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RESEARCH

The Expression of p66Shc Protein in Benign, Premalignant, and Malignant Gastrointestinal Lesions

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Abstract ROS produced from Oxidative stress have long been recognized to be involved in carcinogenesis. p66Shc generates H_2O_2 by oxidizing cytochrome c, and its expression has been reported to be elevated in several tumors. However, the expression of p66Shc in gastric cancer has not been reported, and its role in colorectal cancer has not been well elucidated. This study investigated p66Shc expression in benign, premalignant, and malignant gastric and colorectal lesions. p66Shc expression in 146 gastric tumors, 136 colorectal tumors, 45 gastric hyperplastic polyps, 33 gastric low-grade intraepithelial neoplasias, 41 gastric high-grade intraepithelial neoplasias, 42 colorectal hyperplastic polyps, 21 colorectal low-grade intraepithelial neoplasias, 38 colorectal high-grade intraepithelial neoplasias, and 30 normal gastric and colorectal tissues was measured by immunohistochemistry. Most normal gastric and colorectal tissues exhibited low or no p66Shc expression (93.4 %), while most gastric and colorectal tumors exhibited moderate to high p66Shc expression (78.1 %–80.9 %). The p66Shc expression in normal gastric and colorectal tissues were significantly lower than that in the low-grade intraepithelial neoplasias ($p<0.05$), high-grade intraepithelial neoplasias ($p<0.01$), and gastric adenocarcinomas ($p<0.01$ or <0.001). No differences in p66Shc expression were observed in gastric and colorectal hyperplastic polyps compared to the normal tissues. No statistically significant differences in p66Shc expression were observed between patients with different disease stages, different tumor grades, and with or without lymph node metastasis in gastric and colorectal cancers. In conclusion, p66Shc may be involved in the carcinogenesis of gastric and colorectal cancers and could be a marker for the diagnosis of gastric and colorectal cancers.

Keywords Gastric cancer · Colorectal cancer · p66Shc · Neoplasia · Hyperplastic polyps · Immunohistochemistry

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Introduction

Reactive oxygen species (ROS) are generated during normal mitochondrial metabolism as well as cellular response to oxidative stress. Numerous studies have reported elevated levels of oxidative stress in tumors, which cause direct and indirect ROS-mediated damage of nucleic acids, proteins, and lipids, and have been implicated in carcinogenesis [1]. In tumor cells, ROS can serve as secondary messenger molecules to increase tumor cell proliferation, migration, adhesion and genetic instability, which leads to subsequent tumor growth, invasion, metastasis, angiogenesis, and drug resistance [2]. However, high ROS activity can also induce apoptosis. Importantly, cancer cells can develop mechanisms to evade ROS-induced apoptosis [3, 4]. Generally, intracellular ROS levels can be increased by two main mechanisms: reducing ROS scavenging and increasing ROS production [5]. The increased production of ROS could be a result of enhanced release of O_2^- and/or H_2O_2 from the mitochondria and activation of NADPH oxidase (NOX) systems [6, 7]. p66Shc has been reported to participate in these processes.

p66Shc is an isoform of the ShcA family of adaptor proteins containing four functional domains: a SH2 domain at the COOH-terminal that mediates the formation of multiprotein complexes during signaling [8], two collagen homology (CH1, CH2) domains containing the essential tyrosine phosphorylation sites, and a PTB binding domain separating the

collagen homology domains. In addition to these domains, p66Shc also carries a cytochrome c-binding region within the CH2-PTB domains that regulates mitochondrial oxidative stress [9, 10]. Several lines of evidence indicate that p66Shc mediates diverse biological activities [10]. Among these biological activities, p66Shc's role in mediating redox signaling has prompted further studies ever since it was reported to play a prominent role in oxidative stress-induced apoptosis and in the life span of mammals [11]. It has been demonstrated that p66Shc oxidizes cytochrome c through a region within the CH2-PTB domains, resulting in the generation of H_2O_2 via transferring electrons from reduced cytochrome c to oxygen, which increases intracellular ROS levels [12]. Recent studies demonstrated that the expression level of p66Shc is elevated in estrogen-regulated tumors, such as metastatic breast and ovarian tumors, as well as other types of tumors including thyroid tumors and colon cancers [13–16]. p66Shc has also been reported to be a useful marker for the prognosis of stage IIA colon cancer and tumor progression of prostate cancer [17, 18].

In this study, we investigated the expression of p66Shc in benign, premalignant, and malignant gastric and colorectal tissues and further analyzed its role in the progression and prognosis of gastric and colorectal cancer.

Materials and Methods

Tissue Specimens

One hundred forty six gastric tumors and 136 colorectal tumors were collected from January 2002 to December 2007 at Second Xiangya Hospital, Central South University. The clinicopathologic information, including sex, age, tumor extension, histological types, and lymph node metastasis, was obtained from patient medical records. All diagnoses were made based on the Pathology and Genetics of Tumors in the Digestive System by three pathologists [19]. Among 146 gastric tumors, 38 were well-differentiated, 43 were moderately differentiated, and 65 were poorly differentiated adenocarcinomas. Among the 146 gastric tumors, 70 (47.9 %) were the intestinal types (IT), 67 (45.9 %) were diffuse types (DT), and 9 (6.2 %) were other types of gastric cancer. Among the 136 colorectal tumors, 32 were well-differentiated, 48 were moderately differentiated, and 56 were poorly differentiated adenocarcinomas. No patient received preoperative radiotherapy or chemotherapy. Thirty gastric tissues distal from gastric tumors and 30 colorectal tissues distal from colorectal tumors were collected when patients underwent surgical resection of tumor tissues and were finally diagnosed as normal gastric and colorectal tissues by pathologists. Tissues from 42 colorectal hyperplastic polyps, 21 colorectal low-grade intraepithelial neoplasia, 38 colorectal high-grade intraepithelial neoplasia, 45 gastric hyperplastic polyps, 33 gastric low-grade intraepithelial neoplasia, and 41 gastric high-grade intraepithelial neoplasia were collected from January 2006 to December 2009 from outpatients at the Second Xiangya Hospital. All of these preneoplastic lesions were obtained during endoscopic procedures. This study was pre-approved by The Ethics Committee for Human Research, Central South University.

Immunohistochemistry

Four μm sections were cut from routinely paraffin-embedded tissues. After deparaffinization with xylene and dehydration with alcohol, the sections were incubated with 3 % H_2O_2 for 15 min and then soaked with phosphate buffered saline. Antigen retrieval was performed with EDTA solution (pH 9.0) for 10 min. Sections were incubated with anti-p66Shc antibody (1:400, Abcam, ab75023) overnight at 4 °C, washed with 1× PBS for three times, and then incubated with horseradish peroxidase-conjugated secondary antibody for 30 min. The substrate DAB was added followed by hematoxylin counter-staining. After dehydration, the slides were soaked in xylene for 3×5 min. The positive control was the positive sections purchased from Beijing Zhongshan Biotechnology Company (Beijing, China) while the negative control was designed by replacing the primary antibody with 1× PBS. Histological and IHC evaluations were performed independently by three pathologists. Slides with unequivocal evaluations were reevaluated until a consensus was reached. For each sample, at least 3,000 adenocarcinoma cells were evaluated for the immunohistochemical staining. The percentage of primary adenocarcinoma cells with cytoplasmic staining was determined. The protein expression was classified as "low" (<33 % positive carcinoma cells) expression, "intermediate" expression (≥33 % and <66 % positive carcinoma cells), and "high" (≥66%positive carcinoma cells) expression [20].

Statistical Analysis

The data were analyzed using SPSS13.0 statistical software. The data between two samples were compared using fourfold table χ^2 test. Group comparisons of multiple samples were done using rows × columns χ^2 test. A $p<0.05$ was considered statistically significant.

Results

p66Shc Expression was High in Malignant Gastric Tissues, Moderate in Premalignant Gastric Tissues, and Low or No Expression in Normal Gastric Tissues

As shown in Table 1 and Fig. 1, low p66Shc expression was observed in 2 (6.7 %) normal gastric tissues, moderate

Table 1 p66Shc expression in benign, premalignant and malignant gastric tissues

Clinicopathological characteristics	Case number	p66Shc expression		
		High	Moderate	Low
Normal gastric tissues	30	0(0 %)	2 (6.7 %)	2 (6.7 %)
Intraepithelial neoplasias (low)	33	0(0 %)	6 (18.2 %)	7 (21.2 %)
Intraepithelial neoplasias (high)	41	3(7.3 %)	18 (43.9 %)	13 (31.7 %)
Hyperplastic polyps	45	0(0 %)	6 (13.3 %)	7 (15.6 %)
Gastric cancer	146	32(21.9 %)	82 (56.2 %)	32 (21.9 %)
Differentiation				
Well	38	8 (21.1 %)	23 (60.5 %)	7 (18.4 %)
Moderate	43	11 (25.6 %)	27 (62.8 %)	5 (11.6 %)
Poor	65	16 (24.6 %)	39 (60 %)	10 (15.4 %)
Invasion				
T1+T2	64	12(18.8 %)	43 (67.2 %)	9 (14.1 %)
T3+T4	82	23(28 %)	46 (56.1 %)	13 (15.9 %)
Lymph node metastasis				
N0	24	9 (37.5 %)	12 (50 %)	3 (12.5 %)
N1	67	11 (16.4 %)	43 (64.2 %)	13 (19.4 %)
N2+N3	55	15 (27.3 %)	34 (61.8 %)	6 (10.9 %)

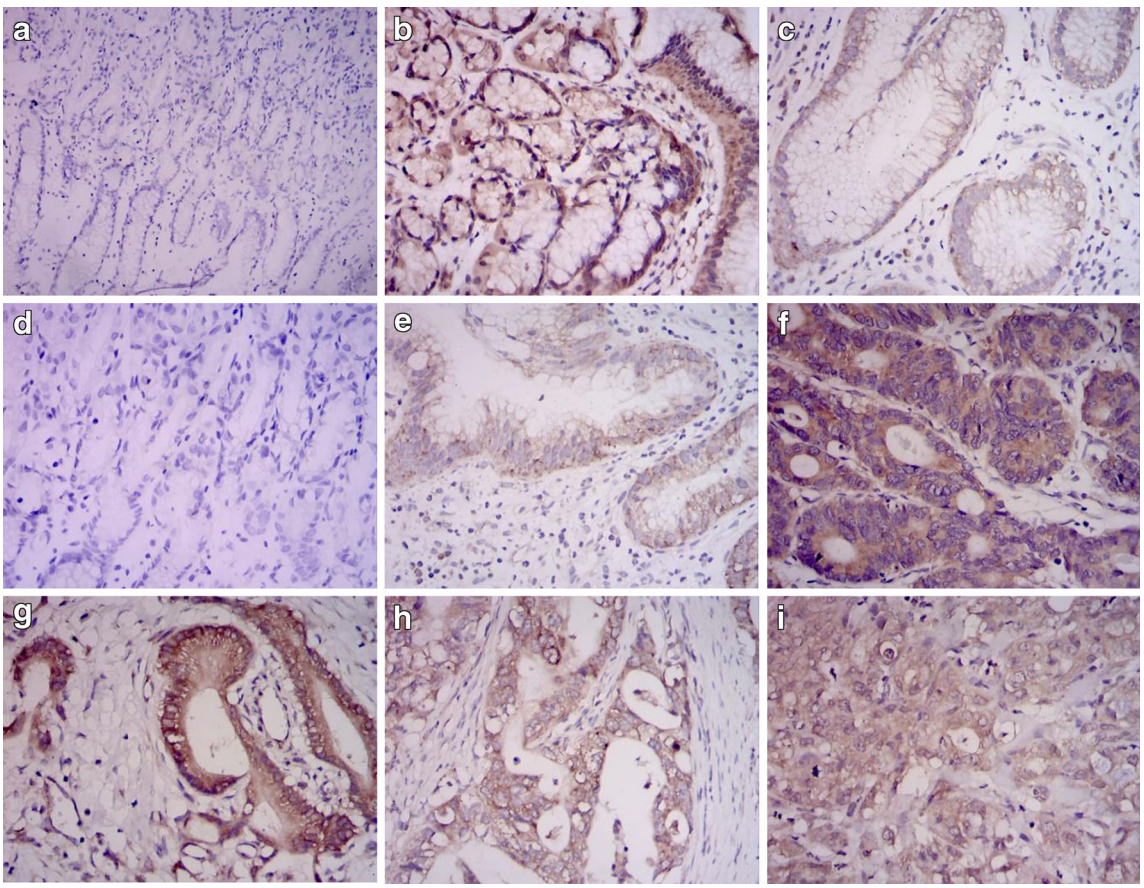

Fig. 1 Immunohistochemistry of p66Shc expression in benign, premalignant and malignant gastric tissues. **a**, **d** normal gastric tissue, 200× and 400×, respectively. **b** gastric hyperplastic polyps, 400×. **c**, **e** low-grade intraepithelial neoplasia (different atypia), 200×. **f** high-grade intraepithelial tumor, 400×. **g**, **h**, **i** well, moderately, and poorly differentiated adenocarcinoma, 400×

expression was observed in 2 (6.7 %) normal gastric tissues, and no expression was observed in 26 (86.7 %) normal gastric tissues. Among the 45 gastric hyperplastic polyps, moderate p66Shc expression was observed in 6 (13.3 %) hyperplastic polyps, low expression was observed in 7 (15.6 %) hyperplastic polyps, and no expression was observed in 31 (68.9 %) hyperplastic polyps. All 33 low-grade intraepithelial neoplasia showed low or no p66Shc expression. Among the 41 high-grade intraepithelial neoplasia, high p66Shc expression was observed in 3 (7.3 %) samples, moderate expression was observed in 18 (43.9 %) samples, low expression was observed in 13 (31.7 %) samples, and no expression was observed in 7 (17.1 %) samples. Among the 146 gastric carcinoma samples, high p66Shc expression was observed in 32 (21.9 %) samples, moderate expression was observed in 82 (56.2 %) samples, and low expression was observed in 32 (21.9 %) samples. The p66Shc expression in normal gastric tissues were significantly lower than that in the low-grade intraepithelial neoplasias ($p=0.066$), high-grade intraepithelial neoplasias ($p<0.01$), and gastric adenocarcinomas ($p<0.01$). The expression of p66Shc in hyperplastic polyps showed no significant difference from normal gastric tissues ($p=0.332$), but was significantly lower in expression compared to gastric adenocarcinomas ($p<0.01$). p66Shc expression showed no statistical differences between patients with different disease stages, different tumor grades, and with or without lymph node metastasis ($p>0.05$). However, p66Shc expression showed a tendency of increase from normal tissue to hyperplastic polyps to low-grade and high-grade intraepithelial neoplasia to gastric adenocarcinomas. This indicated that p66Shc may be involved in carcinogenesis of gastric cancer. We further analyzed p66Shc expression in the intestinal types and diffuse types of gastric cancer. No significant difference was observed (data not shown).

p66Shc was Highly Expressed in Malignant Colorectal Tissues, Moderately Expressed in Premalignant Colorectal Tissues, and Weakly or Not Expressed in Normal Colorectal Tissues

As shown in Table 2 and Fig. 2, p66Shc was moderately expressed in 2 (6.7 %) normal colorectal tissues, weakly expressed in 5 (16.7 %) normal colorectal tissues, and not expressed in 23 (76.7 %) normal colorectal tissues. Among the 38 samples of high-grade intraepithelial neoplasia of colorectal tissues, high p66Shc expression was observed in 3 (7.9 %) samples, moderate expression was observed in 11 (28.9 %) samples, low expression was observed in 20 (52.6 %) samples, and no expression was observed in 4 (10.5 %) samples. In 21 samples of low-grade intraepithelial neoplasia, p66Shc was not expressed in 8 (38.1 %) samples, weakly expressed in 11 (52.4 %) samples, and moderately expressed in 2 (9.5 %) samples. Among the 136 colorectal adenocarcinoma samples, p66Shc was highly expressed in 28 (20.6 %) samples, moderately expressed in 82 (60.3 %) samples, and weakly expressed in 26 (19.1 %) samples. Among the 42 colorectal hyperplastic polyp samples, p66Shc was moderately expressed in 6 (14.3 %) samples, weakly expressed in 13 (30.9 %) samples, and not expressed in 23 (54.8 %) samples. The p66Shc expression in normal

Table 2 p66She expression in benign, premalignant and malignant Colorectal tissues

Clinicopathological characteristics	Case number	p66Shc expression		
		High	Moderate	Low
Normal colorectal tissues	30	0(0 %)	2(6.7 %)	5(16.7 %)
Intraepithelial neoplasias (low)	21	0(0 %)	3(14.3 %)	10(47.6 %)
Intraepithelial neoplasias (high)	38	3(7.9 %)	11(28.9 %)	20(52.6 %)
Hyperplastic polyps	42	0(0 %)	6(14.3 %)	13(30.9 %)
Colorectal cancer	136	28(20.6 %)	82(60.3 %)	26(19.1 %)
Differentiation				
Well	32	7(21.9 %)	21(65.6 %)	4(12.5 %)
Moderate	48	10(20.8 %)	32(66.7 %)	6(12.5 %)
Low	56	11(19.6 %)	29(51.8 %)	16(28.6 %)
Invasion				
T1+T2	64	13(20.3 %)	39(60.9 %)	12(18.8 %)
T3+T4	72	15(20.8 %)	43(59.7 %)	14(19.4 %)
Lymph node metastasis				
N0	23	6(26.1 %)	11(47.8 %)	6(26.1 %)
N1	64	16(25 %)	36(56.3 %)	12(18.8 %)
N2+N3	49	6(12.2 %)	35(71.4 %)	8(16.3 %)

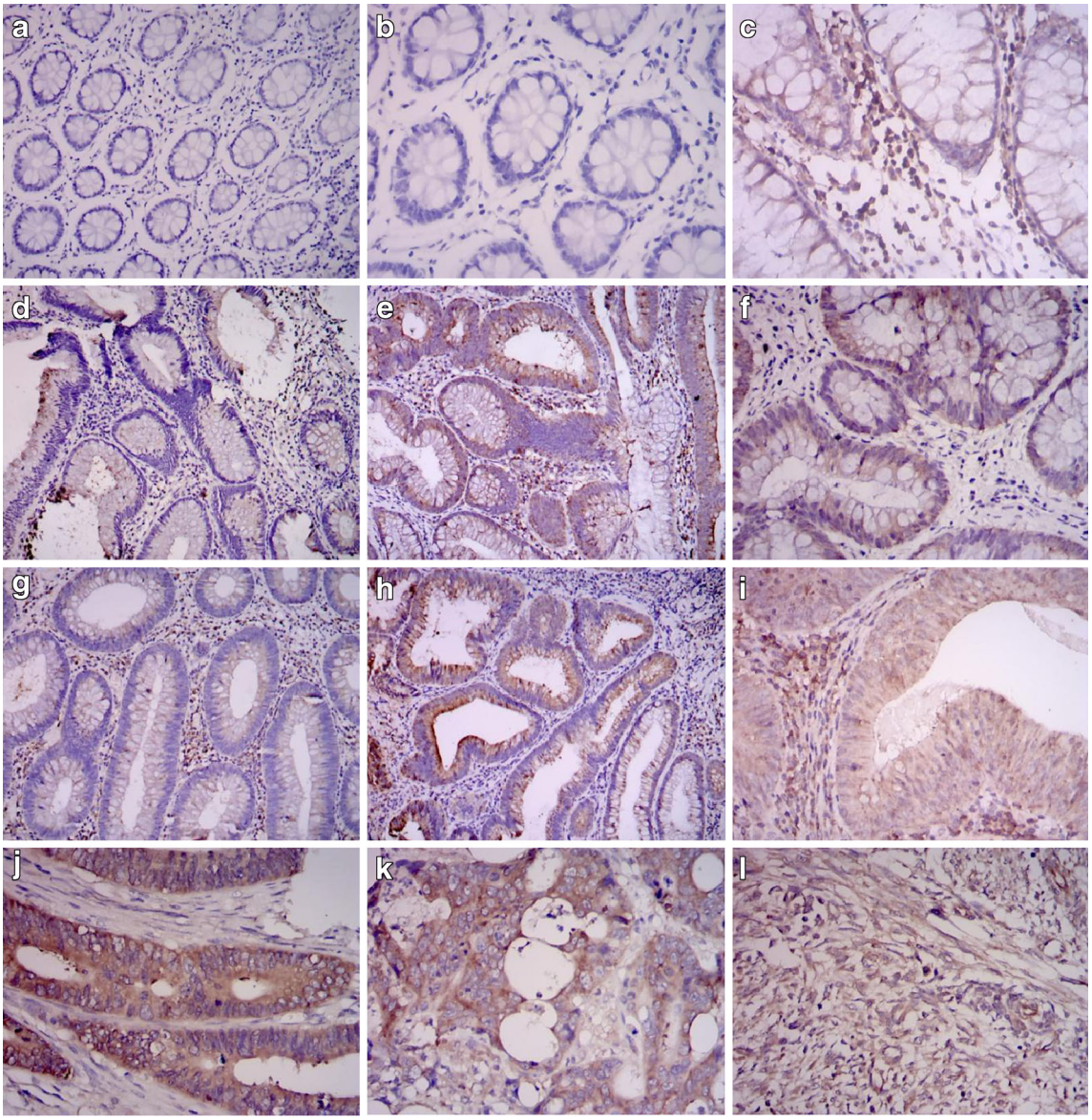

Fig. 2 Immunohistochemistry of p66Shc expression in benign, premalignant and malignant colorectal tissues. **a**, **b** normal colon tissue 200× and 400×, respectively. **c** rectal hyperplastic polyps, 400×. **d**, **g** low-grade intraepithelial neoplasia of rectum and colon, respectively, 200×. **e**, **h** low-grade neoplasia of rectum and colon, respectively, 200×. **f** low-grade intraepithelial neoplasia of colon, 400×. **i** high-level intraepithelial neoplasia of rectum, 400×. **j** well-differentiated rectal cancer, 200×. **k** moderately differentiated rectal cancer, 200×. **l** poorly differentiated rectal cancer, 200×

colorectal tissues were significantly lower than that in the low-grade intraepithelial neoplasias ($p=0.023$), high-grade intraepithelial neoplasias ($p=0.000$), and colorectal adenocarcinomas ($p=0.000$). The p66Shc expression in hyperplastic polyps showed no significant difference from normal tissues ($p=0.187$), but showed significantly lower expression than that in colorectal adenocarcinomas ($p=0.000$). p66Shc expression showed no statistical differences between patients with different disease stages, tumor grades, and with or without lymph node metastasis ($p>0.05$). p66Shc expression showed a tendency of increase from normal tissue to hyperplastic polyps to low-grade and high-grade intraepithelial neoplasias to colorectal carcinoma. This indicated that p66Shc may also be involved in carcinogenesis of colorectal cancer.

Discussion

Although elevated p66Shc level has been observed in metastatic breast, ovarian, and thyroid tumors [13–16], its expression in gastric cancer has not been reported. In addition, a comparative study of its gene expression in benign, premalignant, and malignant tissues could provide useful clues for understanding the role of the p66Shc gene in carcinogenesis and cancer progression. In this study, we first demonstrated

that p66Shc expression is moderate to high in most gastric adenocarcinomas, which is significantly higher than that in normal gastric tissues. p66Shc expression also exhibited an increase from benign to premalignant to malignant gastric tissues. Consistent with previous findings that p66Shc was significantly elevated in colorectal tumors, we further found that p66Shc expression increases progressively from benign to premalignant to malignant colorectal tissues. Our study implicates the involvement of p66Shc in the carcinogenesis of gastrointestinal cancers.

In this study, low or no p66Shc expression was observed in normal gastric and colorectal tissues as well as in hyperplastic polyps. p66Shc expression in low-grade and high-grade intraepithelial neoplasia tissues exhibited a tendency of increase compared to normal tissues. In contrast to 86.7 % of normal tissues without p66Shc expression, p66Shc expression was observed in all gastric and colorectal adenocarcinomas. Although a previous study didn't detect p66Shc expression at all in gastric cancer tissues or the adjacent normal mucosa [21], two other studies detected p66Shc expression in colon cancer cells [16, 17]. It is currently unknown whether the controversial findings were caused by the sensitivity of the antibody. In addition, p66Shc expression is not associated with disease staging, tumor grading, or lymph node metastasis. These observations suggest that p66Shc may be involved in the carcinogenesis of gastric and colorectal cancers, but there is not enough evidence supporting its role in tumor progression. Also, the molecular mechanisms responsible for the involvement of p66Shc in the carcinogenesis of gastrointestinal cancers are currently unclear. As mentioned above, oxidative stress has been widely revealed to cause direct and indirect ROS-mediated damage of nucleic acids, protein, and lipids. These damages have been implicated in carcinogenesis. p66Shc mediates various biological activities and plays a major role in redox signaling [11]. p66Shc produces H_2O_2 through oxidizing cytochrome c and transferring electrons from reduced cytochrome c to oxygen [12]. Moreover, p66Shc can bind to the tyrosine residues of a variety of phosphorylated receptors, including epidermal growth factor receptor (EGFR) and IGFR, mediating the activation of their downstream signaling molecules [22], and subsequently participate in a variety of processes such as tumor development, tumor formation, metastasis, and invasion [9, 13]. The molecular events that regulate p66Shc expression in gastric and colorectal cancer cells require further studies.

A previous study demonstrated that the tumor suppressor p53 is critically involved in oxidative stress-dependent apoptosis, and this process is required for the upregulation of p66Shc expression [23]. At the same time, p66Shc is induced transcriptionally by p53 [24]. A number of stress-activated kinases including PKC-beta and JNK1 can phosphorylate p66Shc at serine 36, which enhances the translocation of p66Shc into the mitochondria. The mitochondrial p66Shc then increases ROS content, thereby inducing permeability transition and cell death [25]. Therefore, p66Shc is involved in moderating the balance between apoptosis and proliferation in tumor cells. Previous studies have demonstrated that different levels of ROS mediate different roles in cells: a small amount of ROS promote cell proliferation and maintain cell viability, elevated ROS stimulate cell transformation and angiogenesis in tumors, even higher levels of ROS can inhibit cell proliferation and arrest cell cycles, and excessive ROS can lead to apoptosis and necrosis [22, 26, 27]. Under normal circumstances, p66Shc is inactivated and does not affect mitochondrial function. In tumor cells, p66shc is a key regulator of ROS production through activation of cytochrome c. A certain amount of ROS can activate tumor cell proliferation [10]. Our study showed that p66Shc expression is low to moderate in 78.1 % of gastric cancers and 79.4 % of colorectal cancers. p66Shc expression may help maintain a level of ROS required for gastric cancer and colorectal cancer carcinogenesis, but not apoptosis.

p66Shc is weakly or not expressed in 93.4 % of normal gastric tissues and 84.5 % of gastric hyperplastic polyps. In contrast, p66Shc is moderately to highly expressed in 78.1 % of gastric cancers. Similarly, p66Shc is weakly or not expressed in 93.4 % of normal colorectal tissues and 85.7 % of colorectal hyperplastic polyps, but p66Shc is moderately to highly expressed in 80.9 % of colorectal cancers. This indicates that p66Shc may serve as a potential tumor marker for the diagnosis of gastric and colorectal cancers. However, p66Shc expression is not associated with the clinicopathological characteristics of gastric and colorectal cancers; therefore, it may not be an ideal predictive marker for disease progression. Although p66Shc is involved in p53-mediated cell apoptosis, elevated p66Shc expression is observed in both gastric and colorectal cancers. More well-controlled studies are needed to determine whether p66Shc could be a biomarker for the target therapy of gastric and colorectal cancers. Overall, our study is the first to examine p66Shc expression in gastric cancers and its possible roles in carcinogenesis. We also confirmed previous findings of elevated p66Shc expression in colorectal cancers, but was first to comparatively analyze its expression in benign, premalignant, and malignant colorectal tissues.

Conflict of Interest All authors declared no conflict of interest.

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