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AKT PLAYS A CRUCIAL ROLE IN GASTRIC CANCER

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Abbreviations : PI3K: phosphatidylinositol-3 kinase, VEGF: vascular endothelial growth factor, pAKT: phosphorylated AKT, *iNOS*: inducible nitric oxide synthase, PTEN: tensin homolog deleted on chromosome 10, NT: nitrotyrosine, *hTERT*: human telomerase reverse transcriptase, CG: chronic gastritis without *H. pylori*, CAG: chronic active gastritis with *H. pylori*, CMG: chronic metaplastic gastritis without *H. pylori*, CGA: chronic gastritis with atypia without *H. pylori*, SHP2: src homology 2 domain-containing protein tyrosine phosphatase-2, *hTR*: human telomerase RNA, EMT: epithelial-mesenchymal transition, NO: nitric oxide, ROS: reactive oxide species

Abstract : The AKT protein is involved in the phosphatidylinositol-3 kinase signaling pathway and is a vital regulator of survival, proliferation, and differentiation of various types of cells. *Helicobacter pylori* induce epithelial cell proliferation and oxidative stress in chronic gastritis. These alterations lead to telomere shortening and resultant telomerase activation. Specifically, AKT is activated by *H. pylori-induced* inflammation; it subsequently promotes expression of human telomerase reverse transcriptase, which encodes a catalytic subunit of telomerase; and induces telomerase activity, which is an essential process of carcinogenesis. AKT activation is increased in gastric mucosa with carcinogenic properties and is associated with low survival of patients with gastric cancer. These findings suggest that AKT is pivotal in gastric carcinogenesis and progression.

Key words : Helicobacter pylori, telomerase, *hTERT*, chronic gastritis

Introduction

Gastric cancer is the fourth most common cancer in the world [1]. Recent advances in molecular analyses of preneoplastic and neoplastic lesions of the stomach have uncovered a large number of epigenetic and genetic alterations that determine a multistep process of stomach carcinogenesis [2]. *Helicobacter pylori* infection is inherently responsible for the pathogenesis of intestinal metaplasia, and its epidemiological contribution to gastric cancer is similar in European and Japanese populations [3]. In 1994, the International Agency for Research on Cancer recognized *H. pylori* as a definite carcinogen of gastric cancer based on a strong

epidemiological correlation between chronic colonization of *H. pylori* and gastric cancer [4]. Moreover, a Mongolian gerbil model showed that *H. pylori* inoculation into the stomach is closely associated with the occurrence of chronic gastritis, intestinal metastasis, and promotion of adenocarcinoma [5].

AKT is a pivotal regulator of cell survival, proliferation, and differentiation and 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 catalyzed by heat shock protein 90, and its dephosphorylation is mediated via protein phosphatase 2A. Subsequently, AKT regulates signaling via various growth factors and cytokines. Upstream of AKT, 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, activates AKT [6; 7]. Hence, AKT is a biomarker that predicts metastasis of human gastrointestinal cancer [8].

Phosphorylation of AKT modulates signals from phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and from the mammalian target of rapamycin (mTOR), resulting in diverse effects on cells [9]. In this regard, AKT1 is recognized as an apoptotic inhibitor, which contributes to cancer progression. Phosphorylation catalyzed by AKT inactivates Bcl-2 antagonist of cell death, resulting in its dissociation from Bcl-2. In addition, AKT activates nuclear factor κB, which in turn upregulates transcription of many survival genes [10]. AKT also promotes angiogenesis through upregulation of vascular endothelial growth factor (VEGF) [11]. The AKT-microRNA regulatory network suggests that microRNA-mediated gene regulation interacts with the AKT signal pathway [12]. Thus, the expression/activation of AKT promotes tumorigenesis, and AKT represents a relevant molecular target for cancer treatment [7].

AKT activation in the gastric mucosa

To establish the role of oxidative stress and 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*, the catalytic component of telomerase) by enzyme-linked immunosorbent assay in non-cancerous gastric mucosa and gastric cancers [13]. We found that the levels of pAKT are directly correlated with the levels of *iNOS*, NT, and *hTERT*. Gastric mucosa is classified into 4 categories: chronic gastritis without *H. 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 CG, CAG, CMG, and CGA. *hTERT* was detected only in CGA. These findings suggest that oxidative stress may be implicated in AKT activation and *hTERT* induction and that CGA mucosa may represent a high-risk status for gastric carcinogenesis [13].

H. pylori infection is the major cause of chronic persistent inflammation of the gastric mucosa [5; 14]. In gastric mucosal pathology, *H. pylori* 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 CagA phosphorylation by src homology 2 domain-

containing protein tyrosine phosphatase-2 (SHP2) [15; 16]. CagA activates SHP2 phosphatase, which inhibits signal transducers and activators of transcription-mediated growth-suppressive signal and activates extracellular signal-regulated kinase-mediated growth signal [17]. The increased growth activity may enhance the risk of gene alteration.

H. pylori-induced inflammation generates morphological changes, i.e., intestinal metaplasia, which is transdifferentiation from gastric epithelium to the intestinal phenotype [18]. In that condition, antral or fundic mucosa of the stomach is replaced with mucosa that resembles intestinal mucosa [19] through ectopic expression of *CDX2* [20]. Intestinal metaplasia differs from normal mucosa in that cell division is accelerated [21; 22]. Intestinal metaplasia shares several genetic and epigenetic alterations with gastric cancer [3]. We reported that mutated APC and abnormal *CD44* transcripts are present in intestinal metaplasia as well as gastric cancer [23]. Furthermore, we explained that microsatellite instability as well as p53 mutation [24] are detected in 33% of intestinal metaplasias [25].

Telomerase

The telomere is a repetitive "TTAGGG" sequence present at the ends of eukaryotic chromosomes to maintain and protect their integrity [26]. As cells divide, the telomere is shortened in length; thus, the length of the telomere behaves like a marker of the division limit for cells and/or for cell death [27]. In stem cells, cancer cells, and cancer stem cells, the telomere is elongated by telomerase—a ribonucleoprotein enzyme—activity, which enables cells to divide endlessly [28]. Thus, through its reactivation, telomerase is a key enzyme to induce immortalization and malignant properties in somatic cells. Telomerase activity is detected in 85% of gastric cancers, regardless of tumor stage and histological types [29]. In addition, telomerase activity is detected in 23% of intestinal metaplasias and 50% of gastric adenomas, whereas no activity in the corresponding gastric normal mucosa has been reported.

Telomerase RNA

Human telomerase RNA (*hTR*) is generated to function as the template RNA component of telomerase, and *hTR* has 11 nucleotides complementary to the telomere sequence (TTAGGG) [30]. *hTR* is expressed in pre-crisis cell lines, non-neoplastic tissues, immortalized cell lines, and tumor specimens, but the expression level is not correlated with the level of telomerase activity [31]. Interestingly, Blasco *et al.* reported that initial upregulation of *hTR* is an early event and that telomerase is activated only in end-stage tumors during multi-stage carcinogenesis [32].

We reported the expression of *hTR* and telomerase activity in gastric cancer and the corresponding non-cancerous mucosa [33]. Telomerase activity is detected in 88% of carcinoma tissues. Although tumor specimens and non-cancerous mucosas express various levels of *hTR*, 81% express *hTR* at higher levels in tumor specimens than that in the corresponding mucosa. Gastric carcinoma cell lines also express *hTR* at high levels. Thirty-five percent of non-cancerous mucosas show telomerase activity, and all non-cancerous mucosas contain intestinal metaplasia. The incidence of telomerase-positive mucosa in moderate intestinal metaplasia is

significantly higher than that in mild intestinal metaplasia, whereas *hTR* overexpression is detected in mild intestinal metaplasia as well as moderate intestinal metaplasia. Interestingly, the degree of metaplasia of *H. pylori*-infected mucosa increased concordantly with the level of *hTR* expression and telomerase activity. These results indicated that *hTR* overexpression is caused by *H. pylori*, which plays a role upstream of telomerase activity as an early event in stomach carcinogenesis [33].

In the stomach, various kinds of inflammation affect the growth of the mucosa. Specifically, intestinal metaplasia and chronic atrophic gastritis exhibit accelerated cell growth and active cell cycling [22; 34]. Although factors that upregulate *hTR* expression are unknown, continuous inflammatory and regenerative processes may stimulate *hTR* expression by affecting stem cells, and, subsequently, these processes may enhance the activity of telomerase in non-cancerous mucosa of the stomach. Therefore, it is likely that *hTR* overexpression in non-cancerous mucosa may reflect an earlier process of multistep carcinogenesis of the stomach.

Telomere shortening

Telomere shortening of non-cancerous tissues is determined by regenerative processes and is specific to the individual organs or tissue components, since regeneration of tissues requires various degrees of cell division, which also occur in chronic gastritis [21; 22]. We examined the difference in telomere shortening of the gastric mucosal epithelium using an *in situ* telomere quantification technique. Then, we investigated the relationship between telomere length and *H. pylori* infection, and we also assessed the difference between telomere length characterizing intestinal metaplasia of cancer patients and that of non-cancer patients [35].

In healthy subjects without *H. pylori* infection, the telomere volumes of gastric epithelial tissues are 70–79% that of intramucosal lymphocytes (internal control). In patients without gastric cancer, the telomere volume of *H. pylori*-infected mucosa is significantly less than that of *H. pylori*-negative mucosa in both metaplastic and non-metaplastic tissues ($P < 0.0001$). In gastric cancer patients, telomere volumes in intestinal metaplasias adjacent to cancer are 75% that of intestinal metaplasias in non-cancer patients ($P = 0.0001$) [35]. *H. pylori* infection is closely associated with shortening of telomeres, which subsequently stimulates necessary elongation of the telomeres by telomerase activity, especially in the gastric epithelium.

Telomere and AKT

The catalytic subunit of *hTERT* is responsible for telomerase activity and telomere elongation and is suppressed in differentiated cells [36]. We reported that telomere shortening is a significant factor for the induction of *hTERT* expression in gastric mucosa [35]. *hTERT* expression is detected in 7% of the corresponding mucosas of cancer-associated intestinal metaplasias and 3% of cancer-negative intestinal metaplasias, in which telomere volume is markedly reduced [35]. We classified the status of gastric mucosa according to inflammation and immortalization by *hTERT* expression. AKT is a key protein that links inflammation and tumorigenesis; hence, it became the focus of our studies. *iNOS* is a significant mediator

of inflammation in the gastric mucosa. Increased expression of *iNOS* is epigenetically induced upon *H. pylori* infection as a host defense [37]. NT is a marker of nitric oxide (NO)-induced protein degradation. In a previous study, 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 are associated with *hTERT* expression in CGA [13]. In addition, in another study we showed that pAKT levels are associated with *hTERT* expression in gastric adenocarcinomas [13]. Taken together, these findings suggest that the immortality of gastric mucosal cells is associated with inflammation-induced AKT activation.

It is notable that pAKT levels are elevated in certain gastric mucosa; specifically CAG, CMG, and CGA. Interestingly, high pAKT levels in CAG are associated with *H. pylori*-induced active inflammation. In contrast, increased pAKT in CMG suggests that persistent *iNOS* upregulation develops during the metaplastic process. The highest pAKT levels in CGA may develop during carcinogenic processes [38]. *In vitro* analysis showed that only CGA and MKN28 cancer cells showed upregulation of pAKT in response to brief NO exposure. In Barrett esophagus, activation of AKT is associated with dysplasia-carcinoma sequence [39]. Moreover, only CGA mucosa expressed *hTERT*. These findings suggest that CGA is likely a distinct category in the gastric carcinogenesis process.

hTERT activity is regulated by phosphorylation and expression of *hTERT*, and protein kinase C and AKT phosphorylate *hTERT* [40; 41]. AKT-catalyzed phosphorylation of *hTERT* induces intranuclear translocation of *hTERT* and, subsequently, activates *hTERT*. In contrast, ring finger protein 1, an E3 ubiquitin ligase, decreases the activity of *hTERT* by ubiquitination [42].

AKT is associated with diverse pro-tumoral responses, e.g., *hTERT* activation. In our study, the significance of AKT phosphorylation and *hTERT* on the prognosis of gastric cancer was examined. AKT activation by epidermal growth factor increased *hTERT* expression and telomerase activity. In contrast, AKT inactivation by inhibitors and knockdown decreased *hTERT* expression and telomerase activity in MKN28 gastric cancer cells. In 40 gastric cancer tissues, significant correlations were found among the levels of pAKT, *hTERT* expression, and telomere length. The pAKT levels or the levels of pAKT/*hTERT* are not associated with clinicopathological parameters, including stage and nodal metastasis. However, survival rates of the high-level pAKT patients or the high-level pAKT and high-level *hTERT* patients are significantly poorer than those of other patients. These findings suggest that AKT and *hTERT* are good molecular targets for the treatment of gastric cancer.

AKT is associated with cancer cell survival via alteration of Bcl-2 antagonist of cell death, p53, forkhead, nuclear factor κB, mTOR, and PTEN [10] [43]. Moreover, dysregulated PTEN/PI3K/AKT signaling interacts with the Wingless-INT pathway to induce epithelial-mesenchymal transition (EMT), which is usually associated with cancer stem cell-phenotype and poor prognosis [44]. A recent report states that *hTERT* promotes transforming growth factor- β -induced and β -catenin-induced EMT by inducing β -catenin nuclear translocation and its transcriptional activity for vimentin, a mesenchymal factor, expression [45]. Therefore, PTEN/PI3K/AKT signaling enhances EMT and stem cell phenotypes. In our study, the association of AKT phosphorylation, *TERT* expression, and telomerase activity was confirmed in MKN28

gastric cancer cells and gastric cancer tissues. These associations could result in poor prognoses in patients with high pAKT levels or high pAKT/*hTERT* levels; further, a multivariate analysis revealed that pAKT levels or pAKT/*hTERT* levels are reliable prognostic factors. The examination of more gastric cancer cases is required to confirm the hypothesis that the EMT/stem cell phenotype affects disease progression.

Angiogenesis is an essential phenotype for cancer progression [46], and VEGF expression is associated closely with neovascularization and cancer progression in many malignancies. The PI3K/AKT pathway induces VEGF response, which includes other downstream inducers such as mitogen-activated protein kinase (extracellular signal-regulated kinases or p38), Src, focal adhesion kinase, Rho family GTPases, and endothelial NO [47]. The PI3K/AKT pathway increases the secretion of VEGF from cancer cells by hypoxia-inducible factor 1-dependent and independent mechanisms [48]; therefore, AKT suppression could result in an anti-angiogenic effect on gastric cancer.

Our data showed that AKT and *hTERT* are present in high levels in gastric cancer, and the concurrent synthesis of these 2 proteins at high levels is associated with a poor prognosis. These results suggest that AKT and *hTERT* are plausible molecular targets for the treatment of gastric cancer.

Conclusions

In gastric carcinogenesis, *H. pylori* is an essential factor stimulating the transformation of gastric epithelial cells. *H. pylori* induces *iNOS*, synthesis of reactive oxide species (ROS), and telomere shortening as a result of repetitive destruction/regeneration cycles in chronic active gastritis (Fig. 1). The inflammatory changes activate AKT, which promotes *hTERT* expression and telomerase activation. Telomerase activity immortalizes epithelial cells to enable uncontrolled cancer cell division and instigates malignant phenotypes advancing cancer. These findings demonstrated that AKT plays a pivotal role in gastric cancer development. Thus, the study of AKT as a promising molecular target to prevent gastric cancer development and/or progression should be reconsidered.

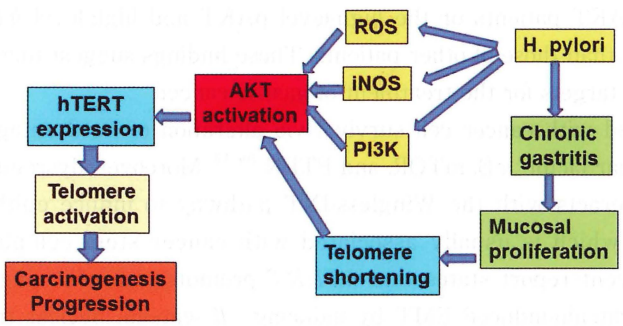

Fig. 1. Central role of AKT in *H. pylori*-induced gastric carcinogenesis
hTERT: human telomerase reverse transcriptase, ROS: reactive oxide species, *iNOS*: inducible nitric oxide synthase, PI3K: phosphatidylinositol-3 kinase, and *H. pylori*: Helicobacter pylori

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