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*Original Article*

# Overexpression of oncoprotein ecotropic viral integration site 1 is an independent prognostic biomarker of gastric cancer

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**Abstract:** Background: Ecotropic viral integration site 1 (EVI1) is a well-recognized oncoprotein in hematopoietic malignancies such as leukemia. In gastric cell lines, EVI1 was demonstrated to inhibit TGF-β signaling, thus promoting cell growth. However, the clinical significance and prognostic values of EVI1 in gastric cancer is still blank. Materials and methods: In our study, we investigated the expression of EVI1 in 190 formalin-fixed gastric cancer tissues and divided them into EVI1 high-expression group and low-expression group. The correlation between EVI1 expression and clinicopathologic factors was analyzed by Chi-square test. The prognostic value of EVI1 expression was calculated by univariate analysis with Kaplan-Meier method and independent prognostic factors were identified by multivariate analysis with Cox-regression model. Results: In our study, the percentages of EVI1 high-expression and low-expression were 18.94% (36/190) and 81.06% (154/190), respectively. EVI1 high-expression was proved to be associated with poor prognosis in gastric cancer (P = 0.012). By multivariate analysis, we demonstrated that EVI1 high-expression could be considered as the independent prognostic factor in gastric cancer (P = 0.008, HR = 2.23, 95% CI = 1.23-4.04). Conclusions: In our investigation, EVI1 high-expression was demonstrated to be an independent prognostic factor in gastric cancer, which may provide new tendency to discover new molecular target in gastric cancer therapy.

**Keywords:** Ecotropic viral integration site 1, gastric cancer, prognosis, prognostic biomarker

## Introduction

Gastric cancer (GC) is the fourth most common cancer and the third lethal cause of cancer worldwide, with an estimated 951,600 new cases and 723,100 deaths in 2012 [1]. Gastric cancer is heterogeneous, and the classification differed based on different criteria. In the Lauren classification, there are two main subtypes- the intestinal type which takes up the majority of GC, and the diffuse type with different histological features and outcomes [2]. In WHO pathological classification, more than 90% of GCs are adenocarcinoma, which can be further divided into tubular, mucinous or papillary type and so on. Less than 10% of GCs are squamous cell carcinoma, carcinoid carcinoma, undifferentiated carcinoma, etc. Current treatment strategies for GC are still based on surgery with conventional chemotherapy and radiotherapy. The overall survival rate of GC is still unsatisfactory, remaining under 30% in some countries, although the surgery equipment and adjuvant therapy developed significantly [3]. However, some breakthroughs were made in GC treatment. For example, the finding of human receptor tyrosine-protein kinase erbB-2 (HER2) as a GC prognostic marker expanded the strategy of GC treatment. More and more similar biomarkers should be investigated and dug out to further find more drug therapies and improve patient's survival rate.

Ecotropic viral integration site 1 (EVI1) is the protein translated by gene MDS1 and EVI1 complex locus (MECOM), functioning as a transcriptional regulator [4]. EVI1 is usually recognized as an oncogene involved in the development, cell proliferation and differentiation of tumor cells [5]. As a nuclear zinc finger protein,

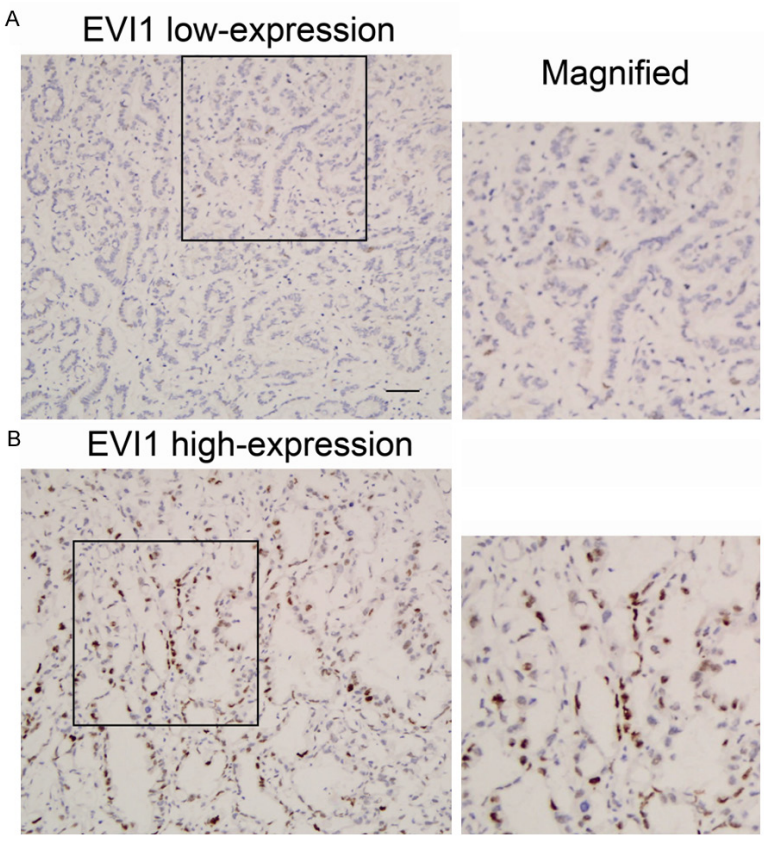


**Figure 1.** Representative immunohistochemical images of EVI1 in gastric cancer. A: Image of EVI1 low-expression. B: Image of EVI1 high-expression; Scale bar: 100 μm.

EVI1 regulates the transcription of target genes via binding to DNA sequences in promoter region [6]. Many cellular signaling pathways were reported to be regulated by EVI, including PI3K-AKT, JNK and TGF-β signaling [5]. In physiological condition, EVI1 plays an important role in proliferation and maintenance of hematopoietic stem cells, therefore, the functions and mechanisms of EVI1 as an oncoprotein are mainly focused in hematopoietic malignancies such as leukemia and lymphoma [7, 8]. The oncogenic role of EVI1 is gradually elucidated in solid tumors such as pancreatic cancer and ependymoma [9, 10]. In gastric cancer cell lines, EVI1 was demonstrated to inhibit TGF-β signaling and thus promote cell growth, which is an important sparkle to gastric cancer study [11]. However, the clinical significance and prognostic value of EVI1 in gastric cancer is still blank.

In our investigation, we detected the expression of EVI1 in 190 cases of gastric cancer and divided these patients into EVI1 high-expression group and EVI1 low-expression group. We further explored the prognostic value of EVI1 by comparing the high-expression group and low-expression group with univariate and multivariate analysis.

## Materials and patients

### *Patients and follow-up*

From 2004 to 2014, 316 patients were diagnosed as gastric adenocarcinoma and underwent radical resection in Yishui Central Hospital, constituting the primary cohort. Total of 190 patients were enrolled into validation cohort according to the criteria: (1) available tissue samples and medical records, (2) available follow-up and post-operation survival time more than 3 months. (3) no severe perioperative complications and other tumors. All the samples were obtained with prior patient consent and approval of the Institutional Clinical Ethics Review Board of Yishui Central Hospital. The tumor TNM stage was identified according to the guideline of 7th American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC).

### *Immunohistochemistry*

All gastric cancer tissues were obtained from the Department of Pathology and the final diagnosis were confirmed by two pathologists. All the tissues were formalin-fixed and paraffin-embedded first in the Department of Pathology. The protocol of immunohistochemistry (IHC) was detailed explained in previous article [12]. Briefly, after deparaffinization with xylene and rehydration with graded ethanol, slides were soaked in 3% hydrogen peroxide for endogenous peroxidase inactivation and then citrate buffer (pH = 6.0) for antigen retrieval. Following by washed with phosphate buffer saline, the tissues were incubated in primary antibody dilution (1:200) (Cell Signaling Technologies,

**Table 1.** Correlations between EVI1 expression and clinicopathologic factors

| Characters | Number | Percentage | EVI1 | | P* |
|---|---|---|---|---|---|
| | | | Low | High | |
| Gender | | | | | |
| Male | 142 | 74.74% | 114 | 28 | 0.831 |
| Female | 48 | 25.26% | 40 | 8 | |
| Age | | | | | |
| < 60 | 81 | 42.63% | 64 | 17 | 0.577 |
| ≥ 60 | 109 | 57.37% | 90 | 19 | |
| Tumor diameter (cm) | | | | | |
| ≤ 5 | 79 | 41.58% | 66 | 13 | 0.574 |
| > 5 | 111 | 58.42% | 88 | 23 | |
| Differentiation | | | | | |
| Well + Moderate | 83 | 43.68% | 66 | 17 | 0.710 |
| Poor | 107 | 56.32% | 88 | 19 | |
| Tumor invasion | | | | | |
| T1 + T2 | 52 | 27.37% | 43 | 9 | 0.837 |
| T3 + T4 | 138 | 72.63% | 111 | 27 | |
| Lymph node metastasis | | | | | |
| No (N0) | 54 | 28.42% | 48 | 6 | 0.101 |
| Yes (N1/2/3) | 136 | 71.58% | 106 | 30 | |
| Distant metastasis | | | | | |
| M0 | 168 | 88.42% | 136 | 32 | 1.000 |
| M1 | 22 | 11.58% | 18 | 4 | |
| TNM stage | | | | | |
| I-II | 72 | 37.89% | 57 | 15 | 0.703 |
| III-IV | 118 | 62.11% | 97 | 21 | |

*means calculated by Chi-square test.

Danvers, MA, USA) overnight at 4°C. All slides were washed by phosphate buffer saline after primary antibody incubation and then incubated in a biotin-labeled secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China) for 30 minutes. After that, streptavidin-peroxidase and 3, 3'-diaminobenzidine substrate (DAB) (Beyotime Institute of Biotechnology, Shanghai, China) was used for antigen visualization.

*Immunohistochemistry score and evaluation*

Slides were blindly scored by two independent pathologists who were previously unaware of the clinical data of the patients. The score system of slides was referring to previous study [13]. Briefly, the final IHC score was the product of staining intensity multiplied positively-stained tumor cells. The score of positively-stained tumor cells was defined as: 0 for < 5% positive tumor cells; 1 for 6%-30% positive tumor cells; 2 for 31%-50% positive tumor cells; 3 for more than 50% positive tumor cells. The staining intensity was defined as: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. ROC curve was drawn and analyzed for setting as the cut-off, which divided the cohort into EVI1 high-expression and EVI1 low-expression group. The cut-off point is the point in ROC curve with the highest sum of sensitivity and specialty.

*Statistical analysis*

All data were analyzed with software SPSS 17.0 (IBM cooperation, Chicago, USA). The correlation between EVI1 expression and clinicopathological factors was analyzed by Chi-square test. Kaplan-Meier method was used to display the overall survival curve and log-rank test was performed to calculate the significance between overall survival rate and clinicopathologic factors including EVI1 expression. Independent prognostic factors were confirmed by Cox proportional hazards regression model. *P* value less than 0.05 was considered as statistically significant.

## Results

*EVI1 is overexpressed in gastric cancer tissues*

Our validation cohort included 190 cases of formalin-fixed and paraffin-bedded gastric cancer tissues, and EVI in these tissues was investigated by IHC to display its expression pattern and location. In our study, we found that EVI1 was mainly expressed in cell nucleus, which was coordinated with its function as transcription regulator. According to the IHC score of EVI1 expression, our cohort was classified into EVI1 high-expression and EVI1 low-expression group (**Figure 1**). In our study, the percentages of EVI1 high-expression and low-expression were 18.94% (36/190) and 81.06% (154/190), respectively.

*Correlation between EVI1 and clinicopathologic factors in gastric cancer*

With Chi-square test, we evaluated the correlation between EVI1 and clinicopathologic factors for better description of the clinical significance

**Table 2.** Univariate analysis

| Characters | 5-year survival rate | P* |
|---|---|---|
| Gender | | |
| Male | 46.1 | 0.156 |
| Female | 49.8 | |
| Age | | |
| < 60 | 45.3 | 0.484 |
| ≥ 60 | 49.6 | |
| Tumor diameter (cm) | | |
| ≤ 5 | 40.8 | 0.292 |
| > 5 | 56.5 | |
| Tumor invasion | | |
| T1 + T2 | 78.3 | 0.006 |
| T3 + T4 | 37.6 | |
| Lymph node invasion | | |
| No (N0) | 59.0 | 0.013 |
| Yes (N1/2/3) | 35.0 | |
| Distant metastasis | | |
| M0 | 49.7 | 0.001 |
| M1 | 36.4 | |
| TNM stage | | |
| I-II | 54.2 | 0.023 |
| III-IV | 40.5 | |
| Differentiation | | |
| Well + Moderate | 52.0 | 0.051 |
| Poor | 42.3 | |
| EVI1 | | |
| Low | 51.5 | 0.012 |
| High | 24.6 | |

*means calculated by log-rank test.

of EVI1 (**Table 1**). The clinicopathologic factors included patients' age, gender, tumor size, differentiation, tumor invasion, lymph node metastasis and distant metastasis. However, no statistically significant correlation between these factors and EVI1 expression was observed in our study, which indicated EVI1 may be an independent factor in gastric cancer progression.

*EVI1 is correlated to overall survival rate*

We performed univariate analysis to explore the prognostic value of EVI1 and other clinicopathologic factors in gastric cancer (**Table 2**). The survival curve was drawn with Kaplan-Meier method while the difference between compared groups was analyzed by log-rank test. In our investigation, EVI1 high-expression was proved to be associated with poor prognosis in gastric cancer (P = 0.012) (**Figure 2**). In addition to EVI1 high-expression, advanced tumor invasion (T stage), positive lymph node invasion (N stage), positive distant metastasis (M stage), and advanced TNM stage were all identified to be associated with unfavorable prognosis significantly. Moreover, tumor differentiation tended to be affect the prognosis, with a statistically insignificant tendency (P = 0.051).

*EVI1 is an independent prognostic factor in gastric cancer*

Multivariate analysis was performed to confirm the prognostic factor in univariate analysis and further identify the independent ones. All the prognostic factors in our experiments were enrolled into the Cox-regression model for multivariate analysis, including EVI1 expression, T stage, N stage, and M stage (**Table 3**). We expanded the criteria to P < 0.1 to include tumor differentiation into our Cox-regression model because tumor differentiation had the most suspicion to be a prognostic factor in univariate analysis (P = 0.051). TNM stage was excluded because of its obvious interaction with T stage, N stage and M stage. As the result, we demonstrated that EVI1 high-expression could be considered as the independent prognostic factor in gastric cancer (P = 0.008, HR = 2.23, 95% CI = 1.23-4.04). Besides EVI1 high-expression, other independent prognostic factors included tumor differentiation (P = 0.022, HR = 1.85, 95% CI = 1.09-3.15), T stage (P = 0.005, HR = 2.93, 95% CI = 1.38-6.21), N stage (P = 0.031, HR = 1.95, 95% CI = 1.06-3.56), and M stage (P < 0.001, HR = 3.81, 95% CI = 1.85-7.82).

## Discussion

In our study, we demonstrated that the oncoprotein EVI1 was significantly associated with overall survival rate and could be identified as an independent prognostic biomarker in patients with gastric cancer for the first time. This may be an important supplement to the study of gastric cancer biomarker and encourage more scientists to focus on the EVI1 function in the oncogenesis, progression and prognosis of gastric cancer.

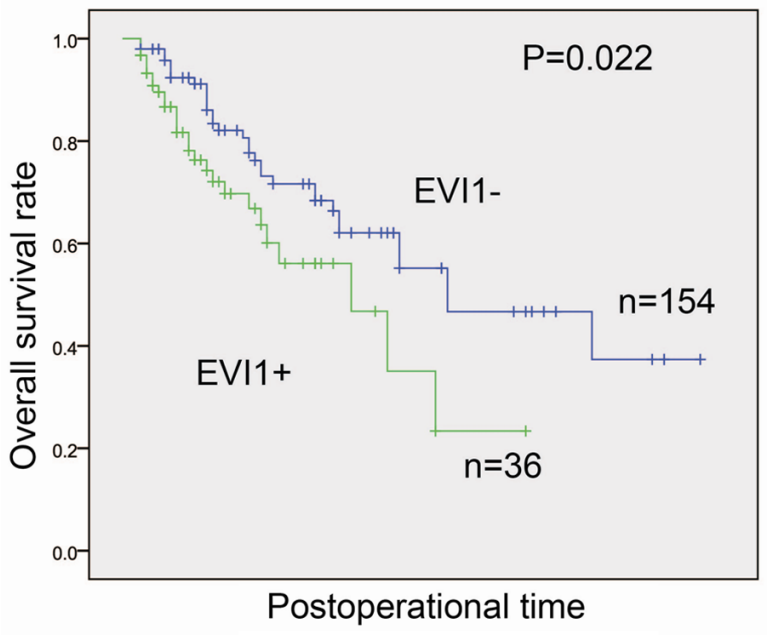


**Figure 2.** Correlation between overall survival rate and EVI1 expression. Survival curves were stratified by EVI1 expression with Kaplan-Meier method and the difference of survival rate was analyzed by log-rank test. Patients with EVI1 high-expression had a significantly poorer overall survival rate than those with EVI1 low-expression (P = 0.012).

**Table 3.** Multivariate analysis

| Characters | HR | 95% CI | P* |
|---|---|---|---|
| Tumor invasion | | | |
| T1 + T2 | 1 | | |
| T3 + T4 | 2.93 | 1.38-6.21 | 0.005 |
| Lymph node invasion | | | |
| No (N0) | 1 | | |
| Yes (N1/2/3) | 1.95 | 1.06-3.56 | 0.031 |
| Distant metastasis | | | |
| M0 | 1 | | |
| M1 | 3.81 | 1.85-7.82 | < 0.001 |
| Differentiation | | | |
| Well + Moderate | 1 | | |
| Poor | 1.85 | 1.09-3.15 | 0.022 |
| EVI1 | | | |
| Low | 1 | | |
| High | 2.23 | 1.23-4.04 | 0.008 |

*means calculated by Cox-regression model.

Previously, most studies of EVI1 as an oncoprotein were focused on myeloid neoplasms such as leukemia [14, 15]. However, more emerging evidence demonstrated the overexpression of EVI1 lead to poor prognosis in solid tumor including prostate cancer and glioblastoma multiforme [13, 16]. Some other studies reported that high frequency of mutations or isoforms of MECOM, the gene translating EVI1, existed in several cancers or cancer cell lines [17, 18]. Taken together, EVI1 may influence cancer oncogenicity, progression or even prognosis in many cellular levels including gene mutation, epigenetic modification, translation or expression directly or by targeting other effector molecules. Our finding that EVI could be considered as an independent prognostic factor in gastric cancer is an important supplement to EVI1 study in solid tumor.

In our study, we identified EVI1 overexpression as a high risk of poorer prognosis in gastric cancer. However, we could not provide the underlying molecular mechanisms in our clinical study. Takahata et al. had explored the function of EVI1 in gastric cell lines previously. In gastric cell line, EVI1 was proved to inhibiting TGF-β signaling, which could suppress tumor growth at early stages of tumorigenesis [11]. The suppression of TGF-β signaling by EVI1 was achieved through multiple mechanisms, mostly by inhibiting SMAD3 promoters [4]. As a transcriptional co-repressor, the molecular interaction network of EVI1 is very complicated. Many molecules were reported to be interacted or regulated by EVI1, such as SUV39H1, SMAD3, TCL1A, CTBP1, etc [19-22]. Our study did not involve the molecular mechanisms of why EVI1 lead to poorer prognosis, but we hope our findings could trigger the interest on EVI1 oncogenic function in gastric cancer, by which new molecular target or even chemotherapy could emerged.

## Disclosure of conflict of interest

None.

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