RESEARCH ARTICLE

# Effects of PTTG Down-regulation on Proliferation and Metastasis of the SCL-1 Cutaneous Squamous Cell Carcinoma Cell Line

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## Abstract

**Aims: To study effects of down-regulation of pituitary tumor-transforming gene (PTTG) on proliferation and metastasis ability of the SCL-1 cutaneous squamous cell carcinoma (CSCC) cell line and explore related mechanisms. Methods: SCL-1 cells were divided into 3 groups (untreated, siRNA control and PTTG siRNA). Cell proliferation assays were performed using a CCK-8 kit and proliferation and metastasis ability were analyzed using Boyden chambers. In addition, expression of MMP-2 and MMP-9 was detected by r-time qPCR and Western blotting. Results: Down-regulation of PTTG could markedly inhibit cell proliferation in SCL-1 cells, compared to untreated and control siRNA groups ($P < 0.05$). Real-time qPCR demonstrated that expression levels of PTTG, MMP-2 and MMP-9 in the PTTG siRNA group were 0.8%, 23.2% and 21.3% of untreated levels. Western blotting revealed that expression of PTTG, MMP-2 and MMP-9 proteins in the PTTG siRNA group was obviously down-regulated. The numbers of migrating cells (51.38±4.71) in the PTTG siRNA group was obviously lower than that in untreated group (131.33±6.12) and the control siRNA group (127.72±5.20) ($P < 0.05$), suggesting that decrease of proliferation and metastasis ability mediated by PTTG knock-down may be closely correlated with down-regulation of MMP-2 and MMP-9 expression. Conclusion: Inhibition of PTTG expression may be a new target for therapy of CSCC.**

**Keywords:** Cutaneous squamous cell carcinoma - pituitary tumor - transforming gene - proliferation - metastasis

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## Introduction

As per the United National Environment Programme (UNEP), globe incidence of non-melanoma skin cancer exceeds 2 million each year (Urba et al., 2012). With a 10% decrease in stratospheric ozone, this number is being expected to increase by 300,000 annually (http://www.who.int/inf-pr-1999/en/pr99-40.html). Seventy-five percent of the non-melanoma skin cancers are basal cell carcinomas (BCC), which are usually effectively treated with excision. The remaining is predominantly cutaneous squamous cell carcinoma (CSCC). CSCC is a malignant tumor of keratinocytes that tends to metastasize and leads to mortality. Spread of neoplastic cells into lymph nodes and distant organs leads to poor therapy and survival of CSCC patients. Accumulating evidence indicates that as a poor prognostic factor (Moore et al., 2005; Mourouzis et al., 2009; Chollet et al., 2012; Goh et al., 2012), lymph node metastasis correlates with CSCC patient survival. Therefore, the identification of target genes for the determination of tumor progression is urgently required for CSCC diagnostic and therapeutic development.

Since pituitary tumor-transforming gene (PTTG) was successfully isolated by Pei etc (Pei and Melmed, 1997) using molecular biology methods such as differential display PCR in 1997, many studies have been taken in investigating its biological functions such as the promotion of cell transformation, angiogenesis, tumor metastasis, etc. and found that it can induce tumorigenesis and tumor progression in many aspects. It has been reported that PTTG is carcinogenic and closely related with most tumorigenesis while in the absence of the auxiliary genes. Recently, there are more studies on the effect of PTTG in cancer, including pituitary tumors (Zhang et al., 1999), breast cancer (Solbach et al., 2004), esophageal cancer (Shibata et al., 2002), ovarian tumors (Chen et al., 2004), etc. The properties of local invasion, lymphatic dissemination, and subsequent metastasis are attributed to the poor prognosis of CSCC (Bhave et al., 2011). Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell-matrix composition. Gelatinases, MMP-2 and MMP-9, are the members of MMPs family that play an important role in the progression and metastasis of human cancers (Roy et al., 2009). In particular, over-expression and activation of MMP-2 and MMP-9 are strongly associated with tumor aggressiveness and decrease survival of patients with CSCC (Ondruschka et al., 2002). In this study, we investigate the effect of down-

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regulation of PTTG on proliferation and metastasis ability of cutaneous squamous cell carcinoma cell line SCL-1 and explore its related mechanism.

## Materials and Methods

*Materials*

PTTG siRNA (human), antibody of MMP -2, MMP-9 and β-actin were purchased from Santa Cruz Inc., California, USA; Cell counting kit-8 (CCK-8) was purchased from Beyotime Bio Co., Ltd., Shanghai, China; One-step real-time qPCR kit was purchased from Tiangen Biotech (Beijing) Co., Ltd., Beijing, China; The primers for PCR were as follows: 1) PTTG F: 5'-CGGCTGTTAAGACCTGCAAT-3', R: 5'-CAGCTTC AGCCCATCCTTAG-3', 106 bp; 2) MMP-2 F: 5'-CTAC GATGGAGGCGCTAATG-3', R: 5'-TGCACTGCCAACT CTTTGTC-3', 160 bp; 3) MMP-9 F: 5'-TTGACAGCGA CAAGAAGTGG-3', R: 5'-ACATGGGTACATGAGCGC C-3', 132 bp; 4) β-actin F: 5'-AGAAAATCTGGCACCA CACC-3', R: 5'-TAGCACAGCCTGGATAGCAA-3', 173 bp. The primers are all synthesized by Shanghai Boshang Biotechnology Co., Ltd., Shanghai, China.

*Cell culture and transfection*

SCL-1 cells were cultured in DMEM with 10% fetal bovine serum, 100U/ml penicillin and 100μg/mL streptomycin at 37°C with 5% $CO_2$ relatively saturated incubator. Experimental cells were in logarithmic phase. Control siRNA and PTTG siRNA were transfected respectively when the SCL-1 cell growth to 80% to 90% confluence and the transfection was operated according to Lipofectamine TM 2000 transfection reagent manual. The transfected cells were then used for the analysis of Real-time qPCR, Western blot, cell proliferation and invasiveness and metastasis ability respectively.

*Cell proliferation analysis*

Cells were collected after 24, 48, 72, 96 and 120 h from transfection, respectively. When the cell growth rate was measured by CCK-8 method, cells were cultured in the equivalent fresh medium containing 10% CCK-8 at 37°C for 1 to 4 hours, and then, the absorbance of the culture medium at 450 nm were measured by the microplate reader.

*Boyden chamber*

Cells were divided into 3 groups (untreated group, control siRNA group and PTTG siRNA group). In each group, about 105 cells were suspended in 800μL nutrient solution containing 0.2% calf serum, then, seeded onto the upper layer of Boyden chamber, cultured for 6 hours. Subsequently, the cells which transferred to the lower layer of the membrane were collected, fixed with methanol and observed by HE stain. Finally, the transferred cell was counted through the number of cells in lower layer of the membrane (30 fields of vision, × 200).

*Real-time qPCR*

Total RNA of cells was extracted by Trizol reagent and the one-step real-time qPCR was taken according to manufacture's protocol in ABI7300 apparatus. The procedure was as follows: reverse transcription at 50 oC for 30 min; pre-denaturation at 94 oC for 2 min; follow by 35 cycles of at 94 oC for 30 s, 58 oC for 30 s, and 65 oC for 30 s. Each sample has 3 repeats, the relative gene expression was calculated according to the formula of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

*Western blotting*

SCL-1 cells (untreated and transfected for 48 h) were collected and lysed in lyses buffer. Cell extracts was collected and centrifuged at 12 000 rpm for 5 min and the protein concentration was determined by Bradford method. The total protein (80 μg) was boiled in sample buffer for 5 min, then underwent by 10% SDS-PAGE electrophoresis and electro-transferred on nitrocellulose membrane. The nitrocellulose membrane containing protein was blocked by TBST with 5% skim milk for 2 h at room temperature, and then incubated with 1st antibody (and β-actin) in TBST containing 5% skim milk at 4 oC overnight. After incubation, the membrane was rinsed 3 times with TBST for 5 min, and subsequently incubated with 2nd antibody for 1 hour. The membrane was exposed after rinsed 3 times for 5 min. The relative protein expression quantity was analyzed by Gene Tools.

*Statistical analysis*

Results of western blotting were analyzed by the gray value with Gene Tools. All the experiments above were repeated 3 times. SPSS 13.0 was used in statistic analysis, and the statistical data was recorded as mean±SD, t test was used to compare the 2 samples' means, One-way ANOVA was used to compare multiple samples' means. Differences were considered significant when $P < 0.05$.

## Results

*Effect of PTTG expression down-regulation on SCL-1 cell proliferation*

The CCK-8 cell proliferation experiment result showed that, there were no significant difference between the untreated group and the control siRNA group ($P > 0.05$) (Figure1). However, compared with other two groups, the cell proliferation of PTTG siRNA group was obviously inhibited ($P < 0.05$) (Figure 1). It indicates that the inhibition of PTTG expression can inhibit SCL-1 cell proliferation significantly.

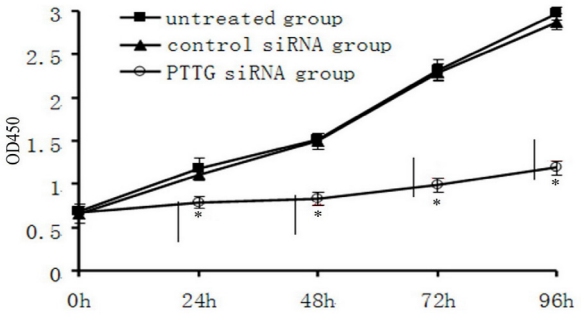


**Figure 1. Effect of PTTG Expression Down-regulation on SCL-1 Cell Proliferation.** *$P < 0.05$ compared with untreated group and control siRNA group

**Table 1. Real-time qPCR Analysis ($2^{-\Delta\Delta Ct}$) of PTTG Gene in SCL-1 Cells**

| Group | PTTG mean Ct | β-actin mean Ct | ΔCt | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|
| Untreated | 23.7 | 16.9 | 6.9 | 0.0 | 1.0 |
| Control siRNA | 24.0 | 17.1 | 6.8 | 0.0 | 1.0 |
| PTTG siRNA | 31.1 | 17.2 | 13.9 | 7.0 | 0.0 |

**Table 2. Real-time qPCR Analysis ($2^{-\Delta\Delta Ct}$) of MMP-2 Gene in SCL-1 Cells**

| Group | PTTG mean Ct | β-actin mean Ct | ΔCt | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|
| Untreated | 25.2 | 16.9 | 8.3 | 0.0 | 1.0 |
| Control siRNA | 25.2 | 16.8 | 8.4 | 0.1 | 0.9 |
| PTTG siRNA | 27.4 | 17.0 | 10.4 | 2.1 | 0.2 |

**Table 3. Real-time qPCR Analysis ($2^{-\Delta\Delta Ct}$) of MMP-9 Gene in SCL-1 Cells**

| Group | PTTG mean Ct | β-actin mean Ct | ΔCt | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|
| Untreated | 24.7 | 16.9 | 7.8 | 0.0 | 1.0 |
| Control siRNA | 25.1 | 17.2 | 7.9 | 0.1 | 1.0 |
| PTTG siRNA | 28.3 | 18.2 | 10.1 | 2.2 | 0.2 |

*Effect of PTTG expression down-regulation on SCL-1 cell metastasis*

After 48 h from transfection, cells from 3 groups were collected respectively and seeded onto the upper layer of chamber. The result showed that in the PTTG siRNA group, the number of migrated cells (51.38±4.71) was significantly less than the untreated group (131.33±6.12) and the control siRNA group (127.72±5.20) ($P < 0.05$). While comparing the latter 2 groups, there was no statistical difference ($P > 0.05$) (Figure 2).

*Effect of PTTG expression down-regulation on mRNA expression of PTTG, MMP-2 and MMP-9*

After 48 h from transfection, the result of real-time qPCR exhibited that the relative mRNA expression of PTTG, MMP-2 and MMP-9 gene dropped obviously in PTTG siRNA group compared with the untreated group and the control siRNA group ($P < 0.05$). However, there was no significant difference ($P > 0.05$) between the latter 2 groups (Table 1, 2, 3).

*Effect of PTTG expression down-regulation on protein expression of PTTG, MMP-2 and MMP-9*

After transfection, Western blotting results exhibited that the relative expression of PTTG, MMP-2 and MMP-9 protein decreased significantly in PTTG siRNA transfected SCL-1 cells compared with the untreated group and the control siRNA group ($P < 0.05$). However, there were no statistic difference ($P>0.05$) between the latter 2 groups (Figure 3).

## Discussion

PTTG which is more than 10 kb in full length and encoding a 26000 protein containing 201 amino acids locates on chromosome 5q33. PTTG gene is a powerful

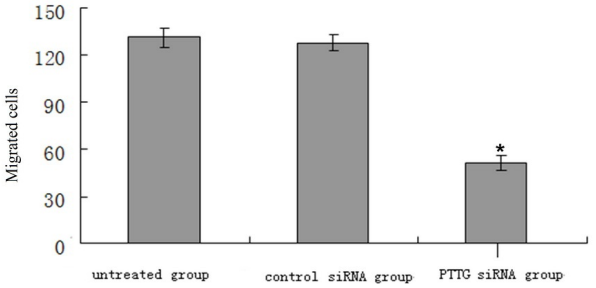


**Figure 2. Effect of PTTG Expression Down-regulation on SCL-1 Cell Metastasis.** *$P < 0.05$ compared with untreated group and control siRNA group

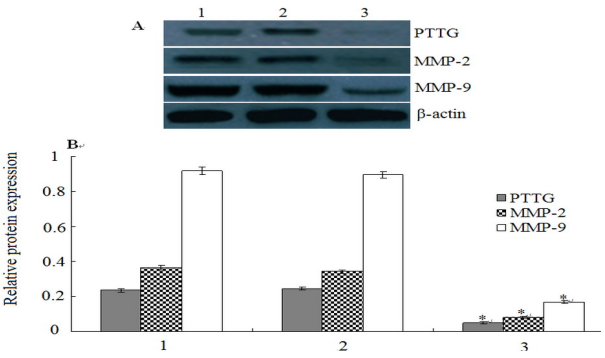


**Figure 3. Effect of PTTG Expression Down-regulation on Expression of PTTG, MMP-2 and MMP-9.** A) Western blotting; B) Relative protein expression level (1, untreated group; 2. control siRNA group; 3, PTTG siRNA group). *$P < 0.05$ compared with untreated and control siRNA group

tumor transforming gene and closely related to most of tumorigenesis. Many studies reveal that PTTG highly expresses in different tumor tissues and cells, and it played an important role in tumorigenesis and progression. In addition, extensive attention was attracted on PTTG's ability of malignant transform cells no matter in vivo or in vitro and this suggests that it may be a molecular target for tumor treatment if its expression can be disrupted and down-regulated by molecular biology methods.

Rapid proliferation, invasion and metastasis are important biological characteristics of tumor cells. Research suggests that the over-expression of PTTG is closely related with tumor proliferation, invasion and metastasis. Kakar and Malik (2006) inhibited PTTG expression by siRNA, then the lung cancer cells growth was inhibited. Akino et al. (2005) reported that PTTG was a molecular marker of rat liver cell regeneration. Jung Cho-Rok et al. (2006) reveal that hepatic cancer cells are inhibited when they are transfected by PTTG gene siRNA through adenovirus vector. These studies showed that PTTG's down-regulation could inhibit cancer cells proliferation. Our study reveal it consistent with the lung and hepatic cancer studies that PTTG's down-regulation can inhibit SCL-1 cells proliferation, and it's proved that the inhibition of PTTG expression is a basic treatment. This study also exhibited that PTTG's down-regulation can significantly reduce the SCL-1 cells invasion and metastasis, suggesting that PTTG may be a molecular marker of CSCC invasion and metastasis.

This study shows that PTTG's inhibition can reduce SCL-1 cells migration, but its mechanism is still unclear.

For cancer metastasis, 3 steps are necessary: cell-to-extracellular matrix (ECM) adhesion, proteolytic cleavage and cell migration (Woodhouse et al., 1997). ECM may be degradated by a variety of proteolytic enzymes, with MMPs playing the important roles. MMPs is a family of enzymes mainly responsible for ECM degradation (the critical step of metastasis) (Itoh and Nagase, 2002). Among them, MMP-2 and MMP-9, namely gelatinase A and B, play a key role in tumor invasion (Bogusiewicz et al., 2003), since they are able to digest collagen type IV, which is the main component of base membrane (Gelse et al., 2003). Improved expression and activity of MMP-2 and MMP-9 have been reported in different kinds of cancers (Coussens et al., 2000), including SCC (Franchi et al., 2002; Patel et al., 2005). The function of MMP-2 and MMP-9 involve not only the breakdown of breakdown of basement membrane but also play a role in the regulation of activities of various growth factors and cytokines which affect the immune response and angiogenesis, resulting in growth initiation and maintenance of primary and metastatic tumors (Kessenbrock et al., 2010). One of the major mediators of PTTG siRNA therapeutic action may be its ability to down-regulate the expression of MMP family member MMP-2 and MMP-9. So we try to illuminate the reason of migration ability's decline by the expression of MMP-2 and MMP-9 which related with tumor invasion and metastasis. MMP-2 and MMP-9 are the main protease in degradation of gelatin IV, V, VII and X, and are the main proteolytic enzymes in tumor invasion and metastasis. A research (Fang et al., 2000) showed that MMP-2 down-regulation could inhibit the seeded subcutaneous melanocyte tumorigenesis. In the mouse prostate cancer model, down-regulated MMP-9 can reduce the angiogenesis, tumor growth and induce tumor retrogression (London et al., 2003). In this study, we down-regulated PTTG's expression, then tested the mRNA of MMP-2 and MMP-9 by Real-time qPCR and Western blot. Our results show that PTTG's down-regulation can reduce the MMP-2 and MMP-9 protein level. So, it can be conjectured that the descendent of skin squamous carcinoma cells proliferation, invasion and metastasis induced by down-regulated PTTG may be related with down-regulated MMP-2 and MMP-9.

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