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Original contribution

# An abnormally high expression of ISL-1 represents a potential prognostic factor in gastric cancer☆,☆☆

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**Summary** Insulin gene enhancer binding protein–1 (ISL-1) is a transcription factor involved in development of the heart, motor neurons, and pancreas. Our previous study indicated that ISL-1 was overexpressed in gastric cancer but not in other gastrointestinal tumors. However, no immunohistochemical or clinicopathological studies of ISL-1 in gastric carcinoma have been performed. The aim of this study was to determine the expression and prognostic value of ISL-1 in gastric carcinoma. A nude mouse xenograft model was established to study the role of ISL-1 on cancer genesis and development in vivo. Overexpression of ISL-1 significantly enhanced the tumorigenicity of NIH3T3 cells in vivo. ISL-1 expression was evaluated using immunohistochemistry in 456 human gastric carcinoma and normal tissues. ISL-1 was significantly overexpressed in gastric adenocarcinoma compared with normal gastric tissues. ISL-1 expression was significantly associated with depth of invasion, lymph node metastasis, TNM stage, and histological grade ($P < .05$, $\chi^2$ test). Positive ISL-1 expression was associated with poorer 5-year overall survival in gastric cancer ($P = .001$, log-rank test). Multivariate Cox regression analysis demonstrated that ISL-1 expression ($P = .047$) could be an independent prognostic factor for overall survival in gastric carcinoma. This study suggests that ISL-1 may be a useful prognostic biomarker and may represent a novel therapeutic target for gastric adenocarcinoma.


## 1. Introduction

Although its incidence has been declining for several decades, gastric cancer was the leading cause of cancer-related deaths worldwide until the mid-1990s. A total of 989 600 new cases and 738 000 deaths due to gastric cancer were estimated to have occurred in 2008, accounting for 8% and 10% of all cases and cancer-related deaths, respectively, with two-thirds of these cases in developing countries [1]. Gastric cancer is now relatively rare in North America and most of Northern and Western Europe; however, the incidence is higher in Eastern Europe, Russia, and selected areas of Central and South America and East Asia [2,3]. In China, gastric cancer is the most common type of cancer and the second leading cause of cancer deaths after lung cancer [4]. The overall morbidity and mortality of gastric cancer are doubled in male patients than female patients in China [1,4].

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Disease stage is the most important factor for predicting the treatment outcome of patients [5,6]. Despite the fact that most cases of early stage gastric carcinoma can be cured by surgical resection, metastasis contributes to the high rate of mortality in gastric carcinoma [7]. Although numerous immunohistochemical markers such as human epidermal growth factor receptor 2, matrix metallopeptidase–2, and epithelium cadherin have been recognized as biomarkers in other types of cancer, these markers are not commonly used to assess biological behavior and prognosis in gastric cancer because of low levels of sensitivity and specificity [8–13]. Therefore, there is an urgent need to identify novel prognostic and predictive biomarkers to improve the diagnosis and clinical management of patients with gastric cancer, which in turn may help to develop more effective treatment strategies.

Insulin enhancer binding protein–1 (ISL-1), a LIM-homeodomain transcription factor, was originally isolated as an insulin-responsive protein that binds to the islet $\beta$-cell–specific enhancer element [14]. The rat, hamster, and human ISL-1 amino acid sequences share 100% homology [15]. ISL-1 has previously been described to play crucial roles in development of the heart, motor neurons, and pancreas [16–18]. Recently, ISL-1 was implicated in the development of cancer, mainly based on the fact that expression of ISL-1 is dysregulated in a range of tumor types [19–22].

In a pilot study, we examined the expression of ISL-1 in a variety of human tumor types including breast, prostate, colon, liver, lung, and esophageal cancer using immunohistochemical staining. Markedly higher expression of ISL-1 was observed in human gastric carcinoma specimens compared with the tumor specimens from other organs [23]. However, no comprehensive immunohistochemical or clinicopathological studies of ISL-1 in gastric carcinoma have been performed. An accurate evaluation of the expression of ISL-1 in gastric cancer is required to investigate its prognostic value in this tumor type.

In the present study, we investigated the expression of ISL-1 in gastric cancer tissues and normal tissues. We found that an abnormally high expression of ISL-1 was significantly associated with the depth of invasion, lymph node metastasis, TNM stage, and histological grade, as well as poorer overall survival, in patients with gastric cancer. The findings of this study indicate that ISL-1 has potential as a diagnostic and prognostic biomarker for gastric carcinoma.

## 2. Materials and methods

### 2.1. Patients and tissue microarray

Human gastric cancer tissues and normal gastric tissue microarray sections with complete stage and grade information were purchased from Biomax (T012A, T014, ST1001, ST2091; Rockville, MD) and Shanghai Biochip Co Ltd (HStm-Ade180Sur-04; Shanghai, People's Republic of China). In total, ISL-1 protein expression was analyzed in 262 gastric cancer tissues, 144 noncancerous tissues (including 9 gastric ulcers, 17 gastric inflammations, 6 hyperplasias, and 112 normal gastric tissues), and 50 gastric adenocarcinoma lymph node metastases.

In addition, tissue specimens from 90 patients with gastric cancer (Shanghai Outdo Biotech Co Ltd, HStm-Ade180-Sur-04) for whom complete overall survival data were available (from the date of surgery until death or August 2013) were analyzed. The clinicopathological characteristics of these patients are shown in Table 1; the patients were staged according to the sixth edition of the American Joint Committee on Cancer manual [24]. Patients were only included in the study if they had provided written consent to participate in the study after receiving oral and written information regarding its nature and purpose. Approval for the study was received from the ethics committee of the host institutions in addition to the company that provided the samples.

### 2.2. Animal experiments

All animals were purchased from the Department of Laboratory Animal Science, Peking University; and all protocols were approved by the Animal Care and Use Committee of Peking University (LA 2010-066). The plasmid pcDNA3-ISL1 and control vector pcDNA3 were separately transfected into NIH3T3 cells to establish stable ISL-1–overexpressing cells (pcDNA3-ISL-1) and control cells (pcDNA3), as previously described [22]. Five-week-old female nude mice were subcutaneously injected on the right side of the dorsum (pcDNA3-ISL-1) and left armpit (pcDNA3) with $1 \times 10^7$ cells suspended in 200 $\mu$L phosphate-buffered saline (PBS). Tumors were measured at the indicated time points using calipers, and tumor volumes were calculated using $1/2 \times \text{length} \times \text{width}^2$. The significance of the differences between groups was determined using 2-way analysis of variance.

### 2.3. Immunohistochemistry

Five-micrometer-thick tissue sections were cut from each tissue microarray block. All subsequent procedures were carried out at room temperature, unless otherwise stated. Tissue sections were rehydrated; and antigen retrieval was performed by submerging the slides in sodium citrate buffer (10 mmol/L sodium citrate, 0.05% Tween 20, pH 6.0) in a pressure cooker, boiling for 3 minutes, followed by cooling for 30 minutes at room temperature. Afterward, the tissue sections were incubated in 3% $H_2O_2$ in PBS-T (0.01% Tween 20 in PBS) for 10 minutes to block endogenous peroxidase activity; and a circular hydrophobic barrier was drawn around each specimen using a Pap pen (ZLI-9305; ZSGB-Bio, Beijing, China). The sections were blocked in 5% bovine serum albumin in PBS-T for 30 minutes and, incubated with mouse polyclonal anti-ISL-1 antibody (1:400; ab86472; Abcam, Hong Kong, China) in 5% bovine serum albumin in PBS-T in a humidified chamber overnight,