

Original Article

Loss of E-cadherin expression in bladder cancer is associated with IL6-STAT3 signaling pathway activation

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Abstract: Introduction: Loss of E-cadherin expression is a frequent event in solid tumors including bladder cancer, and contributes to cancer invasion. The aim of this study is to screen regulatory pathways controlling E-cadherin expression in bladder cancer using The Cancer Genome Atlas (TCGA) database and immunohistochemistry staining. Materials and methods: The mRNA profiles of human bladder cancer samples were downloaded from TCGA database. Samples with low and high E-cadherin expression were subjected to gene set enrichment analysis (GSEA) with hallmark and KEGG gene sets. Immunohistochemistry staining was used to confirm the correlation of E-cadherin expression and signaling pathways. Results: Ten Hallmark signaling pathways and 9 KEGG signaling pathways were enriched in CDH1^{low} samples. Interestingly, the hallmark gene sets "IL6-STAT3 signaling" and "IL2-STAT5 signaling" partially overlapped with the KEGG gene set "JAK-STAT-signaling pathway". However, "epithelial mesenchymal transition" gene set was enriched in IL6^{high} but not IL2^{high} samples. Similarly, E-cadherin mRNA expression was reduced in the IL6^{high} group but not in IL2^{high} group. Immunohistochemistry staining for 75 human bladder cancer samples showed that IL6 expression was positively associated with STAT3 Y705 phosphorylation, while negatively associated with E-cadherin expression. A negative association between STAT3 Y705 phosphorylation and E-cadherin expression was observed as well. In addition, protein profiles from TCPA (The Cancer Proteome Atlas) database showed that STAT3 Y705 phosphorylation was associated with E-cadherin loss and worse prognosis. Conclusion: IL6-STAT3 signaling pathway might be a key regulatory signaling pathway for loss of E-cadherin expression in bladder cancer.

Keywords: Bladder cancer, E-cadherin, EMT, IL6, STAT3

Introduction

Bladder cancer is the most common urinary malignant disease with more than 430,000 newly diagnosed cases worldwide each year. About 75% of the cases are non-muscle invasive and the other 25% are muscle-invasive bladder cancer at presentation [1]. Although nearly half of non-muscle-invasive bladder cancers are low grade, some of them can eventually progress to high-grade invasive tumors [2].

Epithelial-to-mesenchymal transition (EMT) is a process that plays essential roles in development, wound healing and tumor invasiveness. EMT is characterized by loss of epithelial marker such as E-cadherin, and acquisition of mesenchymal marker such as N-cadherin. During the process of EMT, cancer cells acquire high

potential of migration and invasion [3]. E-cadherin-mediated cell-cell adhesion plays a central role on the remaining epithelial phenotype. Loss of E-cadherin resulted in EMT and promoted cancer invasion. In bladder cancer, reduced E-cadherin expression was associated with poor prognosis and advanced clinicopathological characteristics [4]. However, the mechanisms of E-cadherin loss in bladder cancer has not been understood clearly.

In recent years, the development of high throughput data collecting equipment collected more and more data from human cancers. More importantly, the development of bioinformatics analysis makes it possible to search clues of key molecules or pathways involved in cancer initiation and progression using the database. In this study, we screened the IL6-

Table 1. Summary of clinical and pathological data

characteristics	Informative cases
Median age (range), y	67.2 (44-85)
Sex	
Male	57
Female	18
Stage	
Tis	9
T1	16
T2	19
T3	25
T4	6
Grade	
G1	8
G2	41
G3	26

STAT3 signaling pathway as a candidate regulator of E-cadherin expression in bladder cancer by gene set enrichment analysis (GSEA) with TCGA database, and confirmed the association of IL6-STAT3 signaling pathway and E-cadherin expression in bladder cancer tissues by immunohistochemistry.

Materials and methods

Database and data analysis

The mRNA profiles used in this study were downloaded from TCGA database (<http://cancergenome.nih.gov>). Information of the samples with high and low (top 25% for each group) E-cadherin, IL6, and IL2 mRNA expression level was collected in this study for GSEA analysis. GSEA was performed using GSEA 3.0 software with hallmark gene sets or KEGG gene sets deposited in the GSEA MSigDB resource. Significance was assumed for $|NSE| > 1$, NOM p value < 0.05 , FDR q value < 0.25 . Correlation of p-STAT3 Y705 and E-cadherin protein expression and survival data were downloaded from TCPA (The Cancer Proteome Atlas) database (<http://tcpaportal.org/tcpa/index.html>).

Reagents

IL6 antibody (ab9324) was purchased from Abcam (Cambridge, UK). E-cadherin antibody (sc-71007) was purchased from Santa Cruz Biotechnology Inc. (CA., USA). p-STAT3 Y705 antibody (#9145) was purchased from Cell

Signaling Technology, Inc. (Beverly, MA, U.S.). Evison immunohistochemistry kit was purchased from Dako Inc. (CA, USA).

Tissue specimens

Three slides of paraffin-embedded tissue microarrays including 87 human bladder transitional cell carcinoma (TCC) tissues were purchased from Superchip (Shanghai, China, Cat: HBl-a-Uro105Sur-01 and OD-CT-UrBl-a-03-002). The samples were obtained from patients who had undergone transurethral resection or partial/radical cystectomy for primary bladder TCC. The slides were stained immunohistochemically for IL6, p-Stat3 Y705 and E-cadherin. Tissues without cancer tissue were excluded in our study. Finally, 75 TCC tissues were used for protein expression analysis. The clinical and pathological data were summarized as **Table 1**.

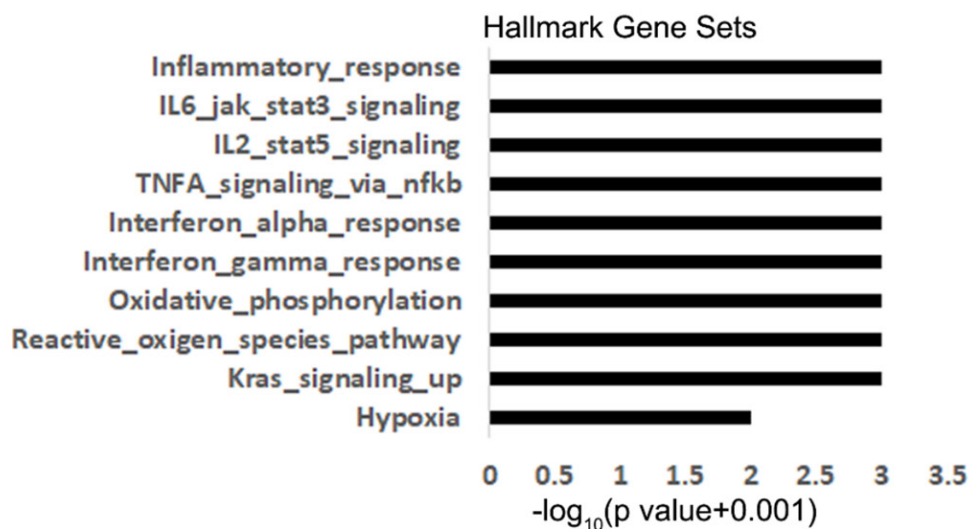
Immunohistochemistry staining

Immunohistochemistry staining was performed with the DAKO EnVision™ System. Tissues were de-paraffinized, rehydrated, and subjected to pressure cooking antigen retrieval for 5 min, endogenous enzyme block for 15 min, primary antibody incubation for 12 h, and incubation with Envision-HRP-labeled secondary antibody for 30 min. Signals were detected by adding substrate hydrogen peroxide using diaminobenzidine (DAB) as a chromogen followed by hematoxylin counterstaining.

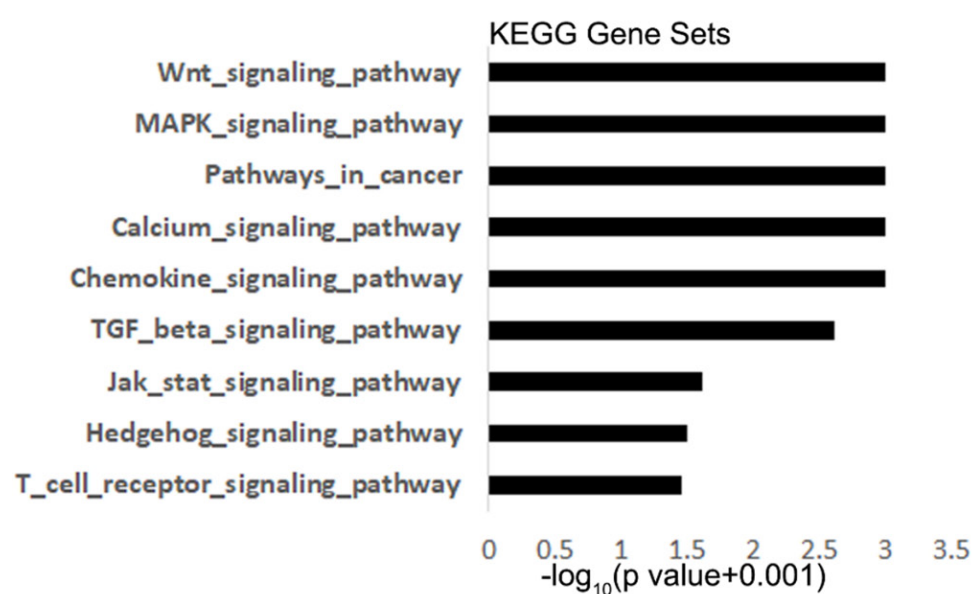
Evaluation of immunohistochemistry staining

Evaluation of immunohistochemistry staining was performed as we previously described [5]. Briefly, each section was examined at a high-power field (400×) in a double-blinded manner by a pathologist. The results collected from every 5 random fields were averaged. Each field was judged by a scoring system that combines intensity and percentage. In each field, staining intensity was scored as follows: 0 for no staining, 1 for weakly positive staining, 2 for moderately positive staining and 3 for strongly positive staining. The staining percentage of the relative number of cells stained was graded as follows: 0 for 0%, 1 for $\leq 25\%$, 2 for 25-50%, 3 for 50-75% and 4 for $\geq 75\%$. The total score of each section was calculated by multiplying the intensity and percentage scores. Sections with total scores ≥ 4 were defined as positive expression.

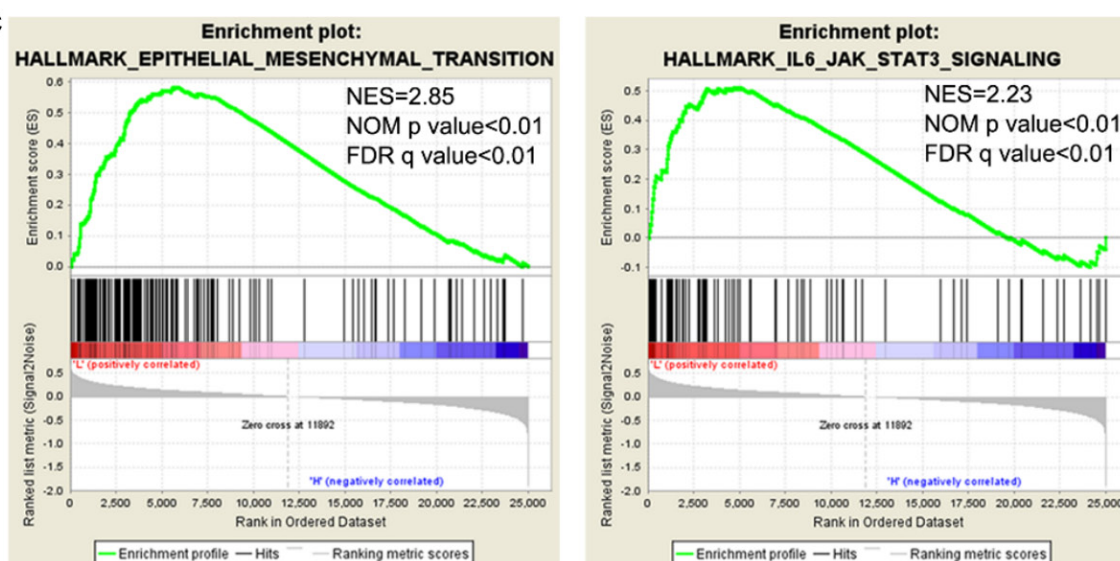
A



B



C



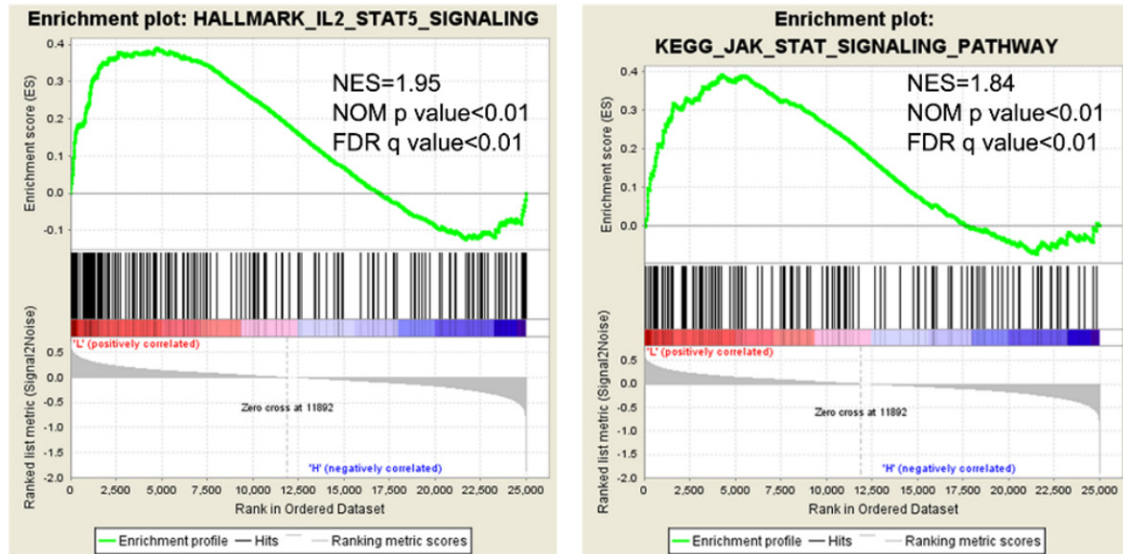


Figure 1. Enrichment of JAK-STAT signaling pathway in E-cadherin^{low} samples in TCGA cohort. The data of TCGA samples with high or low E-cadherin mRNA expression were collected and compared for signaling pathways by GSEA analysis with hallmark gene sets or KEGG gene sets. A. Pathways enriched in E-cadherin^{low} samples analyzed with Hallmark gene sets. B. Pathways enriched in E-cadherin^{low} samples analyzed with KEGG gene sets. C. Enrichment of “epithelial mesenchymal transition”, “IL6-STAT3 signaling”, “IL2-STAT5 signaling” and “JAK-STAT-signaling pathway” in E-cadherin^{low} samples.

Statistical analyses

Statistical analyses were carried out using GraphPad prism 6.0. Correlation analyses were carried out using Pearson’s chi-squared analysis (expected cell frequency >5) or Yates’ continuity corrected chi-square (expected cell frequency <5). Survival analyses were performed using log-rank tests. Significance was assumed for *p* values <0.05.

Results

Enrichment of JAK-STAT signaling pathway in E-cadherin^{low} samples in TCGA cohort

To evaluate the gene set features related to E-cadherin expression, gene set enrichment analysis (GSEA) in TCGA cohort was performed based on the expression level of E-cadherin. Briefly, the data of TCGA samples with high or low E-cadherin mRNA expression were collected and compared for signaling pathways by GSEA analysis with hallmark gene sets or KEGG gene sets. The gene set “epithelial mesenchymal transition” was enriched in E-cadherin^{low} samples, which is consistent with the knowledge that E-cadherin is one of the central pro-

tein for EMT (Figure 1C). Our results showed that 10 hallmark signaling pathways and 9 KEGG signaling pathways were enriched in CDH1^{low} samples (Figure 1A, 1B). Interestingly, the hallmark gene sets “IL6-STAT3 signaling” and “IL2-STAT5 signaling” partially overlapped with the KEGG gene set “JAK-STAT-signaling pathway” (Figure 1C).

High IL6 expression is associated with EMT and E-cadherin loss in TCGA cohort

Next, GSEA was performed to determine the correlation of IL2 and IL6 with EMT and E-cadherin expression in bladder cancer using TCGA database. As shown in Figure 2A and 2B, “epithelial mesenchymal transition” gene set was enriched in IL6^{high} but not IL2^{high} samples. Similarly, E-cadherin mRNA expression in IL6^{high} group was significantly lower than IL6^{low} group (Figure 2C). However, the expression of E-cadherin mRNA showed no difference between IL2^{low} and IL2^{high} group (Figure 2D). Thus, this result indicated that the activation of IL6-STAT3 signaling pathway might confer the reduced E-cadherin expression and EMT in bladder cancer.

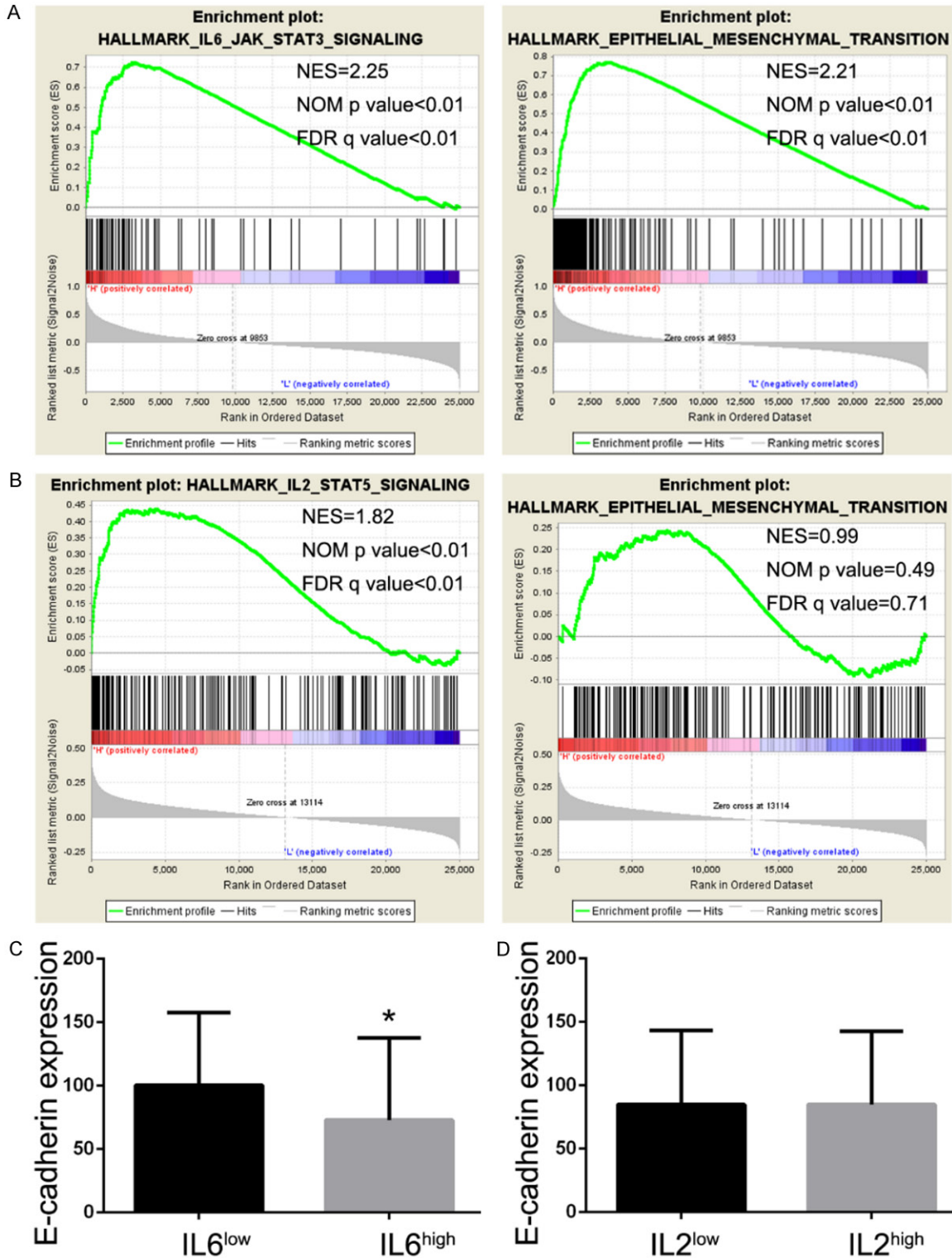


Figure 2. High IL6 expression is associated with EMT and E-cadherin loss in TCGA cohort. A. Enrichment of “epithelial mesenchymal transition” gene set in IL6^{high} samples. B. Enrichment of “epithelial mesenchymal transition” gene set in IL2^{high} samples. C. Comparison of E-cadherin mRNA expression in IL6^{high} and IL6^{low} group. Fragments per kilobase million (FPKM) was used to represent the mRNA expression level. *P<0.05 vs IL6^{low} group. D. Comparison of E-cadherin mRNA expression in IL2^{low} and IL2^{high} group, FPKM was used to represent the mRNA expression level. *P<0.05 vs IL2^{low} group.

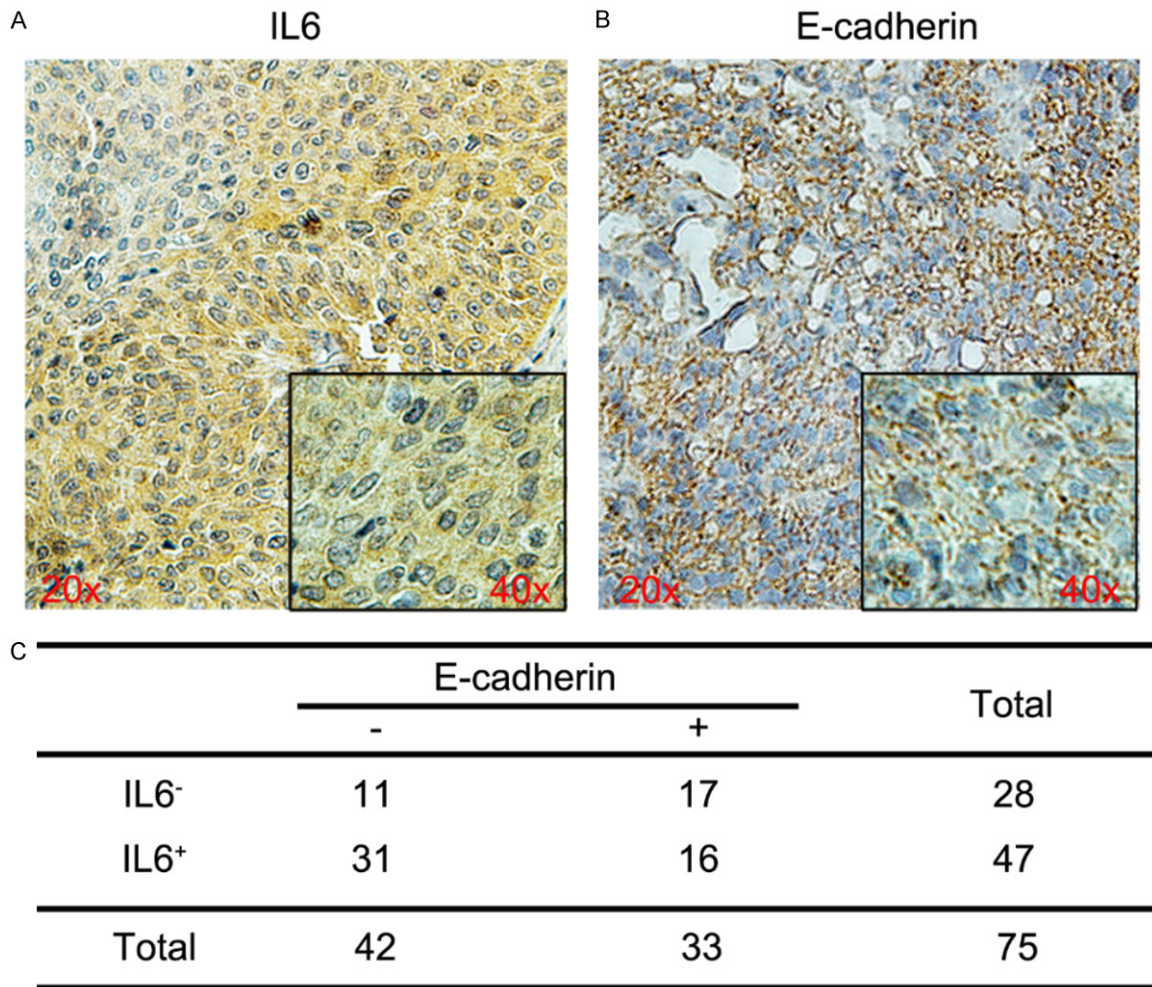


Figure 3. Negative association between E-cadherin expression and IL6 in bladder cancer tissues stained by immunohistochemistry. In total, 75 bladder cancer tissues were immunohistochemically stained for IL6 and E-cadherin expression. A. Representative photo of IL6. B. Representative photo of E-cadherin. C. Statistical analysis for the association between E-cadherin expression and IL6 in bladder cancer, $P < 0.05$.

Negative association between E-cadherin expression and IL6 in bladder cancer tissues stained by immunohistochemistry

To confirm the findings above, 75 bladder cancer tissues were immunohistochemically stained for IL6 and E-cadherin expression (**Figure 3A, 3B**). In our study, 47 (62.7%) bladder cancer tissues were identified as IL6 positive and 33 (44.0%) bladder cancer tissues were identified as E-cadherin positive. There was a significant negative association between E-cadherin and IL6 expression (**Figure 3C**, $P < 0.05$).

Correlation of E-cadherin and IL6-STAT3 signaling pathway in bladder cancer

Phosphorylation of STAT3 Y705 (p-STAT3 Y705) is a key regulator of IL-6-mediated STAT3 acti-

vation. We then detected p-STAT3 Y705 expression in bladder cancer tissues by immunohistochemistry staining (**Figure 4A**). We found that p-STAT3 Y705 expression was positively correlated with IL6 expression but negatively correlated with E-cadherin expression in bladder cancer (**Figure 4B, 4C**, $P < 0.05$). In addition, the analysis from TCPA (The Cancer Proteome Atlas) also showed negative association between p-STAT3 Y705 and E-cadherin in bladder cancer (**Figure 4D**, $P < 0.05$). Patients in the high p-STAT3 Y705 expression group had worse prognosis (**Figure 4E**, $P < 0.05$).

Discussion

The tumor suppressor E-cadherin is a key molecular for cell-cell adhesion. Loss of E-cadherin enables cancer cells to invade neighbor-

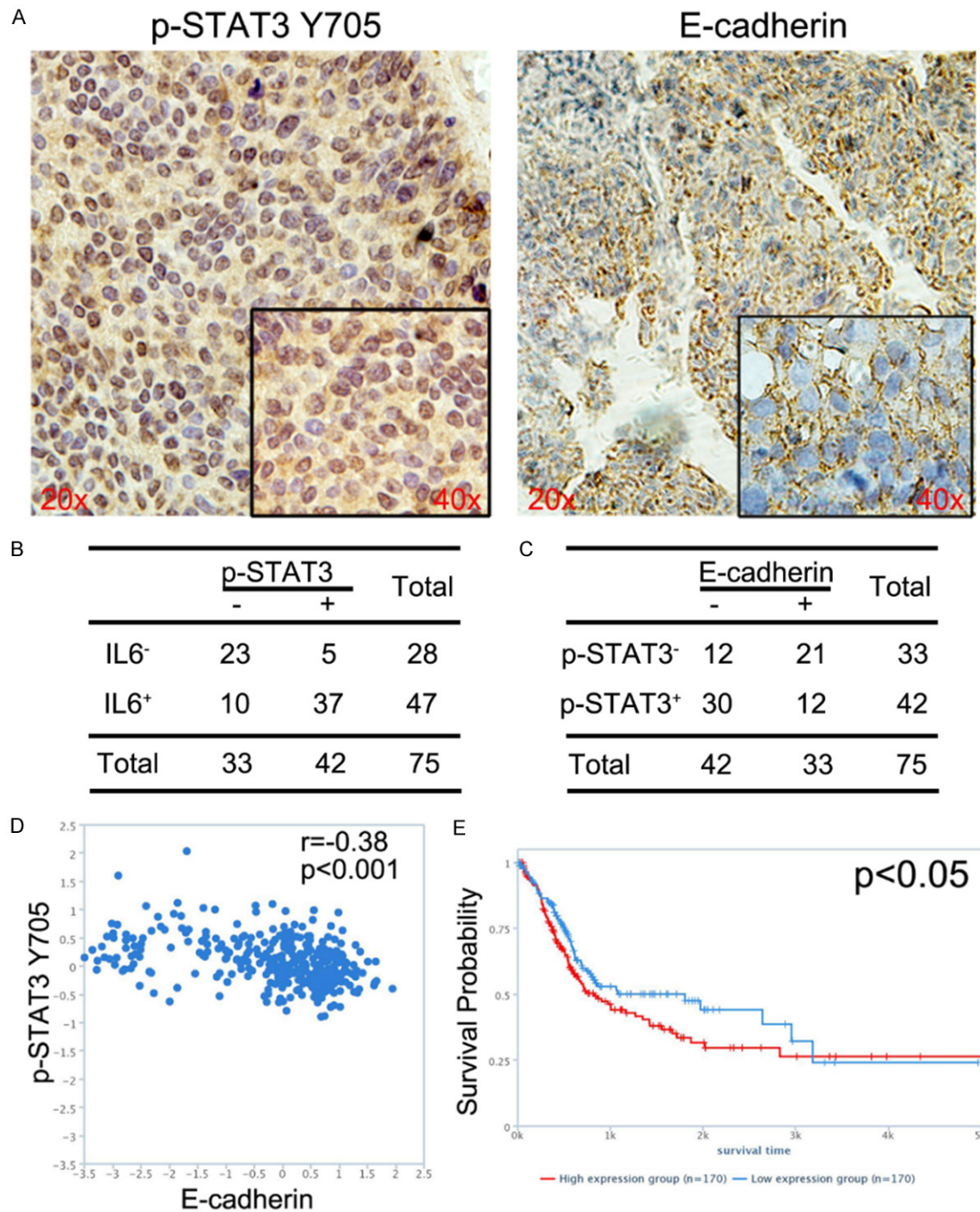


Figure 4. Correlation of E-cadherin and IL6-STAT3 signaling pathway in bladder cancer. A. Representative photos of p-STAT3 Y705 and E-cadherin expression in bladder cancer tissues by immunohistochemistry staining. B. Statistical analysis for the association between IL6 and p-STAT3 Y705 expression in bladder cancer, $P < 0.05$. C. Statistical analysis for the association between E-cadherin and p-STAT3 Y705 expression in bladder cancer, $P < 0.05$. D. Negative association between p-STAT3 Y705 and E-cadherin in bladder cancer analyzed with TCGA database, $P < 0.05$. E. High p-STAT3 Y705 expression was associated with worse prognosis in bladder cancer analyzed with TCGA database, $P < 0.05$.

ing tissues and, ultimately, to metastasize. Knockdown of E-cadherin expression in human

cancer cells is sufficient to induce EMT and a high invasive potential [6]. The loss of

E-cadherin expression in bladder cancer is associated with worse progression-free survival [7]. In the past years, the mechanisms of aberrant E-cadherin expression in bladder cancer, such as DNA methylation [8] and transcription factor Twist [9], were preliminarily investigated. However, the regulatory signaling pathway of E-cadherin expression in bladder cancer remains unknown. Since many inhibitors of signaling pathways have been developed, exploring regulatory signaling pathway of E-cadherin expression is necessary and may provide therapeutic opportunities for bladder cancer treatment.

In this study, we firstly analyzed the gene sets enrichment in E-cadherin^{low} bladder cancer samples using RNA expression profile from TCGA database. Our results showed significant activation of “IL6-STAT3 signaling pathway”, “IL2-STAT5 signaling pathway” and “JAK-STAT signaling pathway” in E-cadherin^{low} bladder cancer. However, when GSEA was performed for gene set enrichment in IL6^{high} and IL2^{high} bladder cancer tissues, we found that the gene set “epithelial mesenchymal transition” was enriched only in IL6^{high} samples. Similarly, we observed reduced E-cadherin mRNA expression in IL6^{high} but not in IL2^{high} bladder cancer tissues. Thus, we focused on IL6-STAT3 signaling pathway in the following study.

Interleukin-6 (IL6) is a multifunctional cytokine widely expressed in normal and cancer cells. As a pro-inflammatory cytokine, IL6 plays a vital role in host defense against pathogens and acute stress [10]. Nevertheless, aberrant expression of IL-6 might cause human diseases. IL6 could activate the transcription factor signal transducer and activator of transcription 3 (STAT3) by a cascade through IL6R and JAK2. In prostate cancer, IL6 promotes cancer progression and metastasis by activating STAT3 [11].

STAT3 is a member of the STAT protein family activated in many cancer types and involved in tumorigenesis, cell proliferation, cell survival and angiogenesis [12]. Upstream cytokines or growth factors can activate STAT3 via JAK/src kinase mediated STAT3 phosphorylation, dimerization and translocation. Tyr 705 is the most well-known phosphorylation site and a key regulator of its transcriptional activity [13].

It is reported that p-STAT3 Y705 was overexpressed in bladder cancer [14] and inhibition of STAT3 impaired bladder cancer migration [15], however, the role of STAT3 on E-cadherin expression and EMT in bladder cancer remains unknown.

To confirm the results of our bio-information analysis, bladder cancer tissue microarray slides were used to detect the expression of IL6, p-STAT3 Y705 and E-cadherin with immunohistochemistry staining. We found that the expression of p-STAT3 Y705 in bladder cancer was positively associated with IL6, indicating that STAT3 is a downstream effector of IL6 in bladder cancer. In addition, E-cadherin expression in bladder cancer was negatively associated with both IL6 and p-STAT3 Y705, suggesting that E-cadherin expression might be inhibited by IL6-STAT3 signaling pathway.

As one part of the TCGA project, the TCPA (The Cancer Proteome Atlas) database was established for protein expression data over a large number of tumor and cell line samples using reverse-phase protein arrays (RPPAs) [16]. The TCPA database includes 344 bladder cancer tissues and expression profile of 223 proteins. We found that there was a significant negative association between E-cadherin and p-STAT3 Y705. Furthermore, high p-STAT3 Y705 expression predicts worse prognosis in bladder cancer. This finding was a complement for our TCGA and immunohistochemistry analysis.

Taken together, in this study, we screened IL6-STAT3 signaling pathway as a candidate regulator of E-cadherin expression in bladder cancer by GSEA with TCGA database, and confirmed the association of IL6-STAT3 signaling pathway and E-cadherin expression in bladder cancer tissues by immunohistochemistry. In vitro studies are needed to determine the role of IL6-STAT3 signaling pathway on bladder cancer invasion and E-cadherin expression in the future. Therapeutic approaches targeting IL6-STAT3 signaling pathway might be promising strategies for bladder cancer therapy.

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Disclosure of conflict of interest

None.

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