytologic diagnosis of 1916 samples are presented in Table 1. Table 1 shows that cellular DNA content was closely related to epithelial changes in esophagus. DI increased as the grade of dysplasia increased. Significant differences in DI were found in the mild, moderate

and severe dysplasia groups. There was no significant difference in DI between the normal and mild dysplasia groups.

Table 1 Relationship between cellular DNA content and cytologic diagnosis

Cellular diagnosis	No. of cases	DI value ($\bar{x} \pm s$)	P value (<i>t</i> test)
Normal	217	1.01 \pm 0.06	>0.2
Mild dysplasia	306	1.03 \pm 0.10	<0.01
Moderate dysplasia	952	1.06 \pm 0.27	<0.01
Severe dysplasia	349	1.09 \pm 0.14	<0.01
Cancer	92	1.24 \pm 0.18	<0.01

Relationship between DNA ploid and cytologic diagnosis

The results of DNA ploid status in different cytologic groups of exfoliated esophageal cells are shown in Table 2, which shows that 21 cases in normal group were DNA heteroploid, but none became cancerous in follow-up. Thus, DNA heteroploidy in normal group was defined as false heteroploid. The rate of DNA heteroploid in dysplasia increased as the dysplasia lesion progressed. The difference in DNA heteroploid among three dysplasia groups was statistically significant ($P < 0.01$).

Relationship between PI value and cytologic diagnosis

The results of cell cycle analysis showed that cell proliferation activity was closely related to epithelial changes in esophagus, PI value was 13.8 \pm 4.3 % in normal group, 16.4 \pm 2.5 % in mild dysplasia group, 17.9 \pm 4.1 % in moderate dysplasia group, 19.8 \pm 2.9 % in severe dysplasia group, 24.6 \pm 4.2 % in cancer group, respectively. PI value increased as dysplasia lesion progressed. The difference in PI value between normal group and the three dysplasia groups had a statistical significance ($P < 0.05$).

Table 2 Relationship between DNA ploid and cytologic diagnosis

Cellular diagnosis	No. of cases	DNA ploid pattern		Rate of heteroploid (%)	P value χ^2 test
		diploid	heteroploid		
Normal	217	196	21	9.7	>0.05
Mild dysplasia	306	280	26	8.5	>0.01
Moderate dysplasia	952	791	161	16.9	<0.01
Severe dysplasia	349	229	120	34.4	<0.01
Cancer	92	20	72	78.3	<0.01

Relationship between P⁵³, P²¹ protein expression and cytologic grade

The results of P⁵³, P²¹ protein expression are shown in Table 3.

Table 3 Expression of P⁵³, P²¹ in various lesions of esophageal epithelial cells

Cytological group	No. of cases	P ⁵³ FI ($\bar{x} \pm s$)	P ⁵³ + cases (%)	P ²¹ FI ($\bar{x} \pm s$)	P ²¹ + cases (%)
Normal	10	1.00 \pm 0.11	0	1.00 \pm 0.19	0
Mild dysplasia	24	1.10 \pm 0.15	5 (20.8 %)	1.09 \pm 0.21	6 (25.0 %)
Severe dysplasia	61	1.36 \pm 0.32	34 (54.8 %)	1.34 \pm 0.35	35 (57.4 %)
Cancer	5	2.28 \pm 0.20	5 (100 %)	1.82 \pm 0.24	5 (100 %)

Expression of P⁵³ and ras P²¹ in Table 3 showed that FI value increased as the grade of cytologic diagnosis of the esophageal epithelia increased. The differences in FI values among the normal, mild and severe dysplasia and cancer groups were statistically significant ($P < 0.01$). The positive

rate of P⁵³ and ras P²¹ expression among the four groups of esophageal epithelial lesion was also statistically significant ($P<0.01$).

Correlation of cyclin D1 and telomerase associated protein (TP1) expression with cytologic diagnosis grading

Table 4 Results of cyclin D1 and TP1 expression in various lesions of esophageal epithelia

Cytologic diagnosis	No. of cases	Cyclin D1, FI ($\bar{x}\pm s$)	Cyclin D1 ⁺ cases	TP FI ($\bar{x}\pm s$)	TP1 ⁺ cases
Normal	7	1.00±0.07	0	0.99±0.07	0
Mild dysplasia	11	0.97±0.12	1 (9.1 %)	1.22±0.15	7 (63.6 %)
Severe dysplasia	76	1.21±0.22	45 (59.2 %)	1.33±0.27	64 (84.2 %)
Cancer	6	1.84±0.18	6 (100 %)	1.70±0.15	6 (100 %)

Table 4 contains the cyclin D1 and TP1 expression data from 100 cases of esophageal epithelial lesion. The results showed that the cyclin D1 and TP1 protein expression level was correlated with cytologic diagnosis grading. FI values of cyclin D1 in normal group were not significantly different from that in mild esophageal dysplasia group. But FI values of TP1 between normal group and mild dysplasia group were significantly different ($P<0.05$).

The difference in FI value of cyclin D1 and TP1 among the mild, severe dysplasia and cancer groups was markedly significant ($P<0.01$), and the positive rate of cyclin D1 and TP1 protein expression among the four groups was also significantly different ($P<0.01$).

DISCUSSION

The results of this study showed that alteration of multiple molecular events occurred during esophageal epithelial carcinogenesis.

In this study, we found that the increased DI value was correlated with the precancerous lesion progression. Cellular DNA content and DNA ploidy status were closely related to the severity of epithelial dysplasia. The rate of FCM DNA heteroploidy increased significantly in the transition of dysplasia from mild to severe. Thus it is clear that cellular DNA content could reflect the pathologic changes in the epithelium of esophagus^[4]. DNA content increase and DNA heteroploidy were the very important early signals of carcinogenesis^[3,4].

Detection of FCM DNA heteroploidy might provide early cancerous information, before the morphologic evidence of cancer occurred^[3,4]. It has been proved in previous studies that proliferation index (PI) is an indicator to reflect cell proliferation activity. In our study, the cell proliferation activity was closely related to the dysplasia degree of esophagus. PI was 16.4 % in mild dysplasia group, 17.9 % in moderate dysplasia group, 19.8 % in severe dysplasia group. The results proved that the rate and speed in cell proliferation were obviously increased during esophageal epithelial carcinogenesis.

In carcinogenesis, the precancerous cell lesion resulted in not only abnormal DNA change but also abnormal change in multiple genes and its product expression^[3-14]. Previous studies demonstrated that lots of genetic alteration existed in esophageal precancer cells. P⁵³ mutation in tumor-suppressor gene and ras P²¹ activated in tumor gene were revealed to be early molecular events during esophageal carcinogenesis^[5,9,10,16]. In our studies, expression of P⁵³ and P²¹ protein varied with dysplasia degree, FI value increased significantly from mild to severe dysplasia. Overexpression of P⁵³ and P²¹ was closely related to early stages of esophageal carcinogenesis. The studies by Yasuda's group also revealed that P⁵³ mutation was a key

molecular event in esophageal carcinogenesis^[6]. The expression of P⁵³ protein in our study accumulated at early stages of esophageal carcinogenesis.

The ras gene in normal cells possesses an important effect on cell growth and proliferation. The ras gene activation can speed up cell proliferation and malignant transformation. Overexpression of ras gene (P²¹) occurred frequently in precancerous lesion of esophagus^[15]. In our studies, expression of ras P²¹ varied with dysplasia degree. FI of ras P²¹ expression was significant among the normal, mild, severe dysplasia and cancer groups ($P<0.01$). It is suggested that ras P²¹ overexpression may be an important factor during esophageal epithelial carcinogenesis, and ras P²¹ may be a valuable marker at the early stages of carcinogenesis.

Recent studies have indicated that cyclin D1 is an oncogene which is related to the regulation of cell cycle^[17-21]. The cyclin D1 gene product is a key protein which makes cells enter into proliferation condition, and cyclin D1 and CDK4 are composed of a complex which could functionally inhibit tumor suppressor gene P¹⁶ and Rb activity^[23], thus promoting the cell proliferation ability. Many cells could enter into proliferation stage from G1 to S, G2M phase, so that excessive cell proliferation could cause carcinogenesis^[19,23]. In this study, the expression of cyclin D1 protein increased in severe degree of esophageal epithelial dysplasia. The results indicate that overexpression of cyclin D1 plays an important role during esophageal carcinogenesis. An increase in cyclin D1 might result in an oncogene^[22-25].

Recently, it has become a new research target of telomerase activity and its relation to carcinogenesis. It has been demonstrated that telomerase activity is increased in esophageal carcinoma. Telomerase activity could be used as an early stage marker of esophageal epithelial carcinogenesis^[25-27]. Telomerase quantitative analysis of esophageal precancerous lesion has not been reported. In this study, telomerase content in esophageal epithelial dysplasia cells was quantitatively analyzed by flow cytometry. The results showed that telomerase content obviously increased in severe dysplasia group and cancer group. The positive rate of telomerase expression was 83.1 % in severe dysplasia group and 100 % in cancer group. Overexpression of telomerase might be an important factor and early molecular event to monitor the patients with high-risk of precancerous lesion during esophageal epithelial carcinogenesis^[28-31]. Therefore, quantitative detection of telomerase content might be an early diagnostic method for esophageal cancer^[27]. It is suggested that inhibition of telomerase activity might be a new therapy for cancer^[29-31].

In summary, the DNA content, P⁵³, ras P²¹, cyclin D1 and telomerase content showed significant changes during esophageal epithelial carcinogenesis, they could also become biomarkers to identify precancerous lesions in high-risk population at high incidence areas of esophageal cancer. These results indicate that combined analysis of multiple parameters can greatly increase the accuracy of early identification of esophageal cancer in the high risk population.

REFERENCES

- 1 **Moreto M.** Diagnosis of esophagogastric tumors. *Endoscopy* 2001; **33**: 1-7
- 2 **Bektas A, Yasa MH, Kuzu I, Dogan I, Unal S, Ormeci N.** Flow cytometric DNA analysis and immunohistochemical P⁵³, PCNA and histopathologic study in primary achalasia: preliminary results. *Hepatogastroenterology* 2001; **48**: 408-412
- 3 **Ma P, Song M, Ren CS.** Significance of P²⁷ expression and DNA contents in esophagus carcinoma. *Shiyong Aizheng Zazhi* 2001; **16**: 262-263
- 4 **Zuo LF, Ling PZ, Qi FY, Wang JX, Ding ZW, Guo JW, Liu JH.** Flow cytometric DNA analyses of epithelial dysplasia of the esophagus. *Analyt Quant Cytol Histol* 2000; **22**: 175-177

- 5 **Zhu MH**. Research advance and significance in biologic function of P⁵³. *Zhonghuan Binglixue Zazhi* 2000; **29**: 60-62
- 6 **Jin YL**, Zhang W, Liu BQ, Wang HP, Han SK, Han ST, Qu P, Li M, Ding ZW, Lin PZ. Abnormal expression of P⁵³ Ki67, and iNOS in human esophageal carcinoma in situ and pre-malignant lesions. *Zhonghua Zhongliu Zazhi* 2001; **23**: 129-132
- 7 **Zuo LF**, Qi FY, Zhang LX, Lin PZ, Liu JH, Guo JW. The role of tumor suppressor gene P⁵³ and cell-cycle related gene produces in the carcinogenesis of esophageal epithelia. *Zhonghua Xin Yixue* 2003; **4**: 1345-1347
- 8 **Yasuda M**, Kuwano H, Watanabe M, Toh Y, Ohno S, Sugimachi K. P⁵³ expression in squamous dysplasia associated with carcinoma of the oesophagus: evidence for field carcinogenesis. *Br J Cancer* 2000; **83**: 1033-1038
- 9 **Geboes K**, Van Eyken P. The diagnosis of dysplasia and malignancy in Barrett's oesophagus. *Histopathology* 2000; **37**: 99-107
- 10 **Shimada Y**, Imamura M, Watanabe G, Uchida S, Harada H, Makino T, Kano M. Prognostic factors of esophageal squamous cell carcinoma from the perspective of molecular biology. *Br J Cancer* 1999; **80**: 1281-1288
- 11 **Zhan XM**, Wang GX, Sun CW, Wang JY, Li L. Expression of P16, P21, P53 and cyclin D1, and their significance in esophageal carcinoma. *Shiyong Aizheng Zazhi* 2001; **16**: 36-38
- 12 **Itoshima T**, Fujiwara T, Waku T, Shao J, Kataoka M, Yarbrough WG, Liu TJ, Roth JA, Tanaka N, Kodama M. Induction of apoptosis in human esophageal cancer cells by sequential transfer of the wild-type P53 and E2F-1 genes: involvement of P53 accumulation via ARF-mediated MDM2 down-regulation. *Clin Cancer Res* 2000; **6**: 2851-2859
- 13 **Kato H**, Yoshikawa M, Fukai Y, Tajima K, Masuda N, Tsukada K, Kuwano H, Nakajima T. An immunohistochemical study of P16, P21 and P53 proteins in human esophageal cancers. *Anticancer Res* 2000; **20**: 345-349
- 14 **Xu M**, Jin YL, Fu J, Huang H, Chen SZ, Qu P, Tian HM, Liu ZY, Zhang W. The abnormal expression of retinoic acid receptor- β , P53 and Ki67 protein in normal, premalignant and malignant esophageal tissues. *World J Gastroenterol* 2002; **8**: 200-202
- 15 **Galiana C**, Fusco A, Martel N, Nishihira T, Hirohashi S, Yamasaki H. Possible role of activated ras genes in human esophageal carcinogenesis. *Int J Cancer* 1993; **54**: 978-982
- 16 **Qiao GB**, Han CL, Jiang RC, Sun CS, Wang Y, Wang YJ. Overexpression of P53 and its risk factors in esophageal cancer in urban areas of Xi'an. *World J Gastroenterol* 1998; **4**: 57-60
- 17 **Ikeda G**, Isaji S, Chandra B, Watanabe M, Kawarada Y. Prognostic significance of biologic factors in squamous cell carcinoma of the esophagus. *Cancer* 1999; **86**: 1396-1405
- 18 **Shinohara M**, Aoki T, Sato S, Takagi Y, Osaka Y, Koyanagi Y, Hatoaka S, Shinoda M. Cell-cycle regulated factors in esophageal cancer. *Dis Esophagus* 2002; **15**: 149-154
- 19 **Ohbu M**, Kobayashi N, Okayasu I. Expression of cell cycle regulatory proteins in the multistep process of esophageal carcinogenesis: stepwise over-expression of cyclin E and P53, reduction of P21(WAF1/CIP1) and dysregulation of cyclin D1 and P27 (KIP1). *Histopathology* 2001; **39**: 589-596
- 20 **Saeki H**, Ohno S, Miyazaki M, Araki K, Egashira A, Kawaguchi H, Watanabe M, Morita M, Sugimachi K. P53 protein accumulation in multiple esophageal squamous cell carcinoma: Relationship to risk factors. *Oncology* 2002; **62**: 175-179
- 21 **Fujii S**, Tominaga O, Nagawa H, Tsuno N, Nita ME, Tsuruo T, Muto T. Quantitative analysis of the cyclin expression in human esophageal cancer cell lines. *J Exp Clin Cancer Res* 1998; **17**: 491-496
- 22 **Shamma A**, Doki Y, Shiozaki H, Tsujinaka T, Yamamoto M, Inoue M, Yano M, Monden M. Cyclin D1 overexpression in esophageal dysplasia: a possible biomarker for carcinogenesis of esophageal squamous cell carcinoma. *Int J Oncol* 2000; **16**: 261-266
- 23 **Liu SX**, Zuo LF, Zhang JY, Guo JW, Liu JH, Li YM. The expression of Rb, P21 and cyclin D1 in 41 esophageal cancers. *Zhonghua Xin Yixue* 2002; **3**: 97-99
- 24 **Toyoda H**, Nakamura T, Shinoda M, Suzuki T, Hatoaka S, Kobayashi S, Ohashi K, Seto M, Shiku H, Nakamura S. Cyclin D1 expression is useful as a prognostic indicator for advanced esophageal carcinomas but not for superficial tumors. *Dig Dis Sci* 2000; **45**: 864-869
- 25 **Zhang LX**, Qi FY, Zuo LF, Lin PZ, Guo JW, Liu JH. The expression of P¹⁶, cyclin D1 and DNA content analysis in the carcinogenesis of esophageal epithelial cells. *Zhonghua Xin Yixue* 2001; **2**: 1-3
- 26 **Wu MY**, Wu XY, Zhuang CX. The expression of human telomerase reverse transcriptase in esophageal squamous cell carcinoma and paracancerous esophageal epithelium. *Shiyong Aizheng Zazhi* 2003; **18**: 132-134
- 27 **Zhang LX**, Qi FY, Zuo LF, Lin PZ, Guo JW, Liu JH. The quantitative analysis of telomerase and DNA ploidy in the carcinogenesis of esophageal epithelium. *Zhonghua Xin Yixue* 2001; **2**: 193-195
- 28 **Shen ZY**, Xu LY, Li EM, Cai WJ, Chen MH, Shen J, Zeng Y. Telomere and telomerase in the initial stage of immortalization of esophageal epithelial cell. *World J Gastroenterol* 2002; **8**: 357-362
- 29 **Koyanagi K**, Ozawa S, Ando N, Takeuchi H, Ueda M, Kitajima M. Clinical significance of telomerase activity in the non-cancerous epithelial region of esophageal squamous cell carcinoma. *Br J Surg* 1999; **86**: 674-679
- 30 **Koyanagi K**, Ozawa S, Ando N, Mukai M, Kitagawa Y, Ueda M, Kitajima M. Telomerase activity as an indicator of malignancy potential in iodine-nonreactive lesion of the esophagus. *Cancer* 2000; **88**: 1524-1529
- 31 **Rudolph P**, Schubert C, Tamm S, Heidorn K, Hauschild A, Michalska I, Majewski S, Krupp G, Jablonska S, Parwaresch R. Telomerase activity in melanocytic lesions: A potential marker of tumor biology. *Am J Pathol* 2000; **156**: 1425-1432

Edited by Wang XL and Zhu LH