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Expression analysis of the TGF-β/SMAD target genes in adenocarcinoma of esophagogastric junction

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Abstract: The TGF-β/SMAD signaling pathway is found to play pivotal roles in cell growth, differentiation and tumorigenesis. Its target genes are closely related to the biological behaviors of some malignancies. The aim of this study was to analyze the expression of the target genes of this pathway, including growth-related c-myc, p21, p15, and metastasis-related Snail, ZEB1 and Twist1 in the adenocarcinomas of esophagogastric junction (AEJ) tissues. Clinical esophagogastric junction tissues from 25 cases of AEJ patients and 10 cases of non-tumorous tissues from the same site were collected. Quantitative real-time poly chain reactions were carried out to analyze the expression of the above referred target genes of TGF-β/SMAD pathway. A notable up-regulation in the mRNA expression of p15, Snail, ZEB1, down-regulation of c-myc, was found whereas there were no significant change of p21 and Twist1. The findings suggests that the TGF-β/SMAD pathway might be abnormally activated in AEJ since most of the target genes of this pathway exhibited altered expression at mRNA level.

Keywords: Adenocarcinoma of esophagogastric junction, TGF-b/SMAD pathway

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1 Introduction

Transforming Growth Factor b (TGF-β), was initially discovered and hence named due to its ability to induce growth of fibroblasts in soft aga, which is a feature associated with oncogenes [1,2]. However, through subsequent studies, people found a contrast function of this molecule, for instance, it has cytostatic effects on normal non-tumoral cells [3]. The roles of TGF-β are is controversial and dependents on different cellular context: in normal non-cancerous cells and tumor initiation phases, TGF-β acts as a suppressor mainly through preventing cell cycle arrest, whereas in the late or advanced phases of tumor progression, TGF-β may transit into a promoter via enhancing invasion, motility and metastasis [4-6]. Hence, TGF-β and components of its signal transduction pathway can play dual roles in the early development and advanced malignant progression of human malignancies.

There are three isoforms of TGF-β ligands: TGF-β1, TGF-β2 and TGF-β3, binding with TGF-β type 1 and type 2 receptors (TβRI and TβRII, respectively). TGF-β signal transduction acts through binding and activation of TβRI and TβRII trans-membrane serine/threonine kinase receptors, which leads to the phosphorylation of SMAD2 and3. This in association with SMAD4 subsequently translocates into the nucleus, finally leading to transcriptional activations of downstream targets [7-8].

The tumor suppressor role of TGF-β in many cell types is mediated by downregulation of c-myc oncogene expression in a SMAD-dependent manner, and by upregulation of $p15^{INK4B}$ and $p21^{CIP1}$. This further leads to cyclin-dependent kinases (CDKs) inhibition [9,10]. Epithelial-to-mesenchymal transition (EMT) is a vital process for morphogenesis. The EMT transcription factors targeted by TGF-β include Snail [11], two-handed zinc finger factors ZEB1 [12] and ZBE2 [13], basic helix-loop-helix Twist1 [14] and Twist2, and so on. Therefore, one of the main mechanisms by which TGF-β promotes cell migration, invasion, and metastasis is through induction of EMT [6,15].

Adenocarcinomas of the esophagogastric junction (AEJ) is now one of the frequent types of gastric diseases with a dramatic increase in the incidence worldwide, especially over recent years [16]. The current prognosis of AEJ patients is not satisfactory, and has not improved. Most of these patients are diagnosed at an advanced stage. Although the roles of TGF-β/SMAD signaling pathway have been extensively discussed in a wide range of human cancers, the expression and function of the components of this pathway remain largely unknown in AEJ.

In this study, we set out to analyze the expression levels of selected targets of this pathway, and to evaluate the possibility and provide directions of utilizing this pathway for future AEJ targeted therapy.

2 Materials and Methods

2.1 Patients and Samples

Twenty-five adenocarcinoma of esophagogastric junction tissue samples and ten normal esophagogastric tissue samples (collected from September 2014 to August 2015) were used in this study and were obtained from First Affiliated Hospital of Anhui Medical University. Each sample was snap-frozen in liquid nitrogen and stored at −80°C prior to RNA isolation and quantitative RT-PCR analysis. All patients provided written informed consent. This study was approved by the Human Ethics Committee of First Affiliated Hospital of Anhui Medical University.

2.2 RNA isolation and cDNA synthesis

RNA was isolated from the above collected tissues using TRIzol reagent (Takara). The quantitation and quality of RNA was determined using Nanodrop 2000c spectrophotometer. Then, 2μg RNA reversely transcribed from each sample to synthesis cDNA using MLV reserve transcriptase (Promega).

2.3 Quantitative RT-PCR

For the quantitative RT-PCR analysis, the reverse-transcribed cDNA was subjected to RT-PCR using SYBR Green master mix (Toyobo) and the ABI7900 quantitative RT-PCR fast system (ABI). Each experiment was performed in triplicates and was repeated at least three times. GAPDH was used as internal controls.

2.4 Statistical Analysis

All results were expressed as mean ± s.d. Data analysis was performed by SAS v8.0. Statistical significance was analyzed using Grouped t-test. Differences with $P<0.05$ were considered as statistically significant.

3 Results

3.1 c-myc was decreased in tested AEJ tissues

Since it was well established that c-myc was negatively targeted by TGF-b/SMAD pathway in various cancers, we first of all applied quantitative RT-PCR to measure its expression in 25 AEJ tissues and 10 non-tumoral esophagogastric tissues. As shown in Figure 1 A and 1B, c-myc was significantly decreased in these AEJ tissues than the control group, in which gapdh was used as normalization ($P<0.01$).

3.2 Expression of p15 and p21

We also analyze the negative modulators of cell cycle progression, p15 and p21 expression levels in the above tissues. We found that for p15, we confirmed a remarkably up-regulation mRNA expression in the AEJ tissues ($P<0.001$). However, for p21, we observed no change at its expression (Figure 2). Although both of p15 and p21 have been clearly characterized as direct transcription targets of SMAD4, we did not observe similar expression patterns in these AEJ tissues.

3.3 Expression of Snail, ZEB1 and Twist1

Finally, we detect the EMT-related the expression of transcription factors targeted by TGF-β, including Snail, ZEB1 and Twist1 by quantitative RT-PCR. As shown in Figure 3, we found elevated expression of Snail ($P<0.001$) and ZEB1 ($P<0.001$), but not significant change for Twist1 in these AEJ tissues and the control tissues. Therefore, we speculated that TGF-β/SMAD mediated EMT and metastasis were

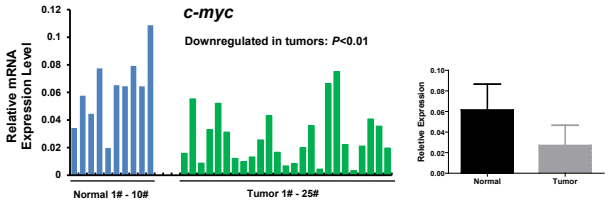

Figure 1: *c-myc* expression in adenocarcinoma of esophagogastric junction tissue samples.

Different expression level of ***c-myc*** gene was tested between adenocarcinoma of esophagogastric junction tissues (Tumor 1#-25#) and non-tumoresophagogastric tissues (Normal 1#-10#). The expression of ***c-myc*** was normalized to ***gapdh***.Green bars represented downregulation in the tumor tissues. Histogram plot for the results was shown in the right panel. The statistical difference between the two groups was analyzed with grouped t-test (n1 = 10, n2 = 25, p < 0.01).

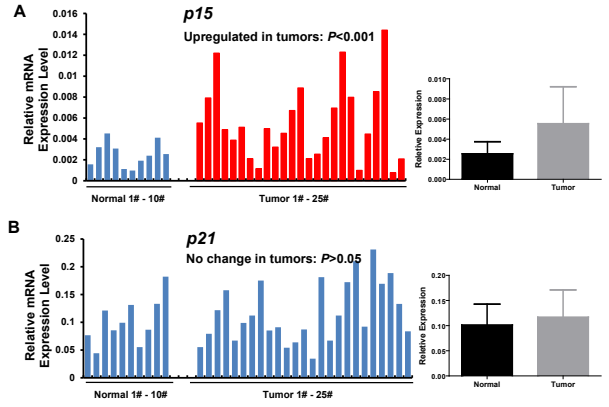

Figure 2: *p15* and *p21* expression in adenocarcinoma of esophagogastric junction tissue samples.

Different expression levels of p15 (A) and p21 (B) between adenocarcinoma of esophagogastric junction tissues (Tumor 1#-25#) and non-tumor esophagogastric tissues (Normal 1#-10#) were tested by qRT-PCR. The expression of p15 and p21 was normalized to gapdh. Red bars represented upregulation in the tumor tissues. Histogram plot for the results in the right panels. The statistical differences between samples were analyzed with grouped t-test (n1 = 10, n2 = 25).

enhanced in the AEJ cells, since most of the responsible transcription factors exhibited abnormal up-regulation.

4 Discussion

The transduction of TGF-β signaling includes complex formation of SMAD2, SMAD3 and SMAD4, nuclear

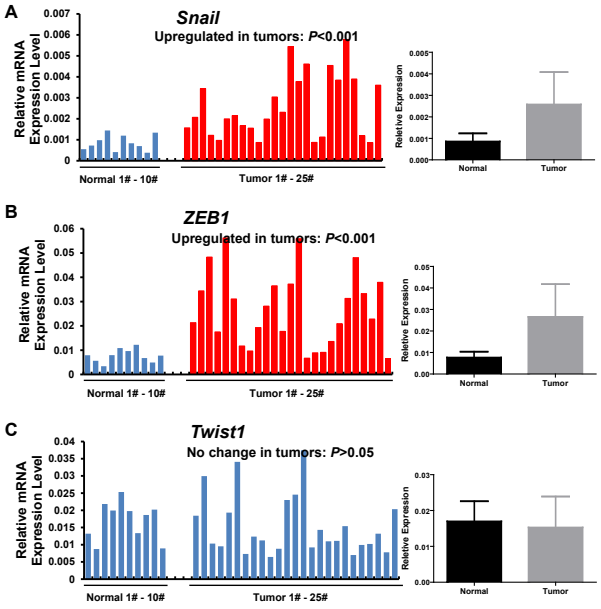

Figure 3: *Snail, ZEB1* and *Twist1* expression in **adenocarcinoma of esophagogastric junction tissue samples**

Different expression levels of Snail (A), ZEB1 (B)and Twist1 (C) genes between adenocarcinoma of esophagogastric junction tissues (Tumor 1#-25#) and non-tumor esophagogastric tissues (Normal 1#-10#) were tested by qRT-PCR. The expression of Snail, ZEB1 and Twist1 was normalized to gapdh. Red bars represented upregulation in the tumor tissues. Histogram plot for the results in the right panels. The statistical differences between samples were analyzed with grouped t-test (n1 = 10, n2 = 25).

translocation of this complex and eventual activation of target genes [17]. Among these target genes, it was already addressed that c-myc was repressed, and p15, p21 as well as EMT-related Snail1, ZEB1, Twist1 could be up-regulated and activated in a wide range of human cancer cells. However, whether it was the same in AEJ remains largely unknown.

In the present study, we collected 25 AEJ tissues and 10 non-tumoral esophagogastric tissues, and analyze the mRNA expression of the above referred targets of TGF-b/SMAD signaling pathway by quantitative RT-PCR. Our results showed that c-myc was indeed down-regulated in AEJ tissues, and p15, Snail, ZEB1 were also up-regulated. However, the expression of p21 and Twist1 were not obviously changed in our system. We explained these results from the following aspects: 1) p21 and Twist1 might not be direct targets of SMAD4 in AEJ cells. 2) Besides the TGF-b signaling, there might be other signaling or factors that impact the expression of the two molecules. Therefore, the down-regulation of c-myc and up-regulation of p15, EMT-related Snail1, ZEB1 were net effects of multiple upstream signaling pathways.

In conclusion, we found that most of the TGF-β/SMAD signaling targts exhibited abnormal expression at mRNA levels, which indicated an activation of this pathway in AEJ cells. However, whether the altered mRNA expression of these targets could exert functional outputs in AEJ cells requires further investigations. Although we were merely able to describe clues regarding the TGF-β/SMAD targts expression at the mRNA levels, the findings still provides a possibility of utilizing this dual-role pathway into the AEJ treatment development.

Conflict of interest statement: Authors state no conflict of interest.

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