Sonic Hedgehog Pathway Promotes Metastasis and Lymphangiogenesis via Activation of Akt, EMT, and MMP-9 Pathway in Gastric Cancer

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Abstract

Activation of sonic hedgehog (Shh) signaling has been implicated in progression of a variety of tumors. In this study, we elucidated a role for Shh in the invasion of gastric tumors and determined the mechanism by which Shh is regulated. Immunohistochemical analysis of 178 primary human gastric tumor biopsies indicated that Shh expression was positively correlated with lymph node metastasis, high lymphatic vessel density, and poor prognosis. In mouse xenograft models of human gastric cancer, enforced expression of Shh significantly enhanced the incidence of lung metastasis compared with nonexpressing controls. Mechanistic investigations revealed that phosphoinositide 3-kinase (PI3K)/Akt inhibition blocked Shh-induced epithelial–mesenchyme transition, the activity of matrix metalloproteinase 9 (MMP-9), and lymphangiogenesis, reducing tumor invasiveness and metastasis. Taken together, our findings establish that Shh signaling promotes the metastasis of gastric cancer through activation of the PI3K/Akt pathway, which leads to mesenchymal transition and MMP-9 activation. These findings offer preclinical validation of Shh as a candidate therapeutic target for treatment of metastatic gastric cancers. Cancer Res; 71(22); 7061–70. ©2011 AACR.

Introduction

The sonic hedgehog (Shh) signaling pathway plays a critical role in stem cell maintenance and specifying patterns of cell growth and differentiation during embryonic development (1). Shh activates its signaling by binding to the receptor, Patched (Ptc), which relieves Ptc-mediated repression of a downstream membrane protein related to G protein-coupled receptors, Smoothened (Smo). Upon activation, Smo promotes nuclear translocation of a family of transcription factors [Ci in Drosophila and Gli (Gli1, Gli2, and Gli3) in vertebrates], and subsequently activates target genes through Glis (2).

Recently, the Shh signaling pathway was shown to not only be involved in induction of angiogenesis in an adult mammalian system but also in the control of the motility and migration of multiple cell types (3). Studies conducted by Hochman and colleagues have suggested that the components of the Shh signaling pathway participate directly in cell migration and angiogenesis, whereas inhibition of this pathway blocks Shh-induced cell migration and angiogenesis (4). In addition, Shh and Gli1 expression has also been shown to affect epithelial-to-mesenchymal transition (EMT) in pancreatic cancer cell lines and lymphatic metastasis in esophageal squamous cell carcinoma (5, 6). These observations imply that disregulated Shh signaling is correlated with the severity of the associated tumor and contributes to maintain metastatic behavior. Therefore, understanding these pathways in tumor metastasis will provide great promise for the discovery of novel therapeutics and the treatment of metastatic disease. Nevertheless, the exact role and regulatory mechanisms of Shh signaling in tumorogenesis and metastatic progression of gastric cancer are controversial and need further classification. To clarify the functional role of Shh signaling in gastric cancer progression, we used multiple approaches in testing the gene, both in vitro and in vivo, and validated the outcome results in a clinical setting.

In this study, we found that the Shh signaling pathway, acting through the phosphoinositide 3-kinase (PI3K)/Akt pathway, may contribute to tumor metastasis by inducing EMT and mediating matrix metalloproteinase 9 (MMP-9) activity.

Materials and Methods

Cell lines and cultures

AGS and KATOIII were obtained from the American Type Culture Collection and MRC-5, SNU-5, SNU-16, SNU-216,
SNU-638, MKN-28, MKN-45, and MKN-74 were purchased from the Korea Cell Line Bank. Adult human dermal lymphatic microvascular endothelial cells (HMVEC-dLyAd) were obtained from Lonza and the cells were maintained according to manufacturer’s instructions.

Authentication and characterization of cell lines

Each cell was characterized by cytogenetic analysis. SNU-5, SNU-16, SNU-216, and SNU-638 were initially characterized by Park and colleagues (7). MKN-28, MKN-45, and MKN-74 were characterized by Kitano and Kitamura (8). Origin of MRC-5 was confirmed by nucleoside phosphorylase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase isoenzyme electrophoresis. HMVECs stain positive for acetylated LDL and von Willebrand’s (factor VIII) antigen and stain negative for smooth muscle α-actin.

Clinical samples

The archival paraffin-embedded tissues were used from 178 patients who had undergone surgical excision for gastric cancer at Korea University Guro Hospital between March 2002 and April 2004. Tissue samples were obtained from Korea Lung Tissue Bank, assigned and supported by the Korea Science & Technology Foundation in the Ministry of Science & Technology. Patient clinical characteristics according to the International Union Against Cancer, as revised in 2002, are shown in Table 1. This protocol was reviewed and permitted by Institutional Review Board of Guro Hospital.

Animal studies using a subcutaneous and tail vein injection model

Exponentially growing cells (1 × 10⁶) in 0.1 mL of PBS were prepared and then injected into the subcutis or tail veins of 6-week-old BALB/c nu/nu mice (Charles River Laboratories). Mice were sacrificed 7 weeks after inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically. Primary tumor tissues were harvested, weighed, and processed for further analyses.

Reverse transcriptase PCR analysis, luciferase assay, immunohistochemical staining, cell proliferation assay, wound healing, Matrigel invasion assays, Western blotting, immunofluorescence staining, and gelatin zymography

The above analyses were conducted as described earlier (9, 10). Antibodies, sources, clones, and dilutions used in immunofluorescence staining are listed in Supplementary Table S1. The sequences of primers used for reverse transcriptase PCR (RT-PCR) analysis are shown in Supplementary Table S2.

In vitro kinase assay and in vitro tube formation assay

Nonradioactive Akt kinase assays were carried out according to the manufacturer’s protocol (Cell Signaling). Quantitative analysis of lymphatic vessel density (LVD) was conducted in sections, which were single stained for D2–40.

Statistical analysis

The similar methods were used for statistical analysis of in vitro and in vivo data described previously (9). The relationships between variables were assessed with a Spearman rank correlation coefficient. Difference in the incidence of lung metastasis was analyzed by Fischer exact test. Kaplan–Meier survival curves were constructed for patients positive and negative for Shh. Differences between survival curves were tested for statistical significance by a log-rank test. P values less than 0.05 were defined as statistically significant.

Results

Elevated expression of Shh is correlated with advanced clinical stage, lymph node metastasis, and poor prognosis in patients with gastric cancer

To investigate the role of Shh pathway in gastric cancer metastasis, we first conducted RT-PCR for components of the Shh pathway in a panel of gastric cells. As expected, the comparison of various gastric cancer cell lines or tissues revealed that all of the components of this pathway were expressed in diverse gastric tumor cells to a different extent (Supplementary Fig. S1A and B). Interestingly, we detected significantly higher expression of Shh and Gli1 in gastric tumor tissues obtained from patients with lymph node metastasis compared with patients without lymph node metastasis (Fig. 1A). Furthermore, we showed that there was a significant positive correlation between the levels of Shh and Gli1 transcripts (P < 0.01, r = 0.766, Fig. 1A).

Next, we conducted immunohistochemical staining for Shh in 178 gastric cancer specimens. Clinicopathologic analysis revealed that the expression of Shh was strongly elevated in the cases with advanced stages (stages II–IV) and with lymph node metastasis compared with early stage (stage I, P = 0.039) and nonmetastatic cases (P = 0.042; Table 1 and Supplementary Fig. S1C). The results also indicate that in advanced stages (stages II–IV) of gastric cancer (n = 147), patients with positive expression of Shh had a significantly worse rate of survival than the patients with low expression of the gene (P = 0.002 by log-rank test; Fig. 1B). On the basis of univariate Cox regression analysis, the death risk of patients with increased Shh expression was 3.2 times higher than the risk of patients with a reduced expression of the gene. Indeed, the overall survival rate was significantly lower in patients with gastric cancer at stages II to IV than those at stage I (P = 0.02 by log-rank test; Fig. 1B). Taken together, these results clearly show that elevated expression of Shh is correlated with advanced clinical stage, lymph node metastasis, and poor prognosis in patients with gastric cancer.

Crucial function of Shh signaling on invasiveness and metastatic potential of gastric cancer cells

Next, we tested whether or not the Shh signaling pathway is involved in progression to metastatic disease in gastric cancer, especially in late tumorigenesis, including migration and invasion. Our results showed that N-Shh treatment significantly stimulated the migration and invasiveness of these cells (Fig. 1C, and D). In contrast, the stimulatory effects...
of N-Shh on cell migration and invasion were completely abolished by cyclopamine (a Shh signaling inhibitor) or Gli1-specific siRNA treatment (Supplementary Fig. S2 A).

The AGS cell line expressing Shh and containing only vector was subcutaneously injected into athymic mice, then the formation and growth rate of the tumor was monitored every 4 days for 24 days. All of the vector only and Shh expressing cells had similar growth rates during the indicated period, suggesting that overexpression of Shh did not significantly affect tumor growth rate (Supplementary Fig. S2B). This result is consistent with data obtained from in vitro BrdU labeling and fluorescence-activated cell sorting analysis that assesses the proliferation and the cell-cycle distribution of the vector only and Shh expressing AGS cells (Supplementary Fig. S2C). On the contrary, tumors stably expressing Shh showed nearly 5 times more colony formation on lung surfaces compared with the tumors containing only vector (Supplementary Fig. S2D). In addition, Shh significantly increased the number of mice with metastasis to the lung, and similar results were also obtained in a tail vein injection model (Fig. 1E). These results strongly suggest that Shh signaling enhances the metastatic potential of gastric cancer cells.

Shh signaling pathway induces lymphangiogenesis and morphologic changes of gastric cancer cells through the EMT process and MMP-9 activity

In the context of tumor progression, EMT is an early event in tumor invasion and increased motility and invasion are positively correlated with EMT (11). Here, we found that treatment of SNU-216 and MKN-74 cells with N-Shh for 72 hours exhibited dramatic changes in cell morphology, from a cuboid, epithelial-like shape to a spindle, fibroblastic-like appearance, consistent with EMT (Supplementary Fig. S2C). On the contrary, tumors stably expressing Shh showed nearly 5 times more colony formation on lung surfaces compared with the tumors containing only vector (Supplementary Fig. S2D). In addition, Shh significantly increased the number of mice with metastasis to the lung, and similar results were also obtained in a tail vein injection model (Fig. 1E). These results strongly suggest that Shh signaling enhances the metastatic potential of gastric cancer cells.

### Table 1. Relationship between Shh expression and other clinicopathologic features in gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathologic feature (number of cases examined)</th>
<th>Number of cases</th>
<th>Shh expression</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (174)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>79</td>
<td>37 (46.8)</td>
<td>42 (53.2)</td>
</tr>
<tr>
<td>&lt;60</td>
<td>95</td>
<td>68 (71.6)</td>
<td>25 (28.4)</td>
</tr>
<tr>
<td>Gender (173)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>124</td>
<td>82 (66.1)</td>
<td>42 (33.9)</td>
</tr>
<tr>
<td>Female</td>
<td>49</td>
<td>23 (46.9)</td>
<td>26 (53.1)</td>
</tr>
<tr>
<td>Lauren’s classification (162)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal type</td>
<td>57</td>
<td>41 (72)</td>
<td>16 (28)</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>105</td>
<td>61 (58.1)</td>
<td>44 (41.9)</td>
</tr>
<tr>
<td>Histologic grade (73)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>4 (80)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>MD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47</td>
<td>36 (76.6)</td>
<td>11 (23.4)</td>
</tr>
<tr>
<td>PD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21</td>
<td>8 (38)</td>
<td>13 (62)</td>
</tr>
<tr>
<td>Lymph node status (174)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No metastasis</td>
<td>46</td>
<td>19 (41.3)</td>
<td>27 (58.7)</td>
</tr>
<tr>
<td>Metastasis</td>
<td>128</td>
<td>88 (68.8)</td>
<td>40 (31.2)</td>
</tr>
<tr>
<td>TNM staging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>31</td>
<td>13 (41.9)</td>
<td>18 (58.1)</td>
</tr>
<tr>
<td>II–IV</td>
<td>147</td>
<td>92 (62.6)</td>
<td>55 (37.4)</td>
</tr>
</tbody>
</table>

NOTE: The values in parenthesis are expressed in percentage.

<sup>a</sup>Statistical analysis was conducted using Student t test. P < 0.05 was considered statically significant.
<sup>b</sup>Well differentiated.
<sup>c</sup>Moderately differentiated.
<sup>d</sup>Poorly differentiated.
Next, we evaluated Shh-mediated cell migration and invasion to determine whether it resulted from elevated levels of MMPs, which are well-documented ECM-degrading enzymes, the activity of which is associated with tumor invasiveness. RT-PCR analysis showed that N-Shh significantly increased the mRNA levels of MMP-9 in AGS and MKN-28 cells (Fig. 2B). In contrast, abrogating the Shh pathway by cyclopamine or Gli1 siRNA reduced induction of MMP-9 by N-Shh. Similar results were obtained in Western blot (data not shown) and gelatin zymographic analysis of conditioned medium obtained from AGS and MKN-28 cells (Fig. 2B). As expected, overexpression of MMP-9 siRNA reduced the ability of Shh to stimulate migration and invasion of those cells compared with the cells expressing control siRNA (Supplementary Fig. S3B and Fig. 2B). Importantly, in the tail vein experiments, MMP-9 knockdown in the cells expressing Shh also resulted in significant reduction in the lung metastasis compared with cells expressing only Shh (Fig. 2C). Collectively, these findings suggest that Shh-mediated induction of EMT or MMP-9 contributes to Shh pathway-related migration and invasive ability of gastric cancer cells.

The metastatic spread of tumors through lymphatic vessels to local or regional lymph nodes is an early event in metastatic disease for a variety of human tumors, and nodal metastasis is considered to be dependent on the tumor-induced
lymphangiogenesis (12–14). These findings suggest that Shh signaling may influence the metastatic capacity of gastric cancer to lymph nodes by inducing lymphangiogenesis. Our results showed that cells treated with N-Shh displayed significantly higher levels of expression of Lyve-1, Podoplanin, and VEGF-D than cells treated without N-Shh (Fig. 2D). The levels of expression of these molecules in cells treated with cyclopamine or Gli1 siRNA were significantly lower than those in the cells treated with control vehicle. Next, we evaluated the responses in terms of migration and tubule formation in HMVECs, both of which are essential steps for lymphangiogenesis in vivo. As shown in Fig. 2E, N-Shh significantly increased the motility and tubule-like structure formation of HMVECs compared with control vehicle. In contrast, pretreatment of HMVECs with cyclopamine completely inhibited Shh-induced cell migration and the generation of tubular networks.
PI3K/Akt signaling pathway is required for Shh-mediated EMT, MMP-9 activity, and lymphangiogenesis in gastric cancer cells

Recently, numerous studies have shown that PI3K/Akt pathway is an essential component of Shh signaling and inhibition of PI3K/Akt pathway with the PI3K/AKT inhibitor, LY294002, results in impairment of Shh morphogenic activity (15–17). Western blot analysis showed that the activation of PI3K-dependent Akt phosphorylation in AGS, MKN-28, and SNU-216 cells was induced as early as 30 minutes after N-Shh stimulation (Supplementary Fig. S4A and Fig. 3A). However, blockade of Shh signaling by cyclopamine completely reversed the enhancement of Akt phosphorylation (Fig. 3A). In vitro Akt kinase assay showed that N-Shh treatment enhanced GSK-3β phosphorylation, whereas the cyclopamine blocked N-Shh-activated increase in the phosphorylated GSK-3β (Supplementary Fig. S4B). Notably, nonphosphorylatable Akt mutant (DN-Akt) significantly reduced the ability of N-Shh to stimulate activity of the Gli-luciferase reporter (Fig. 3A). Similar results were also obtained in real-time RT-PCR analysis for Gli1 target gene (Pten and Gli1; data not shown). In addition, in spite of the presence of added N-Shh, treatment of these cells with LY294002 or DN-Akt suppressed Shh-mediated Akt activation, as well as cell migration and invasion (Supplementary Fig. S4C and Fig. 3B). Similar inhibition was observed with Akt1 siRNA (Supplementary Fig. S4D).

We subsequently examined whether or not Shh-induced EMT, MMP-9 activity, or lymphangiogenesis is mediated through the PI3K/Akt pathway in gastric cancer cells. Immunofluorescence staining and Western blotting showed that the loss of E-cadherin and gain of Snail by N-Shh was completely reversed by LY294002 or DN-Akt (Fig. 3D and E). After treating HMVECs with N-Shh and LY294002, we also found that LY294002 abrogated the Shh-induced cellular motility and tube formation of HMVECs (Fig. 3E). Together, these results showed
that PI3K/Akt signaling pathway is necessary for Shh-mediated EMT, MMP-9 activity, and lymphangiogenesis in gastric cancer cells.

**Shh signaling pathway promotes metastatic potential of gastric cancer cells by activation of the PI3K/Akt pathway *in vivo***

The effects of Akt activation on Shh-mediated metastatic capacity of human gastric cancers were further investigated using a mouse xenograft model. The metastatic potential of AGS cells expressing vector only, Shh, DN-Akt, or Shh/DN-Akt was evaluated by measuring colonization of lung surfaces by tumor cells after subcutaneous or tail vein injection. The tumor growth did not reveal significant differences among all 4 tumor xenografts (Supplementary Fig. S5B). In both the tail vein and subcutaneous experiments, the Shh cells expressing DN-Akt also showed a significant reduction in the number of lung metastatic colonies or mice with lung metastasis compared with cells expressing only Shh (Fig. 4A). In addition, expression of those EMT- and mesenchymal-related proteins was evidently decreased in Shh/DN-Akt tumor cells compared with Shh tumor cells (Fig. 4B). As expected, the levels of expression of MMP-9, Lyve-1, Podoplanin, and VEGF-D mRNA were also significantly decreased in the Shh/DN-Akt tumor cells than in the Shh tumor cells (Fig. 4C and D). Collectively, these results revealed that the Shh signaling pathway promotes metastatic potential of gastric cancer cells by sequential activation of the PI3K/Akt pathway, followed by upregulation of the EMT pathway and MMP-9.

**Correlation between Shh expression and Akt activity, EMT, or lymphangiogenesis in gastric tumor specimens**

Clinicopathologic analysis revealed that phospho-Akt, E-cadherin, or MMP-9 expression levels in tumors with lymph node metastases was higher than that in tumors without lymph node metastases (E-cadherin: \( P = 0.024 \); MMP-9: \( P = 0.019 \); Phospho-Akt: \( P = 0.056 \); Supplementary Table S3). Therefore, we examined whether or not expression of phospho-Akt, E-cadherin, or MMP-9 was associated with expression of Shh in tumors with lymph node metastases. Phospho-Akt and Shh...
had a weak correlation with regard to the level of expression ($r = 0.126$), but the correlations were not statistically significant ($P = 0.183$). Of greater importance, the level of expression of Shh also showed a strong correlation with that of E-cadherin or MMP-9 (E-cadherin: $P < 0.001$, $r = 0.447$; MMP-9: $P < 0.001$, $r = 0.515$; Table 2 and Supplementary Fig. S6A). Finally, to further evaluate the role of Shh signaling with respect to lymph node metastasis and lymphangiogenesis in human gastric tumor specimens, we explored the correlation of expression of Shh with lymphangiogenesis in 174 gastric cancer specimens. To quantitatively analyze the degree of tumor-associated lymphangiogenesis, tumor LVD was determined by quantification of D2-40 (a specific antibody for podoplanin-positive vessels; Supplementary Fig. S6B). Increased LVD was significantly associated with lymph node metastasis ($P < 0.001$) and tumor-node-metastasis (TNM) staging ($P = 0.032$, Supplementary Table S4). Importantly, higher rates of LVD were observed in patients in the Shh-positive groups, whereas low rates of LVD were found in patients in the Shh-negative groups ($P < 0.001$; Table 3). Furthermore, Shh expressions were also significantly correlated with the presence of lymphatic vessel invasion (LVI, $P = 0.014$). We also found that the phospho-Akt-positive group had a higher density of tumor lymphatic vessels compared with the phospho-Akt-negative group ($P = 0.051$). Taken together, these data suggested that the Shh signaling pathway regulates lymphangiogenesis via a mechanism involving the PI3K/Akt pathway.

**Table 2.** Relationship between expression of Shh, E-cadherin, and MMP-9 in lymph node metastatic gastric cancer

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Shh</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>28</td>
<td>10 (35.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>100</td>
<td>73 (73)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>97</td>
<td>68 (70.1)</td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
<td>15 (48.4)</td>
</tr>
</tbody>
</table>

NOTE: The values in parenthesis are expressed in percentage.
The precise mechanisms controlling Shh/Gli-mediated EMT and metastasis through PI3K/Akt signaling need to be elucidated in detail. In addition, the mechanism whereby Shh signaling induces PI3K/Akt activities is also yet to be defined. Interestingly, elevation of Akt is a response to the loss of the PTEN, which occurs frequently in metastatic gastric cancer (24–26). Therefore, downregulation of PTEN mediated by Shh might contribute to enhance the activity of Akt and thereby further promote tumor progression. Although PI3K/Akt activities are essential for ligand-dependent Shh signaling in metastatic function of gastric cancer cell, activation may not be sufficient to drive Gli activation. Recent studies have suggested that oncogenic K-RAS is directly involved in activation of the Shh pathway (20, 27, 28). Activating RAS mutations have been frequently detected in malignant cells in many gastric cancer patients. Therefore, oncogenic RAS, through the RAF/MEK/MAPK pathway, may play an important role in Shh-mediated metastatic potential.

Lymph node metastasis is a common occurrence in cancer, and lymphatic vessels have been reported as an important route contributing to metastasis of solid tumors (12–14). Our data showed that most patients without lymph node metastasis were in the Shh-negative group, whereas patients with lymph node involvement were Shh-positive group. Despite its clinical relevance, surprisingly little is known about the mechanisms of Shh leading to lymphatic metastasis in gastric cancer. Several studies have indicated that one possible molecular mechanism promoting metastasis to regional lymph nodes is tumor-induced lymphangiogenesis. Our findings indicate Shh signaling, via a mechanism involving the PI3K/Akt pathway, promotes tumor lymphangiogenesis and, consequently, lymph node metastasis, which is in good agreement with recent findings (5). However, it is not clear whether or not Shh signaling plays a role in the formation of the lymphatic vessels and whether or not Shh-mediated lymphangiogenesis is accompanied by enhanced lymphatic metastasis. This problem may be solved with the establishment of a new model of lymphangiogenesis being able to capture the entire network of lymphatics and spread of cancer cells from the site of inoculation.

Our observations improve our understanding of the mechanism by which Shh signaling activation occurs as it relates to the metastatic behavior of gastric cancer. Investigating the expression of Shh and its related proteins in gastric cancer cells may be helpful for determining the therapeutic strategy and antagonists of this signaling may be useful in the clinical setting to suppress the spread of tumors through the lymphatic system.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

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