Shiga-like toxin I exerts specific and potent anti-tumour efficacy against gastric cancer cell proliferation when driven by tumour-preferential Frizzled-7 promoter

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Abstract

Objectives: Tumour-targeted gene therapy is a promising approach for effective control of gastric cancer cell proliferation. Our study aims to develop a cancer therapy which combines tumour-targeting promoters with cytotoxins.

Methods: The expression of globotriaosylceramide (Gb3), which is a Shiga-like toxin I (Stx1) receptor, was verified in gastric cancer compared with normal stomach tissues as assessed by flow cytometry and immunohistochemical analysis. We therefore constructed the recombinant pFZD7-Stx1 plasmid vectors with tumour-preferential Frizzled-7 promoter and Stx1. pFZD7-Stx1 was used to treat gastric cancer in vitro and in vivo. The gastric cancer cell proliferation and tumour growth were identified after the transfection with the pFZD7-Stx1.

Results: Globotriaosylceramide was obviously increased in gastric cancer compared with normal stomach. The gastric cancer cell proliferation and tumour growth decreased significantly after the transfection with the pFZD7-Stx1.

Conclusion: Frizzled-7 promoter is preferentially active, and Gb3 is abundant in gastric cancer cells. Frizzled-7 promoter and Stx1 may be used to determine a novel and relatively specific and potent gastric cancer therapeutic strategy.
INTRODUCTION

The incidence of gastric cancer is still very high worldwide and is also associated with poor prognosis. Although new surgical, chemotherapeutic and radiotherapy approaches have improved gastric cancer treatment, standard treatment protocols are ineffective in a considerable number of cases. Tumour-targeted gene therapy may be a complementary treatment for gastric cancer. With the use of various tumour-selective delivery systems, cancer gene therapy has made significant progress. However, in order to improve the targeting by gene therapy, there is still a long way to go to find cancer-specific genes. To achieve this goal, a range of tumour-targeting promoters and cytotoxins have been discovered and used in cancer therapy.

The frizzled receptors (FZDs) are characteristic of seven-pass transmembrane proteins, with WNT glycoproteins as their ligands. Tumour formation can result from aberrant activation of FZDs. Overexpression of human Frizzled type 7 receptor (Frizzled-7) altered cell motility and migration by translocation of a stable wild-type β-catenin into the nucleus. Among the 10 Frizzled family members, Frizzled-7 is the only evolutionarily conserved family member that regulates the development of the gastric system. The expression level of Frizzled protein-7 in gastric cancer cell lines is higher than that in human oesophageal cancer. The WNT-β-catenin-TCF pathway can be activated by elevated Frizzled-7, which may play an important role in the development of human gastric cancer.

Shiga-toxin like I produced by Shigella dysenteriae and certain strains of Escherichia coli. These toxins consist of two non-covalently attached modular parts: a moiety (StxA) contains the enzymatically active A1 fragment and non-toxic pentameric binding moiety (StxB). The Stx1 specifically binds to glycosphingolipid (GSL) globotriaosylceramide (Gb3) on the surface of target cells, and it is internalized by endocytosis. Subsequently, the Stx1/Gb3 complex is transported retrogradely in toxin-sensitive cells to the endoplasmic reticulum via the Golgi, where the enzymatically active portion of the Stx1 translocates to the cytosol, enabling it to irreversibly inhibit protein synthesis by modification of ribosomal 28S RNA. The Gb3 exhibits a relatively restricted expression in normal human tissues, and it has been reported to be highly expressed in many types of cancers, such as lymphoma, breast cancer and colorectal carcinoma. The Gb3 expression in colorectal cancer correlates with invasiveness and metastatic potential. The Gb3 expression in colorectal cancer correlates with invasiveness and metastatic potential. Stx1 harbours several advantageous properties compared to conventional chemotherapeutic drugs. It kills cells in an extremely effective manner (theoretically, one toxin molecule is enough to kill a cell) and therefore may have a therapeutic effect at low doses, requiring only one or a few rounds of treatment.

Several researchers have shown that intratumoral or intraperitoneal injection of the Stx1 inhibited tumour growth in a mouse model of metastatic fibrosarcoma, as well as in mouse xenograft models of human malignant meningiomas, atypical human bladder carcinoma with endothelial characteristics, human renal carcinoma and human astrocytoma. However, the Gb3 expression level in gastric cancer is still controversial and the use of Stx1 has high potential but needs to be further identified in gastric cancer therapy.

Herein, we constructed a recombinant plasmid vector in which the Frizzled-7 promoter was used to drive the expression of Shiga-like toxin I genes (pFZD7-Stx1). The cancer cells were transfected with this vector, resulting in a significant decrease in cancer cell proliferation and cancer-specific cellular apoptosis in vitro. The apoptosis-necrosis rate and cleaved caspase 3 expression for gastric cancer cells transfected with pFZD7-Stx1 were significantly higher than that of normal stomach cells. The average tumour volume of pFZD7-Stx1-treated murine models with stomach tumour xenografts was obviously reduced, while the survival rate was significantly increased. The tumour growth was effectively suppressed by Stx1 driven by FZD7 promoter. Therefore, our tumour-specific targeting promoters combined with cytotoxins demonstrated this new specific and effective gene therapy for gastric cancer in vitro and in vivo.

MATERIALS AND METHODS

2.1 Cell culture

Normal stomach cell line GES-1 and gastric cancer SGC7901 cells were provided by our laboratory. All of the cells were maintained in RPMI-1640 medium (HyClone) supplemented with 10% FBS (GIBCO-BRL) and 1% penicillin/streptomycin (Sigma) at 37°C in a humidified atmosphere containing 5% CO₂.

2.2 Flow cytometry

The cells were adjusted to a concentration of 5 × 10⁵ cells/mL using phosphate-buffered saline (PBS). Three hundred microlitres of cell suspension was incubated with 2% formaldehyde for 1 hour at room temperature, and then washed once with 2% bull serum albumin (BSA) in PBS and twice with PBS-BSA. Cells were subsequently stained with rat anti-Gb3 IgM (diluted 1:20; Beckman Coulter) primary antibody and incubated with Alexa Fluor-labelled goat anti-rat IgM (diluted 1:50; Invitrogen) secondary antibody for 1 hour at 4°C. After washing and centrifugation for 20 minutes, cells were analysed using a FACS flow cytometer (BD) and data were processed using BD CellQuest software. Every assay in this paper was performed in triplicate.

2.3 Immunohistochemical analysis

Human normal kidney tissues, normal stomach tissues and corresponding gastric carcinoma tissue specimens were clinically obtained from the pathology file of the Affiliated Drum Tower Hospital of Nanjing University Medical School. All harvested tissues were fixed in 10% buffered formalin, embedded in paraffin and sectioned into a thickness of 6 μm. Gb3 expression was then detected using a rat anti-Gb3 antibody (diluted 1:20; Beckman
Reagent (Roche). Fifty microlitres of MTT substrate was added into continued for 4 hours. The cell culture medium was removed, and the cell suspension after 24, 48 and 72 hours, and incubation was referred from the previously reported papers. 25,26

2.4 | Construction of Frizzled-7 promoter and recombinant vectors

The design of Frizzled-7 promoter driving Stx1 vector (pFZD7-Stx1), the mutated Frizzled-7 driving Stx1 vector (Mut pFZD7-Stx1), the EF1a promoter driving Stx1 vector (pEF1a-Stx1) and control pFZD7 vector were referred from the previously reported papers. 25,26

2.5 | Stx1 expression analysis

Shiga-like toxin I expressions of the transfected GES-1 and SGC7901 cells were measured by quantitative real-time PCR (qRT-PCR). The method for mRNA extraction and qRT-PCR was performed as mentioned above. 25

2.6 | Cellular proliferation assay

Cell proliferation was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). Two hundred microlitres of cell suspension was seeded into three 96-well microplates at a cell density of 8000 cells/well. The cells were then transfected with different plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche). Fifty microlitres of MTT substrate was added into the cell suspension after 24, 48 and 72 hours, and incubation was continued for 4 hours. The cell culture medium was removed, and the cells were suspended with 150 μL of dimethyl sulfoxide. The absorbance was measured at a wavelength of 550 nm, and inhibition rate was defined as 1−(transfected OD value/unprocessed SGC value SGC7901) × 100%.

2.7 | Morphology and ultrastructure analysis

The SGC7901 and GES-1 cells were seeded at 3 × 10^5 cells/well density into 6-well plates. The cells were then transfected with pDZD7-Stx1, Mut pDZD7-Stx1 or pDZD7. Cell morphology changes were examined under DMIRB phase contrast microscope (Leica) after 48 hours of incubation in DMEM plus 10% FBS (GIBCO-BRL). The cells were fixed with glutaraldehyde and stained with osmium tetroxide, and the morphology of the cells was observed by transmission electron microscopy. After a series of pre-treatment procedures, the cell block was sectioned and collected on a carbon-coated grid, and then stained with uranyl acetate and Reynold lead citrate, and the cell superstructure change was observed with TEM-1200EX (JEOL).

2.8 | Detection of apoptosis and proliferation

Apoptosis was assessed by flow cytometry (FCM) with annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. Apoptotic cells were defined as PI negative (representing intact plasma membrane) and annexin-V-FITC positive population. At the same time, apoptosis and proliferation levels were assessed by DNA fragmentation analysis and Western blotting of proliferating cell nuclear antigen (PCNA) and cleaved caspase 3 content. The above three experimental methods also referred to the previously published articles. 25

2.9 | Animal studies

Thirty-two nude mice bearing tumour xenografts were randomly divided into four groups (eight in each group). Subcutaneous tumour xenografts from the pFZD7-Stx1, Mut pFZD7-Stx1, pFZD7 and control groups were established by subcutaneous inoculation with SGC7901 cells on left flank (1 × 10^6 cells in 100 μL serum-free media mixed with the corresponding 20 μg of each plasmid and cationic lipid, no plasmid was added in control group). Nude mice were used for the experiment about 10 days after the injection, and the growing tumours from mice were injected from three directions and continued for 3 weeks at a frequency of twice a week. The subcutaneous growth rate was determined by measuring the tumour size weekly using a slide card scale. The volume of tumour was calculated as per previously published article. 25 The expression levels of PCNA and cleaved caspase 3 in tumour resected tissues from mice injected with different vectors were evaluated by Western blot analysis. Our animal studies were in line with institutional guidelines and the National Institutes of Health Guidelines for animal ethical use.

2.10 | Statistical analysis

The experimental data are presented as mean ± SD. Statistical analysis was performed using SPSS 19.0 software. Each assay was performed at least three times. Both Student’s t test and ANOVA were used, and the statistical significance was set at P < 0.05.

3 | RESULTS

3.1 | The expression of Gb3 was significantly increased in gastric cancer

Aberrant glycosylation appears to be a universal feature in carcinogenesis and may alter cell signalling, growth, adherence and motility. Remarkably, all experimental and human cancers are essentially found to exhibit alterations in GSL composition and metabolism. The Gb3 is one of the GSLs that have been found to be tumour-associated antigens. 12 We therefore assessed the level of Gb3 expression in gastric cancer using the FACS and immunohistochemistry analysis. HEK-293 cells, known for high Gb3 levels, were used as positive controls. Flow cytometry scattergrams showed that the Gb3 was detected in 29.6% from the SGC7901 cells, 43.8% from the normal kidney HEK-293 cells and 10.3% from the GES-1 cells (Figure 1A). Consistent with results from the flow cytometry analysis, the gastric cancer tissues exhibited high levels of Gb3 as compared with normal stomach tissues by the immunohistochemistry analysis (Figure 1B).
FIGURE 1  The level of globotriaosylceramide (Gb3) expression in gastric cancer. A, Expression levels of Gb3 were evaluated by FACS analysis in GES-1 cells and SGC7901 cell lines. HEK-293 cells were used as positive control. Error bars indicate SD. B, Status of Gb3 expression was assessed by immunohistochemistry analysis in normal stomach and gastric cancer tissues. Normal kidney tissues were used as positive control. *P value < 0.01

FIGURE 2  Construction of recombinant vectors and Stx1 expression. A, Frizzled-7 promoter comprising of nucleotides -1718 to +95 was designed according to the DNA sequence from the human Frizzled-7 (accession number: AB017365). Several evolutionarily conserved transcription factor-binding sites, including PU.1-, SP1-, CCAAT box- and TCF/LEF/SOX-, were all encompassed in the Frizzled-7 promoter region. The mutated Frizzled-7 promoter (Mut FZD7) was generated by deleting the predicted conserved region in the sequence. B, pFZD7-Stx1 vectors were constructed by replacing the pFZD7-Luc luciferase gene with Stx1 genes using the Ncol and Xbal restriction endonucleases. C, qRT-PCR analysis of in vitro Stx1 expression in GES-1 cells and SGC7901 cells after pFZD7-Stx1 transfection. The Stx1 was highly expressed in SGC7901 cells after transfection with pFZD7-Stx1. Error bars indicate SD
These results open room for the possibility of using the Stx1 specific binding to Gb3 in targeted gastric cancer therapy.

### 3.2 Stx1 driven by Frizzled-7 promoter was highly expressed in SGC7901 cell lines

As much as it is in the stomach tumour tissues and SGC7901 cell, the Frizzled-7 promoter has preferential activity and Gb3 is highly expressed. We next assessed whether the Frizzled-7 promoter-driven Stx1 therapy could be used for targeted gene therapy in gastric cancer cells. We in this study constructed the pFZD7-Stx1 vector and two control vectors (pFZD7 and Mut pFZD7-Stx1) to transfect the SGC7901 and GES-1 cells (Figure 2A,B). In addition, we constructed the pEF1a-Stx1 to replace the Frizzled-7 promoter from the pFZD7-Stx1, using the well-characterized EF1a promoter as positive control (data not shown). The results from qRT-PCR showed that the Stx1 expression was significantly higher in SGC7901 cells transfected with pFZD7-Stx1 than in GES-1, while Stx1 gene expression was almost undetectable in Mut pFZD7-Stx1-transfected SGC7901 and GES-1 cells (Figure 2C).

### 3.3 pFZD7-Stx1 transfection selectively inhibited SGC7901 cell proliferation

The impact of pFZD7-Stx1 on proliferation of gastric cancer cells and cytotoxicity was evaluated by MTT assay (Figure 3A). Cell viability of pFZD7-Stx1-transfected SGC7901 cells was significantly decreased when compared with pFZD7-transfected (P < 0.01), Mut pFZD7-Stx1-transfected (P < 0.01) and untreated SGC7901 cells (P < 0.01) after 48 hours. Moreover, no significant decrease was observed in cell viability in GES-1 cells. In summary, Stx1 showed significant selective inhibition and cytotoxicity on gastric cancer cell proliferation compared to normal gastric cells.

The cells treated with different vectors were observed under a light microscope. SGC7901 cells transfected with pFZD7-Stx1 showed more obvious morphological changes than the other three groups of SGC7901 cells and GES-1 cells after 48 hours after transfection. The pFZD7-Stx1-transfected SGC7901 cells were observed to have apoptotic features on ultrastructure by electron microscopy, including chromatin condensation and nuclear fragmentation.

![Figure 3](image-url)
other three groups of SGC7901 and GES-1 cells showed no significant morphological and ultrastructural changes (Figure 3B). These results indicated that the SGC7901 cells transfected with pFZD7-Stx1 may have undergone apoptosis.

3.4 Frizzled-7 promoter-driven Stx1 therapy specifically induces cellular apoptosis in vitro

Flow cytometry analysis was used to verify that transfection with pFZD7-Stx1 may specifically kill cancer cells or induce apoptosis by certain pathways. The degree of apoptosis was assessed by FCM analysis of annexin-V-FITC and PI staining. Figure 4A shows that the apoptosis-necrosis rate for SGC7901 cells transfected with pFZD7-Stx1 was significantly higher than that of the other three groups \( (P < 0.01) \). There was no significant difference in apoptotic rates among the control group, pFZD7- and Mut pFZD7-Stx1-transfected SGC7901 cell groups or between normal GES-1 cells.

The results from the DNA fragmentation analysis further validated the results from the FCM analysis. DNA fragmentation is the process by which a DNA strand separates or breaks into pieces during the process of apoptosis. As shown in Figure 4B, DNA fragmentation occurred in pFZD7-Stx1-transfected SGC7901 cells and not in the other groups.

Cleaved caspase 3 is an active form of caspase 3 and elevated during apoptosis. PCNA is closely related to cell DNA synthesis and is a good indicator reflecting the state of cell proliferation. Both caspase 3 and PCNA act as important downstream effector molecules for the apoptotic pathway. As shown in Figure 4C, PCNA

FIGURE 4 Frizzled-7 promoter-driven Stx1 therapy specifically induces cellular apoptosis in vitro. A, Apoptosis in the GES-1 and SGC7901 cells after pFZD7-Stx1 transfection, as detected by FCM. B, DNA fragmentation in the GES-1 and SGC7901 cells after pFZD7-Stx1 transfection, as detected by agarose gel electrophoresis. C, Western blot analysis of apoptosis and proliferation signalling in GES-1 and SGC7901 cells after pFZD7-Stx1 transfection. Each experiment was done in triplicate.
expression in the pFZD7-Stx1-transfected group was lower than that of the other three SGC7901 cells groups. However, no differences in the levels of PCNA and cleaved caspase 3 were found in the GES-1 cell groups. These results strongly suggest that the Stx1 gene therapy system regulates caspase 3 and PCNA in the SGC7901 gastric cancer cells, to downregulate cell proliferation and induce apoptosis.

3.5 | pFZD7-Stx1 reduced tumour burden and improved survival of nude mice with gastric tumour xenografts in vivo

The in vivo anti-tumour effect of the vectors was evaluated in a murine model of stomach tumour xenografts. The average tumour volume at 5 weeks after pFZD7-Stx1 treatment was about 0.19 cm³, which was comparable to 1.54, 1.46 and 1.21 cm³ average tumour volumes after the PBS (P < 0.01), Mut pFZD7-Stx1 and pFZD7 transfection, (P < 0.01) respectively (Figure 5A, B). As demonstrated in Figure 5C, the survival rate for pFZD7-Stx1-treated mice was significantly increased. Seven of the pFZD7-Stx1-treated groups in each group of eight mice survived 7 weeks after injection of the vectors, while only one of the other three groups survived.

3.6 | pFZD7-Stx1 transfection induced tumour cellular apoptosis in vivo

In order to evaluate the tumour cell proliferation in mice, the mice were sacrificed after 8 weeks of vector injection, xenograft tumours were collected, and tumour cell PCNA and cleaved caspase 3 levels from each group were examined. Figure 6 shows that, consistent with the in vitro results, the PCNA levels for the tumour cells in the pFZD7-Stx1 group were lower than in the other groups, while the cleaved caspase 3 expression in the pFZD7-Stx1 group was higher.
than other groups. These results indicate that pFZD7-Stx1 has an in vivo anti-tumour effect of inhibiting cell proliferation and inducing apoptosis.

4 | DISCUSSION

The urgent need for a better gastric cancer therapy and our previous work on tumour-target promoters and cellular toxins led us to explore the potent anti-tumour efficacy of this gene therapy in the treatment of gastric cancer. Previous studies on specific cancer gene therapy paid close attention to tumour-selective delivery systems, but they had difficulties in achieving satisfactory effect because of side effects. We therefore proposed a new strategy to deal with the limitation from the use of vectors. The tumour-preferential Frizzled-7 promoter and Shiga toxin receptor (Gb3) caught our attention and the effect of combined application of the Frizzled-7 promoter and Stx1 in gastric cancer therapy surprised us. In this study, we proved overexpression of Gb3 in gastric cancer and firstly reported that the Stx1 can specifically and significantly suppress the gastric cancer cell proliferation and tumour growth when driven by the Frizzled-7 promoter.

The Frizzled-7 has been demonstrated as a critical receptor for the Wnt signalling and is involved in tumorigenesis and metastasis in many cancer types, including gastric cancer. Dr Masaru Katoh's group found that the expression level of Frizzled-7 mRNA was higher in primary gastric cancer than in the corresponding non-cancerous gastric mucosa, which was in accordance with our study. In addition, the Frizzled-7 was demonstrated to be a downstream target for β-catenin in cancer cells and overexpression of the Frizzled-7 leads to activation of the WNT-β-catenin-TCF pathway. The Frizzled-7 promoter has been verified to be highly active in liver, lung, stomach, breast, prostate and throat cancer in vitro and in vivo. It was found in our current study that the expression of Stx1 was significantly increased in the gastric cancer cells when driven by the Frizzled-7 promoter, indicating that the Frizzled-7 promoter is tumour-specific and can be effectively used in recombinant vectors. Furthermore, when the predicted conserved region of Frizzled-7 promoter (Mut FZD7) was deleted in the assay plasmid, there existed a significant reduction in the expression of the GFP and Stx1. This observation is consistent with the notion that the region is not only a conserved part but also a functionally important factor for maintenance of the Frizzled-7 promoter activity. For transcriptional regulation of the Frizzled-7, previous research showed that the Frizzled-7 promoter activity is increased in a dose-dependent manner by the β-catenin and that some extracellular matrix (ECM) components at the invasive front may influence the transcriptional regulation.

The Stx1 and other Shiga toxins are composed of two non-covalently attached parts: the enzymatically active A-subunit and non-toxic pentameric B-subunit. In our study, a mass of the Stx1 protein existed in the gastric cancer cell cytoplasm as driven after the pFZD7-Stx1 transfection. Moreover, the A-subunit of the Stx1 was cleaved and the enzymatically active A1 part was released to the cytosol. The A1 fragment irreversibly modified ribosomal 28S RNA, leading to inhibition of protein biosynthesis and cell death by apoptosis. In addition, some of the Stx1 protein may be transported outside of the cells through exocytosis but its STxB maybe specifically bound to the sugar moiety of the Gb3 in the plasma membrane from the target cancer cells. The Stx1 protein was then internalized by clathrin-independent endocytosis and transported in a retrograde fashion through the Golgi apparatus and endoplasmic reticulum. Cellular glucosylceramide is required to maintain the Gb3 in the Stx1-detectable plasma membrane lipid rafts and is also required for ER retrograde transport of the Stx1. Cells without the lipid raft expression of the Gb3 are thus insensitive to the Stx1. These ensure that our strategy has a good performance in killing the gastric cancer cells and that very little non-specific toxicity was observed upon the normal stomach tissues. Furthermore, researchers found that the major neutral GSLs, such as Gb3 from the scirrhous gastric cancer tissues, were derived from the orthotopic fibroblasts and not from cancer cells, which is quite different from non-scirrhous gastric cancer. It is possible that the orthotopic fibroblasts in the scirrhous gastric cancer tissues have been affected by cancer cells and transformed specifically to synthesize Gb3 in high quantities.

In summary, our findings suggest that the Frizzled-7 promoter is preferentially active and Gb3 is abundant in gastric cancer cells when compared with normal stomach cells. On the basis of this finding, a novel therapeutic strategy that combines the use of the Frizzled-7 promoter, driving the Stx1 and specially binding to the Gb3 of gastric cancer cells, will obviously be able to result in specific and potent outcomes in inhibiting the cancer cell proliferation and tumour growth both in vitro and in vivo.
5 | CONCLUSIONS

Taken together, the Frizzled-7 promoter is preferentially active and Gb3 is abundant in gastric cancer cells. An improved and comprehensive understanding of these and future methods that combine use of the Frizzled-7 promoter and Stx1 will define possibly definitive inhibiting of gastric cancer cell proliferation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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