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Overexpression of DLL3 Promotes Proliferation of Human Gastric Cancer Cells (Postprint)

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Abstract

Objective: To construct a eukaryotic expression plasmid of full-length human DLL3 and analyze the effects of up-regulating and down-regulating DLL3 on the proliferation of human gastric cancer cells. Methods: The full-length human DLL3 gene was amplified by PCR and cloned into the eukaryotic expression vector pCMV-Tag4. Following identification via restriction enzyme digestion and sequencing, the plasmid was transiently transfected into HEK293T cells, with pCMV-Tag4-transfected and non-transfected HEK293T cells serving as negative and blank controls, respectively. Expression of full-length human DLL3 was verified by quantitative real-time PCR (qRT-PCR) and Western blot analysis. Additionally, qRT-PCR and Western blot were employed to detect differential expression of human DLL3 in human normal oral epithelial cells GES-1 and three gastric cancer cell lines including AGS. After transient transfection of human DLL3/pCMV-Tag4 into three gastric cancer cell lines, MTT assay was utilized to assess proliferation of gastric cancer cells upon DLL3 overexpression. Concurrently, specific human DLL3 siRNA was transfected into human gastric cancer cells MGC803 and MKN45, and MTT assay was used to evaluate proliferation of gastric cancer cells following DLL3 downregulation. Results: The recombinant plasmid of full-length human DLL3/pCMV-Tag4 was successfully constructed. Twenty-four hours after transfection into HEK293T cells, qRT-PCR and Western blot demonstrated that DLL3 expression at both mRNA and protein levels was significantly elevated compared to control groups. MTT cell proliferation assay revealed that overexpression of DLL3 promoted proliferation of gastric cancer cells, whereas downregulation of DLL3 inhibited proliferation of gastric cancer cells. Conclusion: The eukaryotic expression plasmid of full-length human DLL3/pCMV-Tag4 was successfully constructed and expressed in HEK293T cells. Overexpression of DLL3 promoted proliferation of gastric cancer cells, while downregulation of DLL3 inhibited proliferation of gastric cancer cells. This study provides novel insights for targeted therapy of gastric cancer

with DLL3 as a new target.

Full Text

Preamble

Over-expression of human Notch ligand Delta-like 3 promotes proliferation of human gastric cancer cells in vitro

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Abstract

Objective: To construct a eukaryotic expression plasmid carrying human full-length Notch ligand Delta-like 3 (DLL3) gene and investigate the effects of DLL3 overexpression and knockdown on the proliferation of gastric cancer cells in vitro.

Methods: Human full-length DLL3 gene was amplified by PCR and cloned into the eukaryotic expression vector pCMV-Tag4. After verification by restriction enzyme digestion and sequencing, the recombinant DLL3/pCMV-Tag4 vector was transiently transfected into HEK293T cells. Empty pCMV-Tag4 vector and untransfected HEK293T cells served as negative and blank controls, respectively. Expression of human full-length DLL3 was verified by real-time quantitative PCR (qRT-PCR) and Western blotting. Furthermore, qRT-PCR and Western blot were used to detect differential DLL3 expression in human normal oral epithelial cells GES-1 and three gastric cancer cell lines. After transient transfection of human DLL3/pCMV-Tag4 into three gastric cancer cell lines, MTT assay was performed to assess cell proliferation upon DLL3 overexpression. Additionally, specific human DLL3 siRNA was transfected into human gastric cancer cells MGC803 and MKN45, and MTT assay was used to evaluate proliferation after DLL3 knockdown.

Results: The human full-length DLL3/pCMV-Tag4 recombinant plasmid was successfully constructed. Twenty-four hours after transfection into HEK293T cells, qRT-PCR and Western blot showed that DLL3 expression at both mRNA and protein levels was significantly higher than in control groups. MTT proliferation assays demonstrated that DLL3 overexpression promoted gastric cancer cell proliferation, while DLL3 downregulation inhibited it.

Conclusion: We successfully constructed the human full-length DLL3/pCMV-Tag4 eukaryotic expression plasmid and achieved protein expression in HEK293T cells. DLL3 overexpression promotes gastric cancer cell proliferation,

while its downregulation inhibits proliferation. This study provides new insights for targeted gastric cancer therapy using DLL3 as a novel therapeutic target.

Keywords: human Delta-like ligand 3; eukaryotic expression; gastric cancer cell line; proliferation

Introduction

The Notch signaling pathway is widely conserved in higher organisms and represents a critical regulator of cell fate determination and development. Humans and mammals express four Notch receptors (Notch1-4) and five ligands (Jagged1, Jagged2, DLL1, DLL3, and DLL4) [1]. Since the initial report in 2004 identifying Notch1 mutations in approximately 50% of T-cell acute lymphoblastic leukemia patients [2], accumulating evidence has demonstrated that dysregulation of Notch signaling is closely associated with the initiation and progression of various tumors [3-6]. Consequently, the Notch pathway has emerged as a promising target for cancer diagnosis and therapy [7-9].

Gastric cancer ranks as the third leading cause of cancer-related mortality worldwide [10]. Previous studies have shown that aberrant expression of Notch ligands correlates with gastric carcinogenesis. For instance, Jagged1 is significantly overexpressed in diffuse-type and poorly differentiated gastric cancers, while DLL4 upregulation has been detected in gastric cancer patients at T, N, and TNM stages [11]. DLL4 overexpression enhances the self-renewal capacity of gastric cancer stem cells and predicts poor prognosis through activation of the Notch1 signaling pathway [12]. Additionally, intratumoral injection of siRNA targeting Jagged1 and DLL4 has been shown to inhibit tumor growth [13].

DLL3 overexpression has been reported in small cell lung cancer [14], acute myeloid leukemia [15], and large-cell neuroendocrine tumors [16], with elevated DLL3 expression in lung adenocarcinoma patients correlating with decreased overall survival [17]. However, the role and mechanism of DLL3 in gastric cancer remain unclear. Recently, we analyzed the TCGA (The Cancer Genome Atlas) database of 393 gastric cancer (GC) patients and identified genetic mutations and copy number variations in the human DLL3 gene in 5% of samples. Analysis of RNAseq data from 478 gastric cancer patients in the UCSC (University of California, Santa Cruz) database revealed reduced DLL3 expression in most tumor tissues, while a subset showed increased expression. Kaplan-Meier survival analysis demonstrated that high DLL3 expression was associated with reduced patient survival, suggesting that human DLL3 may be closely involved in gastric cancer pathogenesis. Therefore, elucidating the role and molecular mechanisms of DLL3-mediated Notch signaling in gastric cancer development is warranted.

To date, no reports have described human DLL3 gene expression. Building upon our previous work expressing human Notch ligands DLL1 [18], DLL4 [19], and Jagged1 [20], this study aimed to construct a human full-length DLL3 eukaryotic expression plasmid, achieve expression in HEK293T cells, transfect

three gastric cancer cell lines including AGS, and investigate the effects of DLL3 overexpression on gastric cancer cell proliferation. Additionally, specific siRNA was used to knock down DLL3 expression in gastric cancer cells to further validate its functional role.

Materials and Methods

1.1.1 Plasmids, Strains, and Cell Lines

The human DLL3 (NM_016941) cDNA clone (DLL3/pOTB7) plasmid was purchased from YouBio. The pCMV-Tag4 vector and HEK293T, GES-1, AGS, MGC803, and MKN45 cell lines were maintained in our laboratory. Competent *E. coli* DH5 and JM109 were obtained from Tiangen.

1.1.2 Reagents

Taq DNA polymerase, pMD18-T Vector Cloning Kit, restriction endonucleases SalI, T4 DNA ligase, RNAiso Plus, gel extraction kit, plasmid extraction kit, and PrimeScript RT reagent Kit with gDNA Eraser were purchased from TaKaRa. The ChamQ SYBR qPCR Master Mix for RT-qPCR was from Vazyme. Lipofectamine™ 2000 was from Invitrogen. Human DLL3-siRNA (sc-62206) and Control siRNA (sc-37007) were from Santa Cruz. Mouse anti-human DLL3 antibody was from R&D Systems. Rabbit anti-human GAPDH antibody and mouse anti-human -actin antibody were from Bioworld. RIPA cell lysis buffer, PMSF, DL 5000 DNA marker, 170 kDa protein marker, PVDF membrane, EZ-ECL chemiluminescent reagent, HRP-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG were from Fude Biological. Bradford protein assay kit was from Beyotime. Primers synthesis and DNA sequencing were performed by Guangzhou Aiji Biotechnology.

1.1.3 Instruments

PCR amplification was performed using a Veriti 96-well Thermal Cycler, and real-time quantitative PCR was conducted on an ABI 7500 system (Applied Biosystems).

1.1.4 Culture Media

Calf serum was from Sijiqing, and DMEM medium was from Gibco.

1.1.5 Primer Design

Based on the human DLL3 (NM_016941.3) gene sequence in GenBank, PCR primers were designed to amplify the full-length human DLL3 gene: forward primer 5' -GCCACCATGGTCTCCCCACGGATGT-3' and reverse primer 5' -TTTCACGGACAAGAATCGAGGAAGGGTA-3' . For qRT-PCR detection of DLL3 mRNA expression using -actin as internal control,

the primers were: DLL3 forward 5' -GACCCTCAGCGCTACCTTTT-3' , DLL3 reverse 5' -TACATCTTCAGGGCGATTCC-3' ; -actin forward 5' -TGG CACCCAGCACAATGAA-3' , -actin reverse 5' -CTAAGTC ATAGTCCGCCTAGAAGCA-3' . All primers were synthesized by Guangzhou Aiji Biotechnology.

1.2.1 Amplification of Human Full-Length DLL3 Gene

Using the human full-length DLL3/pOTB7 plasmid as template, PCR amplification was performed under the following conditions: initial denaturation at 95°C for 5 min; 34 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min; final extension at 72°C for 15 min. PCR products were analyzed by 1% agarose gel electrophoresis.

1.2.2 Construction and Identification of Human Full-Length DLL3/pCMV-Tag4 Recombinant Plasmid

PCR products were gel-purified and ligated with pMD18-T vector at a 9:1 molar ratio using T4 DNA ligase at 16°C for 30 min. The ligation mixture was transformed into competent *E. coli* JM109 and plated on LB agar containing X-Gal, IPTG, and ampicillin. White colonies were selected for culture and plasmid extraction. Positive plasmids were digested with BamHI and SalI to recover the full-length DLL3 gene, which was then cloned into the eukaryotic expression vector pCMV-Tag4. The recombinant plasmid was verified by BamHI/SalI double digestion and DNA sequencing.

1.2.3 Transient Transfection of HEK293T Cells with Human Full-Length DLL3/pCMV-Tag4 Recombinant Plasmid

HEK293T cells were cultured in DMEM complete medium (10% FBS) and passaged every 2-3 days. Cells were seeded at 2.5 × 10 cells/mL in 6-well plates. When cells reached 90% confluence, transfection was performed using Lipofectamine™ 2000 according to the manufacturer' s protocol. Empty pCMV-Tag4 vector served as negative control, and untransfected HEK293T cells as blank control. At 24 h post-transfection, cells were harvested for total RNA and protein extraction to verify expression at mRNA and protein levels.

1.2.4 qRT-PCR Detection of Human DLL3 mRNA Expression

Twenty-four hours after transfection with recombinant human full-length DLL3/pCMV-Tag4 plasmid, cells were harvested and washed three times with PBS. Total RNA was extracted and reverse-transcribed to cDNA following the kit instructions. qRT-PCR was performed using ChamQ SYBR qPCR Master Mix with -actin as internal control. The 20 L reaction mixture was run in triplicate, with ultrapure water as negative control. The cycling conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s; melting curve analysis at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s.

1.2.5 Western Blot Analysis of Human Full-Length DLL3 Protein Expression

Twenty-four hours post-transfection, cells were harvested and washed three times with PBS. Cell pellets were lysed in RIPA buffer on ice with vortexing every 5 min. After 20 min, lysates were centrifuged and supernatants collected. Protein concentration was determined by Bradford assay. Samples were mixed with 5× loading buffer, boiled for 10 min, and separated by 10% SDS-PAGE (18 mA, 90 min). Approximately 10 g protein was loaded per lane. Proteins were transferred to PVDF membrane at 200 mA for 90 min. Membranes were blocked with 5% skim milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.08% Tween-20) for 1.5 h at room temperature, then incubated with mouse anti-human DLL3 antibody or rabbit anti-human -actin antibody for 30 min at room temperature followed by overnight incubation at 4°C. After washing with TBST, membranes were incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies for 1 h at room temperature. Following washing, protein bands were visualized using EZ-ECL chemiluminescent reagent.

1.2.6 Analysis of DLL3 Expression in Gastric Cancer Cell Lines

When gastric cancer cells AGS, MGC803, and MKN45, as well as normal gastric epithelial cells GES-1, reached 80-90% confluence, total RNA was extracted and reverse-transcribed for qRT-PCR analysis as described in section 1.2.4. For protein analysis, cells were lysed with RIPA buffer containing PMSF (1:100), and total protein was extracted for Western blot detection using mouse anti-human DLL3 antibody as described in section 1.2.5.

1.2.7 Effect of Human DLL3 Overexpression on Gastric Cancer Cell Proliferation

Gastric cancer cells AGS, MGC803, and MKN45 were transfected with recombinant DLL3/pCMV-Tag4 plasmid or empty pCMV-Tag4 vector using Lipofectamine™ 2000. After confirming DLL3 overexpression by Western blot (as described in section 1.2.5), cells were seeded in 96-well plates at 60% confluence and transfected with DLL3/pCMV-Tag4 for 48 h. Medium was removed, and 200 L MTT working solution was added to each well. After 2 h incubation, the solution was removed, 200 L DMSO was added to dissolve the formazan crystals, and absorbance (A) was measured at 490 nm using a microplate reader.

1.2.8 Downregulation of Human DLL3 Expression and Its Effect on Cell Proliferation

Human DLL3-siRNA and Control siRNA were transfected into gastric cancer cells MGC803 and MKN45 using Lipofectamine™ 2000. After 24 h, cells were harvested and protein expression was analyzed by Western blot as described in section 1.2.5. Following confirmation of DLL3 knockdown, cells were seeded in

96-well plates at 60% confluence and transfected with DLL3-siRNA or Control siRNA for 48 h. MTT assay was performed as described in section 1.2.7.

Statistical Analysis

Data were analyzed using SPSS 20.0 software and expressed as mean ± standard deviation. For qRT-PCR results, one-way ANOVA was used for multi-group comparisons followed by LSD post-hoc test (= 0.05). For MTT results, independent samples t-test was performed, with $P < 0.05$ considered statistically significant.

Results

2.1 Construction and Identification of Human Full-Length DLL3/pCMV-Tag4 Recombinant Plasmid

Using human full-length DLL3/pOTB7 cDNA as template, PCR amplification yielded a specific band at approximately 1800 bp (Figure 1 [Figure 1: see original paper]A), consistent with the expected size. The full-length DLL3 gene was obtained by BamHI/SalI double digestion and cloned into pCMV-Tag4. Restriction analysis of the recombinant plasmid produced two fragments of 4300 bp and 1800 bp (Figure 1B), matching the expected sizes. DNA sequencing of positive clones confirmed the correct open reading frame, and the construct was designated DLL3/pCMV-Tag4.

2.2 Detection of DLL3 Expression at mRNA and Protein Levels by qRT-PCR and Western Blotting

HEK293T cells were transfected with human full-length DLL3/pCMV-Tag4 recombinant plasmid, empty pCMV-Tag4 vector, or left untransfected as blank control. After 24 h, total RNA was extracted and analyzed by qRT-PCR. Results showed that DLL3 mRNA expression was significantly elevated in cells transfected with DLL3/pCMV-Tag4 compared to control groups (Figure 2 [Figure 2: see original paper]A). Western blot analysis of total protein from transfected cells revealed a specific band at approximately 78 kDa, while no band was detected in control groups (Figure 2B), confirming successful expression of the DLL3/pCMV-Tag4 recombinant plasmid in HEK293T cells.

2.3 Expression Analysis of Human DLL3 in Normal Gastric Epithelial Cells GES-1 and Gastric Cancer Cells

Total RNA and protein were extracted from gastric cancer cells AGS, MGC803, and MKN45, as well as normal gastric epithelial cells GES-1, at 80-90% confluence. qRT-PCR and Western blot analyses showed that DLL3 mRNA expression was significantly higher in all three gastric cancer cell lines compared to GES-1 (Figure 3 [Figure 3: see original paper]A). Consistently, Western blotting demonstrated elevated DLL3 protein expression in the three gastric cancer

cell lines relative to GES-1 (Figure 3B).

2.4 Overexpression of Human DLL3 in Gastric Cancer Cell Lines and Its Effect on Cell Proliferation

DLL3 was overexpressed in three gastric cancer cell lines. Western blot analysis showed two specific bands at approximately 68 kDa and 78 kDa in cells transfected with DLL3/pCMV-Tag4, whereas only a single 68 kDa band was observed in empty vector controls, confirming successful overexpression of exogenous DLL3 (Figure 4 [Figure 4: see original paper]A). MTT assays demonstrated that DLL3 overexpression significantly promoted proliferation of gastric cancer cells (Figure 4B).

2.5 Downregulation of Human DLL3 Expression and Its Effect on Gastric Cancer Cell Proliferation

Specific human DLL3-siRNA was transfected into gastric cancer cells MGC803 and MKN45 to knock down DLL3 expression. Western blot analysis showed markedly reduced DLL3 protein levels in DLL3-siRNA transfected cells compared to control siRNA groups (Figure 5 [Figure 5: see original paper]A). MTT results revealed that DLL3 downregulation significantly inhibited proliferation of both gastric cancer cell lines (Figure 5B).

Discussion

Human DLL3 ligand is a single-pass transmembrane protein composed of 619 amino acids, containing one DSL domain, one intracellular domain, and six epidermal growth factor-like domains. Previous studies have suggested that DLL3 may inhibit Notch receptor-mediated signaling, distinct from DLL1 and DLL4. For example, DLL3 does not bind to or activate Notch receptors expressed on adjacent cells [21]; conversely, when co-expressed with other Notch signaling components on the cell surface, DLL3 attenuates signaling induced by other DSL ligands [22]. In hepatocellular carcinoma, DLL3 gene methylation silencing has been observed, and treatment with demethylating agents or exogenous DLL3 gene introduction induces apoptosis [23]. In a rat model of advanced neuroendocrine tumors, a DLL3-targeted antibody-drug conjugate (SC16LD6.5) eliminated tumor-initiating cells [16]. Recent studies have shown that DLL3 is highly expressed in over 80% of small cell lung cancer patients, and Phase I clinical trials of Rova-T (an anti-DLL3 antibody conjugated to the cytotoxin tesirine) have demonstrated promising antitumor activity [24]. Therefore, as a potential target for clinical diagnosis and therapy, in-depth investigation of DLL3' s role and molecular mechanisms in gastric cancer holds significant value.

In this study, we first confirmed successful construction of the human full-length DLL3/pCMV-Tag4 recombinant plasmid through restriction digestion and sequencing analysis. Following transfection into HEK293T cells, qRT-PCR revealed significantly elevated DLL3 mRNA levels compared to empty vector con-

trols ($P < 0.05$). Western blot analysis detected a specific DLL3-Flag fusion protein band at approximately 78 kDa, confirming protein expression in 293T cells. HEK293T cells are human-derived and capable of post-translational modifications, facilitating production of recombinant proteins with native conformation, while the Flag tag in pCMV-Tag4 enables subsequent affinity purification.

Our preliminary analysis of TCGA and UCSC databases suggested that human DLL3 may be closely associated with gastric cancer pathogenesis. To investigate this, we examined DLL3 expression differences between normal human gastric epithelial cells GES-1 and gastric cancer cells. Results showed elevated DLL3 expression in three gastric cancer cell lines compared to GES-1 ($P < 0.05$), with the highest expression in AGS and lowest in MKN45. To further explore DLL3 function in gastric cancer, we overexpressed DLL3 in three gastric cancer cell lines. Western blot analysis revealed two specific bands at approximately 68 kDa and 78 kDa in cells transfected with DLL3/pCMV-Tag4, while empty vector controls showed only the 68 kDa endogenous band, indicating successful overexpression of exogenous DLL3 in addition to endogenous expression. MTT assays demonstrated that DLL3 overexpression promoted proliferation of all three gastric cancer cell lines ($P < 0.05$). To validate these findings, we used specific DLL3-siRNA to knock down DLL3 expression in MKN45 and MGC803 cells. Western blot confirmed reduced DLL3 protein levels, and MTT assays showed that DLL3 knockdown inhibited gastric cancer cell proliferation ($P < 0.05$).

In summary, this study successfully achieved human DLL3 expression in HEK293T cells and demonstrated that DLL3 overexpression promotes gastric cancer cell proliferation, suggesting that DLL3-mediated Notch signaling may play an important role in human gastric cancer development.

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