Thrombopoietin is associated with a prognosis of gastric adenocarcinoma

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SUMMARY

OBJECTIVE: *Thrombopoietin (THPO) is well-known as a megakaryocyte growth and development factor (MGDF) involved in megakaryocyte proliferation and maturation. To explore the biological effects of THPO in gastric adenocarcinoma, we conducted this study. Methods: By accessing the TCGA database, the expression level of THPO was determined in tumor tissues. The association between THPO expression and clinical features, or prognostic significance was described by Cox regression analysis and Kaplan-Meier. The SiRNA method was used to decline the THPO expression; then cell viability, invasion, and migration were detected to verify the effects of the knockdown of THPO. qPCR and western blotting were implemented to examine the expression level of THPO. Results: The expression of THPO was increased in tumor tissue and cells, its high-regulation was associated with a poor prognosis in patients with gastric adenocarcinoma. Cell viability, invasion, and migration were suppressed in AGS with the down-regulation of THPO. Furthermore, on the basis of si-THPO transfection, E-cadherin was promoted while N-cadherin and Vimentin were attenuated.*

CONCLUSION: *Our results revealed that THPO may be a potent marker of gastric adenocarcinoma, providing a novel potential screening method for gastric adenocarcinoma.*

KEYWORDS: *Adenocarcinoma. Stomach neoplasms. Thrombopoietin. Prognosis. Cell movement. Epithelial-mesenchymal transition.*

INTRODUCTION

Gastric cancer is a type of universal malignancy, which ranks fifth regarding cancer incidence rate and third in mortality worldwide[1]. Different regions cause different occurrences. It is well known that China is also a region with a frequent occurrence of gastric cancer[2,3].

Thrombopoietin (THPO) is a key protein, also regarded as megakaryocyte growth and development factor (MGDF), which is encoded by *the THPO* gene in human[s4,5]. Increasing evidence has demonstrated that THPO levels could affect diverse diseases that consist of hematological diseases, acute coronary syndromes, cardiovascular damage, and sepsis[6-8]. In hepatoblastoma cells, THPO can promote cell migration and adjust various signaling pathways[9]. Nevertheless, there is seldom an understanding of the potential effect of THPO in gastric adenocarcinoma.

Hence, in this exploration, we analyzed the THPO expression level in gastric adenocarcinoma tissue and gastric cancer cells.

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METHODS

Cell lines and clinical samples

The study was supported by the TCGA database, which provided us with THPO expressional profiles, including 32 human normal tissues and 375 tumor tissues. Based on the data derived from the TCGA dataset, the overall survival curve of 366 patients with gastric adenocarcinoma was drawn, and analysis of clinical features and Cox regression were elaborated. Four gastric cancer cell lines (BGC-823, MKN-45, and AGS) and one human normal gastric epithelial cell line GES-1 were purchased from the Cell Biology Department of the Chinese Academy of Sciences (Shanghai, China).

Cell culture and transfection

All the cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1g/mL streptomycin. The cell culture environment should be held at 37º and 5% CO2. For the following experiments, the siRNA strategy was used to deal with cells and conducted by Lipofectamine2000 in line with the manufacturer's instructions. At 24 h post-transfection, the expression level of THPO could be observed. The sequences of siRNAs were as follows: si-THPO#1: 5'-CGGACATTTCCTCAGGAACATCTC-3'; si-THPO#2: 5'-AGCTAGCTCTTTGGTCTATTTC-3'; si-con: 5'-CTTCTCCTAACTGCAAGGCTA-3'.usedd

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To examine the relative expression of THPO, we carried out the qRT-PCR method. Briefly, total RNA was collected from cells using TRIzol solution (Invitrogen, Carlsbad, CA, USA). Then, the first-strand cDNA was synthesized by SuperScript III RNase H Reverse Transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Lastly, cDNA was subjected to RT-PCR on ABI 7500 fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc. Waltham, MA, USA) with SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA). GAPDH was considered as a standard control. The qRT-PCR was run at 95º for 5min, then 95º for 30s and 40 cycles, 60º for 45s, and 72º for 30min. The following primers were presented: THPO: 5'-GATACTCGAAGGACAGGCCG-3', 5'-CAGAGTAGGGTGGGGCAAAG-3'; GAPDH: 5'-GCTCTCTGCTCCTCCTGTTC-3', 5'-CGACCAAATCCGTTGACTCC-3'. They were synthesized by GenePharma Co., Ltd (Shanghai, China) and the expression level was calculated through $2^{-\Delta\Delta CT}$ method.

Protein isolation and western blot analysis

After 48 h transfection, cells were lysed with RIPA lysis buffer (protease inhibitor; Thermo Fisher Scientific, MA, USA) to extract protein on ice. The concentration was identified by the BCA method followed by centrifugation, and 5 × SDS loading buffer was used to denature protein at 95º for 5 min. Each sample containing 20μg protein was resolved by SDS-PAGE (Sigma-Aldrich, MO, USA), transferred to a PVDF membrane, and blocked with 5% non-fat milk for 1 h. Subsequently, the membrane was incubated in primary antibodies (at a 1:1, 000 dilution, Abcam, Cambridge, UK) overnight and secondary antibody (at a 1:2, 000 dilution, Abcam, Cambridge, UK) for 1 h. Finally, the membrane was washed using TBST and developed via adding ECL. These antibodies were purchased from Abcam, including THPO (ab196026), E-cadherin (ab40772), N-cadherin (ab18203), Vimentin (ab92547), and GAPDH (ab181602).

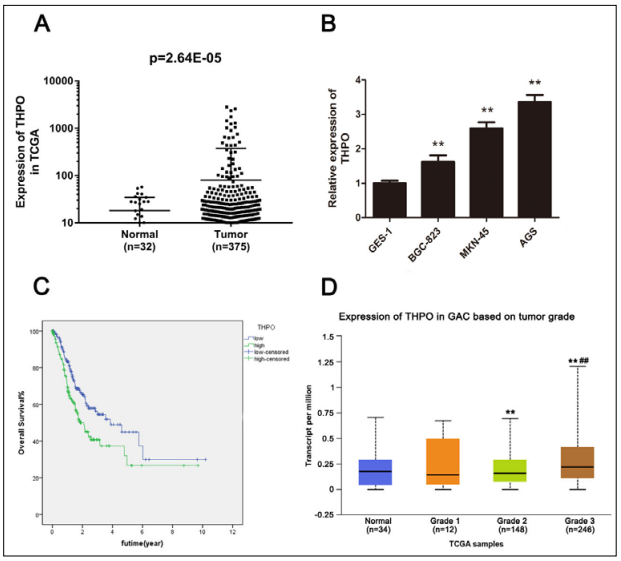

FIGURE 1. THPO WAS UP-REGULATED IN GASTRIC ADENOCARCINOMA TISSUE AND CELL; MEANWHILE, THE HIGH EXPRESSION OF THPO COULD CAUSE POOR SURVIVAL.

(A) The specimens of THPO expression from the TCGA dataset, P = 2.64E-05. (B) Different expression level of THPO in five cell lines, *P < 0.05 and **P < 0.01 versus GES-1. (C) The overall survival curve of gastric adenocarcinoma patients was plotted by Kaplan-Meier, P = 0.004. (D) Relative expression of THPO in gastric adenocarcinoma based on tumor grade, **P < 0.01 versus Normal and ##P < 0.01 versus Grade 1.

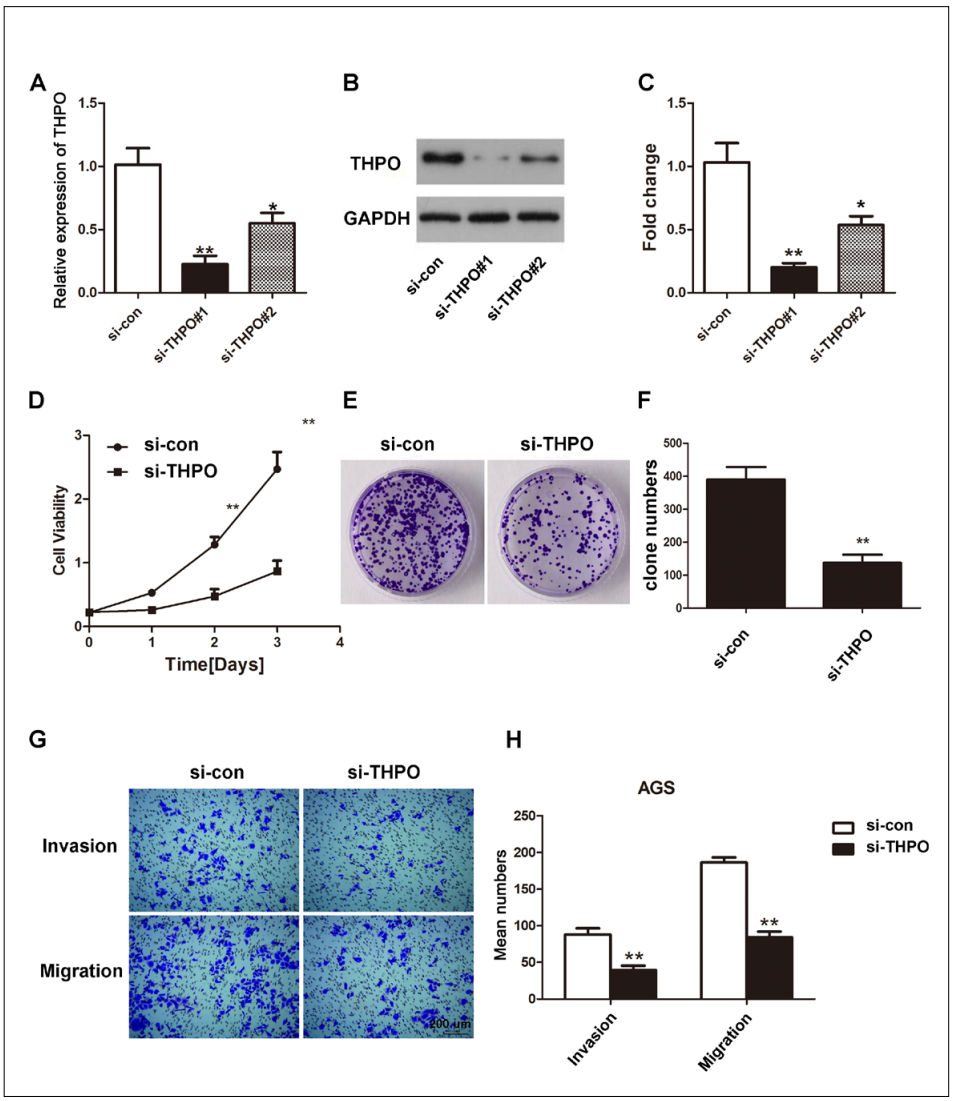

FIGURE 2. THE KNOCKDOWN EFFICIENCY WAS ASSESSED, AND THE REDUCTION OF THPO REPRESSED CELL MALIGNANT BIOLOGICAL PROPERTIES IN AGS.

(A) Detection of transfection efficiency by qRT-PCR, *P < 0.05 and **P < 0.01 versus the si-con group. (B) THPO expression was examined by western blot and (C) quantified, *P < 0.05 and **P < 0.01 versus the si-con group. (D) The reduction of THPO inhibited cell proliferation by MTT assay, **P < 0.01 versus the si-con group. (E) Clone ability was indicated using colony formation assay and (F) the clone numbers were counted, **P < 0.01 versus the si-con group. (G) The representative pictures of invasion and migration, bar = 200 μm, and (H) the number of migratory and invasive cells were calculated, **P < 0.01 versus the si-con group.

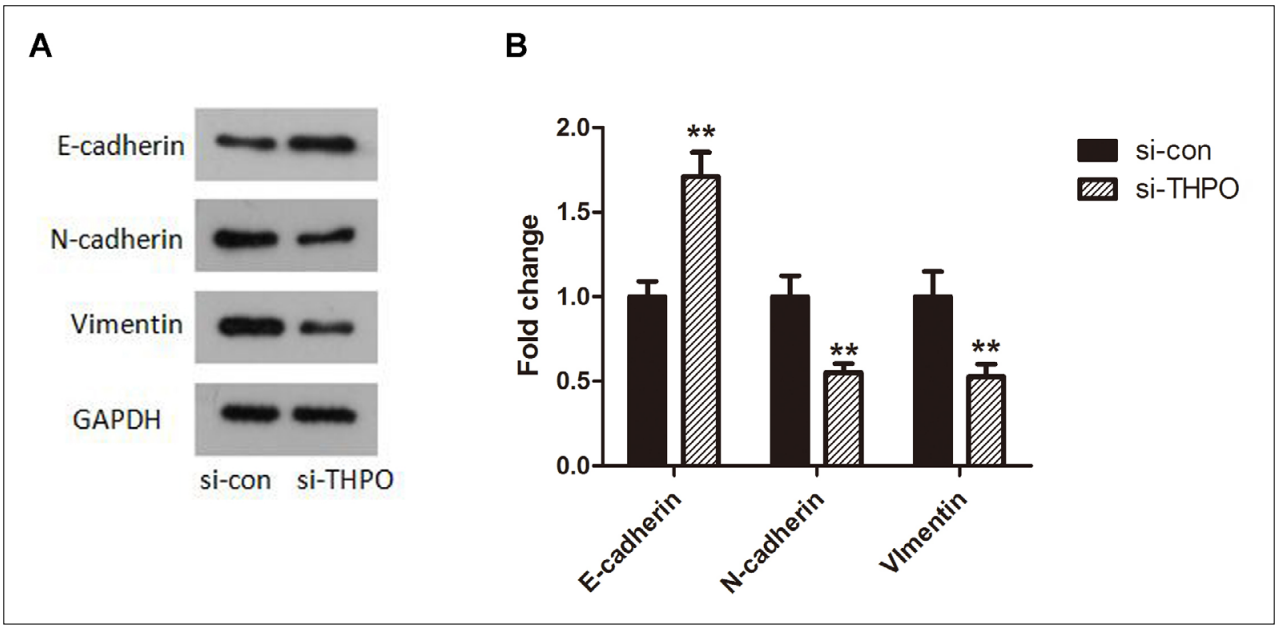

FIGURE 3. THE IMPACT OF THPO SILENCE ON THE EMT PROCESS WAS ASSESSED.

(A) The protein expression level of EMT markers. (B) The comparison of fold change between the si-con group and si-THPO group, **P < 0.01 versus the si-con group.

Colony formation assay

Briefly, cells were digested and blown into single cells to make cell suspension, inoculated in a 60 mm dish at a density of 500 cells at 37° with 5% CO2. After maintained for two weeks, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min. Then the staining solution was gently removed and pictures were captured randomly.

Cell proliferation, migration and invasion assay

To assess the proliferative ability, cells were cultured in a 96-well plate, until reaching 3×10^3 cells/hole. Adding 100 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent, we detected the absorbance at 1 d, 2 d, 3 d with 590 nm.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS22.0 (IBM Corp., Armonk, NY, USA) was applied to conduct statistical analysis. The overall survival was analyzed based on the Kaplan-Meier method with a log-rank test. Data were determined as mean ± standard deviation (SD) and p-value < 0.05 was considered significant.

RESULTS

THPO was highly regulated in gastric adenocarcinoma tissues and cells

Data of qRT-PCR obtained from the TCGA database was applied to evaluate the differential mRNA expression level of THPO in human normal tissue and gastric adenocarcinoma tissue. Compared with normal tissue, THPO was dramatically up-regulated in gastric adenocarcinoma tissue (Figure. 1 A, $P < 0.001$). To verify this result, THPO expression in gastric cancer cell lines (BGC-823, MKN-45, and AGS) and one normal human gastric epithelial cell line GES-1 was examined by the qRT-PCR method. As shown in Figure. 1 B, we observed that all gastric cancer cell lines had higher expression of THPO when the THPO expression in GES-1 was considered as standard control ($P < 0.05$). Analyzed together, these results indicated that THPO might be a cancer-promoting gene in gastric adenocarcinoma, which is implicated in tumorigenesis.

The over-expression of THPO was associated with poor prognosis of gastric adenocarcinoma patients

To further confirm the role of THPO, the prognostic value of THPO in patients with gastric adenocarcinoma was investigated. By analyzing the data from the TCGA database using the Kaplan-Meier method, gastric adenocarcinoma patients with a high expression of THPO presented a lower survival rate than those with a low expression of THPO (Figure. 1 C, $P = 0.004$). The low and high expressions of THPO were remarkably linked with grade (data not shown, $P = 0.001$). Figure 1 D shows that the THPO expression level varied in the different grades of tumor samples ($P < 0.05$).

Knockdown of THPO impaired aggressive behaviors of gastric adenocarcinoma cells

To completely confirm the function of THPO, we performed MTT, transwell, and colony formation assays in gastric adenocarcinoma cells after transfection with si-THPO. We compared with the si-con group, si-THPO#1 and si-THPO#2 sharply interfered with the THPO expression in both the mRNA and protein levels (Figure 2 A-C, $P < 0.05$). Next, MTT assay was implemented to measure the influence of the knockdown of THPO, and the results demonstrated that the down-regulation of THPO inhibited cell proliferation in AGS cells (Figure 2 D, $P < 0.01$). Moreover, the transfection of si-THPO also blocked cell viability through colony formation assay (Figure 2 E and 2 F, $P < 0.01$). As seen in Figure 2 G and 2 H, the migratory and invasive abilities were all impaired when compared with the si-con group ($P < 0.01$).

The reduction of THPO induced the inactivation of the EMT process in AGS cells

The image of protein bands revealed that E-cadherin expression was enhanced (Figure 3 A). By contrast, N-cadherin and Vimentin expression were minified (Figure. 3 A). In addition, the fold change of protein intensity also confirmed the effect of the down-regulation of THPO on EMT markers (Figure 3 B, $P < 0.01$).

DISCUSSION

We predicted that THPO may be a potential independent risk factor in cancer progression. Moreover, a large number of studies have verified the significance of THPO in sets of cancers: THPO was able to facilitate cell growth and survival in acute myeloid leukemia[10,11]; the self-renewal of colorectal cancer cells was promoted by THPO and then diffused into the liver[12]; the platelet produced under THPO regulation could contribute to cell proliferation,

migration, and invasion in cancers, both intracellularly and extracellularly[13].

We found that THPO was highly expressed in gastric adenocarcinoma tissue compared with normal tissue. Meanwhile, we depicted the overall survival curve in patients with gastric adenocarcinoma and found that high expression of THPO shortened lifespan. Functional experiments *in vitro* showed the knockdown of THPO inhibited the biological behaviors in AGS cells in comparison to the si-con control. The EMT process is a vital initial step in cancer and many studies have inferred that EMT induction is the essential molecular mechanism by which tumor cells achieved malignant phenotypes and metastasis[14,15].

Here, we detected the role of THPO expression in gastric adenocarcinoma and indeed had positive results. Consistent with these findings, other relevant cytokines of platelet have been revealed to be associated with the biological viability of tumor cells, such as MMP-2, GPIIb/IIIa, and P-selectin[16-18]. But in line with research by Lang et al.[19], no evident significance was proven in clonal growth when four types of human tumor cells (prostate, breast, cervix, colon) were exposed to thrombopoietin. Therefore, subsequent tests should pay attention to the reason for this difference.

CONCLUSION

We concluded that THPO can promote the progression of gastric adenocarcinoma and may be a potential candidate for tumor diagnosis as well as targeted treatment.

Declaration of conflicting interest

The author(s) declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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Author Contributions

Chang-Lin Zhou and Hai-Long Su contributed equally to the work. Conceptualization, Chang-Lin Zhou; formal analysis, Chang-Lin Zhou; writing—original draft preparation, Hai-Long Su; writing—review and editing, Hong-Wei Dai; supervision, Hai-Long Su; funding acquisition, Hong-Wei Dai.

RESUMO

OBJETIVO: *Trombopoetina (THPO) é um conhecido fator de desenvolvimento e crescimento megacariócito (MGDF) envolvido na proliferação e maturação de megacariócitos. Realizamos este estudo para explorar os efeitos biológicos do THPO no adenocarcinoma gástrico. Metodologia: O nível de expressão do THPO em tecidos tumorais foi determinado acessando a banco de dados TCGA. A associação entre a expressão de THPO e características clínicas ou relevância no prognóstico foi descrita através da análise de Kaplan-Meier e regressão de Cox. O método SiRNA foi utilizado para reduzir a expressão da THPO e, em seguida, a viabilidade, invasão, e migração celular foram detectadas para verificar os efeitos da redução do THPO. qPCR e western blotting foram utilizados para examinar o nível de expressão do THPO. Resultados: A expressão do THPO estava aumentada em tecido e células tumorais, esse aumento estava associado com um prognóstico negativo para pacientes com adenocarcinoma gástrico. A invasão e migração celular foram suprimidos em AGS com a redução do THPO. Além disso, com base na transfecção de si-THPO, a E-caderina foi promovida, enquanto a N-caderina e Vimentina foram atenuadas. Conclusão: Nossos resultados demonstram que o THPO pode ser um potente marcador de adenocarcinoma gástrico, com potencial para ser um novo tipo de triagem para adenocarcinoma gástrico.*

PALAVRAS-CHAVE: *Adenocarcinoma. Neoplasias gástricas. Trombopoetina. Prognóstico. Movimento celular. Epithelial-mesenchymal transition.*

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