

## RESEARCH ARTICLE

# Golgi Phosphoprotein 2 Down-regulates the Th1 Response in Human Gastric Cancer Cells by Suppressing IL-12A

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## Abstract

Golgi phosphoprotein 2 (GOLPH2) is a very important biomarker in a variety of diseases. Its biological function is not clear, particularly in gastric cancer. To investigate the role of GOLPH2 in human gastric cancer, and determine its effect on the Th1 lymphocyte response, its expression and that of IL-12A were measured by real-time PCR and immunohistochemistry. The relationship between GOLPH2 and IL-12A was analysed statistically. The effect of GOLPH2 on the Th1 lymphocyte response was investigated with an *in vitro* co-culture system. The results showed that in human gastric cancer, the expression of GOLPH2 was significantly higher and the expression of IL-12A was lower than in normal gastric mucosal tissues, and the expression levels of GOLPH2 and IL-12A were negatively correlated. In addition, obvious down-regulation of the Th1 response was observed when lymphocytes were co-cultured with gastric cancer SGC7901 cells over-expressing GOLPH2. GOLPH2 down-regulated the expression of IL-12A, and inhibited the expression of TNF- $\alpha$  and IFN- $\gamma$ . The results indicated that GOLPH2 down-regulates the Th1 response via suppression of IL-12A in human gastric cancer, and this might provide a target for the prevention and treatment.

**Keywords:** Golgi phosphoprotein 2 - Th1 response - human gastric cancer - IL-12A

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## Introduction

Golgi phosphoprotein 2 (GOLPH2), discovered in 2000, is a type II trans-membrane protein located in the Golgi (Gong et al., 2012). It was subsequently found in many vertebrates, such as zebra fish, chicken, mice, cattle, dogs, gorillas, and others. Under normal conditions, GOLPH2 is mainly localized in the cis Golgi; however, it can be secreted into the blood through the membrane transport system (Hu et al., 2011). GOLPH2 is a very important biomarker in a variety of diseases (Kim et al., 2012). GOLPH2 was detected in the lesions and sera of patients with liver disease (Riener et al., 2009), and in the lesions of patients with kidney cancer (Fritzsche et al., 2008). Patients with prostate cancer also had significantly higher expression of GOLPH2 (Kristiansen et al., 2008; Wei et al., 2008). The expression of GOLPH2 was also significantly higher in the urine of patients with prostate cancer than in normal individuals (Laxman et al., 2008; Roobol et al., 2011). In addition, GOLPH2 is also associated with seminomas and lung adenocarcinomas (Fritzsche et al., 2010; Zhang et al., 2010). However, the biological function of GOLPH2 is not clear, particularly in gastric cancer. Therefore, this study is focused on GOLPH2 expression in human gastric cancer and its impact on the Th1 lymphocyte response.

## Materials and Methods

### *Tissues and cells*

Paraffin-embedded tumours and adjacent normal tissue samples from 60 patients (30 males with an average age of 47.8 years and 30 females with an average age of 42.9 years) with gastric cancer who underwent tumour resection at Pu Tuo Hospital, Shanghai University of Traditional Chinese Medicine between 2011 and 2012 were collected. Each tissue sample was obtained under sterile conditions by removing the gastric cancer tissue and normal colon mucosa along the surgical margin. This study was approved by the ethics committee of Pu Tuo Hospital, Shanghai University of Traditional Chinese Medicine. All patients provided written informed consent. SGC-7901 cells obtained from ATCC were cultured in 1640 RPM supplemented with 10% foetal calf serum, 100 U/mL penicillin, and 100 g/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### *Real-time PCR*

Total RNA was extracted from tissues with TRIzol, and cDNA was synthesized using a reverse transcription kit from Takara according to the manufacturer's instructions. All the primers were designed and synthesized by Shanghai Biological Engineering Co., and the primer sequences and

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**Table 1. Primer Sequences and the Length of the Fragments Amplified by RT-PCR**

Gene	Sequences (5'-3')	Amplified fragments
T-bet	Forward: GGTTGCGGAGACATGCTGA Reverse: GTAGGCGTAGGCTCCAAGG	213 bp
GOLPH2	Forward: TGGCCTGCATCATCGTCTTG Reverse: CCCTGGAACCTCGTTCTTCTCA	151 bp
GAPDH	Forward: CAACTGGTCGTGGACAACCAT Reverse: GCACGGAACTCACAATGTTTC	130 bp

amplified fragments are shown in Table 1. A SYBR green I mix kit from Takara was used, and the total reaction volume was 20  $\mu$ L, which contained 10  $\mu$ L of SYBR green premix, 0.3  $\mu$ L of correction dye, 1.5  $\mu$ L of complementary strand DNA (cDNA), 0.5  $\mu$ L of each primer (forward and reverse), and water to 20  $\mu$ L. The reaction program was 94°C for 5 min, followed by 35 cycles of 94°C for 15 s, 58°C for 15 s, and 72°C for 15 s. Real-time PCR was performed using the Applied Biosystems 7300 System (Applied Biosystems, Deutschland GmbH).

#### Immunohistochemistry

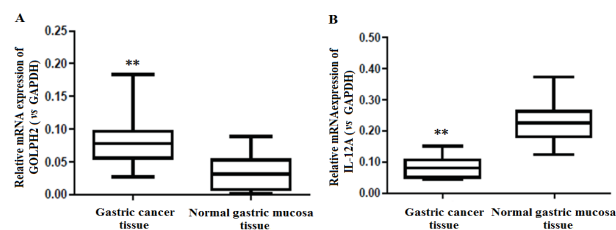
Paraffin-embedded tumour tissues from 60 patients with gastric cancer were cut into 4- $\mu$ m thick sections, dewaxed with xylene and graded ethanol, and then soaked in a solution containing 30 mL/L hydrogen peroxide in methanol for 30 min at room temperature. The sections were heated in an EDTA solution (0.01 mol/L, pH 8.0) for 20 min to fix the antigen. The sections were washed 3 times with PBS, and then incubated with normal goat serum for 10 min to block nonspecific binding. The sections were then incubated with the primary monoclonal antibody (1:500) at 4°C overnight, rinsed 3 times with PBS, and incubated with the secondary antibody (1:5000) at room temperature for 10 min. The sections were rinsed 3 times with PBS, and then streptavidin-peroxidase solution was added and the sections were incubated at room temperature for 10 min. The sections were rinsed with PBS 3 times, and then stained with DAB and hematoxylin. A DMI3000 B microscope connected to a digital imaging system was used to analyse the sections. Immunohistochemical staining of GOLPH2 and IL-12A was quantitatively evaluated using the professional image analysis software Image-Pro Plus (version 5.0). Five fields (200 $\times$ ) of each section were selected for analysis, and the integrated optical density (IOD) was measured.

#### Cell transfection

All the transfections into SGC-7901 cells were performed using Lipofectamine 2000 transfection reagent from Invitrogen as follows: 1 day before transfection, SGC-7901 cells were seeded in 24-well plates at  $5 \times 10^4$ /well. Twenty-four hours later, the control pcDNA3.3 plasmid and the pcDNA3.3-GOLPH2 recombinant plasmid were transfected separately into SGC-7901 cells according to the manufacturer's instructions. Western blotting was used to verify the efficiency of transfection.

#### Western blotting

Whole-cell proteins from SGC-7901 cells transfected



**Figure 1. Measurement of GOLPH2 and IL-12A mRNA Expression in Human Gastric Cancer Tissues and Adjacent Normal Gastric Mucosa by Real-time PCR.** (A) mRNA expression of GOLPH2 and (B) mRNA expression of IL-12A, \*\* $P < 0.01$  vs. mRNA expression in normal gastric mucosa

with either the control pcDNA3.3 plasmid or the pcDNA3.3-GOLPH2 recombinant plasmid were prepared according to the instructions for the ProteoJET Cytoplasmic Protein Extraction Kit (Fermentas, USA). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blocked with 5% BSA prior to incubation with the indicated primary GOLPH2 antibody, and then the secondary antibody. Protein loading was normalized to GAPDH expression. The resulting immunocomplexes were visualized by enhanced chemiluminescence.

#### Flow cytometry

Before fluorescent staining, lipopolysaccharide (LPS) was used to stimulate freshly isolated peripheral blood lymphocytes for 5 h. The cells were then collected and immunostained with a fluorescent antibody. After staining, the cells were resuspended in a PBS solution containing 1% FBS. Expression of the cell-associated molecules was detected by flow cytometry. Each sample contained more than  $1 \times 10^5$  cells. Flow cytometry was conducted using a FACSCalibur flow cytometer (BD Biosciences, USA), and the data were analysed with CellQuest software.

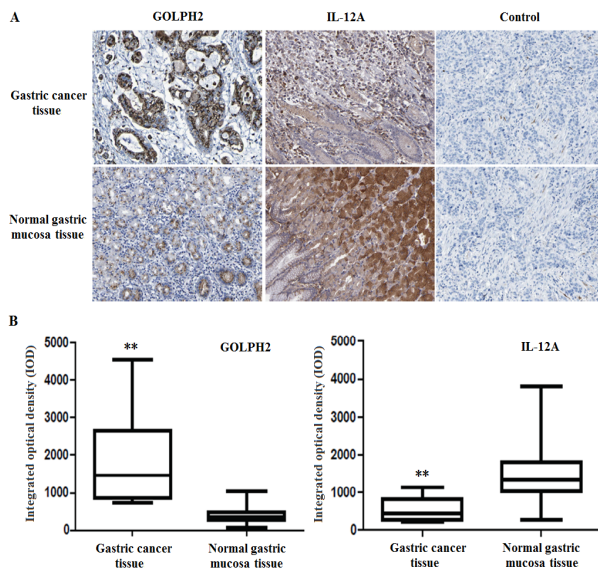
#### Statistical analysis

All data are expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm S$ ), and the data were analysed with SPSS18 Software. Expression of each gene in gastric cancer tissues and normal tissues was compared using one-way ANOVA. The correlation between GOLPH2 gene expression and IL-12A in gastric cancer tissues was assessed with Spearman's rank correlation combined with an F test. A  $P$  value of less than 0.05 was considered statistically significant.

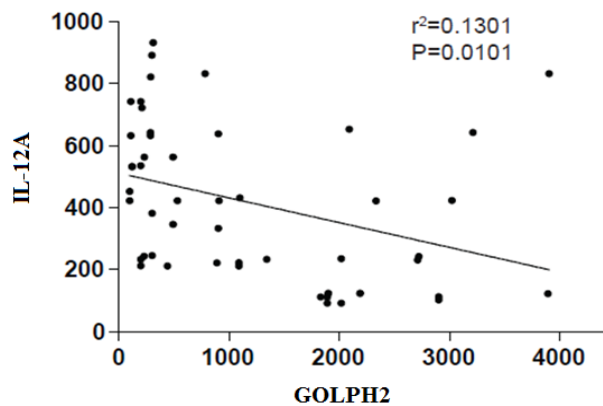
## Results

### *GOLPH2 was up-regulated and IL-12A was down-regulated in human gastric cancer tissues*

The real-time PCR results showed that GOLPH2 was significantly higher in human gastric cancer tissues than in the adjacent normal gastric mucosa ( $P < 0.01$ ) (Figure 1), whereas IL-12A expression was significantly lower in gastric cancer tissues than in the adjacent normal tissues ( $P < 0.01$ ). Immunohistochemistry showed that GOLPH2 was mainly expressed in gastric cancer cells, whereas IL-12A was only expressed in mesenchymal cells and not in the gastric cancer cells. Additionally, higher IL-12A expression was observed in normal gastric mucosa (Figure 2).



**Figure 2. Detection of GOLPH2 and IL-12A Protein in Human Gastric Cancer Tissues and Adjacent Normal Gastric Mucosa by Immunohistochemistry.** (A) Images of GOLPH2 and IL-12A immunohistochemical staining. (B) Immunohistochemical staining of GOLPH2 and IL-12A was quantitatively evaluated using the professional image analysis software Image-Pro Plus (version 5.0). Five fields (200 $\times$ ) in each section were selected to measure integrated optical density (IOD). \*\* $P < 0.01$  vs. the adjacent normal gastric mucosa



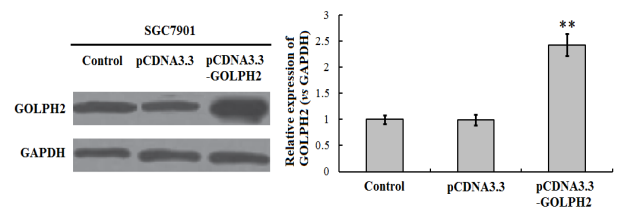
**Figure 3. Negative Correlation Between GOLPH2 and IL-12A Expression in Gastric Cancer Tissues.** Results of IHC staining were evaluated, and the correlation between GOLPH2 and IL-12A protein expression was analyzed with Spearman's rank correlation combined with an F test. \* $P < 0.05$  was considered statistically significant (vs. the control group)

*The expression of GOLPH2 and IL-12A in gastric cancer tissues was negatively correlated*

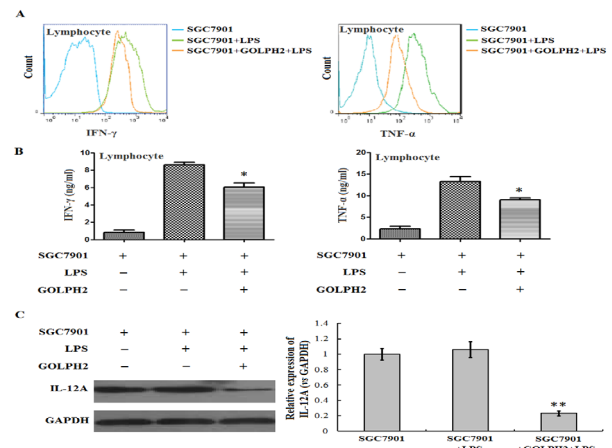
The IHC staining results were evaluated, and the correlation between GOLPH2 and IL-12A expression was analysed. The data analysis indicated that in gastric cancer tissues, GOLPH2 and IL-12A expression was negatively correlated ( $r^2 = 0.1301$ ,  $P = 0.01$ ) (Figure 3).

*Overexpression of GOLPH down-regulated the Th1 response in the gastric cancer cell line SGC7901*

To investigate the potential effect of GOLPH2, the pCDNA3.3-GOLPH2 plasmid was transfected into human gastric cancer SGC7901 cells. GOLPH2 expression in pCDNA3.3-GOLPH2-transfected SGC7901 cells was significantly higher than in control pCDNA3.3-transfected



**Figure 4. Overexpression of GOLPH2 Reduced the Expression of IL-12A.** The control pCDNA3.3 plasmid and the pCDNA3.3-GOLPH2 plasmid were transfected separately into human gastric cancer SGC7901 cells. Expression of GOLPH2 was confirmed by western blotting. \*\* $P < 0.01$  vs. the control pCDNA3.3-transfected cells



**Figure 5. Overexpression of GOLPH Down-regulated the Th1 Response in Gastric Cancer SGC7901 Cells.** pCDNA3.3 or pCDNA3.3-GOLPH2-transfected SGC7901 cells were co-cultured in vitro with human peripheral blood lymphocytes at a ratio of 1:100 in a transwell culture system. The co-cultures were stimulated with lipopolysaccharide (LPS) for 6 h. (A) The expression of TNF- $\alpha$  and IFN- $\gamma$  was measured in the lymphocyte cells by flow cytometry; (B) Expression of TNF- $\alpha$  and IFN- $\gamma$  in the supernatant of the co-culture system as detected by ELISA, \* $P < 0.05$  vs. pCDNA3.3-transfected SGC7901 cells stimulated with LPS; (C) Expression of IL-12A in whole-cell lysates from lymphocytes was measured by western blot, \*\* $P < 0.01$  vs. pCDNA3.3-transfected SGC7901

SGC7901 cells (Figure 4).

pCDNA3.3-GOLPH2-transfected SGC7901 cells were co-cultured with human peripheral blood lymphocytes at a ratio of 1:100 in a transwell culture system. LPS was added to stimulate the cells for 6 h, and then several key experiments were performed.

First, lymphocyte cells were collected and the expression of TNF- $\alpha$  and IFN- $\gamma$  (cytokine markers of the Th1 response) was measured by flow cytometry. The results demonstrated that overexpression of GOLPH2 in gastric cancer SGC7901 cells significantly reduced the secretion of the Th1-type cytokines TNF- $\alpha$  and IFN- $\gamma$  from lymphocytes (Figure 5A). Second, the expression of TNF- $\alpha$  and IFN- $\gamma$  in the supernatant of the co-culture system was detected by ELISA. Similar to the flow cytometry results, overexpression of GOLPH2 in gastric cancer SGC7901 cells significantly reduced the secretion of the Th1-type cytokines TNF- $\alpha$  and IFN- $\gamma$  from lymphocyte (Figure 5B). Third, the lymphocytes were collected and whole-cell proteins were extracted, and then the expression of IL-12A in the Th1 lymphocytes

was measured by western blot. The results suggested that overexpression of GOLPH2 in gastric cancer SGC7901 cells significantly reduced the expression of IL-12A in co-cultured lymphocytes (Figure 5C).

## Discussion

The human GOLPH2 protein is encoded by the GOLM1 gene, which is located on chromosome 9 (9q21.33) (Gong et al., 2012). It is a conserved protein comprised of 401 amino acids, of which the N-terminal domain is located in the cytoplasm, and a large fragment of the trans-membrane region is located inside the Golgiosome (Hu et al., 2011).

GOLPH2 expression is closely correlated with many diseases (Kim et al., 2012), including hepatocellular carcinoma (Riener et al., 2009), kidney cancer (Fritzsche et al., 2008), prostate cancer (Kristiansen et al., 2008; Wei et al., 2008; Laxman et al., 2008; Roobol et al., 2011), seminoma (Fritzsche et al., 2010), lung adenocarcinoma (Zhang et al., 2010), and others. GOLPH2 expression was significantly higher in the urine of patients with prostate cancer than in normal controls (Laxman et al., 2008; Roobol et al., 2011), and both the mRNA and protein levels of GOLPH2 were significantly higher than in the normal controls.

However, there are very few studies on GOLPH2 in gastric cancer. Gastric cancer is a common malignant tumour, and reduced apoptosis, abnormal differentiation of tumour cells, and excessive cell proliferation are the main causes of this disease (Correa et al., 2013). Gastric cancer is associated with reflux disease (Kim et al., 2013), and many premalignant conditions often lead to the development of gastric cancer (Sugano et al., 2013). Many potential biomarkers (Pietrantonio et al., 2013) have been found, and their possible use in the diagnosis and treatment of gastric cancer has been reported. GOLPH2 may be an important biomarker of gastric cancer. Our findings suggested that GOLPH2 could regulate the immune status of gastric cancer through down-regulation of IL-12A.

Our study was the first to show that GOLPH2 was highly expressed in human gastric cancer, whereas IL-12A expression was significantly reduced, which implies that GOLPH2 was closely related to the down-regulation of IL-12A. IL-12A is a protein encoded by the IL12A gene in humans (Wolf et al., 1991). IL12A is required for T-cell-independent induction of IFN- $\gamma$ , and is important for the differentiation of both Th1 and Th2 cells. IL-12A can activate the cytotoxicity of MHC-I-type non-specific antigens in NK cells, induce Th1 immune effector cells, and activate cytotoxic T lymphocytes, thereby specifically eliminating tumour cells and providing long-term protective immunity (Airolidi et al., 2002; Peluso et al., 2006). Our results suggested that overexpression of GOLPH2 in gastric cancer SGC7901 cells could significantly reduce the expression of IL-12A in co-cultured lymphocytes.

In our experiment, LPS was used to stimulate freshly isolated peripheral blood lymphocytes. LPS, also known as lipoglycan, is found in the outer membrane of Gram-negative bacteria, and acts as an endotoxin and elicits

strong immune responses in animals. LPS acts as a prototypical endotoxin due to its binding to the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in a variety of cell types (Reid et al., 1997; Boes et al., 1998; Said et al., 2010).

TNF- $\alpha$  and IFN- $\gamma$  are cytokine markers of the Th1 response (Romer et al., 1995; Rival et al., 1996; Lei et al., 2013). TNF is a cytokine involved in inflammation that stimulates the acute phase reaction (Old et al., 1985). Dysregulation of TNF production has been implicated in a variety of human diseases, including Alzheimer's disease, cancer, major depression, and inflammatory bowel disease (IBD) (Wajant et al., 2003; Clark et al., 2007). IFN- $\gamma$  is a dimerized soluble cytokine that is the only member of the type II class of interferons. In humans, IFN $\gamma$  is encoded by the IFNG gene (Gray et al., 1982). This interferon, which early in its history was known as immune interferon, was first reported in 1970 (Schroder et al., 2004; Schoenborn et al., 2007). Our data demonstrated that overexpression of GOLPH2 in gastric cancer SGC7901 cells significantly reduced the secretion of the Th1-type cytokines TNF- $\alpha$  and IFN- $\gamma$  from lymphocytes.

In conclusion, GOLPH2 could down-regulate the Th1 response in human gastric cancer cells through suppression of IL-12A. This implies that high expression of GOLPH2 in gastric cancer might promote the formation of an immune escape state in the gastric tumour microenvironment through inhibition of the Th1 lymphocyte response via down-regulation of IL-12A expression. However, further research is needed to obtain more data to support our present results, which might provide a target for the prevention and treatment of gastric cancer.

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