AKT Activation and Telomerase Reverse Transcriptase Expression are Concurrently Associated with Prognosis of Gastric Cancer

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**Abstract**

AKT is a protein in the phosphatidylinositol-3 kinase (PI3K) pathway and associated with diverse pro-tumoral responses. Activation of the human telomere reverse transcriptase (hTERT) is one of AKT’s tumorigenic effects. In this study, the significance of AKT phosphorylation and hTERT on prognosis of gastric cancer were 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 phosphorylated AKT (pAKT), hTERT expression, and telomere length. The pAKT levels or the levels of pAKT/hTERT were not associated with clinicopathological parameters, including stage and nodal metastasis. However, survival rates of the pAKT-high patients or the pAKT-high and hTERT-high patients were significantly poorer than those in other patients. These findings suggest that AKT and hTERT are good molecular targets for the treatment of gastric cancer.

**Introduction**

The telomere is a repetitive ‘TTAGGG’ sequence present at the ends of eukaryotic chromosomes to maintain and protect their integrity [1]. 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 [2]. In stem cells and cancer cells, the telomere is elongated by telomerase activity, which enables them to divide endlessly [3].

The catalytic subunit of human telomeras reverse transcriptase (hTERT) is responsible for telomerase activity.
and telomere elongation and is suppressed in differentiated cells [4]. In our previous study, we reported that telomere shortening is a significant factor for the induction of TERT expression in gastric mucosa [5].

AKT is a protein in the phosphatidylinositol-3 kinase (PI3K) 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. Thus, AKT is involved in signaling mediated by various growth factors and cytokines. In particular, insulin-like growth factor-1, epidermal growth factor receptor, and human epidermal growth factor receptor 2, which are important in cancer-progression, activate AKT [6, 7]. Hence, AKT is a one of biomarkers for predicting metastasis of human gastrointestinal cancer [8].

The phosphorylation of AKT modulates signals from phosphatase, tensin homolog deleted on chromosome 10 (PTEN), and the mammalian target of rapamycin to provide diverse effects on cells [9]. In this regard, AKT1 is recognized as an apoptotic inhibitor, which enhances cancer promotion. Phosphorylation via AKT inactivates Bcl-2 antagonist of cell death resulting in its dissociation from Bcl-2. Nuclear factor xB is also activated by AKT, which in turn up-regulates transcription of many survival genes [10]. AKT also induces angiogenesis through the up-regulation 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]. Hence, the expression of AKT is a pivotal tumorigenic factor and AKT is recognized as a relevant molecular target of cancer treatment [7].

The activity of hTERT is regulated by hTERT expression and phosphorylation. Protein kinase C and AKT can phosphorylate hTERT [13, 14]. AKT 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 ubiquitylation [15].

In the present study, AKT phosphorylation is correlated with clinicopathological parameters such as TERT expression and telomerase activity in gastric cancer.

**Materials and Methods**

**Cell Culture and Reagents**
The human gastric cancer cell line MKN28 (kindly gifted from 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% CO2 in air and 37°C. Wortmannin and triciribine were from Biovision LTD. (Milpitas, Calif., USA), and human epidermal growth factor was from Peptrotec EC LTD. (Rocky Hill, N.J., USA).

**Clinical Materials**
Forty gastric tissues (approximately 30 mm3) were randomly selected from cases diagnosed at the Department of Molecular Pathology, Nara Medical University, between 2001 and 2010. The tissues had been frozen quickly in liquid nitrogen and stored at −80°C. The tissue contents were confirmed by microscopic observation of the adjacent tissues, which were prepared for histopathological examination.

**Short Interference RNA**
FlexiTube short interference RNAs (siRNAs) for AKT was purchased from Qiagen Genomics, Inc. (Bothell, Wash., USA). All Stars Negative Control siRNA was used as control (Qiagen Genomics, Inc.). Cells were transfected with 50 nM siRNA for each gene using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, Calif., USA) according to the manufacturer’s instructions.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)**
Total RNA (1 µg) was used for cDNA synthesis with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). qRT-PCR were performed on the StepOne Real-Time PCR System (Applied Biosystems, Foster City, Calif., USA) using the Fast SYBR Green Master Mix (Applied Biosystems) and analyzed by employing the relative standard curve quantification method. The PCR parameters were set according to the manufacturer’s instructions and the beta-actin mRNA level was used as internal control. All amplifications were evaluated by melting curve analysis and PCR products were electrophoresed on 2% agarose gels. All PCRs were performed at least in triplicate. Primer sets were purchased from Santa-Cruz.

**Immunoblot Analysis**
Cell lysates were prepared as described previously [16]. Next, 25 µg (total protein) from lysates were electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel followed by electrotransfer to nitrocellulose membranes, which were subjected to immunoblot analysis. The membranes were incubated with primary antibodies and then probed with the appropriate peroxidase-conjugated secondary IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan). Anti-tubulin antibody (LifeSpan Biosciences, Inc., Seattle, Wash., USA), anti-AKT antibody (Rockland Immunochemicals Inc., Gilbertsville, Pa., USA) and anti-phosphorylated AKT antibody (pSer463, Biorbyt, Cambridge, UK) were used as primary antibodies. The immune complex was visualized with the Enhanced Chemiluminescence Western-blot detection system (Amersham, Aylesbury, UK).

**Enzyme-Linked Immunosorbent Assay (ELISA)**
The lysates prepared as described above were also used for ELISA. Concentrations of pAKT and TERT were evaluated using specific ELISA kits, i.e., Akt (pS473) ELISA kit (Abcam, Cambridge,
UK) and human telomerase reverse transcriptase (HTERT) ELISA kit (Oxford Expression Technology, Oxford, UK), according to the manufacturers’ instructions.

**Telomerase Activity and Telomere Length**

Telomerase activity was examined by using the Quantitative Telomerase Detection Kit (US Biomax Inc., Rockville, Md., USA). The telomere length was examined by employing the TeloTAGGG Telomere Length Assay (Roche Applied Science, Indianapolis, Ind., USA). These kits were used according to the manufacturers’ instructions.

**Statistical Analysis**

Statistical analyses of experimental data were carried out using the Spearman r test and ANOVA. The positivities of pAKT and TERT were compared using the two-tailed chi-squared test (InStat, Graphpad Software Inc., La Jolla, Calif., USA). Survival analysis was performed by using the Kaplan-Meier method along with the Logrank test. Univariate and multivariate analyses were calculated by using Cox’s hazard model (SPSS Statistics, IBM Japan, Tokyo, Japan). Statistical significance was defined as a two-sided p value of less than 0.05.

**Results**

To examine the effect of AKT phosphorylation on hTERT expression and telomerase activity, MKN28 gastric cancer cells were analyzed after treatment under different conditions (fig. 1a). Epidermal growth factor stimulated AKT phosphorylation and increased hTERT expression and telomerase activity. In contrast, wortmannin, a PI3K inhibitor, triciribine, an AKT inhibitor, and siRNA-induced AKT knockdown inhibited AKT phosphorylation and decreased hTERT expression and telomerase activity. Total AKT protein levels were not affected by any treatment. These results suggest that AKT phosphorylation is associated closely with hTERT expression and telomerase activity.

Phospho-AKT (pAKT) levels, hTERT protein levels, and telomerase activity were examined in 40 cases of gastric cancer (fig. 1b–d). The pAKT level correlated with the hTERT level and telomerase activity, and the hTERT level correlated with telomerase activity (p < 0.0001, p =
These correlations were compatible to those found in figure 1a.

Next, pAKT levels were compared with clinicopathological parameters (table 1). Parameters in the pAKT-High cases showed no significant difference when compared with those in the pAKT-Low cases. However, survival analysis showed that the pAKT-High cases had a significantly poorer prognosis than the pAKT-Low cases (fig. 2a, p = 0.0498).

Next, the cases were divided into 3 categories, i.e., pAKT high and TERT high (Both High), pAKT low and TERT low (Both Low), and other cases (Intermediate), to examine the concurrent effect of pAKT and hTERT on disease progression (table 2). As shown in figure 2c, the Both-High cases were the 9 highest cases of the product of pAKT by hTERT. In contrast, the Both-Low cases were the 7 lowest cases of the product. These cases were distinguishable by distribution of the products. Parameters of the Both-High cases showed no significant differences compared to those of the Both-Low cases. However, the survival analysis showed that the Both-High cases had a significantly poorer prognosis than the Both-Low and Intermediate cases (fig. 2b, p = 0.0339), and the Both-Low cases had a significantly better prognosis than the Intermediate cases. Thus, the levels of pAKT and TERT could be useful prognostic markers.

Finally, the significance of pAKT levels or pAKT/hTERT levels was examined by univariate and multivariate analyses to compare stage and nodal metastasis (table 3). Nodal metastasis emerged as an independent factor for the prognosis, whereas pAKT levels, pAKT/hTERT levels, and stage were dependent factors.

**Discussion**

In our study, the pAKT level or pAKT/hTERT levels showed no association with any clinicopathological parameters; however, the levels correlated well with disease prognosis.
AKT is associated with cancer cell survival through altering Bcl-2 antagonist of cell death, p53, forkhead, nuclear factor κB, mammalian target of rapamycin, and PTEN [10] [17]. 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 [18]. It has been recently reported that hTERT promotes transforming growth factor-β and β-catenin-induced EMT by inducing β-catenin nuclear translocation and its transcriptional activity for vimentin expression [19]. Therefore, PTEN/PI3K/AKT signaling enhances EMT and stem cell phenotypes. In the present study, the association of AKT phosphorylation, TERT expression, and telomerase activity was confirmed in MKN28 gastric cancer cells and tissues of 40 gastric cancer patients. These associations could result in poor prognoses in cases with high pAKT levels or high pAKT/hTERT levels. Multivariate analysis revealed that pAKT levels or pAKT/hTERT levels were dependent 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 [20]. VEGF expression is associated closely with neovascularization and cancer progression in many malignancies. The PI3K/AKT pathway is one of the inducers of a VEGF response, which includes other inducers such as mitogen-activated protein kinase (extracellular signal-regulated kinases or p38), Src, focal adhesion kinase, Rho family GTPases, and endothelial nitric oxide [21].

**Table 3.** Univariate and multivariate analyses of pAKT and/or hTERT levels

<table>
<thead>
<tr>
<th></th>
<th>Coefficient</th>
<th>95% CI</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>0.0252</td>
<td>0.005–0.524</td>
<td>0.0296</td>
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<tr>
<td>Nodal metastasis</td>
<td>7.613</td>
<td>1.727–33.562</td>
<td>0.0008</td>
</tr>
<tr>
<td>pAKT (High, Low)</td>
<td>2.453</td>
<td>0.945–6.368</td>
<td>0.0498</td>
</tr>
<tr>
<td>pAKT/hTERT</td>
<td>0.159</td>
<td>0.020–1.243</td>
<td>0.0198</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>1.077</td>
<td>0.236–4.916</td>
<td>0.9351</td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td>0.132</td>
<td>0.028–0.627</td>
<td>0.0108</td>
</tr>
<tr>
<td>pAKT (high, low)</td>
<td>0.572</td>
<td>0.236–1.389</td>
<td>0.2172</td>
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<tr>
<td>pAKT/hTERT</td>
<td>0.793</td>
<td>0.267–2.353</td>
<td>0.7995</td>
</tr>
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CI = Confidence interval. Both high, both low, intermed.
PI3K/AKT pathway increases the secretion of VEGF from cancer cells by hypoxia-inducible factor 1-dependent and -independent mechanisms [22]. Therefore, AKT suppression could result in an anti-angiogenic effect on gastric cancer.

Our data showed that AKT and hTERT were widely expressed in gastric cancer. The concurrent expression of these 2 proteins at high levels is associated with a poor prognosis. These results suggest that AKT and hTERT are good molecular targets for the treatment of gastric cancer.

References