The loss of endothelin-2 exhibits an anticancer effect in A549 human lung adenocarcinoma cell line

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学 位 論 文 の 内 容 要 旨

The loss of endothelin-2 exhibits an anticancer effect in A549 human lung adenocarcinoma cell line

ヒト肺腺癌細胞 A549 におけるエンドセリン-2 欠失による抗がん効果

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A. Background

Lung cancer is the leading cause of death in both sexes, according to the estimated data GLOBOCAN 2020. Based on its histological type, lung cancer is divided into small cell lung cancer (SCLC, 20% of all lung cancers) and non-small lung cancer (NSCLC, 80% of all lung cancers). The major types of NSCLC include adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma is the most common subtype of lung cancer, accounting for 40% of lung cancers and 60% of NSCLCs. Despite the development of numerous novel treatments, lung cancer remains a serious problem. Therefore, more effective treatment strategies are required.

Endothelin (ET) consists of three isoforms (ET-1, ET-2, and ET-3), which act on two G-protein coupled receptors, ET receptor type A and type B (ET_A and ET_B). Among these three isoforms, ET-1 has been the most extensively studied and is involved in the pathophysiology of various diseases, including cancer. Unlike ET-1, only a few studies have reported the role of ET-2 in cancer. ET-2 is upregulated in human breast cancer, and its expression of ET-2 mRNA is three times higher in basal cell carcinoma than in normal skin. Additionally, ET-2 reduces apoptosis under hypoxia and potentiates the invasiveness of cancer. It is hypothesized that ET-2 may have distinct pathophysiological roles in cancer.

In the present study, analysis from public online databases of human cancer identified that ET-2 mRNA was significantly higher in lung adenocarcinoma (LUAD) tissues compared to normal lung tissues. In addition, high ET-2 expression was associated with poor overall survival (OS) in patients with LUAD. Therefore, the aim of this study was to explore the role of ET-2 in human adenocarcinoma cells A549 by silencing ET-2 and further investigate the underlying mechanisms.

B. Methods

Bioinformatics Analysis. Measurement of ET-1 and ET-2 mRNA levels was conducted from Gene Expression of Normal and Tumor tissues 2 (GENT2) (http://gent2.appex.kr/gent2/) and Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (http://gepia2.cancer-pku.cn/#analysis), interactive web-based tools

that comprise normal and tumor samples. Overall survivals (OS) of patients with LUAD cancer (all stages) from the datasets were analyzed using the Kaplan–Meier plotter web tool.

For in vitro studies, ET-2 was silenced in A549 human lung adenocarcinoma cells. Several functional assays were performed to evaluate the proliferation, migration, and invasion ability of ET-2 depleted A549 cells compared with control. Apoptosis was examined by caspase and PARP protein analysis. The cells were then subjected to mRNA and protein analysis.

C. Results

1. High Endothelin-2 (ET-2) Expression in Lung Adenocarcinoma

Analysis from the GEPIA2 (LUAD sample, 483; normal, 59) and GENT2 (LUAD sample, 254; normal, 509) databases identified significantly higher ET-2 expression in LUAD tissues than in normal lung tissues. In contrast, ET-1 expression was significantly lower in LUAD tissues than in normal lung tissues.

2. High ET-2 mRNA Levels Were Associated with a Poor Prognosis of Lung Adenocarcinoma

The Kaplan–Meier analysis and log-rank test evaluating the median expression values of ET-2 and ET-1 mRNA demonstrated that the survival rate of patients with LUAD with high ET-2 expression, in terms of OS, was significantly worse than that of patients with low expression. The survival rate of patients with LUAD with low ET-1 expression in terms of OS was significantly worse than that in the high-expression group. Accordingly, these findings suggest that the role of ET-2 in LUAD progression needs to be further elucidated.

3. ET-2 Downregulation Decreased A549 Cell Proliferation

ET-2 silenced A549 cells showed a decreased cell growth rate compared to the negative control.

4. ET-2 Downregulation Retarded A549 Cell Migration and Invasion

Migration of A549 cells was performed using wound healing and transwell migration assays. While for invasion assay, transwell insert

membranes coated with Matrigel were used. A549 cells transfected with ET-2 siRNA exhibited significantly decreased migration and invasion abilities.

5. ET-2 Silencing Facilitated A549 Cell Apoptosis

During apoptosis, cleavage of PARP-1 is a useful hallmark of this type of cell death. This cleavage is well studied and is generated by caspase-3 and caspase-7, proteases activated during apoptosis. The levels of cleaved PARP, caspase-3, and caspase-7 increased in the ET-2 silencing A549 cells. Taken together, these data showed that ET-2 plays a role in proliferation, migration, invasion, and apoptosis.

6. ET-2 Silencing Reduced X-linked Inhibitor of Apoptosis–Survivin and Epithelial-Mesenchymal Transition Marker in A549 cells

Survivin and XIAP form a complex that binds directly to caspase-9 and plays a role in cancer cell migration. ET-2 ablation reduced the mRNA and protein levels of survivin and XIAP. Epithelial-mesenchymal transition (EMT) was important for A549 cells migration. E-cadherin, an epithelial marker, was increased by silencing of ET-2. Vimentin, SM22, and Twist mRNA, mesenchymal markers, were reduced by silencing ET-2.

D. Discussion

In the present study, ET-2 mRNA expression was significantly elevated in LUAD tissues of patients compared to that in normal lung tissues, according to two bioinformatics human cancer databases. In addition, high ET-2 mRNA levels are associated with poor OS in patients with LUAD. Moreover, ET-2 knockdown in human LUAD A549 cells markedly reduced proliferation, migration, invasion, and enhanced apoptosis. These anticancer effects were mediated by the dual inhibition of XIAP–survivin mRNA and protein levels and suppression of EMT.

XIAP and survivin are inhibitors of the apoptosis protein (IAP) family members, a group of proteins that modulate the balance between proliferation and apoptosis. In addition, IAPs play an important role in cancer cell migration. XIAP and survivin are significantly increased in NSCLC and LUAD according to bioinformatics analyses. Survivin is a well-known regulator of cell proliferation and division. In particular, XIAP is the most explored and potent member of the

IAP family. XIAP interacts with and inhibits the activation of caspase-3, caspase-7, and caspase-9.

EMT is an important phenomenon involved in the metastasis, recurrence, and drug resistance of lung cancer. In addition, EMT progression is associated with a significantly poor prognosis in patients with LUAD (Sowa et al., 2015). XIAP has been reported to play a role in the migration of cancer cells, partly via the inhibition of EMT (Jin et al., 2019). Eventually, ET-2 depletion reduced the migration and invasion of A549 LUAD cells, partly through the inhibition of EMT.

More *in vitro* experiments using other LUAD cell lines, *in vivo* studies, and associated molecular mechanisms are necessary to further explore the pathophysiological functions of ET-2 in cancer. Nevertheless, these findings provide insights into the participation of ET-2 in LUAD and suggest a plausible therapeutic target for LUAD.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨

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論文題目 Title of Dissertation	ヒト肺腺癌細胞A549におけるエンドセリン-2欠失による抗がん効果 The loss of endothelin-2 exhibits an anticancer effect in A549 human lung adenocarcinoma cell line		
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（要旨は１，０００字～２，０００字程度）

【目的】
肺がんは、世界におけるがん関連死亡の第一の原因であり、腺がんは肺がんの中で最も一般的なサブタイプである。エンドセリン-2（ET-2）は肺胞の上皮に発現しており、肺腺がんではその発現が増加していることが知られている。しかし、肺腺癌における ET-2 の役割は不明なままである。そこで本研究では、ET-2 の肺腺がんにおける病態生理学的機能を明らかにすることを目的に研究を行った。

【結果】
肺腺癌組織と非腫瘍肺組織における ET-2 mRNA の発現を、オンラインデータベースを用いて解析したところ、ET-2 mRNA レベルは、肺腺癌組織で発現が増加していることを確認した。また、ET-2 の発現量増加は肺腺癌患者の生存率の低さと相関した。
次に A549 細胞における ET-2 の機能を siRNA を用いて検討した。
生理学的には、ET-2 の発現抑制は，A549 細胞の増殖，遊走，浸潤を減少させ，アポトーシスを促進するとともに、上皮間葉転換を阻害した。
また生化学的には ET-2 サイレンシングは、アポトーシス阻害タンパク質ファミリーのメンバーである X-linked inhibitor of apoptosis および survivin の発現レベルを低下させ、カスパーゼ 7/9/3 の活性化をみた。

【結論】
このように ET-2 の発現は肺腺癌の進展を促進し、ET-2 は肺腺癌の治療標的となる可能性がある。

以上本研究は、 ET-2 が肺腺癌の進展を促進させるという学術的な発見のみならず、
肺腺癌の治療標的となりうるという臨床応用への可能性を提示するもので、非常に価値が高いことから、本研究者は、博士（医学）の学位を得る資格があると認める。