ELSEVIER

• *ESOPHAGEAL CANCER* •

Value of endoscopic methylene blue and Lugol's iodine double staining and detection of GST-π and telomerase in the early diagnosis of esophageal carcinoma

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Abstract

AIM: To explore the expressions of GST-π and telomerase activity in esophageal carcinoma and premalignant lesions and to investigate the value of endoscopic methylene blue (MB) and Lugol's iodine double staining.

METHODS: Seventy-two patients with esophagopathy were sprayed endoscopically with MB and Lugol's iodine in proper order and the areas stained blue and brown, and the area between the blue and brown stains were obtained. Depending on the pattern of mucosal staining, biopsy specimen was obtained. GST-π and telomerase activity in specimens were examined by immunohistochemistry and PCR-based silver staining telomeric repeat amplification protocol, respectively.

RESULTS: After MB and Lugol's iodine staining, the area between both the colors was obtained in 64 of the 72 patients and the areas were stained blue and brown in all of the 72 patients. Association test of two simultaneous ordinal categorical data showed a correlation between the esophageal mucosal staining and the esophageal histology ($P<0.005$). The expression of GST-π and telomerase activity in esophageal carcinoma and premalignant lesions increased. The expression of GST-π and telomerase activity in dysplasia and carcinoma was significantly higher than that in normal epithelium ($P<0.005$). The expression in hyperplasia was slightly higher than that in normal epithelium. With the lesions progressing from low- to moderate- to high-grade dysplasia, the positive rate increased ($P<0.025$). Expression of GST-π was correlated with that of telomerase activity in dysplasia and carcinoma ($\varphi = 0.4831$, $P<0.005$; $\varphi = 0.3031$, $P<0.025$, respectively); but there was no correlation between them in normal epithelium and hyperplasia.

CONCLUSION: The expression of GST-π and telomerase may be an early event in the carcinogenesis of esophagus. They may play an induced and synergistic role with each other in the carcinogenesis of esophagus. Endoscopic MB and Lugol's iodine double staining and detection of GST-π and telomerase activity may contribute to the early diagnosis of esophageal carcinoma.

INTRODUCTION

The early diagnosis of esophageal carcinoma correlates closely to improvement in prognosis. Much dependent on endoscopy, esophageal carcinoma is clinically diagnosed till now, but the choice of biopsy site, partly, has a limitation. The esophageal mucosal staining technique not only obtains the characteristic of the morphology of the lesions, but also helps to direct its biopsy in esophageal carcinoma. Carcinogen is one of the induced etiological factors. The glutathione *S*-transferases (GSTs) are a family of enzymes, which participate in metabolic detoxication and play important roles in the prevention of carcinogenesis by detoxifying numerous potentially carcinogenic compounds[1]. The present studies showed that telomerase activity had a close correlation with cell immortalization and carcinogenesis. The length of telomerase extends to maintain a proportional balance because telomerase is activated[2,3], therefore, the cells may unlimitedly proliferate to induce carcinogenesis. The aim of this study was to examine the expressions of GST-π and telomerase in esophageal carcinoma and premalignant lesions using immunohistochemistry and telomeric repeat amplification protocol (TRAP)-silver staining assay, respectively, and to investigate the value of endoscopic methylene blue (MB) and Lugol's iodine double

staining and detection of GST-п and telomerase in the early diagnosis of esophageal carcinoma.

MATERIALS AND METHODS

Specimen collection and processing

Tissue specimens were obtained endoscopically from in- and outpatients with abnormal findings, such as mucosal congestion, erosions, swelling or masses in the esophagus in the Affiliated Hospital of Jiangxi Medical College from 2002 to 2003. A total of 72 patients were included in this study, aged between 27 and 78 years, composed of 49 men and 23 women. All 72 patients underwent upper endoscopy and MB and Lugol's iodine staining with biopsy. After the endoscopic examination, the esophageal lumen was washed with 50 mL of distilled water. At first, 5 g/L MB solution was sprayed on the esophageal lesion. After 1 min, approximately 120-200 mL of distilled water was sprayed on the esophageal mucosa to wash off excess dye. Then 30 mL/L Lugol's iodine solution was sprayed on the esophageal lesion. After MB and Lugol's iodine staining, the pattern of mucosal staining was classified as blue, brown and the areas between both the colors. Depending on the color, two to four biopsy specimens of positive staining were obtained using the large cup biopsy forceps. Endoscopic specimens were immediately placed in the Eppendorf tube and a 10% buffered formalin solution, but immediately these specimens in the Eppendorf tube were kept in liquid nitrogen, and after 24 h, were stored at -80 ℃ until extraction for telomerase.

GST-п determination

Expression of GST-п was examined using indirect immunohistochemical staining accompanied by positive and negative controls. The key steps in the staining procedure were as follows: 4-μm-thick sections cut from each formalin-fixed and paraffin-embedded tissue specimen; deparaffinized in xylene, dehydrated through graded alcohol and washed thrice with distilled water; incubated for 5 min with 30 mL/L hydrogen peroxide to block endogenous peroxidase activity; again incubated with citrate buffer (pH 6.0) for 3 min using a household microwave oven at 800 W, followed by incubation for 1 h at 37 ℃ with the primary GST-п antibody at a dilution of 1:100, and then washed thrice with PBS. After a second incubation with the GST-п anti-rabbit antibody HRP, 3, 3'-diaminobenzidine tetrachloride was used for color development and finally counterstained with hematoxylin. The immunohistochemical expression of GST-п was examined by means of light microscopy. The staining results were assigned to one of the following groups: (-), less than 25% of cells stained or the cells staining intensity being consistent with that of the background; (+), 25-50% of cells stained with light yellow fine granule showing in cells; (++), 50-70% of cells stained with dark brown granule showing in cells; (+++), more than 70% of cells stained with a great amount of dark brown granule showing in cells. Positive staining provided an internal positive control for GST-п staining. The primary antibody was replaced by PBS for the negative control.

Telomerase activity assay

Expression of telomerase activity was measured using TRAP-silver staining assay. Frozen specimens were homogenized and incubated with 20 μL of ice-cold lyses buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EGTA, 0.1 mmol/L benzamidine, 5 mmol/L β-mercaptoethanol, 5 g/L CHAPS, 100 mL/L glycerol). After 30 min of incubation on ice, the lysate was centrifuged at 14 500 *g* for 30 min at 2-8 ℃, and the supernatant was frozen and stored at -80 ℃. A total volume of PCR reactions was 25 μL. TRAP reaction was performed using 2 μL extracts, 2.5 μL 10× TRAP buffer (200 mmol/L Tris-HCl, pH 8.3, 15 mmol/L MgCl$_2$, 630 mmol/L KCl, 5 g/L Tween 20, 10 mmol/L EGTA, 1 g/L BSA), 1 μL of 4 mmol/L dNTP, 0.5 μL TS primer, and 18 μL double-distilled water. After 10 min incubation at 23 ℃ for telomerase-mediated extension of the TS primer, the reaction mixture was heated for 5 min at 94 ℃ to inactivate the telomerase, then added 2 U of Taq, 0.5 μL CX primer and finally 30 μL wax barrier. All the above steps were performed on ice. PCR reactions were performed for 27 amplification cycles, each cycle consisting of denaturation at 94 ℃ for 30 s, primers annealing at 50 ℃ for 30 s and extension at 72 ℃ for 90 s, followed by an extra incubation at 72 ℃ for 10 min to ensure full extension of the products. The PCR products were electrophoresed on a 120 g/L gel polyacrylamide for 2-3 h and then gel was stained using AgNO$_3$ and was photographed. The extract, which showed a ladder of products with 6-bp increments after being stained on the gel, was considered telomerase positive. For the negative controls of telomerase, the extract was heat-treated by incubation at 65 ℃ for 10 min prior to TRAP assay to inactivate the telomerase, and no PCR product was observed in the negative controls of telomerase.

Criteria of pathological histology

This was performed by evaluating the H&E-stained tissue sections under a microscope according to the criteria described previously by Qiu and Yang[4]. We classified the tissue sections into eight groups: normal epithelium; pure hyperplasia; low-grade dysplasia; moderate-grade dysplasia; high-grade dysplasia; carcinoma *in situ*; squamous cell carcinoma; and adenocarcinoma.

Statistical analysis

Proportion (relative number, %) was used for result analysis, and statistical significance was tested using χ^2 test, exact method analysis, rank sum test or association test of categorical data. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Endoscopic methylene blue and Lugol's iodine double staining

After staining with MB and Lugol's iodine, the area between both the colors was obtained in 64 of the 72 patients. We observed areas stained blue and brown in all the 72 patients. Association test of two simultaneous ordinal categorical data showed a significant correlation between the esophageal mucosal staining and the esophageal histology (*P*<0.005, Table 1, Figure 1).

Table 1 Endoscopic MB and Lugol's iodine double staining (*n*, %)

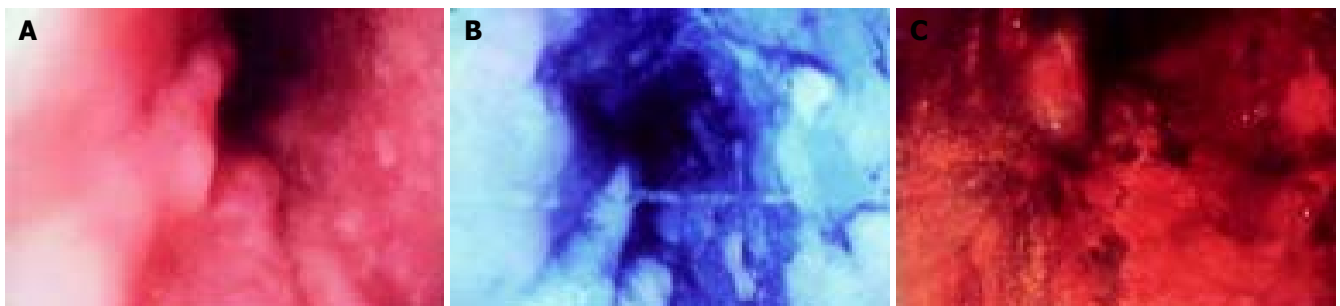| Histology	*n*	Areas stained blue (%)	Areas between both the colors (%)	Areas stained brown (%)
Normal epithelium	53	49 (92.5)	4 (7.5)	0
Hyperplasia	21	13 (61.9)	6 (28.6)	2 (9.5)
Dysplasia	79	10 (12.6)	45 (57.0)	24 (30.4)
Low-grade	17	7 (41.2)	7 (41.2)	3 (17.6)
Moderate-grade	21	3 (14.3)	13 (61.9)	5 (29.4)
High-grade	41	0	25 (61.0)	16 (39.0)
Carcinoma	55	0	9 (16.4)	46 (83.6)
Carcinoma *in situ*	1	0	1 (100)	0
Squamous cell carcinoma	51	0	8 (15.7)	43 (84.3)
Adenocarcinoma	3	0	0	3 (100)
Total	208	72	64	72

$\chi^2 = 170.58$, $P<0.005$.

Figure 1 Endoscopic view of esophageal wall after MB and Lugol's iodine double staining. **A**: Before staining, a large broad-based erosion was observed on esophageal wall and the outlines of erosion were unclearly discernible; **B**: after MB staining, the same lesion stained more deeper blue than surrounding mucosa; **C**: after Lugol's iodine double staining, the normal mucosa appeared brown, the same lesion stained more deeper blue than surrounding mucosa and the areas between both the colors were not stained.

Expression of GST-π in esophageal carcinoma and premalignant lesions

GST-π was expressed in 5.7% normal epithelium (3/53), 62.0% dysplasia (49/79) and 83.6% carcinoma (46/55, $P<0.005$); the positive rate of hyperplasia was slightly higher than that of normal epithelium. With the lesions progressing from low- to moderate- to high-grade dysplasia, the positive rate increased ($P<0.025$, Tables 2 and 3, Figure 2).

Table 2 GST-π protein expression in esophageal carcinogenesis (*n*, %)

Histology	*n*	GST-π expression				Positive rate (%)
		–	+	++	++	
Normal epithelium	53	50	3	0	0	5.7
Hyperplasia	21	19	2	0	0	9.5[1]
Dysplasia	79	30	34	12	3	62.0[b]
Carcinoma	55	9	30	13	3	83.6[b]

[1]$P>0.9$, [b]$P<0.001$ *vs* normal epithelium.

Table 3 GST-π protein expression in various histology mucosa of esophagus (*n*, %)

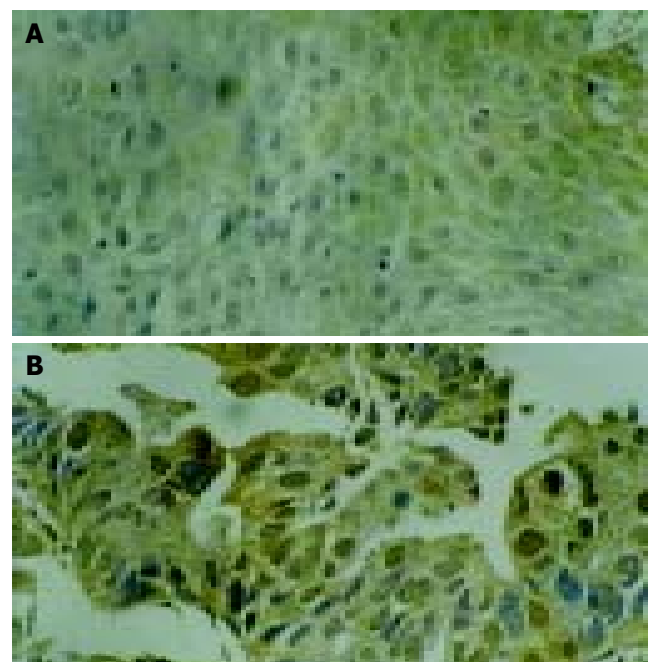| Histology	*n*	GST-π expression				Positive rate (%)
		–	+	++	++	
Low-grade dysplasia	17	11	5	1	0	35.3
Moderate-grade dysplasia	21	9	8	3	1	57.1
High-grade dysplasia	41	10	21	8	2	75.6

$P<0.025$.

Figure 2 Expression of GST-π in histological esophageal tissues (×400). **A**: Esophageal dysplasia showing yellow or brown granule in cell substance and nucleus; **B**: esophageal carcinoma showing yellow or brown granules in cell substance and nucleus.

Expression of telomerase in esophageal carcinoma and premalignant lesions

Telomerase activity was expressed in 3.8% normal epithelium

(2/53), 51.9% dysplasia (41/79), and 89.1% carcinoma (49/55, $P<0.005$); the positive rate of hyperplasia was slightly higher than that of normal epithelium. With the lesions progressing from low-to moderate-to high-grade dysplasia, the positive rate increased ($P<0.005$, Tables 3 and 4, Figure 3).

Table 4 Telomerase activity expression in esophageal carcinogenesis (*n*, %)

Histology	*n*	Telomerase activity expression		Positive number of telomerase (%)
		+	–	
Normal epithelium	53	25	1	2 (3.8)
Hyperplasia	21	1	20	1 (4.8)[1]
Dysplasia	79	41	28	41 (51.9)[a]
Carcinoma	55	49	6	49 (89.1)[c]

[1]$P = 0.998$, hyperplasia *vs* normal epithelium; [a]$P<0.005$, $\chi^2 = 33.45$, dysplasia *vs* normal epithelium; [c]$P<0.005$, $\chi^2 = 78.83$, carcinoma *vs* normal epithelium.
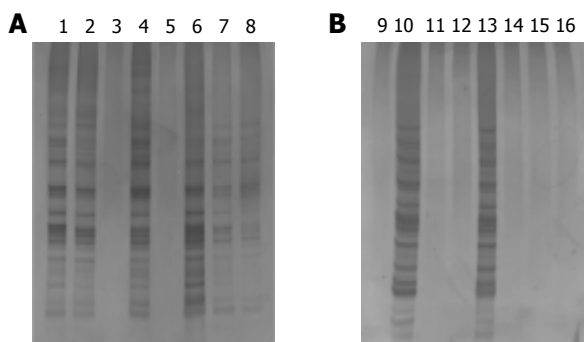

Figure 3 Analysis of telomerase activity of esophageal wall tissues using TRAP-silver staining assay. **A**: Esophageal carcinoma showing telomerase positivity (lanes 1, 2, 4, 6-8) and telomerase negativity (lanes 3 and 5); **B**: esophageal dysplasia showing telomerase positivity (lanes 10 and 13), and telomerase negativity (lanes 9, 11, 12, 14, and 15), and negative control (lane 16).

There was a positive correlation between the expression of GST-π and telomerase activity in esophageal carcinoma and premalignant lesions ($r = 0.9687$, $P<0.001$, Table 5). Expression of GST-π correlated to that of telomerase activity in dysplasia and carcinoma ($\varphi = 0.4831$, $P<0.005$; $\varphi = 0.3031$, $P<0.025$, respectively); but there was no correlation between the expression of GST-π and telomerase activity in normal epithelium and hyperplasia. Expression of GST-π was higher than that of telomerase activity in low- and moderate-grade dysplasia (Table 6).

DISCUSSION

In recent years, most scholars have advocated that the

Table 5 Telomerase activity expression in various histological mucosa of esophagus (*n*, %)

Histology	*n*	Telomerase activity expression		Positive number of telomerase (%)
		+	–	
Low-grade dysplasia	17	2	15	2 (11.8)
Moderate-grade dysplasia	21	7	14	7 (33.3)
High-grade dysplasia	41	32	9	32 (78.0)

Comparing among dysplasias $\chi^2 = 25.10$, $P<0.005$; there was significant difference between high- and low- or moderate-grade dysplasia ($\chi^2 = 21.77$, $P<0.005$; $\chi^2 = 11.90$, $P<0.005$, respectively), but no obvious difference between low- and moderate-grade dysplasia ($\chi^2 = 1.37$, $P>0.1$).

esophageal mucosal staining technique can be used for diagnosis of esophageal carcinoma to increase the sensitivity of the detection. Endoscopic MB and Lugol's iodine staining is one of the mucosal staining technique. The basis of the mucosal double staining technique is that MB stains lesion blue and Lugol's iodine reversibly stains glycogen brown. Normal squamous epithelium appears unstained because it does not absorb MB, but in abnormal mucosa, including intestinal metaplasia cells, columnar cells, erosions, ulcers and squamous carcinoma, the superficial epithelium is often stained blue because it absorbs MB. In normal squamous esophageal mucosa, the superficial epithelium contains large glycogen, so the mucosa stains dark brown, but in abnormal mucosa, the superficial epithelium often loses much of its glycogen, and remains partially or totally unstained. Therefore, the area stained blue indicates the existence of carcinoma; the area stained brown belongs to normal squamous esophageal mucosa and the area between both the colors clarifies the invasive lesion of carcinoma. Canto[5] reported that the diagnostic yield in 43 patients with Barrett's esophagus using MB-directed biopsies were compared with surveillance using a random biopsy technique in a controlled trial and the result indicated MB-directed biopsy was a more accurate technique than random biopsy for diagnosing specialized columnar epithelium and dysplasia and cancer. By means of the analysis of comparing the routine endoscopy to the endoscopy with 12 mL/L Lugol's iodine solution, Dawsey *et al*.[6], found that mucosal iodine solution improved endoscopic detection and this simple technique was highly sensitive in identifying these precursors and invasive squamous carcinoma. Our findings showed a correlation between the esophageal mucosal staining and esophageal histology ($P<0.005$), i.e. the areas between both the colors was a majority of dysplasia, especially moderate- and high-grade dysplasia, and the area stained blue was mostly carcinoma, and the area stained brown was mainly normal epithelium and hyperplasia. Overall,

Table 6 Expressions of GST-π and telomerase activity in esophageal carcinoma and premalignant lesions

	Normal epithelium		Hyperplasia		Dysplasia		Carcinoma	
	GST-π (+)	GST-π (–)	GST-π (+)	GST-π(–)	GST-π(+)	GST-π (–)	GST-π (+)	GST-π (–)
TA (+)	0	2	0	1	36	5	43	6
TA (–)	3	48	2	18	13	25	3	3

TA: telomerase activity. Expression of GST-π correlated to that of telomerase activity in dysplasia and carcinoma ($\varphi = 0.4831$, $P<0.00$; $\varphi = 0.3031$, $P<0.025$ respectively); but there was no correlation between the expression of GST-π and telomerase activity in normal epithelium and hyperplasia ($P>0.05$).

endoscopic MB and Lugol's iodine double staining improves our ability to see the true size and borders of these esophageal lesions and to take biopsy accurately, which contributes to the early diagnosis of the high risk population and patients with esophageal carcinoma and premalignant lesion, thereby preventing the carcinogenesis of esophagus and reducing the mortality of the esophageal carcinoma.

In 1984, Sato et al.[7] firstly reported high level expression of GST-п protein in preneoplastic foci of rat liver, and subsequently the evidence suggested GST-п as an important marker of rat hepatocarcinogenesis. With a close crossing-immunity between GST-п in human tissues and GST-п in rat tissues, GST-п was regarded as a major research focus on the study of human carcinogenesis. A recent study has suggested that GST-п closely correlates with human alimentary carcinoma and is overexpressed in carcinoma and premalignant lesions. Ishioka et al.[8], showed that the RNA transcript levels of GST-п in tumor tissues were obviously higher than those in normal tissues in 80% cases (20/25), but no correlation between GST-п mRNA level and clinical stage or histologic characteristics was apparent. Li et al.[9], reported that the expression of GST-п increased in esophageal carcinoma and premalignant lesions and was associated with tumor differentiation, but no correlation between GST-п expression and clinical stage or lymphatic metastasis, while found that the mean serum GST-п levels in esophageal squamous cell carcinoma patients were significantly higher than those in normal subjects ($P<0.05$). Our data showed increased expression of GST-п in esophageal carcinoma and premalignant lesions, and with the lesions progressing from low- to moderate- to high-grade dysplasia, the positive rate obviously increased ($P<0.005$). In conclusion, when the cells are in the course of premalignancy, it is possible that some preneoplastic changes in metabolic enzyme have taken place, which are undetectable by using routinely stained sections and it is consistent with the fact of their increased GST-п expression level. Therefore, increased GST-п levels may be recognized as a useful marker of esophageal carcinogenesis.

Telomerase is a ribonuclear protein complex, which contains human telomerase RNA component and binding protein. Telomerase binding protein controls cell senescence, immortalization, and carcinogenesis by means of regulating telomerase activity and changing telomere length[10,11]. Normal somatic cells express undetectable levels of telomerase activity, whereas malignant cells express high levels[12]. Telomerase activity is strongly expressed in most human carcinomas, such as thyroid, breast, esophageal, gastric, lung, pancreatic, hepatocellular, and colorectal carcinoma[13-20]. Lord et al.[21], demonstrated that telomerase activation was an important early event in the development of Barrett's esophagus and esophageal adenocarcinoma, suggesting that very high telomerase levels might be a clinically useful biomarker for the detection of occult adenocarcinoma. Using microdissection-TRAP-silver staining assay, Li et al.[22], reported telomerase activity was high in both esophageal squamous cell carcinoma and preneoplastic lesions and was related to lymphatic metastasis, but not to cancer differentiation. The data from our studies showed that the positive rates of telomerase activity in low-,

moderate-, high-grade dysplasia and carcinoma were 11.8%, 33.3%, 78.0% and 89.1%, respectively, but the positive rates of that in normal epithelium and hyperplasia were 3.8% and 4.8%. These observations reveal a crucial role for telomerase in the esophageal carcinogenesis. High expression levels in dysplasia show that telomerase may be activated in the early stage of esophageal carcinogenesis, indicating that telomerase activation is an early event in the carcinogenesis of the esophagus. It is, therefore, concluded that esophageal premalignant lesion may be effectively prevented from the progression to invasive malignancy in the light of irregularly surveying the variations of expression levels of telomerase activity in esophageal dysplasia patients and the prognosis of early esophageal carcinoma and premalignant lesion will improve significantly.

Our study provided evidence that expression of GST-п was higher than that of telomerase in low-moderate dysplasia. We infer that GST-п may be an early event in the carcinogenesis of esophagus in comparison with telomerase, but the relationship between GST-п and telomerase activity was not statistically significant. With the lesion progressing from dysplasia to carcinoma, expression level of GST-п showed an increased trend in degrees, whereas telomerase activity remained unchanged. We found that expression of GST-п was correlated to that of telomerase activity in dysplasia and carcinoma; but there was no correlation between the expression of GST-п and telomerase activity in normal epithelium and hyperplasia. Consequently, the results suggest that GST-п and telomerase may play an induced and synergistic role with each other in the carcinogenesis of esophageal carcinoma, but we do not know at present the mechanism to correlate GST-п with telomerase.

At present, many methods are widely used for the early diagnosis of esophageal carcinoma, but the sensitivity and specificity of a single method make it difficult to clinically diagnose an esophageal carcinoma. So, several authors have been exploring whether the combined method may improve the early diagnosis of esophageal carcinoma. Koyanagi et al.[23], reported that 10 of 18 iodine-nonreactive samples obtained from surgically resected specimens showed telomerase activity and in all 10 telomerase-positive samples, carcinoma in situ were observed in iodine-nonreactive mucosa by light microscopy and in 8 telomerase-negative samples, no tumor tissue were observed in iodine-nonreactive lesions. Inai et al.[24], also reported similar findings. Our study demonstrated that the expression levels of GST-п and telomerase were high in areas stained blue which was mostly carcinoma and the area between both the colors which was a majority of moderate- and high-grade dysplasia, and with the lesions progressing from low- to moderate- to high-grade dysplasia, the positive rate increased. In summary, the combined method of endoscopic MB and Lugol's iodine double staining and the detection of GST-п and telomerase activity are helpful to the early diagnosis of esophageal carcinoma and provide the chosen method for the physician.

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