Increased Phosphorylation of AKT in High-risk Gastric Mucosa

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Abstract. *Aim: To establish the role of oxidative stress and v-akt murine thymoma viral oncogene homolog (AKT) activation in gastric cancer development, we examined the levels of phosphorylated AKT (pAKT), inducible nitric oxide synthase (iNOS), nitrotyrosine (NT), and human telomerase reverse transcriptase (hTERT) by enzyme-linked immunosorbent assay in 73 non-cancerous gastric mucosa and 10 gastric carcinomas. We found that the levels of pAKT were associated with the levels of iNOS, NT, and hTERT. Gastric mucosa was classified into four categories: chronic gastritis without Helicobacter pylori (CG), chronic active gastritis with H. pylori (CAG), chronic metaplastic gastritis without H. pylori (CMG), and chronic gastritis with atypia without H. pylori (CGA). We found increasing levels of pAKT, iNOS, and NT in the order of CG, CAG, CMG, and CGA. hTERT was detected only in CGA. These findings suggest that oxidative stress might be associated with AKT activation and hTERT induction and that mucosa in CGA might confer a high-risk status for gastric carcinogenesis.*

v-Akt murine thymoma viral oncogene homolog (AKT) is a pivotal regulator of cell survival, proliferation, and differentiation. It is a member of the phosphatidylinositol-3 kinase (PI3K) signaling pathway. Stimulation of receptor tyrosine kinases or G-proteins activates PI3K, which in turn activates AKT. AKT phosphorylation is maintained by heat-shock protein-90, and AKT is dephosphorylated by protein phosphatase-2A. AKT regulates signaling *via* various growth factors and cytokines. In particular, activation of insulin-like growth factor-1 receptor, epidermal growth factor receptor, and human epidermal growth factor receptor-2, which are important in cancer progression, activate AKT (3, 15). AKT is also a biomarker that predicts metastasis of human gastrointestinal cancer (11).

Phosphorylation of AKT modulates signals by phosphatase, tensin homolog deleted on chromosome 10 (PTEN), and the mammalian target of rapamycin (mTOR), resulting in diverse effects on cells (4). In this regard, AKT1 is recognized as an apoptotic inhibitor, which contributes to cancer progression. Phosphorylation *via* AKT inactivates B-cell lymphoma-2 (BCL-2) antagonist of cell death resulting in its dissociation from BCL-2. In addition, AKT activates nuclear factor (NF)-κB, which in turn up-regulates transcription of many survival genes (6). AKT also promotes angiogenesis through up-regulation of vascular endothelial growth factor (12). The activation and expression of AKT promote tumorigenesis, thus AKT represents a relevant molecular target for cancer treatment (3).

In the present study, alteration of AKT phosphorylation in gastric mucosa and the role of oxidative stress in AKT phosphorylation were examined for the evaluation of high-risk gastric mucosa.

Materials and Methods

Cell culture and reagents. The human gastric cancer cell line MKN28 (kindly donated by Professor Wataru Yasui, Hiroshima University, Japan) was maintained in Dulbecco's modified essential medium (Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal bovine serum (Sigma Chemical Co.) at 5% CO_2 in air and 37˚C.

Clinical materials. Eighty-three biopsied gastric tissue samples obtained from patients histologically-diagnosed at the Department of Molecular Pathology, Nara Medical University, were examined. One half of each tissue specimen was used for pathological diagnosis and the other half was used for enzyme-linked immunosorbent assay (ELISA). The tissues had been frozen quickly in liquid nitrogen and stored at –80˚C. Seventy-three of the tissue samples were obtained from non-cancerous cases were classified into four categories: [i] Chronic gastritis without *Helicobacter pylori* (CG); [ii] chronic active gastritis with *H. pylori* (CAG): chronic gastritis with regenerative epithelial change and polymorphonuclear cell infiltration in the mucosa; [iii] chronic metaplastic gastritis without *H. pylori* (CMG):

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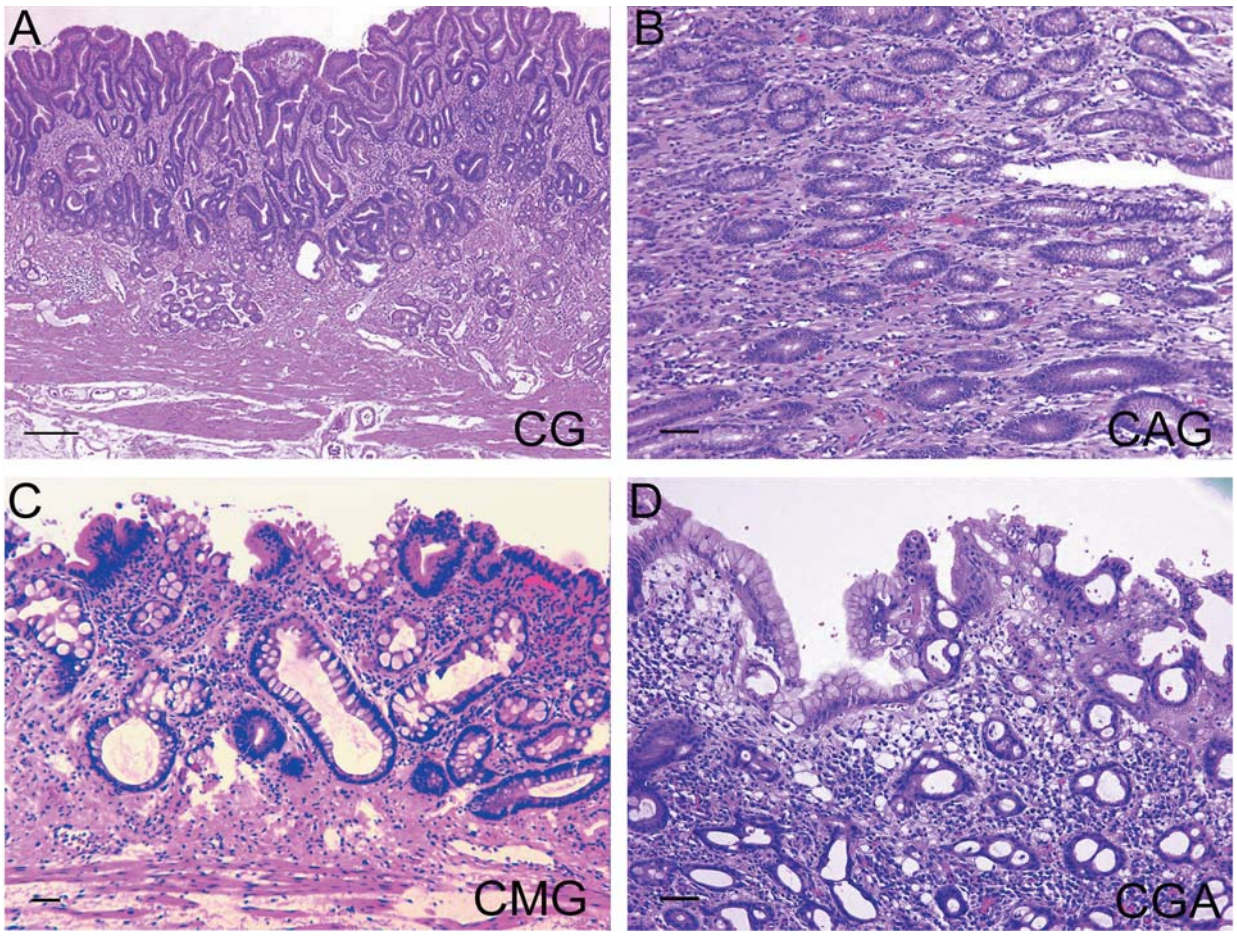


Figure 1. *Histopathological findings of the gastric mucosa (hematoxylin and eosin staining). A: Chronic gastric mucosa without Helicobacter pylori infection (CG). B: Chronic active gastritis with H. pylori infection (CAG). C: Chronic metaplastic gastritis without H. pylori (CMG). D: Chronic gastritis with atypia (CGA). Bar=100 μm.*

atrophic mucosa with diffuse intestinal metaplasia; and [iv] chronic gastritis with atypia without *H. pylori* (CGA): chronic gastritis with regenerative change and nuclear swelling, chromatin increment, or nucleoli formation (nuclear atypia). Ten of the tissues were adenocarcinomas diagnosed as papillary adenocarcinoma (n=3) or well-differentiated tubular adenocarcinoma (n=7). The diagnosis was performed by two pathologists (H.K. and H.O.).

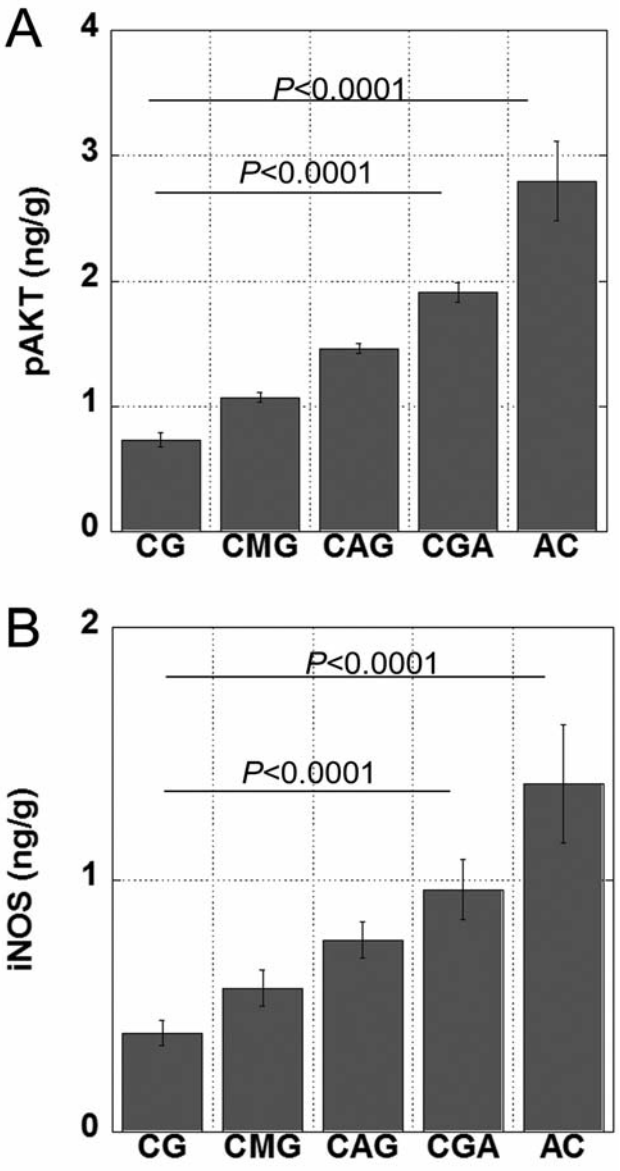
ELISA. The levels of pAKT, TERT, nitrotyrosine (NT), and inducible nitric oxide synthase (iNOS) were determined using specific ELISA kits according to the manufacturer instructions: AKT (pS473) ELISA kit (Abcam, Cambridge, UK), human telomerase reverse transcriptase (HTERT) ELISA kit (Oxford Expression Technology, Oxford, UK) (14), ELISA for nitrotyrosine (Hycult Biotechnology BV, Uden, the Netherlands) (5) and CytoGLOW iNOS Colorimetric Cell-Based ELISA kit (Assay Biotechnology Co. Inc., Sunnyvale, CA), respectively. Whole-tissue lysates were extracted from liquid nitrogen-frozen tumor materials using a lysis buffer (9). ELISA was performed in triplicate.

Nitrite. Sodium nitroprusside (SNP) was used as a NO donor using concentrations designated in the results section. NO concentration was detected as a nitrite concentration, which was measured using the Griess method. Cultured medium was mixed with an equal amount of Griess solution (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid), and OD540 was measured 10 minutes after incubation. Nitrite concentration was determined on the basis of a standard curve generated using different concentrations of sodium nitrite.

Statistical analysis. Statistical analyses of experimental data were performed using Spearman's r and analysis of variance (InStat; Graphpad Software Inc., La Jolla, CA, USA). Statistical significance was defined as a two-sided *p*-value of less than 0.05.

Results

Histological classification of gastric mucosa. In the present study, the gastric mucosa status was classified into four categories; CG, CAG, CMG, and CGA (Figure 1). On the basis of the *H. pylori* infection status, temporal alteration of gastric mucosa is reflected in CG, CAG, and CMG.



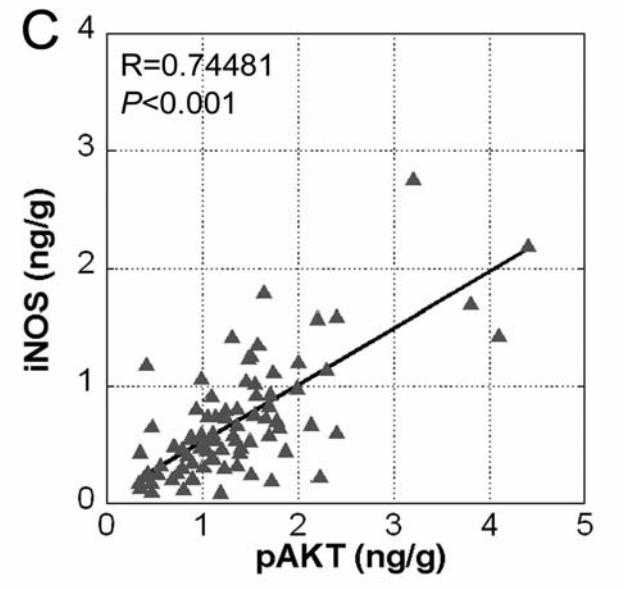


Figure 2. *Phosphorylated v-akt murine thymoma viral oncogene homolog (AKT) level and the association with inducible nitric oxide synthase (iNOS) expression. The level of pAKT (A) and iNOS (B) were examined by enzyme-linked immunosorbent assay. The relationship between pAKT and iNOS was compared by Spearman's correlation analysis (C). Error bar=SE. CG: Chronic gastric mucosa without Helicobacter pylori infection, CAG: chronic active gastritis with H. pylori infection, CMG: chronic metaplastic gastritis without H. pylori, CGA: chronic gastritis with atypia. Levels of pAKT and iNOS increased from CG to CMG to CAG to CGA (A, B). Levels of pAKT and iNOS were significantly correlated (C).*

Levels of pAKT and iNOS in gastric mucosa. As shown in Figure 2A, the level of pAKT gradually increased in the order of CG, CMA, CAG, and CGA. The level of pAKT in CGA was 2.6-times higher than in samples classified as CG.

iNOS is responsible for the production of NO and mediates oxidative stress in the gastric mucosa. Similarly to pAKT, the level of iNOS increased in the order of CG, CMG, CAG and CGA (Figure 2B). Because NO has been shown to up-regulate the level of pAKT, we examined the relationship between pAKT and iNOS. As shown in Figure 2C, we found a significant correlation ($p<0.001$) between the levels of pAKT and iNOS.

Levels of pAKT and NT in gastric mucosa. The level of NT increased in the order of CG, CMG, CAG and CGA (Figure 3A) and level were correlated with the level of pAKT (Figure 3B).

Levels of pAKT and hTERT in gastric mucosa. We have already reported that AKT activation is associated with hTERT activation. Among the four mucosal categories, only samples of CGA exhibited hTERT expression, albeit at lower levels than those of cancer cases (Figure 4A). The levels of hTERT were correlated with the levels of pAKT in the analysis of all patients (Figure 4B).

Nitric oxide induces AKT phosphorylation. Data obtained from clinical samples suggested that NO induces AKT phosphorylation. Therefore, we examined the effect of NO on AKT phosphorylation in gastric epithelial cells. Primary cultured gastric mucosal cells were treated with different concentrations of SNP, a NO donor, and the levels of AKT and pAKT were examined. As shown in Figure 5, the levels of pAKT were significantly increased by SNP treatment in CGA samples and in MKN28 cancer cells but not in CG, CAG, or CMG.

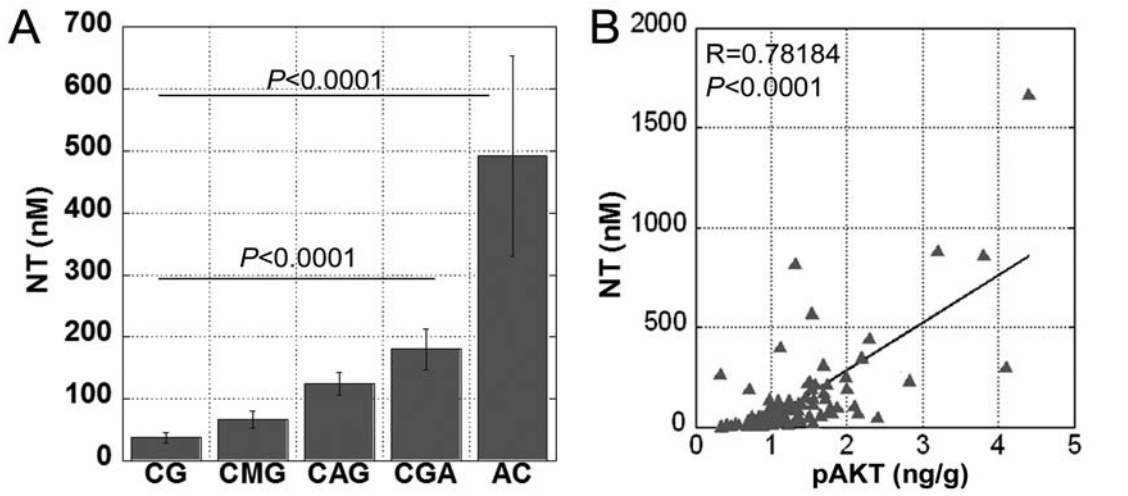


Figure 3. *Nitrotyrosine (NT) level and association with phosphorylated v-akt murine thymoma viral oncogene homolog (AKT) expression. The level of NT was examined by enzyme-linked immunosorbent assay (A). The relationship between NT and pAKT was compared using Spearman correlation analysis (B). Error bar=SE. CG: Chronic gastric mucosa without Helicobacter pylori infection, CAG: chronic active gastritis with H. pylori infection, CMG: chronic metaplastic gastritis without H. pylori, CGA: chronic gastritis with atypia. The levels of NT increased from CG to CMG to CAG to CGA (A) and were correlated with the levels of pAKT (B).*

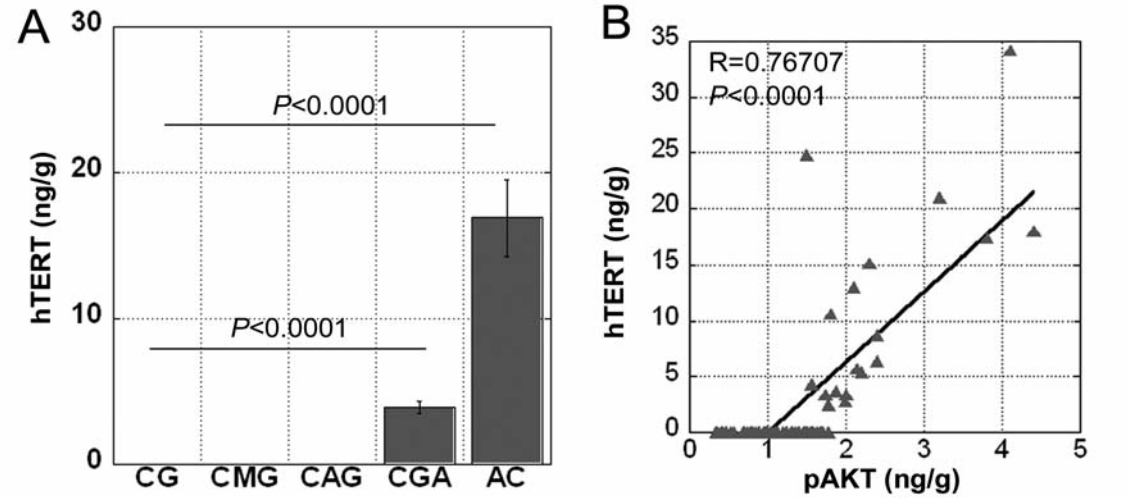


Figure 4. *human telomerase reverse transcriptase (hTERT) level and the association with phosphorylated v-akt murine thymoma viral oncogene homolog (AKT) expression. The level of hTERT was examined by enzyme-linked immunosorbent assay (A). The relationship between hTERT and pAKT was compared using Spearman correlation analysis (B). Error bar=SE. CG: Chronic gastric mucosa without Helicobacter pylori infection, CAG: chronic active gastritis with H. pylori infection, CMG: chronic metaplastic gastritis without H. pylori, CGA: chronic gastritis with atypia. Among the 4 mucosal categories, only CGA samples showed hTERT expression (A). Levels of hTERT were correlated with the levels of pAKT (B).*

Discussion

Our data showed that AKT phosphorylation was associated with the expressions of iNOS and hTERT and with increased level of NT. The levels of pAKT, iNOS, NT, and hTERT were increased in CGA, which is considered to be a high-risk status for gastric carcinogenesis.

In gastric mucosal pathology, *H. pylori* plays a pivotal role, because it induces chronic inflammation and increases the production of reactive oxygen species. *H. pylori* stimulates proliferation of gastric mucosal cells *via* type-IV secretion of CagA followed by its phosphorylation by Schmidt-Ruppin A-2 avian sarcoma viral oncogene (src) homology 2 domain-containing protein tyrosine phosphatase-

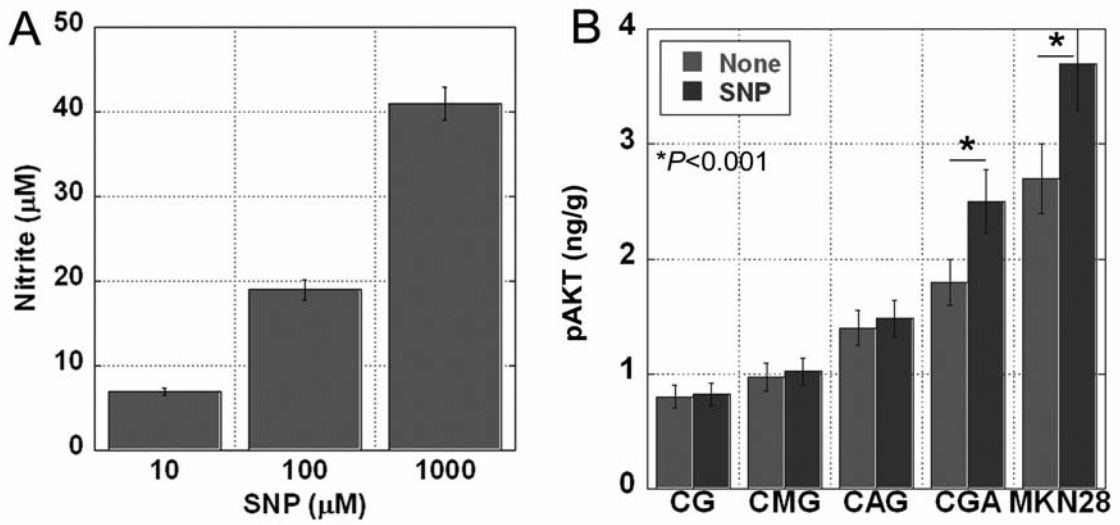


Figure 5. *Phosphorylation of v-akt murine thymoma viral oncogene homolog (AKT) by nitric oxide (NO) in gastric mucosa. A: The levels of nitrite generated by the treatment of cells with the NO donor sodium nitroprusside (SNP). B: Fresh-tissue biopsies (n=3 in each category) were incubated with SNP (100 μM) and the level of pAKT was determined by enzyme-linked immunosorbent assay. MKN28 cells are transformed gastric cells. Error bar, SD. CG: Chronic gastric mucosa without Helicobacter pylori infection, CAG: chronic active gastritis with H. pylori infection, CMG: chronic metaplastic gastritis without H. pylori, CGA: chronic gastritis with atypia. Levels of pAKT were increased by SNP treatment in CGA samples and in MKN28 cancer cells but not in CG, CAG, or CMG.*

2 (SHP2) (7, 8). CagA activates SHP2 phosphatase, which inhibits signal transducers and activators of transcription (STAT)-mediated growth-suppressive signal and activates extracellular signal-regulated kinase (ERK)-mediated growth signal (16). The increased growth activity might enhance the risk of gene alteration.

We classified the status of gastric mucosa according to inflammation and immortalization by hTERT expression. Since AKT is a key protein that links inflammation and tumorigenesis, it is the focus of our study. iNOS is a significant mediator of inflammation in the gastric mucosa. Increased expression of iNOS is epigenetically induced by *H. pylori* infection as a host defense (1). NT is a marker of NO-induced protein degradation. We examined the expression of iNOS and NT to evaluate inflammation and the expression of hTERT as a marker of immortality. Our data showed that the levels of pAKT were associated with hTERT expression in CGA. Our previous study showed that pAKT levels were associated with hTERT expression in gastric adenocarcinomas (14). These findings suggest that the immortality of gastric mucosal cells maybe associated with inflammation-induced AKT activation.

Our results demonstrated that the levels of iNOS and NT were associated with pAKT (13). pAKT, iNOS, and NT were higher in CAG, CMG, and CGA. Interestingly, high pAKT level in CAG associated with *H. pylori*-induced active inflammation. In contrast, increased pAKT in CMG suggests that persistent iNOS up-regulation develops during the metaplastic process. The highest pAKT level in CGA might develop during carcinogenic processes (10). *In vitro* analysis showed that only CGA and MKN28 cancer cells exhibited up-regulation of pAKT in response to short exposure to NO. In Barrett esophagus, activation of AKT is associated with the dysplasia–carcinoma sequence (2). Moreover, only CGA mucosa expressed hTERT. These findings suggest that CGA is likely to be a distinct category in the process of gastric carcinogenesis.

Our data suggest that CGA should be viewed as high-risk mucosa for gastric cancer and that patients with CGA should be evaluated by further endoscopic and histopathological methods.

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