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• GASTRIC CANCER •

Expression of NF-kB and human telomerase reverse transcriptase in gastric cancer and precancerous lesions

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Abstract

AIM: To investigate the expression of NF-κBp65 protein and human telomerase reverse transcriptase (hTERT) and their correlation in gastric cancer and precancerous lesions.

METHODS: Forty-one patients with primary gastric cancer, 15 with dysplasia, 23 intestinal metaplasia and 10 with normal gastric mucosa were included in this study. Expression of NF-κBp65 protein, hTERT mRNA and protein were determined by immunohistochemistry and *in situ* hybridization.

RESULTS: The rate of p65 expression in normal gastric mucosa, intestinal metaplasia, dysplasia and carcinoma was 0%, 34.78%, 53.33% and 60.98%, respectively, while the rate of hTERT mRNA expression was 10.00%, 39.13%, 66.67% and 85.37% and the rate of hTERT protein expression was 0%, 30.43%, 60.00% and 78.05%, respectively. All the three parameters were significantly increased in dysplasia and carcinoma compared to normal mucosa, while the expression levels were also significantly higher in carcinoma than in intestinal metaplasia ($P<0.05$). In gastric cancer tissues, nuclear staining rates of p65 and hTERT protein were both significantly associated with the degree of differentiation, lymph node metastasis, clinical stage and invasion depth ($P<0.05$). However, hTERT mRNA expression was only significantly associated with clinical stage. There was a positive correlation between p65 and hTERT mRNA ($r_s=0.661$-0.752, $P<0.01$), and between hTERT protein and hTERT mRNA ($r_s=0.609$-0.750, $P<0.01$).

CONCLUSION: NF-κBp65 and hTERT expressions are upregulated at the early stage of gastric carcinogenesis. NF-κB activation may contribute to hTERT expression and thereby enhance telomerase activity, which represents an important step in carcinogenesis progress.

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INTRODUCTION

NF-κB is a family of dimeric transcription factors that play a critical role in host defense by regulating the expression of immune and inflammatory genes[1-3]. The most common dimer is RelA (p65)/NF-κB1 (p50) heterodimer. In resting cells, NF-κB is localized in the cytoplasm, which is noncovalently associated with the cytoplasmic inhibitory protein IκB. Upon stimulation with a variety of pathogenic inducers such as viruses, mitogens, bacteria, agents providing oxygen radicals, and inflammatory cytokines, IκB is phosphorylated, ubiquitinated, and degraded in the cytoplasm, and the NF-κB complex migrates into the nucleus and binds to DNA recognition sites in the regulatory regions of target genes[4]. Recently, there were several reports on the role of NF-κB gene products in cell proliferation, transformation, and tumor development[5-9]. Recent studies have also indicated that NF-κB is constitutively activated in several tumors[10-13]. However, the biological significance of NF-κB activation remains unclear in gastric carcinogenesis although gastric cancer is one of the most aggressive forms of cancer.

Telomerase is a key enzyme that catalyzes the synthesis of telomere DNA participating in cell immortalization through stabilization of chromosomal structure[14]. Telomerase is expressed in germ tissues as well as in majority of human tumors, including gastrointestinal carcinomas, but is low and difficult to detect in somatic cells generally[15-19]. So it may be a useful molecular marker for cancer diagnosis and therapeutic strategies. Human telomerase reverse transcriptase (hTERT) has been identified as a putative catalytic subunit of human telomerase[20]. Recent reconstitution experiments, both *in vitro* and *in vivo*, also strongly suggest that hTERT is the major determinant of human telomerase activity[21,22]. Overexpression of hTERT in cancer cells is thought to contribute to tumor development and angiogenesis[23-25]. However, the mechanism by which hTERT is overexpressed in cancer cells remains unclear. There are evidences that hTERT expression may be regulated by the highly inducible NF-κB transcription factor[26]. So it is interesting to investigate the relation between NF-κB and hTERT at cellular level.

We undertook the present study to determine whether NF-κB was constitutively activated in precancerous and cancerous tissues of the stomach, to examine whether expression of hTERT gene correlated with NF-κB activation, and to evaluate the relationship between clinicopathological features and NF-κB activation as well as hTERT gene expression.

MATERIALS AND METHODS

Tissue samples

Gastric tissues were obtained by surgical resection from 89 patients: 10 with normal gastric mucosa, 23 with intestinal metaplasia, 15 with dysplasia and 41 with gastric cancer. Patients with gastric cancer were admitted to Renmin Hospital of Wuhan University from February to December in 2000, and had not been treated with chemotherapy or radiation therapy before operation. Histological examinations were performed, according to the criteria of the Japanese Gastric Cancer Association[27]. All specimens were fixed in 10 % buffered neutral formalin and embedded in paraffin. Serial tissue sections (4.5 μm thick) were placed on glass slides coated with 3-aminopropyltriethoxysilane (Sigma, USA), and then used in the following experiments.

Immunohistochemistry for NF-κB p65 and hTERT proteins

Immunostaining was performed as described previously with slight modification[28]. Briefly, slides were treated overnight at 4 ℃ with either anti-p65 mAb (4 μg/ml) or anti-hTERT polyclonal antibody (3 μg/ml). Both antibodies were from Santa Cruz Biotechnology (USA). Then, slides were incubated with secondary antibody by using a streptavidin-peroxidase kit (Maixin-Bio, Fujian). Finally, the antigen sites were visualized by incubating with diaminobendizine (DAB) solution (Zhongshan Biotechnical Co, Beijing), and the nuclei were weakly counterstained with Mayer' s hematoxylin (Maixin-Bio, Fujian). The specificity of immunostaining was determined by replacement of the primary antibody with PBS. Positive reaction was detected as nuclear stain presenting in brown-yellow color. Slides positively stained were further stratified from 1+ to 3+ based on the overall intensity and percentage of the stained tumor cells, with an estimated scale of <25% cell positive =1+, 25% to 50% =2+, and >50% =3+. Staining was defined as negative if positive cells were <5%.

In situ hybridization for hTERT mRNA

hTERT mRNA ISH detection kit and antisense polyoligonucleotide probe (digoxin-labeled) were purchased from Boster Biological Technology Ltd. (Wuhan). In brief, deparaffinized sections were incubated with 3% hydrogen peroxide for 30 min and then with 1 μg/ml pepsin for 15 min. The prehybridization was performed at 37 ℃ for 2 h, and the hybridization was conducted in a 42 ℃ water bath for 18 h with each section covered with a soil coverslip. After thorough washing, tissue sections were preblocked for 20 min with blocking solution. Then, rabbit anti-digoxin antibody was added for 60 min at 37 ℃. After washed in PBS, the sections were visualized according to the manufacturer' s instructions. A negative control was prepared by using a hybridization solution without the probe. A positive reaction was detected as plasmatic stain presenting in brown-yellow color. Slides positively stained were further stratified from 1+ to 3+ as described above.

Statistics

Statistical analysis was performed using chi-squared test, Fisher' s exact test, and spearman rank test. A P value <0.05 (one-sided) was accepted as statistically significant.

RESULTS

Staining of NF-κBp65 protein, hTERT mRNA and protein

P65 immunostaining was significantly enhanced both in cytoplasm and in nuclei of the tumor cells in comparison to that in normal epithelial cells (Figure 1). Since nuclear staining, which indicated nuclear transportation of p65, was considered as a marker of NF-κB activation, we only counted the number of nuclear stained cells and took them into calculation of the percentage of positively stained cells. Similarly, hTERT protein was mainly localized in the nuclei of tumor cells, and only a small number of cells showed a positive reaction in the cytoplasm (Figure 2). Stromal cells such as endothelial cells and smooth muscle cells (except lymphocytes) showed no reaction for hTERT. Weak staining of p65 and hTERT proteins was observed in some epithelial cells in the lower two-thirds of the glands of nonneoplastic mucosa.

In situ hybridization

Revealed that hTERT mRNA was significantly enhanced in the cytoplasm of tumor cells (Figure 3). Only a few signals were seen in nonneoplastic cells, which were slightly increased in the replicating basal layer, and in intestinal metaplastic cells as well as in activated lymphocytes, while the surface epithelia were negative (Figure 4).

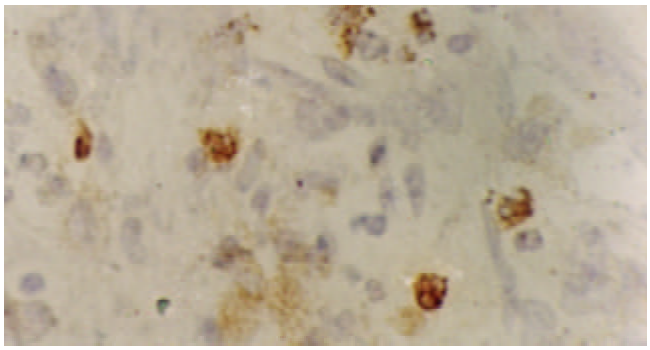

Figure 1 Immunohistochemical detection of NF-κBp65 protein in gastric carcinoma showing cytoplasmic and nuclear staining SP×400.

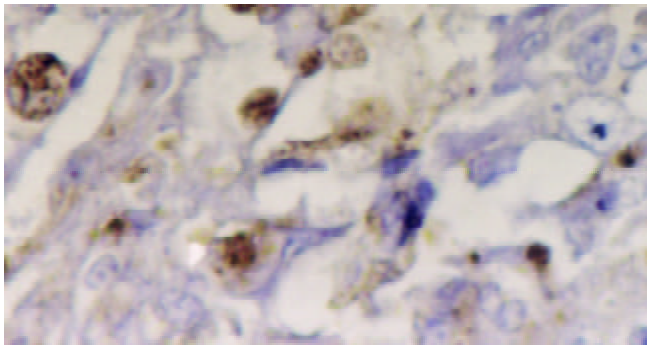

Figure 2 Immunohistochemical detection of hTERT protein in poorly differentiated gastric carcinoma. A positive reaction was shown in the nuclei of cancer cells. SP×400.

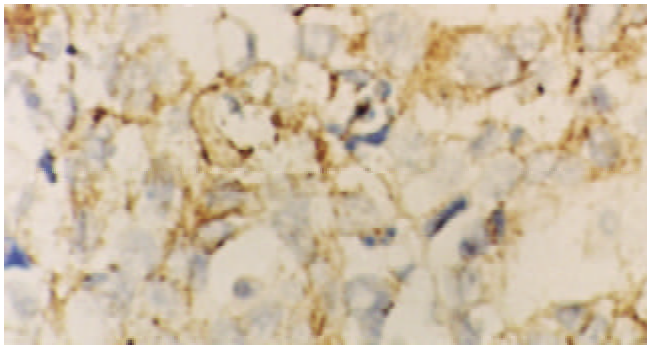

Figure 3 Detection of hTERT mRNA in a mucinous adenocarcinoma. Most tumor cells displayed strong signals that were localized in the cytoplasm. ISH×400.

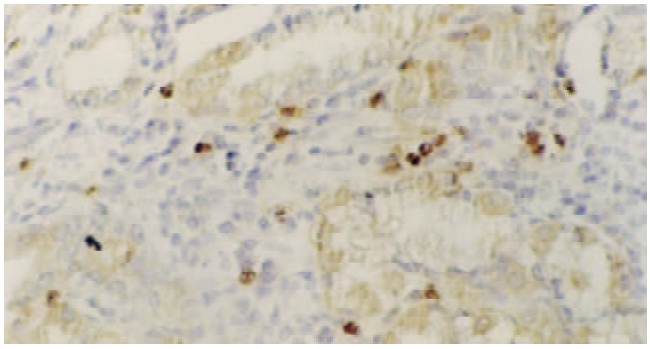

Figure 4 Detection of hTERT mRNA in intestinal metaplasia. The reaction was localized in the cytoplasm of intestinal metaplastic cells, activated lymphocytes, as well as replicating cells in basal layer, while the surface epithelia were negative. ISH×100.

Table 1 Expression of NF-κBp65 protein, hTERT mRNA and protein in precancerous lesions and gastric cancer

Lesion	Cases	NF-κBp65				hTERT mRNA				hTERT protein			
		+	++	+++	Total (%)	+	++	+++	Total (%)	+	++	+++	Total (%)
Normal	10	0	0	0	0 (0)	1	0	0	1 (10.00)	0	0	0	0 (0)
Intestinal metaplasia	23	7	1	0	8 (34.78)[b]	6	3	0	9 (39.13)[b]	5	2	0	7 (30.43)[b]
Dysplasia	15	7	1	0	8 (53.33)[a]	7	3	0	10 (66.67)[a]	7	2	0	9 (60.00)[a]
Cancer	41	7	14	4	25 (60.98)[a]	6	20	9	35 (85.37)[a]	6	20	6	32 (78.05)[a]

[a]P<0.05, *vs* normal mucosa group; [b]P<0.01, *vs* cancer group.

Expression of NF-κBp65 protein, hTERT mRNA and protein

Expression of p65 protein, hTERT mRNA and protein in gastric precancerous and cancerous tissues was increased compared with normal mucosa (Table 1). The nuclear staining rates of p65 (active form) were higher in gastric cancer than in intestinal metaplasia (P<0.05), or normal mucosa (P<0.05). The rates were also significantly higher in dysplasia than in normal mucosa. Similarly, expression of hTERT mRNA and protein was increased in precancerous and cancerous tissues (Table 1).

The relations between clinicopathological features and expression of p65 and hTERT in the 41 patients with gastric carcinoma were further analyzed (Table 2). Nuclear staining rates of p65 and hTERT proteins were both significantly associated with the degree of differentiation, lymph node metastasis, clinical stage and the depth of invasion (P<0.01-0.05). Furthermore, the nuclear staining of p65 was significantly higher in tumors ⩾5 cm than in those <5 cm. With respect to expression of hTERT mRNA, we found that carcinomas at advanced stage showed a significantly higher level than those at early stage. However, there was no significant association between expression of hTERT mRNA and other clinicopathological features.

Table 2 Correlation between expression of NF-κBp65 protein, hTERT mRNA and protein and clinicopathological features

Factor	Cases	NF-κB p65 (%)	hTERT mRNA (%)	hTERT protein (%)
Gender				
Male	30	16 (53.33)	24 (80.00)	24 (80.00)
Female	11	9 (81.82)	11 (100.00)	8 (72.73)
Age (years)				
<60	24	14 (58.33)	21 (87.50)	20 (83.33)
⩾60	17	11 (64.71)	14 (82.35)	12 (70.59)
Histology (type)				
Intestinal	16	7 (43.75)	12 (75.00)	10 (62.50)
Diffuse	25	18 (72.00)	23 (92.00)	22 (88.00)
Differentiation				
Well	13	3 (23.08)[b]	9 (69.23)	7 (53.85)[a]
Poor	28	22 (78.57)	26 (92.86)	25 (89.29)
Lymph node metastasis				
Negative	16	4 (25.00)[b]	12 (75.00)	9 (56.25)[a]
Positive	25	21 (84.00)	23 (92.00)	23 (92.00)
Stage				
Early	12	2 (16.67)[b]	7 (58.33)[b]	5 (41.67)[b]
Advanced	29	23 (79.31)	28 (96.55)	27 (93.10)
Tumor size (cm)				
<5	19	6 (31.58)[b]	14 (73.68)	13 (68.42)
⩾5	22	19 (86.36)	21 (95.45)	19 (86.36)
Depth of invasion				
m, sm	10	2 (20.00)[a]	6 (60.00)	4 (40.00)[a]
ms, ss	17	11 (64.71)	16 (94.12)	15 (88.24)
se, si	14	12 (85.71)	13 (92.86)	13 (92.86)

[a]P<0.05, [b]P<0.01, From comparison in each group of clinicopathologic feature by chi-square test or Fisher's exact test. m: mucosa, sm: submucosa, mp: muscularis propria, ss: subserosa, se: invasion to serosa, si: invasion to other organ. Tumor size was defined as the largest size in extension on the gastric mucosa.

Correlation between NF-κB activation and expression of hTERT

There was a positive correlation between NF-κB activation and hTERT mRNA expression in patients with intestinal metaplasia (r_s=0.665), dysplasia (r_s=0.661) and gastric cancer (r_s=0.752). Similarly, hTERT mRNA expression was also positively correlated with hTERT protein in these three groups (P<0.05), with the r_s values of 0.609, 0.750 and 0.730, respectively.

DISCUSSION

The present study reported the detection of NF-κB activation and hTERT gene expression, as well as their correlation, in cancerous and precancerous tissues of stomach.

NF-κB, an important transcription factor, consists of dimeric complexes. We used immunohistochemistry to detect NF-κB activation. The polyclonal antibody we used, could bind to the active and inactive forms of p65, one of NF-κB subunits, as shown in a previous study[29]. We only quantified the nuclear staining (active form) to evaluate NF-κB activation. With such an analysis, nuclear translocation of p65 could be estimated at single-cell level. We found that active NF-κB was not only in the nuclei of tumor cells but also in the nuclei of infiltrating inflammatory cells, especially lymphocytes, which was accordant with the role of NF-κB involved in immune and inflammatory responses[30,31]. Isomoto *et al*[32] also reported that in *Helicobacter pylori*-associated gastritis, activated NF-κB was expressed in macrophages, vascular endothelial cells and B lymphocytes in addition to epithelial cells, which might be involved in the inflammation process.

Using *in situ* hybridization and immunohistochemistry methods, we detected the expression of hTERT at both RNA and protein levels. Importantly, the *in situ* detection enabled us to differentiate whether telomerase activity was due to proliferative normal cells or lymphocytes. Therefore immunohistochemical detection of hTERT might be a novel tool for the diagnosis of gastric cancer[33]. In this study, we demonstrated that, besides tumor cells, hTERT mRNA and protein were also expressed, albeit weakly in normal gastric fundic mucosa. These epithelial cells were terminally differentiated. It is possible that low levels of hTERT expression might be the characteristics of physically regenerating tissues containing stem cells, and hTERT-positive cells might be competent for regeneration if severe mucosal damage occurred[28]. Although the level of hTERT mRNA correlated significantly with that of hTERT protein in our study, their expression levels were not always coincident in precancerous and cancerous lesions. It is likely that post-translational modifications, such as phosphorylation by akt kinase, might be involved in hTERT expression[28,34].

The present study revealed that NF-κBp65 nuclear staining rates were higher in cancer tissues, followed by dysplasia,

intestinal metaplasia and normal mucosa. This is the first report on NF-κBp65 detection in gastric precancerous tissues. As to the expression of hTERT gene, previous studies revealed that carcinomas could express hTERT more frequently than noncancerous tissues[25,35-38]. Our findings support these results. The present study indicated that NF-κB and telomerase were both activated in precancerous lesions, suggesting that NF-κB activation and increased hTERT gene expression emerge at the early stage of gastric carcinogenesis, and that they may be the prerequisites for malignant transformation. Therefore, NF-κB and telomerase might be potentially important targets for the development of anti-tumor therapies for gastric carcinoma[15,39,40].

Sasaki *et al*[29] reported that NF-κB activation was positively correlated with tumor size, lymphatic invasion, depth of invasion and peritoneal metastasis. Besides that, our study also revealed significantly positive correlations between nuclear staining of NF-κB p65 and differentiation degree and clinical stage. Therefore, NF-κB activation might be associated with tumor growth, invasion and metastasis.

It has been reported that hTERT expression might be downstream of NF-κB activation[26,41]. Yin *et al*[26] found a potential NF-κB binding site at 350 base pairs upstream from the translational start site of mouse TERT promoters and NF-κB was found to contribute to the activation of TERT expression. In addition, Akiyama *et al*[41] showed that NF-κB interacted directly with hTERT protein in multiple myeloma cells. In the present study we examined the relationship between nuclear staining of NF-κBp65 and hTERT mRNA at the cellular level by immunohistochemistry and *in situ* hybridization. We observed that both p65 and hTERT mRNA were overexpressed in gastric cancer with the positive staining rates of 60.98% and 85.37%, respectively. In fact, most specimens showing a high p65 level also showed high hTERT mRNA expression and *vice versa*. Furthermore, the expression of p65 and hTERT mRNA showed a significant correlation in cancerous and precancerous tissues (intestinal metaplasia and dysplasia). And in nonneoplastic mucosa, the positive staining of p65 and hTERT mRNA were detected simultaneously in proliferative glands and activated lymphocytes. All of these suggested that NF-κBp65 cooperated with hTERT in gastric carcinogenesis. We speculate that NF-κBp65 may induce hTERT promoter activity and upregulate telomerase activity and contribute to the aggressiveness of gastric carcinoma.

However, our study was limited by the high specificity of p65 mAb for the p65 subunit, and thus we could not evaluate other NF-κB dimers. The relationship between other NF-κB subunits and gastric cancer needs further studies.

In conclusion, we demonstrated the presence of activated NF-κBp65 and increased hTERT expression in gastric cancerous and precancerous tissues. The levels of p65 and hTERT expression were higher in gastric cancer than in normal mucosa, and NF-κB activation was associated with tumor growth, invasion and metastasis. Our findings suggest that NF-κBp65 cooperates with hTERT in gastric carcinogenesis.

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