RESEARCH ARTICLE

ppGalNAc T1 as a Potential Novel Marker for Human Bladder Cancer

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Abstract

Objectives: To investigate the effect of glycopeptide-preferring polypeptide GalNAc transferase 1 (ppGalNAc T1) targeted RNA interference (RNAi) on the growth and migration of human bladder carcinoma EJ cells in vitro and in vivo. Methods: DNA microarray assays were performed to determine ppGalNAc Ts(ppGalNAc T1-9) expression in human bladder cancer and normal bladder tissues. We transfected the EJ bladder cancer cell line with well-designed ppGalNAc T1 siRNA. Boyden chamber and Wound healing assays were used to investigate changes of shppGalNAc T1-EJ cell migration. Proliferation of shppGalNAc T1-EJ cells in vitro was assessed using [3H]-thymidine incorporation assay and soft agar colony formation assays. Subcutaneous bladder tumors in BALB/c nude mice were induced by inoculation of shppGalNAc T1-EJ cells and after inoculation diameters of tumors were measured every 5 days to determine gross tumor volumes. Results: ppGalNAc T1 mRNA in bladder cancer tissues was 11.2-fold higher than in normal bladder tissues. When ppGalNAc T1 expression in EJ cells was knocked down through transfection by pSUPER-shppGalNAc T1 vector, markedly reduced incorporation of [3H]-thymidine into DNA of EJ cells was observed at all time points compared with the empty vector transfected control cells. However, ppGalNAc T1 knockdown did not significantly inhibited cell migration (only 12.3%). Silenced ppGalNAc T1 expression significantly inhibited subcutaneous tumor growth compared with the control groups injected with empty vector transfected control cells. At the end of observation course (40 days), the inhibitory rate of cancerous growth for ppGalNAc T1 knockdown was 52.5%. Conclusion: ppGalNAc T1 might be a potential novel marker for human bladder cancer. Although ppGalNAc T1 knockdown caused no remarkable change in cell migration, silenced expression significantly inhibited proliferation and tumor growth of the bladder cancer EJ cell line.

Keywords: Bladder carcinoma - RNAi - ppGalNAc T1 - glycosylation - glycosyltransferase

Asian Pacific J Cancer Prev, **13 (11)**, 5653-5657

Introduction

Cancer is the leading cause of deaths worldwide with bladder cancer being eighth in the list of cancer related deaths, whose molecular mechanisms of oncogenesis have not been elucidated.

Glycosylation is a common posttranslational modification of proteins important for stability, solubility, secretion of signal, regulation of interactions, extracellular recognition, and folding (Banks et al., 2011; Boscher et al., 2011). Glycopeptide-preferring polypeptide GalNAc transferases (polypeptide N-acetylgalactosaminyltransferases, ppGalNAc-T's) catalyse the attachment of the first N-acetylgalactosamine (GalNAc) monosaccharide to the polypeptide at the initiation of O-linked glycosylation of proteins. Some members of the family are broadly expressed while others are more restricted in their distribution, their expression and activity being confined to certain cells or tissues, being associated with physiological states or differentiation (Brooks et al., 2007; Perrine et al., 2009; Gao et al., 2011; Gerken et al., 2011; Tran et al., 2012). Kato K et al. made a research that the expression levels of murine ppGalNAc-Ts (mGalNAc-Ts), T1, T2, T3, T4, T6, and T7 were compared between mouse colon carcinoma colon 38 cells and variant SL4 cells. They found that the expression levels of mGalNAc-T1, T2, and T7 were slightly higher in the SL4 cells than in the colon 38 cells, whereas the expression level of mGalNAc-T3 in the SL4 cells was 1.5% of that in the colon 38 cells (Kato et al., 2010). Sellers TA et al. investigated associations between polymorphisms in 26 glycosylation-associated genes and epithelial ovarian cancer risk. Results based on 829 cases and 939 controls suggested that a polymorphism in the ppGalNAc T1 gene was statistically significantly ($P = 0.00017$) inversely associated with epithelial ovarian

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cancer under a recessive model.

ppGalNAc-ts have been seen to correlate with the degree of proliferation and recurrence in carcinomas of human breast cancer (Brooks et al., 2007)), human ovarian cancer, mouse colon carcinoma (Kato et al., 2010), et al. However, there was no report regarding ppGalNAc Ts in human bladder carcinoma.

In order to explore the function of the ppGalNAc T1 gene in human bladder cancer, we genotyped this ppGalNAc T1 variant in 20 independent study populations from the the Second Affiliated Hospital of Kunming Medical University and performed the relevant experiments.

Materials and Methods

DNA microarray assay

This study was approved by the Ethics Committee of Kunming Medical University, and all the patients in this study were informed consent. The mRNA samples were extracted from 10 early stage patients and 10 normal bladder tissues obtained from the Second Affiliated Hospital of Kunming Medical University. DNA microarray assay was performed on bladder tumor tissues of patients and normal bladder tissues to identify the glycosyltransferase responsible for glycosylation. Microarray analysis was used to reveal ppGalNAc ts (ppGalNAc T1-9) mRNA expression in bladder tumor tissues and normal bladder tissues. The housekeeping gene GAPDH was served as a control.

Cell culture and seeding

Human bladder cancer cell line EJ was grown in 25 cm^2 culture flask in RPMI-1640, supplemented with 1% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. When the EJ cells had reached 80%-90% confluency, we removed the medium and washed the cells with phosphate-buffered saline (PBS). Trypsin-EDTA was added to detach the cells from the flask and the suspended cells were collected in a fresh medium and transferred to new flasks.

Silencing of ppGalNAc T1

The RNA sequence against ppGalNAc T1 (N-acetylgalactosaminyltransferase type1) for RNAi was designed based on pSUPER system instructions (Oligoengine) and cloned into pSUPER-puro that expresses 19 nt hairpin-type short hairpin RNA (shRNA) with a 9 nt loop. ppGalNAc T1 shRNA-encoding sequences were as follows: 5'-GATCCCCCCAGTACAAAA GCCTCA TG TTCAAGAGACATGAGGCTTTTGTACTGGTTTTTA -3' (ppGalNAc T1, sense); 5'-AGCTTAAAAACCAGTA CAAAAGCCTCATGTCTCTTGAACATGAGGCTTT TGTACTGGGGG-3' (ppGalNAc T1, antisence). The inserted shRNAs (pSUPER-shppGalNAc T1) were confirmed by DNA sequencing. EJ cells were transfected by using Lipofectamin 2000 (Invitrogen, US) as described by the manufacturer. ppGalNAc T1 silenced cells were selected with puromycin (Sigma, Germany). Empty vectors transfected cells were used as a control.

Cell invasion and migration assay

Transwell assays were performed using a modified Boyden chamber assay using transwells (8 μm pore size; Costar, US). The transwells were coated with matrigel solution at 100 μg/ml and then blocked with 1% BSA in PBS. shppGalNAc T1 silenced EJ cells were trypsinized, washed, and resuspended in serum-free medium containing 1% BSA. Lower chambers contained medium containing 10% FBS or 1% BSA (negative control). After incubation for 4 hours at 37°C, cells remaining at the upper surface of the membrane were removed using a swab, while the cells that migrated to the lower membrane surface were fixed with ethanol and stained with Giemsa solution. The number of cells migrating through the filter was counted and plotted as the number of migrating cells per optic field (×20). Wound-healing assay was performed as described. The distance covered by a cell moving into the scratch wound area was measured, and the images were analyzed.

Cell proliferation assay in vitro

Proliferation of cells was measured using a [3H]-thymidine incorporation assay. Wide type EJ cells, shppGalNAc T1 silenced EJ cells (2×10^3 cells/well) were seeded on 96-well culture plates, cultured until the cells reached 70% to 80% confluency, and then serum starved in RPMI-1640 for 24 hours. Change serum-free medium with RPMI-1640 containing 10% FBS. After 24, 48 and 72 hours, cells were pulsed with [3H]-thymidine for 4 hours. Cells were harvested and their [3H]-thymidine incorporation was measured in the liquid scintillation counter LKB1219.

Colony formation assay

Two 12-well plates were taken, and 0.6% agar was put at the bottom of the hole. The primary Vector-EJ and shppGalNAc T1-EJ cells (0.5×10^4, 1 ml, respectively) were mixed with 0.6% agar 25ml, 2 × containing penicillin streptomycin and 20% fetal calf serum RPMI1640 24 ml, and each hole with 3ml. The 12-well plates were incubated in 37°C, 5%CO_2 incubator. The growth medium was changed every other day. Ten days later, the cell clone counts were observed under the microscope, and the clone formation rates were calculated. Three independent experiments were conducted and analyzed.

Cancerous proliferation and tumor growth assay in vivo

BALB/c nude mice were obtained from the Animal Center of the Chinese Academy of Medical Science, Beijing. Female 6-week-old BALB/c nude mice with a body weight of approximately 16 g were used and kept under specific pathogen-free conditions. To determine in vivo effects of ppGalNAc T1 knockdown on cancerous growth, we established xenografted tumor models by subcutaneously injecting Vector -EJ cells and shppGalNAc T1-EJ cells into the back of BALB/c nude mice. Xenografts of EJ cells (include Vector -EJ cells, and shppGalNAc T1 silenced EJ cells) were produced by injecting tumor cells (1×10^7 resuspended in PBS) subcutaneously into the back of mice. Tumor size was measured every 5 days. The approximate size of the tumor is: $V=\pi/6\times m1^2\times m^2$, and m1 is the shorter length of the path line. The mean tumor volume of each group

DOI:http://dx.doi.org/10.7314/APJCP.2012.13.11.5653

Table 1. The result of Glycopeptide-preferring Polypeptide GalNAc Transferase (ppGalNAc T) Expressed in the Bladder Cancer Compared to the Normal Bladder Tissues

GenBank No.	Gene name	Normal bladder tissues		Bladder tumor tissues		Fold-change[3]
		Signal[1]	Detection[2]	Signal[1]	Detection[2]	
NM_020474	ppGalNAc T 1	303±26	P	3384±142	P	11.2
NM_004481	ppGalNAc T 2	748±23	P	703±35	P	0.9
NM_004482	ppGalNAc T 3	1854±72	P	1315±118	P	0.7
NM_003774	PpGalNAc T4	36±3	A	42±7	A	1.2
NM_014568	ppGalNAc T 5	12±1	A	16±1	A	1.3
NM_007210	ppGalNAc T 6	815±39	P	902±45	P	1.1
NM_017423	ppGalNAc T 7	735±43	P	836±72	P	1.1
NM_017417	ppGalNAc T 8	87±9	A	107±23	A	1.2
NM_021808	PpGalNAc T 9	172±26	A	166±18	A	0.9
NM_002046	GAPDH	80942±294	P	81409±367	P	1

Gene expression profile of human normal bladder tissues and bladder tumor tissues; A, absent; P, present; [1]Calculated mean fluorescence intensity signal; [2]Classification of gene expression as absent or present; [3]Proportion of the signals from experiments with bladder tumor tissues to normal bladder tissues for genes with changed expression (fold change >1 for increased and <1 for decreased gene expression)

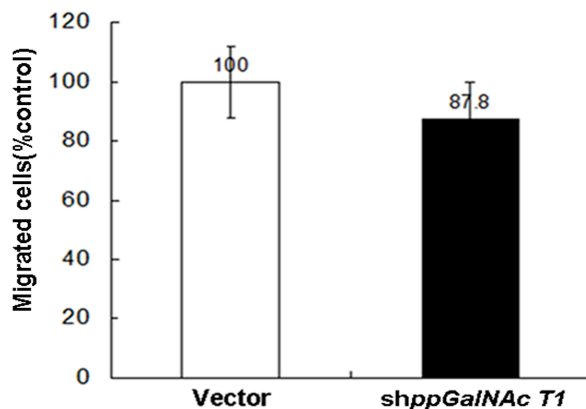


Figure 1. The Migration of ShppGalNAc T1-EJ Cells was Investigated with Transwell

was calculated, and the death time of the nude mice were recorded.

Statistical analysis

Statistical analysis was performed using one-way ANOVA or Student's t test. Values of $P<0.05$ were considered significant. Data were represented as the mean±SD. SPSS v17.0 software was used for all data analysis.

Results

Glycopeptide-preferring polypeptide GalNAc transferase 1 (ppGalNAc T1) is highly expressed in the bladder cancer tissues

Microarray analysis revealed that ppGalNAc T1 mRNA in bladder tumor tissues was 11.2-fold higher than in normal bladder tissues (Table 1). While other ppGalNAc T (ppGalNAc T2-9) had no significant changes in bladder tumor tissues and normal bladder tissues.

ppGalNAc T1 knockdown inhibits cancerous invasion in vitro

Transwell invasion results showed that EJ cells can pass through the Matrigel matrix covered with artificial plastic poly-carbonate membrane. In the empty vector-

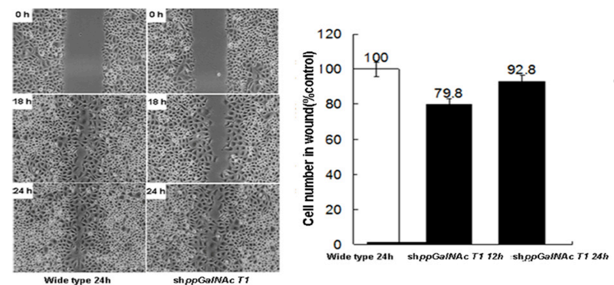
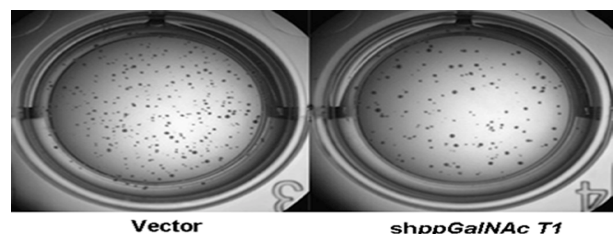


Figure 2. Migration of ShppGalNAc T1-EJ Cells Investigated with Wound Healing Assay



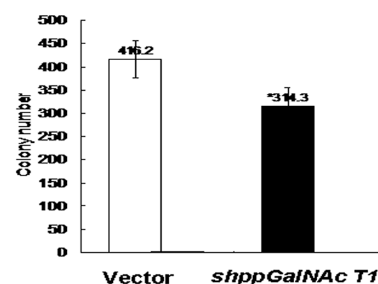


Figure 3. Proliferation of ShppGalNAc T1-EJ Cells were Measured Using Soft Agar Colony Formation Assay

transfected EJ cells group, a large number of EJ cells permeated to the other side of Transwell membrane, but In shppGalNAc T1-EJ cells group, the number of EJ cells that permeated to the other side of Transwell membrane was reduced (Figure 1).

ppGalNAc T1 knockdown inhibits cancerous migration in vitro

Wound healing assay showed different results. We found that ppGalNAc T1 knockdown had no remarkable

100.0
75.0
50.0
25.0
0
6
56
31

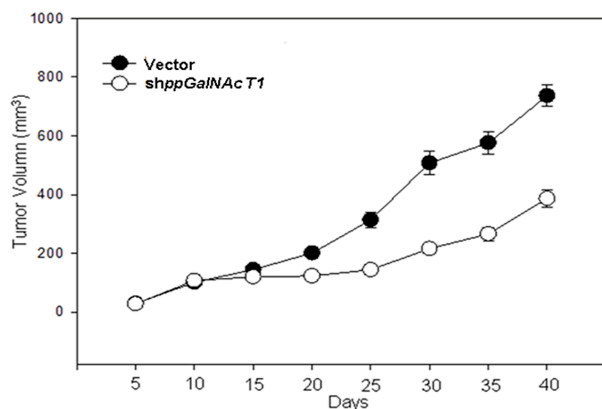


Figure 4. Inhibitory Rate of Cancerous Growth for ppGalNAc T1 Knockdown

changes in cell migration (Figure 2). These indicate that ppGalNAc T1 silencing can not inhibit cell migration in human bladder cancer cell line EJ.

ppGalNAc T1 knockdown inhibits cancerous proliferation in vitro

ppGalNAc T1 knockdown markedly reduced incorporation of [3H]-thymidine into DNA of EJ cells at all time points compared with the empty vector transfected control cells. ppGalNAc T1 knockdown significantly decreased colony formations of EJ cells compared with the empty vector transfected control cells. Silenced ppGalNAc T1 expression significantly inhibited subcutaneous tumor growth compared with the control groups by injection of the empty vector transfected control cells. At the end of observation course (40 days), the inhibitory rate of cancerous growth for ppGalNAc T1 knockdown was 42%. These data indicate that silenced ppGalNAc T1 expression significantly inhibits proliferation and tumor growth of bladder cancer. While ppGalNAc T1 knockdown significantly decreased colony formations of EJ cells compared with the empty vector transfected control cells. (Figure 3)

ppGalNAc T1 knockdown inhibits cancerous proliferation and tumor growth in vivo

Silenced ppGalNAc T1 expression significantly inhibited subcutaneous tumor growth compared with the control groups by injection of the empty vector transfected control cells (Figure 4). At the end of observation course (40 days), the inhibitory rate of tumor volumn for ppGalNAc T1 knockdown was 52.5%, and the inhibitory rate of tumor weight for ppGalNAc T1 knockdown was 45.1%. These data indicate that silenced ppGalNAc T1 expression significantly inhibits proliferation and tumor growth of bladder cancer.

Discussion

Bladder cancer is one of most common malignant cancers. Its main pathological features are multiple and easily recurrent. Increasing evidence showed tumorigenesis of bladder cancer contributes to alterations in molecular pathways that modulate cellular homeostasis (Platt et al., 2009; Gallagher et al., 2010). However, the molecular mechanisms of malignant transformation of bladder cancer have not been elucidated.

There were a few researches on ppGalNAc ts (Topaz et al., 2005; Brooks et al., 2007; Perrine et al., 2009; Kato et al., 2010; Zlocowski et al., 2011; Liu et al., 2011; Tran et al., 2012). Brooks S A, et found that expression of normally tightly restricted ppGalNAc-Ts may result in initiation of O-linked glycosylation at normally unoccupied potential glycosylation sites leading to altered glycoforms of proteins with changed biological activity which may contribute to the pathogenesis of cancer.Expression of ppGalNAc T1 gene was statistically significantly inversely associated with epithelial ovarian cancer. However, expression and potential role of ppGalNAc T1 gene in other cancers are still largely unknown. This is the first study investigating the expression and prognostic relevance of ppGalNAc T1 in human bladder cancer.

In our study, DNA microarray showed that the glycosyltransferase ppGalNAc T1 in the bladder tissues was expressed eleven-fold higher than the normal bladder tissues. Silenced ppGalNAc T1 expression suppressed cancerous proliferation and tumor growth in vitro and in vivo, and the glycosyltransferase ppGalNAc T1 may responsible for the aberrant glycosylation.

ppGalNAc T1 expression was related to cancerous proliferation and tumor growth in vitro and in vivo. We have provided the initial observations that knockdown of ppGalNAc T1 expression by siRNAs attenuates tumorigenicity of bladder cancer cells. Silenced ppGalNAc T1 expression significantly inhibits proliferation and tumor growth of bladder cancer.

This study supports the role of ppGalNAc T1 as a tumor-related gene, and its potential utility as a clinical prognostic marker in human bladder carcinoma.

ppGalNAc T1 may be a useful indicator of the tumor proliferation and tumor growth However, given the ppGalNAc T1 gene may be a potential tumor-related gene in human bladder cancer, as well as other cancers, future studies will need to focus on how ppGalNAc T1 is regulated and the mechanisms of its anticancer functions, with the goal being to critically examine the possibilities of exploiting ppGalNAc T1 as a therapeutic target.

In conclusion, using both DNA microarray and biological behaviour researches, expression of the ppGalNAc T1 gene in human bladder carcinoma has been confirmed, and ppGalNAc T1 gene may be a prognostic factor in this carcinoma.

Acknowledgements

This research is supported by the Doctor Innovation Fundation of Kunming Medcial University (No. 2009003).

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