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ORIGINAL ARTICLE

Loss of MGAT3 Amplifies TGF-β Signaling to Promote Bronchiolitis Obliterans Progression

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ABSTRACT

Background: **Bronchiolitis obliterans syndrome (BOS), a severe complication following allogeneic hematopoietic stem cell transplantation (allo-HSCT), presents as chronic graft-versus-host disease characterized by inflammation and fibrosis of the small airway epithelium. This progressive fibrosis obstructs bronchiolar airways, leading to respiratory distress in patients. Due to the lack of effective treatments, a deeper understanding of the underlying mechanisms is crucial. This study explored the molecular mechanisms underlying the abnormal glycosylation regulation in BOS, aiming to provide new insights for early diagnosis and treatment of this disease.**

Methods: **Bronchoalveolar lavage fluid (BALF) was collected from patients with and without BOS following allo-HSCT. N-glycans from the BALF were enriched using a solid-phase extraction method. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) was used to detect N-glycosylation characteristics in the BALF of BOS patients. This phenomenon was validated using a graft-versus-host disease (GVHD) mouse model. The impact of MGAT3 knockdown on the biological functions of airway epithelial cells (BEAS-2B) was then examined using lentivirus transfection. The molecular mechanism by which the loss of MGAT3 enhances TGF-β signaling was explored using western blotting, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence (IF).**

Results: **Our study investigated N-glycosylation changes in BALF of BOS patients and identified a strong correlation between the loss of bisecting-GlcNAc N-glycans and BOS progression. We found a novel mechanism where MGAT3, the enzyme synthesizing bisecting-GlcNAc structures, critically regulates TGF-β signaling. MGAT3 and bisecting-GlcNAc deficiency significantly enhance TGF-β signaling by increasing TGF-β storage through upregulation of latent TGF-β binding protein 1 (LTBP1) and by increasing the availability of TGF-β receptors.**

Conclusions: **Our discovery highlights the crucial role of aberrant glycosylation in BOS progression and holds significant promise for developing novel biomarkers for early diagnosis, identifying new therapeutic targets, and ultimately improving the quality of life for BOS patients.**

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Supplementary Data

Experimental Methods

Cell culture and cellular EMT establishment

Human bronchial epithelial cells (BEAS-2B), purchased from Servicebio (Wuhan, China), were cultured in Dulbecco's Modified Eagle Medium (DMEM, PM150210, Procell) supplemented with 10% fetal bovine serum (FBS) at 37°C. To induce epithelial-to-mesenchymal transition (EMT), BEAS-2B cells were cultured for 48 hours in FBS-free DMEM medium containing 5 ng/mL TGF-β1 (HY-P7118, MCE).

Cell counting Kit-8 (CCK-8) assay

Cell proliferation capacity was assessed using the Cell Counting Kit-8 (CCK-8). 100 μL BEAS-2B cell suspensions containing 3,000 cells were seeded in 96-well plates and allowed to adhere. After 24 hours, 10 μL of CCK-8 reagent was added to each well at specific time points: 0, 24, 48, 72, and 96 hours. The plate was then incubated in a CO_2 incubator at 37°C for additional 2 hours. Following incubation, the absorbance of each well was measured at 450 nm using a microplate reader. The proliferation curve was then plotted based on the absorbance values.

Wound healing assay

BEAS-2B cells were seeded in a 6-well culture plate and grown to approximately 90% confluency. A scratch wound was then created on the cell surface using the tip of a 200 μL pipette. The cells were subsequently washed three times with 1 × PBS and placed in FBS-free medium. Images of the scratch wound were taken at 0, 24, and 48 hours. ImageJ was used to quantify the migration area of cells at different time points.

Cell adhesion assay

To assess cell adhesion, 50 μL of 10 ng/mL fibronectin (F8180, Solarbio) was pre-added to each well of a 96-well plate and incubated for 3 hours. Next, 200 μL of 1% denatured BSA solution was added, and the plate was incubated at 37°C for 1 hour. Each well was then seeded with 100 μL of cell suspension containing 50,000 cells, mixed gently, and incubated for an additional hour. The experimental group and control group each contained more than five repetitive wells. After washing the plate with PBS, one well was left unwashed for total cell count. Finally, CCK-8 assay was used to determine the remaining cell count in each well and calculated the cell adhesion rate.

Cell invasion assay

BEAS-2B cells were cultured in serum-free medium for 24 hours. A cell suspension was then prepared, and 5 × 10^4 cells were seeded in the upper chamber of a transwell insert coated with Matrigel (8 μm pore size, 14347, LABSELECT). In the lower chamber, 500 μL of DMEM medium supplemented with 10% FBS was added. The cells were incubated at 37°C for 24 hours. Afterward, the upper chamber was washed three times with PBS. Then, the cells were fixed with 4% paraformaldehyde for 15 minutes and stained with crystal violet for 10 minutes. The cells were examined and photographed under a bright-field microscope.

Western blotting analysis

Protein samples were collected from BALB/c mice and BEAS-2B cells. RIPA lysis buffer was used to extract the proteins for subsequent experiments. The protein solution was then mixed with 5 x SDS-PAGE loading buffer and boiled for denaturation. Denatured protein samples were separated by SDS-PAGE electrophoresis. Following electrophoresis, the membrane was transferred to an activated PVDF membrane.

The membrane was blocked in rapid blocking buffer at room temperature for 30 minutes. After blocking, the membrane was incubated overnight at 4°C with specific primary antibodies. These antibodies included anti-MGAT3 (1:2,000 dilution, 17869-1-AP, Proteintech), anti-E-cadherin (1:10,000 dilution, 20874-1-AP, Proteintech), anti-Vimentin (1:2,000 dilution, AF7013, Affinity), anti-Smad2/3 (1:1,000 dilution, AF6367, Affinity), anti-pSmad2/3 (1:1,000 dilution, AF3367, Affinity), anti-Smad6 (1:1,000 dilution, AF5160, Affinity), anti-Smad7 (1:1,000 dilution, AF5147, Affinity), anti-LTBP1 (1:1,000 dilution, 26855-1-AP, Proteintech), anti-TGF-β1 (1:1,000 dilution, AF5160, Affinity), anti-TGFBR1 (1:1,000 dilution, 30117-1-AP, Proteintech), anti-TGFBR2 (1:2,000 dilution, 66636-1-Ig, Proteintech), anti-TGF-β1 (1:1,000 dilution, AF1027, Affinity), anti-MGAT4A (1:1,000 dilution, 30718-1-AP, Proteintech), anti-MGAT4B (1:1,000 dilution, 30117-1-AP, Proteintech), and anti-MGAT5 (1:1,000 dilution, DF14627, Affinity). The membrane was then washed three times with 1 x TBST buffer. Subsequently, the membrane was incubated with appropriate HRP-conjugated secondary antibodies at room temperature for 1 hour. Two secondary antibodies were used: HRP-labeled Goat Anti-Rabbit IgG (H+L) (1:1,000 dilution, A0208, Beyotime) and HRP-labeled Goat Anti-Mouse IgG (H+L) (1:1,000 dilution, A0216, Beyotime). Following incubation with secondary antibodies, the membrane was washed three times with 1 × TBST buffer. Finally, ECL reagents were used for protein detection, and the signals were visualized using a gel imaging system (CHEMIDOC, Bio-Rad). ImageJ was used for quantitative analysis of protein expression levels.

Bisecting-GlcNAc lectin blotting

Similar to WB, lectin blotting involved transferring the membrane following electrophoresis. The PVDF membrane was then incubated with biotinylated PHA-E (1:3,000 dilution, B-1125-2, Vector Lab) for 1 hour at room temperature to detect specific glycoproteins. After

washing, HRP-streptavidin conjugate (1:2,000 dilution, A0305, Beyotime) was added and incubated for another hour at room temperature. Finally, ECL reagents were used for subsequent detection of the lectin-bound glycoproteins.

Enzyme-Linked Immunosorbent Assay (ELISA)

A Human Transforming Growth Factor Beta 1 (TGF-β1) ELISA kit was purchased from Boster (Wuhan, China). The TGF-β1 standard solutions were prepared at various concentrations. Cell supernatant samples from BEAS-2B cells were activated according to the kit instructions. The diluted standard TGF-β1 and the activated cell supernatants were then added to an ELISA plate pre-coated with TGF-β1 antibody. The plate was incubated for 90 minutes at 37°C. Afterward, 100 µL of biotin-labeled anti-TGF-β1 antibody was added to each well, followed by another incubation for 60 minutes at 37°C. The plate was then washed three times. 100 µL of enzyme-conjugated working solution was added to each well, and the plate was incubated for 30 minutes at 37°C. Subsequently, the plate was washed five times. Next, 90 µL of substrate solution was added to each well, and the plate was incubated in the dark for 15 minutes at 37°C. The reaction was then stopped by adding 50 µL of stop solution. Finally, the optical density (OD) at 450 nm was immediately measured using an ELISA reader. ELISACalc software was used to fit the standard curve and construct a logarithmic four-parameter fitting equation to calculate the TGF-β1 concentration based on the OD values.

Gene correlation analysis

We used the GEPIA database to conduct the gene correlation between *MGAT3* and 27 genes regulated by TGF-β1, including *CTGF, SMAD7, ID1, MMP2, IL11, PTGS2, CCND1, IL6, VEGFA, SNAI1, TWIST1, FN1, THBS1, COL1A1, SOX9, BMP7, CDKN1A, CCNE1, CDH1, CDKN2A, PTEN, TP53, RB1, CLDN4, CLDN3, SERPINE1* and *ADAM12*. The gene correlation results were visualized with ggplot2 R package (version 4.2.2).

The prediction of N-glycosylation sites

We extracted the amino acid sequence of human LTBP1 protein from the Uniprot database (http://www.uniprot.org/) and then searched for potential N-glycosylation sites using the NetNGlyc database (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/).

Table S1. Expression analysis of glycosyltransferases in lung tissue of BOS-mice after lung transplantation.

Gene	Log_2FC	p adj
Fut1	4.765294	0.002561
Fut4	1.184773	0.004534
Fut7	1.594604	0.008613
B3gnt7	1.951554	0.010158
B4galnt4	1.646159	0.002465
B4galnt1	1.146562	0.007035
B4galnt2	1.11242	0.0132
Galnt12	1.066286	0.009102
Galnt6	1.271853	0.013376
Galnt7	1.129031	0.003125
Galnt14	1.253177	0.043341
B4galt6	1.640837	0.018881
St8sia1	1.004342	0.014339
Mgat3	-1.88717	0.003722
Gcnt2	-1.26214	0.00042
B3gnt4	-1.51011	0.012947
B3gnt6	-1.36566	0.046145
Galnt18	-1.45044	0.000416
Galnt5	-1.79954	0.000551
Galnt13	-1.38567	0.041025
Galnt15	-1.06409	0.041073
St6galnac3	-1.65965	0.017896
St3gal2	-1.02897	0.001591

FC: Fold change, p adj: Adjusted p-value.

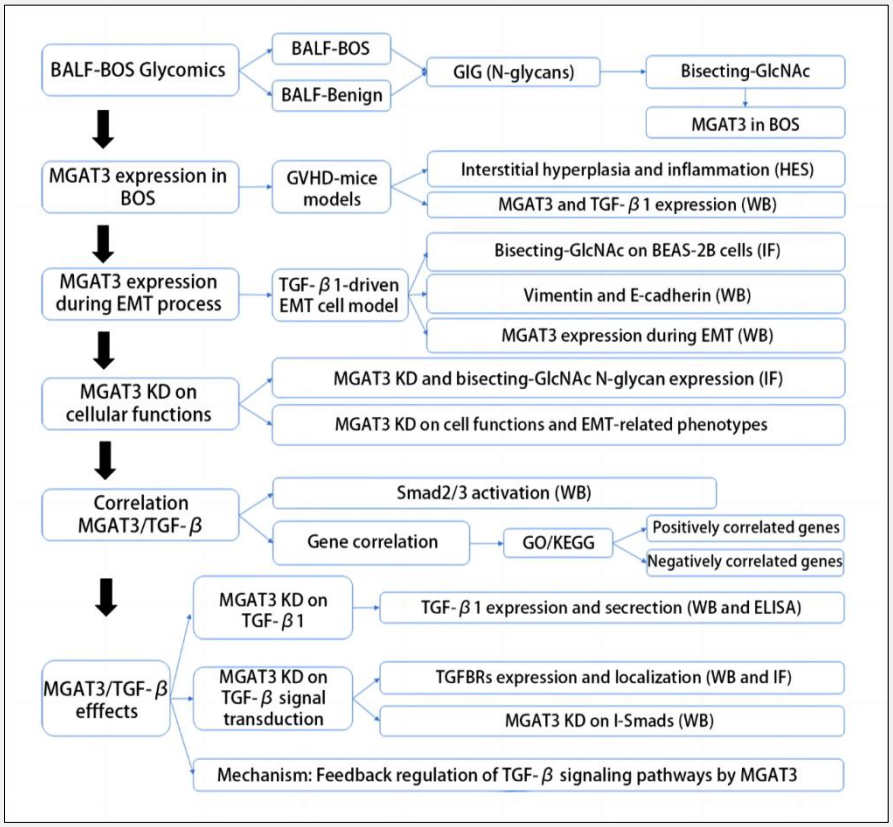

Figure S1. An experimental design to elucidate the role of MGAT3 in the development of bronchiolitis obliterans syndrome (BOS)-related airway fibrosis.

GIG: Glycoprotein immobilization for glycan analysis, HES: Histochemistry staining, IF: Immunofluorescence, WB: Western blot, ELISA: Enzyme-linked immunosorbent assay.

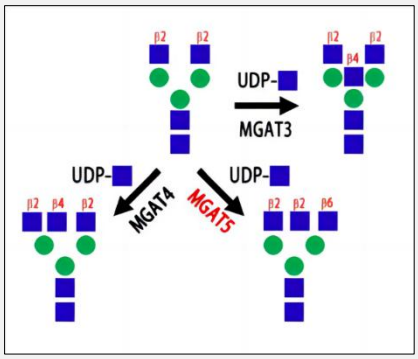

Figure S2. The biosynthesis of bisecting- and branching-GlcNAc N-glycans.

The presence of specific glycosyltransferases can be used to deduce the formation of branching- or bisecting-GlcNAc structures. Specifically, *MGAT3* catalyzes the synthesis of β1,4-bisecting-GlcNAc, MGAT4 catalyzes the synthesis of β1,4-branching-GlcNAc, and MGAT5 catalyzes the synthesis of β1,6-branching-GlcNAc.

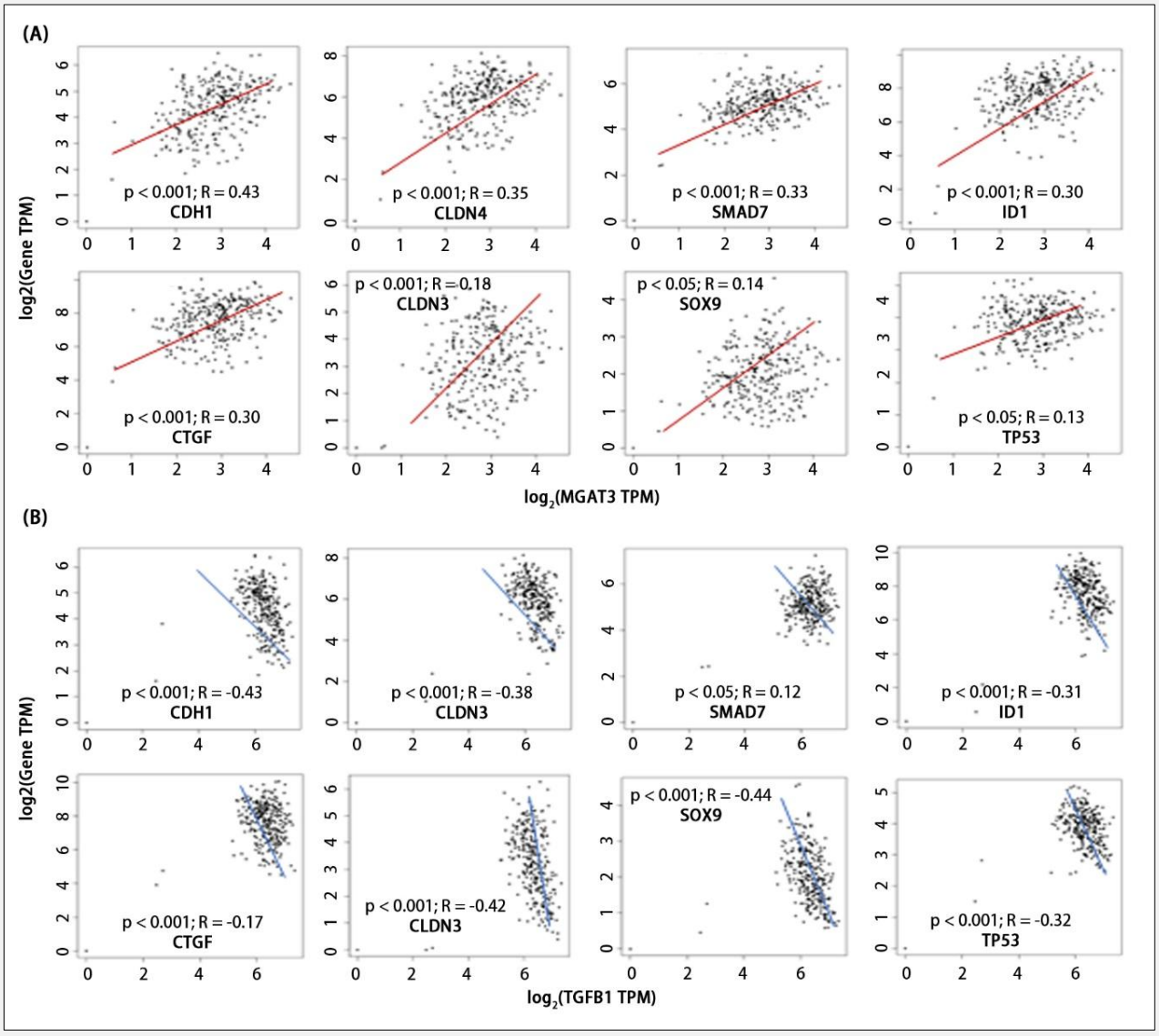

Figure S3. Correlation of MGAT3 and TGF-β1 with genes regulated by TGF-β signaling.

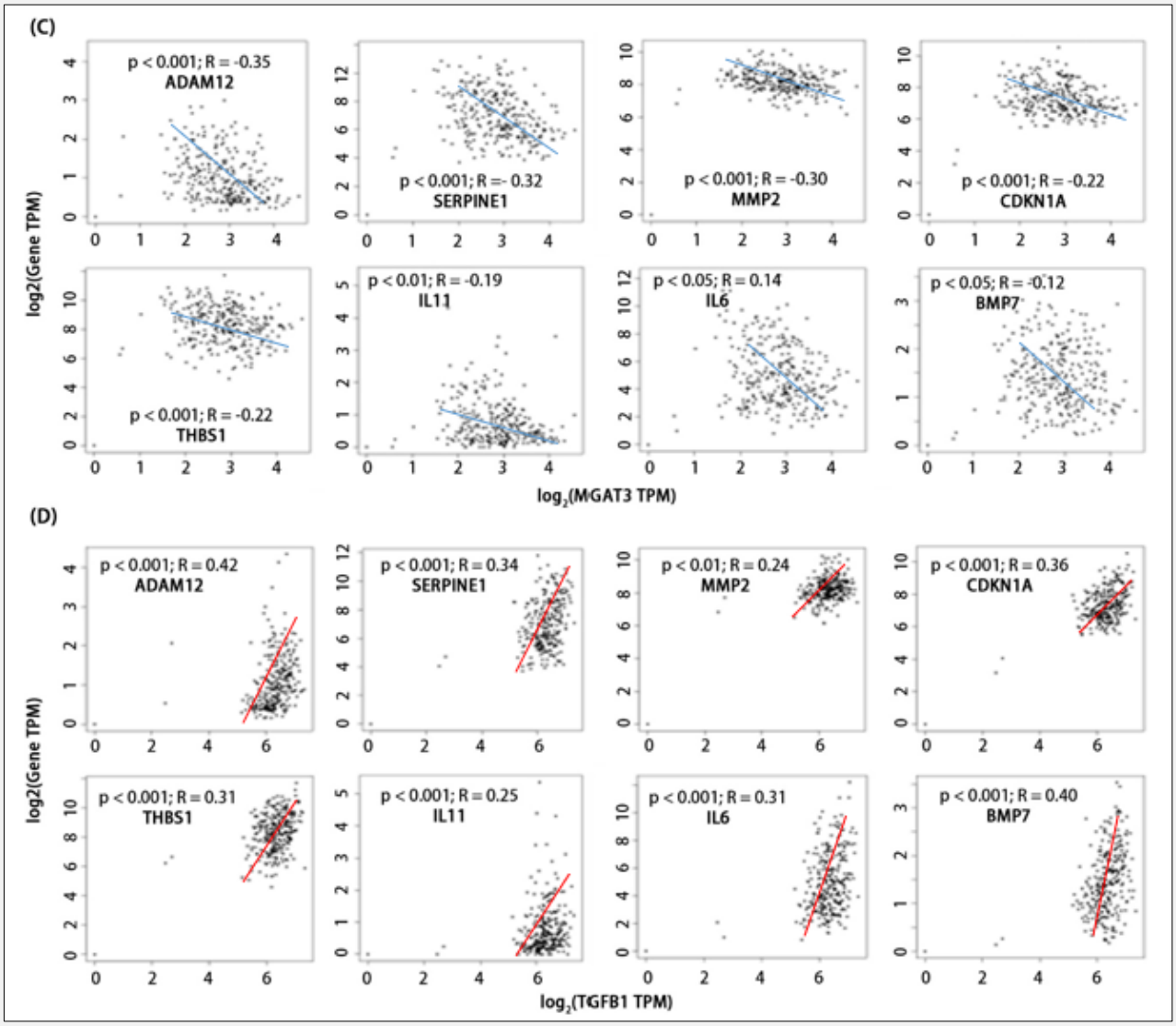

Figure S3. Correlation of MGAT3 and TGF-β1 with genes regulated by TGF-β signaling (continued).

Twenty-seven genes were screened that directly or indirectly participate in the regulation of the TGF-β1, including cytokines, cell membrane receptors, intracellular signaling proteins, and some transcription factors. The screened genes were: *CTGF*, *SMAD7*, *ID1*, *MMP2*, *IL11*, *PTGS2*, *CCND1*, *IL6*, *VEGFA*, *SNAI1*, *TWIST1*, *FN1*, *THBS1*, *COL1A1*, *SOX9*, *BMP7*, *CDKN1A*, *CCNE1*, *CDH1*, *CDKN2A*, *PTEN*, *TP53*, *RB1*, *CLDN4*, *CLDN3*, *SERPINE1* and *ADAM12*. Using the GEPIA website, we analyzed the correlation of these genes with *MGAT3* and TGFB1 (TGF-β1) in the GTEx database. Interestingly, we found that among them, there were 16 genes showed an opposite correlation pattern with *MGAT3* versus TGFB1, suggesting that *MGAT3* may be involved in inhibiting the activation of the TGF-β pathway. A - B: Correlation analyses of *MGAT3* and TGF-β1 with 8 genes: *CDH1*, *CLDN4*, *SMAD7*, *ID1*, *CTGF*, *CLDN3*, *SOX9*, *TP53*. Although *SMAD7* is positively correlated with *MGAT3* and *TGFB1*, it is a protein that inhibits TGF-β signaling. The positive correlation between *MGAT3* and *SMAD7* suggests that *MGAT3* may also negatively regulate TGF-β signaling. C - D: Correlation analyses of *MGAT3* and TGF-β1 with 9 genes: *ADAM12*, *SERPINE1*, *MMP2*, *CDKN1A*, *THBS1*, *IL11*, *IL6*, *BMP7* and *PTGS2*.