Snail Enhances Glycolysis in the Epithelial-Mesenchymal Transition Process by Targeting FBP1 in Gastric Cancer

Jie Yu, Jing Li, Yong Chen, Wenmiao Cao, Yuanyuan Lu, Jianqi Yang, Enmin Xing

Department of Oncology, Clinical Medical College of Yangzhou University, Subei People’s Hospital of Jiangsu Province, Yangzhou, Department of Oncology, The Affiliated hospital of Yangzhou University, Yangzhou University, Yangzhou NO.1 People’s Hospital, Yangzhou, China

Key Words
Snail • FBP1 • Glycolysis • Epithelial-mesenchymal transition • Gastric cancer

Abstract
Background: Snail is a key regulator of epithelial-mesenchymal transition (EMT) in cancer. However, the regulatory role and underlying mechanisms of Snail in gastric cancer metabolism are unknown. In this study, we characterized the regulation of aerobic glycolysis by Snail in gastric cancer. Methods: The impact of Snail on glucose metabolism was studied in vitro. Combining maximum standardized uptake value (SUVmax), which was obtained preoperatively via a PET/CT scan, with immunohistochemistry staining, we further analyzed the correlation between SUVmax and Snail expression in gastric cancer tissues. Results: Increased expression of Snail promoted lactate production, glucose utilization, and decreased FBP1 expression at both mRNA and protein level. The expression level of Snail was positively associated with SUVmax in gastric cancer patients (P=0.022). Snail and FBP1 expression were inversely correlated at both mRNA and protein level (P=0.002 and P=0.015 respectively) in gastric cancer tissues. Further studies demonstrated that Snail inhibited the FBP1 gene expression at the transcriptional level. Restoring FBP1 expression reversed the effects of glycolysis and EMT induced by Snail in gastric cancer cells. Conclusions: Our results thus reveal that Snail serves as a positive regulator of glucose metabolism through regulation of the FBP1 in gastric cancer. Disrupting the Snail-FBP1 signaling axis may be effective to prevent primary tumor EMT and glycolysis process.

Introduction
Gastric cancers is one of the most common and deadliest malignancies globally, especially in east Asia [1]. Despite recent improvement in diagnosis and therapeutic methods, the overall prognosis is still poor and the 5-year survival rate for patients with gastric cancer...
has remained 20–25% [2]. Approximately 50% of gastric cancer patients suffer from tumor recurrences or metastases after curative resection [3]. Thus, a better understanding of the mechanisms that promote the progression of gastric cancer will be helpful to improve the prevention, diagnosis and treatment of gastric cancer.

Epithelial-mesenchymal transition (EMT), a well-characterized embryological process, has been identified to play a critical role in tumor progression, including invasion and metastasis, by which cancer cells could gain aggressive characteristics [4, 5]. In the process of EMT, the hallmark is the loss of epithelial cells phenotypic characteristics by losing their cell polarity and the epithelial markers (E-cadherin) and gaining of mesenchymal cells characteristics through acquiring the mesenchymal markers (N-cadherin, vimentin). Therefore, these transformed epithelial cells acquire fibroblast-like properties and exhibit reduced cell-cell adhesion and increased motility [4, 6, 7]. The enhanced motility and invasiveness afforded by EMT is critical in the initiation of metastasis for cancer progression [4, 8, 9]. However, the metastasis process induced by EMT is a highly inefficient process during cancer progression [10]. Experimental evidence indicated that only 0.02% of B16F1 murine melanoma cells injected intraportally was capable of making a macroscopic tumor [11].

The matrix detached cancer cells rapidly leads to metabolic stress, and metabolic reprogramming is required for cancer cell survival [10, 12]. Cancer cells convert glucose to lactate through aerobic glycolysis as main energy supply, and this phenomenon was also named as Warburg effect [13]. However, the reprogram of glucose metabolism in EMT process is remained unknown in gastric cancer. The aim of the present study was to assess the effect of the important EMT regulator Snail on glycolysis and explore its underlying molecular mechanisms.

Materials and Methods

Cell lines and transfection

Human gastric adenocarcinoma cell lines, AGS and MGC-803 were used in this study. The cell lines were originally obtained from Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China), and were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C with humidified 5% CO₂.

2 × 10⁵ cells were plated in 6-well plates and transfected with Snail or FBP1 expression vectors or their empty control using Lipofectamine® 3000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. The coding sequences Snail and FBP1 were amplified by PCR and inserted into pCDH-CMV-MCS-EF1-Puro vector to generate Snail or FBP1 overexpression vectors.

RNA extract and quantitative real-time PCR

Total RNA was extracted from gastric cancer cell lines using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using the PrimeScript™ RT Master Mix (RR036A, Takara), and quantitative real time PCR was performed using SYBR Premix Ex Taq II (TaKaRa). All reactions were performed in triplicate. The sequences of the PCR primers were listed in Table 1. β-actin was used as an internal reference. The relative expression levels were calculated using the RQ value.

Western blot

Total proteins were isolated by lysing cells in ice-cold radioimmunoprecipitation (RIPA) buffer containing protease and phosphatase inhibitors (Roche). Total proteins were separated by SDS-PAGE gel and blotted onto PVDF membranes (Bio-rad). After blocked with 5% non-fat milk, the membranes were probed with primary antibodies, anti-Snail rabbit polyclonal antibody (1: 1000 dilution; Abcam, USA), anti-FBP1 polyclonal antibody (1: 1000 dilution; Abcam, USA), anti-E-cadherin (1: 1, 000 dilution; Abcam, USA), anti-N-cadherin (1:1000 dilution; Abcam, USA), and Vimentin (1: 1000 dilution; Abcam, USA). After being thoroughly washed, membranes were further incubated with corresponding secondary antibodies. Finally, the bands were visualized using enhanced chemoluminescence (Pierce, Thermo Scientific, USA).
**Immunohistochemistry (IHC)**

A total of 101 patients who had a histological diagnosis of gastric adenocarcinoma and received initial radical surgery were enrolled in this study. Thirty-two gastric cancer tissues with PET/CT scan results from the same patient were studied, and the remaining 69 gastric cancer tissues from surgical resection were also collected for studying the relationship between Snail and FBP1. None of these patients included in the study had received neoadjuvant therapy. All the subjects involved in this study provided written informed consent. This project was approved by the Ethics Committee of Clinical Medical College of Yangzhou University. The paraffin-embedded primary tumor tissue sections (4 μm in thickness) were subjected to IHC assays as previously described [14]. Briefly, paraffin sections were baked overnight at 50°C, de-paraffinized in xylene, rehydrated through graded ethanol, quenched for endogenous peroxidase activity in 0.3% hydrogen peroxide at 37°C for 15 min, and processed for antigen retrieval by high pressure cooking in citrate antigen retrieval solution (pH = 6.0) for about 10 min. Sections were incubated at 37°C for 1.5 h with rabbit polyclonal antibodies against Snail (1:200, ab109020, Abcam, Cambridge, MA, USA) and FBP1 (1:100, ab109020, Abcam, Cambridge, MA, USA) in a moist chamber. Immunostaining was performed using the EnVision+System-HRP (AEC) (K4005, Dako, Glostrup, Denmark), which resulted in a brown-colored precipitate at the antigen site. Subsequently, sections were counterstained with hematoxylin (Sigma-Aldrich, St Louis, MO, USA) and mounted in a non-aqueous mounting medium. All runs included a no primary antibody control.

The immunohistochemically stained tissue sections were scored separately by two pathologists blinded to the clinicopathological parameters. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) or 3 (strong). Extent of staining was scored as 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%) and 4 (>75%) according to the percentages of the positive staining areas in relation to the whole carcinoma area. Scores for staining intensity and percentage positivity of cells were then multiplied to generate the immunoreactivity score (IRS) for each case. Samples having a final staining score of ≤ 4 were considered to be low and those with score of > 4 were considered to be high [15].

**Glycolysis analysis**

Glucose Uptake Colorimetric Assay Kits (Biovision) and Lactate Colorimetric Assay Kits (Biovision) were purchased to examine the glycolysis process in gastric cancer cells, according to the manufacturer’s protocols.

**Luciferase assays**

For the luciferase assays, the FBP1 promoter was cloned into the pGL3 basic vector (Promega, Madison, WI, USA). Then, AGS and MGC803 cells (8 × 10^5 cells/well) were cultured in 96-well plates and co-transfected with the pGL3-FBP1, pCDH-Snail/pCDH-vector, and renilla plasmid using Lipofectamine™ 3000 (Invitrogen, USA). Forty-eight hours after transfection, cells were lysed using 20 μl of passive lysis buffer. Next, a dual-luciferase assay was carried out using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA). The firefly luciferase activity of transfected cells was normalized to the renilla luciferase activity for each sample.

**Table 1. Primers for quantitative real-time PCR analysis**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5'to3')</th>
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<tbody>
<tr>
<td>FBP1</td>
<td>AGTGGCGACCAGTCATGGAG</td>
</tr>
<tr>
<td>FBP1R</td>
<td>TATGTTGCGAATGCTTTCTCAG</td>
</tr>
<tr>
<td>FBP1F</td>
<td>GATGGCGACCAGTCATGGAG</td>
</tr>
<tr>
<td>FBP1R</td>
<td>TATGTTGCGAATGCTTTCTCAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GACCCATGCTTCAGCTGCTAA</td>
</tr>
<tr>
<td>GAPDHF</td>
<td>GACCCATGCTTCAGCTGCTAA</td>
</tr>
<tr>
<td>GAPDHR</td>
<td>GACCCATGCTTCAGCTGCTAA</td>
</tr>
<tr>
<td>PGK1</td>
<td>CAGGTGGAAGCCGAAGGCAAC</td>
</tr>
<tr>
<td>PGK1R</td>
<td>GCCCTCTCTCTGGAGATATTCC</td>
</tr>
<tr>
<td>PGK1F</td>
<td>CAGGTGGAAGCCGAAGGCAAC</td>
</tr>
<tr>
<td>PGK1R</td>
<td>GCCCTCTCTCTGGAGATATTCC</td>
</tr>
<tr>
<td>PGK1F</td>
<td>CAGGTGGAAGCCGAAGGCAAC</td>
</tr>
<tr>
<td>LDHA</td>
<td>TGGAGATCTTCCATGGTCTAGG</td>
</tr>
<tr>
<td>LDHAR</td>
<td>TGCCCTTCTCTCTCTGCTAGT</td>
</tr>
<tr>
<td>LDHAR</td>
<td>TGCCCTTCTCTCTCTGCTAGT</td>
</tr>
<tr>
<td>LDHBF</td>
<td>GAGAGGATATCCTGATGTTGG</td>
</tr>
<tr>
<td>LDHBR</td>
<td>CCCTCCTCTCTCTCTGCTAGG</td>
</tr>
<tr>
<td>PDK1F</td>
<td>CCCTCCTCTCTCTCTGCTAGG</td>
</tr>
<tr>
<td>PDK1R</td>
<td>CCCTCCTCTCTCTCTGCTAGG</td>
</tr>
<tr>
<td>actinF</td>
<td>GATGGAGGCGCCGATCCACAGG</td>
</tr>
<tr>
<td>actinR</td>
<td>GATGGAGGCGCCGATCCACAGG</td>
</tr>
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out as directed by the manufacturer (Promega, Madison, WI, USA). The ratio of firefly to Renilla luciferase activity was used to express luciferase activities. All experiments were performed in triplicate.

**Statistical analysis**

All data are presented as the mean ± SD. Experiments were repeated at least three times. One-way analysis of variance was used to evaluate the data from functional study. Chi-square test was used to analyze the relationship between Snail and FBP1 expressions. SPSS version 21.0 software (IBM) was used for the data analysis. Differences were considered significant when $P < 0.05$.

**Results**

**Snail enhanced glycolysis in gastric cancer cells**

Snail is one of the key regulators of EMT process, and the aerobic glycolysis is the primary aspect of altered in cancer metabolism. Hence, we attempted to explore the effect of Snail on metabolism regulation of glycolysis in gastric cancer cells. We transfected AGS and MGC803 cells with Snail plasmid and empty vector. As shown in Fig. 1a, Snail could successfully induced EMT process as indicated by decreased expression of epithelial markers E-cadherin and increased expression of mesenchymal markers vimentin detected by Western blot. Meanwhile, Snail also could significantly increase cancer cells’ glucose uptake and lactate production through glucose metabolism analysis ($P<0.05$, Fig. 1b).

**Snail expression correlated with glycolysis in patients with gastric cancer**

As positron emission tomography (PET) technology using 18F-fluoro-2-deoxyglucose (FDG) is a manifestation of glycolysis in vivo [16, 17], the SUVmax has been widely used as a surrogate marker for the prognosis of disease in numerous types of cancer, including gastric cancer [18]. To confirm the relationship between Snail and glucose metabolism, we further analyzed the correlation between the Snail IHC staining and the PET/CT SUVmax data from gastric cancer patients. As expected, patients with high expression of Snail exhibited a higher SUVmax value (Fig. 2a), and the correlation is statistically significant ($P=0.022$, Fig. 2b). These results indicate that Snail might play a critical role in glucose metabolism in gastric cancer.

**Transcriptional activation of FBP1 expression is inhibited by Snail in gastric cancer cells**

Given that we observed a correlation between Snail expression and glycolysis activity, we then detected the mRNA levels of glycolysis related enzymes in Snail transfected cells. We found that most of the glycolytic enzyme mRNA expressions were changed after transfection, with the most significantly decreased FBP1 in both AGS and MGC803 Snail-transfected cells (Fig. 3a). These results were further validated by Western blot analysis for FBP1 expression. Ectopic Snail expression could significantly decreased FBP1 protein expression (Fig. 3b). Therefore, we assumed that the effect of Snail on glycolysis might be through the regulation...
Fig. 3. Snail inhibited the transcriptional activation of FBP1. (a), Snail affected the expression of rate-limiting glycolytic enzymes determined by quantitative real-time RT-PCR analysis with the most significantly decreased FBP1. (b), Overexpression of Snail decreased FBP1 protein expression determined by Western blot analysis. (c), Snail decreased the FBP1 promoter activity in a dose dependent manner.

We then generated FBP1 promoter pGL3-FBP1 plasmid and cotransfected the reporter plasmids with Snail expression vectors into AGS and MGC803 cells. As shown in Fig. 3c, overexpression of Snail decreased the FBP1 promoter activity in a dose dependent manner. These data suggested that Snail could transcriptionally regulated FBP1 expression.

**Restore FBP1 expression reverse the effect of glycolysis induced by Snail in gastric cancer cells**

To further confirm whether FBP1 was associated with Snail mediated regulation of EMT process and glycolysis, we restored FBP1 expression in ectopic Snail expression cells.
Fig. 4. Restoring FBP1 expression reversed the effect of glycolysis and EMT induced by Snail in gastric cancer cells. (a), Restoring FBP1 expression significantly decreased glucose utilization and lactate production. (b), Restoring FBP1 reversed the EMT process by Western blot analysis.

results showed that restoring FBP1 expression significantly decreased glucose utilization and lactate production ($P<0.05$, Fig.4a). Western blot analysis also found that restoring FBP1 reversed the EMT process which was induced by snail (Fig.4b). Collectively, Snail could induce EMT process and enhance glycolysis through regulating FBP1 in gastric cancer cells.

Snail reversely correlated with FBP1 expression in gastric cancer

We provide evidence that Snail negatively regulated FBP1 expression in gastric cancer cells. To further confirm our hypothesis, we investigated expressions of Snail and FBP1 in gastric cancer specimens. We first reviewed the relationship between Snail and FBP1 transcriptional levels from TCGA database and found that Snail mRNA levels were inversely correlated with FBP1 mRNA levels ($r=-0.165$, $P=0.002$, Fig.5a). Furthermore, we detected Snail and FBP1 protein expression by IHC in 69 gastric cancer patients’ specimens and also found a directly inverse correlation between Snail and FBP1 expression, which was consistent with our findings from gastric cancer cell lines ($\chi^2=5.927$, $P=0.015$, Fig.5b and 5c).
Discussion

In the present study, we determined the roles of Snail in gastric cancer aerobic glycolysis and its related mechanism. We provided convinced evidences supporting a critical role for Snail in regulation of aerobic glycolysis through the transcriptionally inactivated FBP1 gene. First, we found that overexpression of Snail increased the glucose utilization, lactate production in two gastric cell lines. Then we indicated that Snail expression in gastric cancer tissues was positively correlated with glycolysis as assessed by SUVmax. Moreover, ectopic expression of Snail influenced the activities of glycolytic enzymes, with the most significant change of FBP1, and FBP1 was further validated as a transcriptional target of Snail. Importantly, restoring FBP1 expression could reverse the glycolysis and EMT process which were induced by Snail. Furthermore, the expression of Snail and FBP1 in gastric cancer was inversely correlated at both mRNA and protein levels from TCGA database and our own gastric cancer samples respectively. These data indicated that Snail induced aerobic glycolysis via inhibiting the expression of FBP1. This novel Snail-FBP1 signaling critically contributed to the Warburg effect in gastric cancer and to gastric cancer progression.

Snail is a key regulator of EMT, and plays critical roles in cancer development and progression, but little evidence related to the function of Snail in gastric cancer metabolism were reported. Metastasis is a highly inefficient process during cancer progression and metabolic reprogramming is essential for cancer cell survival [10]. The Warburg effect, also known as aerobic glycolysis, is a shift from oxidative phosphorylation to glycolysis, a feature of which is increased lactate production even at normal oxygen concentrations, and has been considered to be at the root of cancer development and progression [19, 20]. The Warburg effect not only provides cancer cells with ATP and nutrients, but also creates an acidic environment that leads to destruction of extracellular matrix and facilitates metastasis. Also, the Warburg effect provides the basis for the most sensitive and specific imaging technique (such as PET) available for the diagnosis and staging of solid cancers. Our study demonstrated that Snail induced glucose metabolism reprogramming, which was accompanied by EMT process. It fact, Snail has been reported to be involved in metabolic reprogramming in some tumors. In breast cancer, loss of FBP1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer [21]. Furthermore, Snail reprogramed glucose metabolism by repressing phosphofructokinase PFKP allowing MCF-6 and MDA-MB-231 cell survival under metabolic stress [22].

It must be noted that the experimental design of our current study had certain limitations. First, the number of patients included in the study was small, which may cause limited statistical power. Second, although we got the conclusions from in vitro study and human gastric tissues, in vivo experiments using animal model were also needed to observe the effects of Snail on glycolysis. Thus, further in vivo animal studies are warranted to confirm our findings. Third, we mainly focused on glycolysis in EMT process, some other metabolic reprogramming might also happened, such as fatty acid oxidation, oxidative phosphorylation, which deserve further study.

Conclusion

In summary, this study for the first time provided critical insight into the role of Snail in gastric cancer aerobic glycolysis and identified a role for novel Snail-FBP1 signaling in gastric cancer progression. Snail-FBP1 signaling axis may serve as a potential therapeutic target for gastric cancer treatment by disrupt both EMT and glycolysis process.

Acknowledgements

This work is supported by The National Natural Science Foundation of China (81302015), the project of Jiangsu Provincial Medical Youth Talent (QNRC2016319), the Natural Science Foundation of Yangzhou City (YZ2014044) and Jiangsu Government Scholarship for Overseas Study.
Disclosure Statement

The authors declared no conflicts of interest.

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