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Original Article

IFIH1-mediated post-transcriptional regulation of PTTG1 promotes proliferation and affects PHA-848125 sensitivity and prognosis in oropharyngeal carcinoma

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Abstract: The pituitary tumor-transforming gene 1 (PTTG1) is an oncogene involved in chromosomal segregation, DNA repair, apoptosis, and metabolism. PTTG1 can be used for clinical diagnosis and treatment and is a potential target for oropharyngeal carcinoma. The proliferation and viability of Cal27 and FaDu cells were assessed using the CCK-8 assay. Real-time PCR and western blotting, respectively, were used to analyze the mRNA and protein expression levels of PTTG1 and IFIH1. The interaction between *PTTG1* mRNA and the translational regulatory protein IFIH1 was analyzed using RNA pull-down, RNA immunoprecipitation, and luciferase reporter assays. PTTG1 protein was significantly overexpressed in oropharyngeal carcinoma, whereas *PTTG1* mRNA was not. We hypothesized that a translation regulatory protein plays a post-transcriptional role in PTTG1. The IFIH1 protein specifically bound to the 42-52 nt region of *PTTG1* mRNA, promoted the translation of PTTG1, and promoted the proliferation of oropharyngeal cancer cells. Administration of the PTTG1 inhibitor PHA-848125 and silencing of IFIH1 synergistically decreased the expression of PTTG1, inhibited the proliferation of oropharyngeal cancer cells, and indicated a good prognosis. We found that the IFIH1-PTTG1 axis could regulate the PHA-848125 response and functionally mediate inter-individual oropharyngeal cancer susceptibility and prognosis. This study aimed to confirm the upstream regulatory genes of PTTG1 and further investigate the specific interactions in this signaling pathway, which will provide a new approach for the treatment of oropharyngeal carcinoma.

Keywords: IFIH1, PTTG1, PHA-848125, oropharyngeal carcinoma, post transcriptional regulaiton

Introduction

The incidence of oropharyngeal cancer has continued to increase over the last six consecutive years in the United States. More than 54,000 new cases of oropharyngeal cancer were reported in 2022 and accounted for 2.82% of all new cancer cases in the US [1]. The pituitary tumor-transforming gene (PTTG1), also known as securin, is overexpressed in many human cancers, such as pituitary tumor [2], breast cancer [3], laryngeal cancer [4], osteosarcoma [5], lung cancer [6], and liver cancer [7]. The level of PTTG1 is directly related with the degree of malignancy and invasive tumor progression [8], DNA repair, apoptosis, metabolism, and gene transcription [9]. Therefore, PTTG1 has been identified as a key oncogene.

However, PTTG1 has also been reported as a tumor suppressor. In human breast cancer, PTTG1 is downregulated, and the degree of reduction is significantly correlated with the tumor grade [10]. PTTG1 inhibits cell cycle progression during mitosis [11]. and induces apoptosis through p53-dependent and -independent pathways [12]. Ectopic expression of PTTG1 could impair the proliferation of the choriocarcinoma cell line JEG-3, cervical cancer cell line HeLa, and lung cancer cell line A549 [9]. and PTTG1 could reduce the occurrence of

pituitary tumors. However, the effect of PTTG1 in oropharyngeal carcinoma has not yet been reported.

There are also different reports on the role of PTTG1 in the prognosis of patients with tumors. High expression of PTTG1 is associated with poor prognosis in patients with laryngeal cancer [4]. A high proportion of cancer cells expressing PTTG1 is predictive of poor clinical outcomes in patients with breast cancer [13]. However, PTTG1 expression did not significantly correlate with the prognosis of oral tongue squamous cell carcinoma [14]. Moreover, PTTG1 is a good prognostic factor for osteosarcoma [5]. However, the effect of PTTG1 on the prognosis of oropharyngeal carcinoma has not yet been reported.

The mRNA and protein levels of PTTG1 were effectively downregulated 48 h after transfection, by 80% and 50%, respectively; however, the mRNA and protein changes in PTTG1 were not completely consistent [3]. Therefore, we speculated that PTTG1 may have an important translational regulatory mechanism. However, there have been few reports on the translational regulation of PTTG1.

IFIH1 (interference induced with helicase C domain 1), also known as MDA5, induce double-stranded RNA and participate in antiviral immunity [15, 16]. It promotes transcriptional reprogramming through the PSAT1-IRF1-IFIH1 axis and affects lung cancer cell metastasis [17, 18]. IFIH1 is differentially expressed in testicular cancer and is associated with disease-free survival in patients with TGCTs [19]. Additionally, IFIH1 is highly expressed in drug-resistant ovarian cancer cells [3]. However, the effect of IFIH1 on oropharyngeal carcinoma has not yet been reported.

In this study, we explored whether PTTG1 protein expression is associated with the susceptibility to and prognosis of oropharyngeal carcinoma. The translational regulator IFIH1 upregulated PTTG1 at the translational level. Specifically, both IFIH1 and PTTG1 positively correlated with the susceptibility to and prognosis of oropharyngeal carcinoma. Administration of the PTTG1 inhibitor PHA-848125 and silencing of IFIH1 could synergistically inhibit the proliferation of oropharyngeal carcinoma cells, providing a new therapeutic target for oropharyngeal carcinoma.

Materials and methods

The study was subject to approval by the Institutional Review Board of The Fourth Affiliated Hospital of China Medical University, and all participants gave their written informed consent before inclusion in the ongoing study. A total of 118 patients with histopathologically confirmed oropharyngeal carcinoma were consecutively recruited from The Fourth Affiliated Hospital of China Medical University between January 2010 and December 2015. Clinical data were obtained from medical records and interviewer-administered health-risk questionnaires.

Quantitative real-time (qRT-PCR) assays

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, USA). For the qRT-PCR assay, reverse transcription was performed, and qRT-PCR was performed using SYBR Green mix (Toyobo, Japan) on the Applied Biosystems 7500HT Real-Time PCR System. Relative mRNA expression was normalized to that of the reference gene GAPDH. The reaction conditions and primers used for qPCR are listed in Table S1.

Immunohistochemistry

Paraffin-embedded tissues were cut into 4 μm sections. The samples were then transferred onto polylysine-coated glass slides. The samples were deparaffinized in xylene, rehydrated in a series of alcohol solutions, and washed with tap water. Samples were incubated in sodium citrate buffer (pH 6.0) for 15 min for antigen retrieval. Endogenous peroxidase activity was blocked by 3% H_2O_2 at 37°C for 30 min. The samples were blocked to avoid nonspecific binding by the addition of goat serum at 37°C for 30 min, followed by incubation overnight at 4°C with the primary antibody (PTTG1, 1:100, Abcam, ab79546, USA; IFIH1, 1:100, Abcam, ab283311, USA). The next day, samples were washed three times with PBS (pH 7.4) thrice 5 min each and incubated with secondary antibody for 1 h at 37°C. Finally, the samples were stained with DAB for 1 min. Immunostaining was performed by two pathologists who were blinded to the experimental

conditions. The intensity of immunoreactivity was scored as follows: the percentage of stained cells was scored in 5% increments (0%, 5%, 10%, ..., 100%). 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was determined by multiplying the percentage of positively stained cells with the intensity score.

Cell culture

Human oropharyngeal carcinoma Cal27 and FaDu cells were purchased from Peking Union Medical College Cell Resource Center (Beijing, China). The cell lines were verified using short tandem repeat analysis (Shanghai Genechem Co., Ltd.). The cells were found to be free of Mycoplasma contamination by PCR. The Cal27 cells were cultured in DMEM culture medium (Invitrogen, USA) containing 10% FBS (HyClone), 100 U/mL penicillin (Invitrogen, USA), 100 U/mL streptomycin (Invitrogen, USA) with culture environment of 5% CO_2 and 37°C. The FaDu cells were cultured in MEM culture medium (Invitrogen, USA) containing 10% FBS (HyClone), 100 U/mL penicillin (Invitrogen, USA), 100 U/mL streptomycin (Invitrogen, USA) with a culture environment of 5% CO_2 and 37°C. Cells were maintained in the logarithmic growth phase by routine passaging every three days at a 1:2 split ratio. Cells between passages three and eight were used for the experiments.

Plasmid transfection

Cal27 and FaDu cells were transferred to 6-well plates (1×10^5 cells/well) or 96-well plates (1×10^4 cells/well), and the plasmid was transfected at a final concentration of 1 μg/mL according to the instructions for Lipofectamine 3000 (Invitrogen, USA) kit. After 48 h, the cells were collected for subsequent experiments.

Luciferase reporter assay

A region containing the PTTG1 translation regulatory sequence was inserted after the transcriptional start site of the pGL3 luciferase reporter gene plasmid. The cells were transferred to 96-well plates (1×10^4 cells/well), and the IFIH1 overexpression plasmid pcDNA3.1-IFIH1 was transfected into 293T, Cal27, and FaDu cells. After 48 h, 25 μL of ONE-Glo™ EX reagent was added per well; the samples were mixed by placing the plate on an orbital shaker at 100 rpm for 15 min. Next, 100 μL of ONE-Glo™ EX Reagent was added per well, and the samples were mixed by placing the plate on an orbital shaker 200 rpm for 2 min. FLuc luminescence was measured at intervals of 0.5 seconds using the Dual-Luciferase® Reporter Assay System (Promega, USA). Assays were conducted in triplicate in a single experiment, followed by three independent experiments.

RNA pull-down

Wild-type and mutant biotin-linked RNA probes were mixed with Pierce Streptavidin Agarose Resin (Thermo, 20347, USA) for 30 min. Pierce Streptavidin Agarose Resin without RNA was used as a negative control (NC). After the cells were freeze-thawed 3 times, the lysate was collected to centrifuge at 12000 g/min for 10 min at 4°C. The supernatant (250 μL) was mixed with agarose resin for 2 h and centrifuged at 1000 g/min for 10 min. The supernatant was discarded, and the cells were washed three times with PBS. 6X loading buffer (50 μL) was added to the agarose resin, boiled for 10 min, and centrifuged at 12000 g/min for 10 min. The supernatants were collected for western blot analysis.

RNA immunoprecipitation (RIP)

Plasmids containing the wild-type and mutant sequences were transfected into Cal27 cells. The Cal27 cells (10^7) were cross-linked with 1% formaldehyde for 30 min at 37°C. The cells were sonicated thrice for 10 s, followed by centrifugation at 12,000 × *g* for 10 min. Supernatants were collected and subjected to immunoprecipitation with IFIH1 antibody (IFIH1, Abcam, ab283311, USA) overnight at 4°C. Simultaneously, the supernatants were incubated with rabbit IgG antibodies as NC. Thereafter, the samples were incubated overnight with protein A/G magnetic beads (Beyotime P2078, China) at 4°C. The beads were washed and pelleted using a magnetic separator and heated with Proteinase K at 65°C for 2 h to reverse the cross-links of protein/RNA complexes. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo, USA). Reverse transcription solution (2 μL) was subjected to 35 cycles of amplification for PCR assay. The

PCR products were detected by DNA electrophoresis.

Cell proliferation and viability assay

Cal27 and FaDu cells were seeded onto 96-well plates (1×10^4 cells/well). The cells were incubated with 10 μL of CCK-8 (Cell Counting Kit-8, Beyotime, China) at 37°C for 2 h, after 0, 24, 48, and 72 h of transfection. The absorbance was measured at 450 nm. The experiment was repeated thrice.

Clonogenic assays

Cal27 and FaDu cells were seeded in 6-well plates (5×10^2 cells/well). After transfection, the cells were incubated at 37°C for 20 d. The medium was replaced with fresh medium at 3 d intervals. Colonies were fixed with 4% paraformaldehyde for 15 min at room temperature, incubated with 1% crystal violet for 20 min at room temperature, and observed under a field of view.

Green fluorescent protein (GFP) detection

The region containing the PTTG1 translational regulatory sequence was inserted after the transcription start site (TSS) of the pGFP plasmid. The 293T cells were seeded at a density of 1×10^5 cells/well in a chamber slide. After 24 h, the pGFP-WT, pGFP-Mut, and pcIFIH1 (IFIH1 overexpression) plasmids were transfected into the 293T cells. GFP was detected using a confocal laser scanning microscope (FV1000S-SIM/IX81; Olympus) 48 hours after transfection.

Western blot

Total protein was extracted from Cal27 and FaDu cells. Protein (30 μg) was separated using 10% SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. The silencing group primary antibodies and the final antibody concentrations were as follows: PTTG1, 1:1000, Abcam, ab79546, UK; IFIH1, 1:1000, Abcam, ab283311, UK; and GAPDH, 1:1000, Boster, BM3876, China. They were incubated overnight at 4°C. The overpression group primary antibodies and the final antibody concentrations were as follows: PTTG1, 1:1000, Abcam, ab79546, UK; IFIH1, 1:1000, Abcam, ab283311, USA; and GAPDH, 1:1000, Boster, BM3876, China. They were incubated for 2 h at room temperature. The secondary antibody (1:1000; Boster, BA1054, China) was incubated at room temperature for 2 h. Chemiluminescence was detected using enhanced chemiluminescence reagent (Amersham, Freiburg, Germany). Data were statistically analyzed using FluorChem V2.0 (Alpha Innotech Corp., USA).

Immunofluorescence

Cal27 and FaDu cells were seeded at a density of 1×10^5 cells/well in a chamber slide. After 48 h of transfection, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min. The silencing group primary antibodies (with the final antibody concentrations as follows: PTTG1, 1:100, Abcam, ab79546, UK and IFIH1, 1:100, Abcam, ab283311, UK) were incubated overnight at 4°C. The silencing group primary antibodies (with the final antibody concentrations as follows: PTTG1, 1:100, Abcam, ab79546, UK and IFIH1, 1:100, Abcam, ab283311, UK) were incubated for 2 h at room temperature. The nuclei were stained with DAPI (diamidino-2-phenylindole). Fluorescence was detected using a confocal laser-scanning microscope (FV1000S-SIM/IX81, Olympus).

In vivo xenograft experiments

Transplanted tumor models with BALB/c nude male mice (4-5-week-old) were established by subcutaneously injecting Cal27 cells into the scapula. Cal27 cells (1×10^7) were suspended in 200 μL PBS and MatrigelTM (1:1) (BD Biosciences). Nude mice in the experimental group were orally administered PHA-848125 (20 $mg \cdot kg^{-1}$) once daily, and the control group was administered normal saline. The weights of the xenograft mice were recorded every 5 days. Approximately 10 days after cell injection, the tumor was formed and was measured at intervals of 5 d. Tumor volume was then calculated from the width (W) and length (L) according to the following formula: $V = (L \times W^2) \times 0.5$. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Ethics Committee of the Fourth Affiliated Hospital of the China Medical University. All the mice were bred under pathogen-free conditions

at the Animal Center of the Fourth Affiliated Hospital of China Medical University.

Statistical analysis

All data analyses were performed using the SPSS software (version 16.0; SPSS Inc., Chicago, Illinois, USA). Associations between cancer and adjacent tissues of oropharyngeal carcinoma and clinical variables were analyzed using odds ratios (OR), 95% confidence intervals (CI), and unconditional logistic regression models. Overall survival (OS) was defined as the time between surgery and death or the last known follow-up. Disease-free survival (DFS) was measured from surgery until recurrence, death, or the last known follow-up. Survival curves were generated using the Kaplan-Meier method, and the log-rank test was used to estimate the association between DFS and OS. Data from qRT-PCR, reporter assays, cell proliferation assays, and *in vivo* xenograft experiments were analyzed using two-tailed Student's t-test, and a P value < 0.05 was considered statistically significant.

Results

PTTG1 protein, IFIH1 protein, and IFIH1 mRNA are highly expressed in oropharyngeal carcinoma tissues

PTTG1 protein levels in 118 pairs of oropharyngeal cancerous and paracancerous tissues were detected using immunohistochemical techniques, and the levels of PTTG1 in the carcinoma tissues was significantly higher than that in the paracancerous tissues (**Figure 1A**, **1B**). The mRNA levels of PTTG1 in cancerous and paracancerous tissues were detected using qRT-PCR, and there was no significant difference between the cancerous and paracancerous tissues (**Figure 1C**).

We speculate that PTTG1 may have a post-transcriptional regulatory mechanism, resulting in increased protein expression, whereas mRNA remains unchanged. To identify the genes that regulate PTTG1 expression, the Catrapid algorithm (http://service.tartaglialab.com/page/catrapid group) was used to predict the binding of IFIH1 to the translation regulatory region of PTTG1. The RNA-protein interaction prediction tool (http://pridb.gdcb.iastate.edu/RPISeq/) was used to verify the results, and they were consistent. According to the bioinformatics analysis using TCGA and GEPIA, IFIH1 was highly expressed in oropharyngeal carcinoma (**Figure 1D**, **1E**). The mRNA and protein levels of 118 pairs of oropharyngeal carcinoma and adjacent tissues were analyzed, and we found that the mRNA and protein of IFIH1 were significantly overexpressed (**Figure 1F**, **1G**). In addition, there was no correlation between the mRNA levels of IFIH1 and PTTG1; however, their protein levels were positively correlated (**Figure 1H**, **1I**).

The expression of IFIH1 and PTTG1 is associated with the prognosis of oropharyngeal carcinoma

To further explore the clinical utility of PTTG1 and IFIH1 as independent prognostic factors for this cohort of 118 patients with oropharyngeal carcinoma, each low- or high-expression index was determined using the cutoff point value determined by the ROC curve method (**Figure 2A-D**). Notably, higher levels of PTTG1 protein was significantly correlated with poorer DFS (P < 0.001, high expression: MST = 23 months, low expression: MST = 66 months) and OS (P < 0.001, high expression: MST = 26 months, low expression: MST = 70 months) (**Figure 2E**, **2F**). Similarly, higher levels of IFIH1 protein was significantly correlated with poorer DFS (P < 0.001, high expression: MST = 31 months, low expression: MST = 70 months) and OS (P < 0.001, high expression: MST = 35 months, low expression: MST = 72 months) (**Figure 2G**, **2H**). After adjusting for confounding factors such as age and sex, the independent prognostic values of PTTG1 and IFIH1 in diagnosing OS and DFS in 118 patients with oropharyngeal carcinoma were further confirmed using multivariate Cox regression analysis (P < 0.001) (Tables S2, S3). In addition, unconditional logistic regression analysis demonstrated that the protein expression of PTTG1 was significantly correlated with tumor size and family history (P < 0.01), and the protein expression of IFIH1 was significantly correlated with tumor size and family history (P < 0.01) (Tables S4, S5).

Translation was triggered by targeted binding of IFIH1 to the translation regulatory region 42 nt-52 nt sequence of PTTG1 mRNA

Using the catrapid bioinformatics (http://service.tartaglialab.com/page/catrapid_group) and the RNA protein interaction predic-

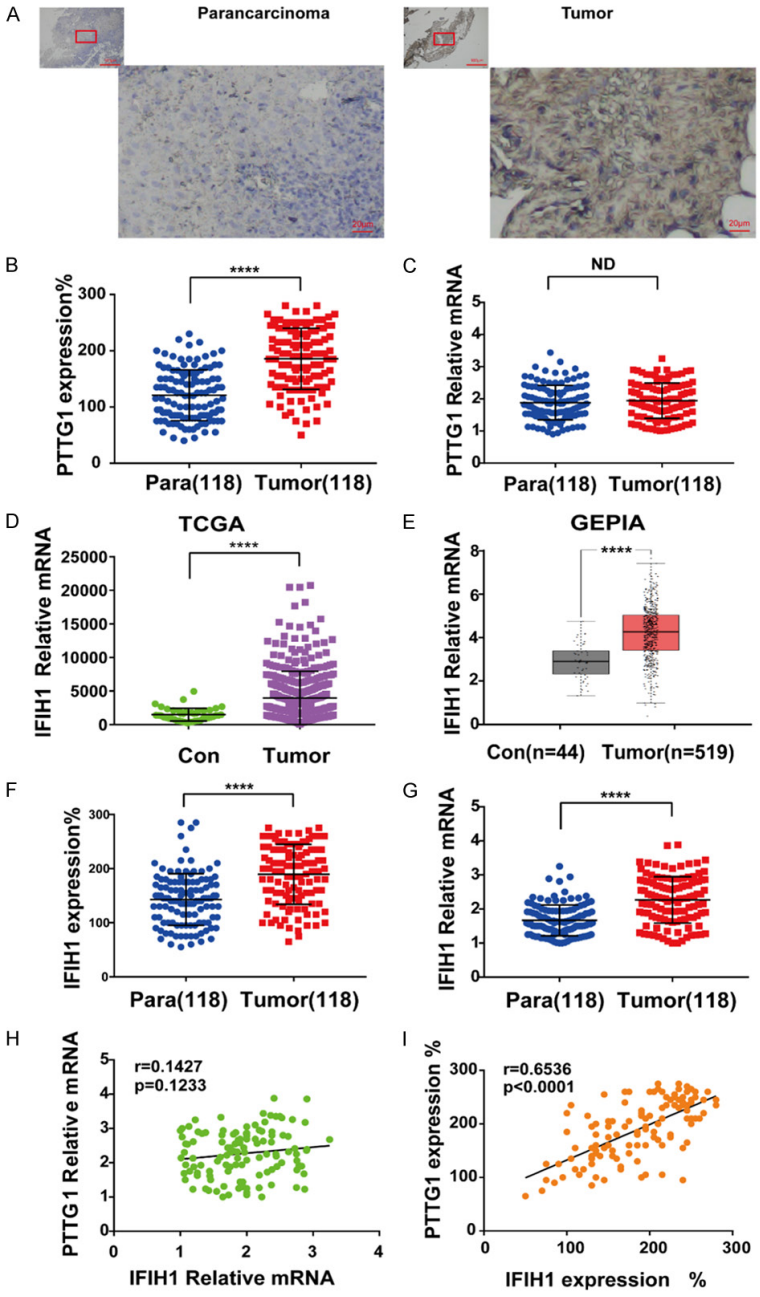


Figure 1. PTTG1 protein, IFIH1 protein and *IFIH1* mRNA are highly expressed in oropharyngeal carcinoma tissues. A. The protein expression of PTTG1 in paracancerous tissues and cancer tissues of oropharyngeal carcinoma immunohistochemistry images. B. The protein expression of PTTG1 statistic in paracancerous tissues and cancer tissues of oropharyngeal carcinoma, in which cancer tissues carriers are associated with higher expression of PTTG1 proteins. C. PTTG1 mRNA levels detected by qPCR analysis showed that there was no significant difference between paracancerous tissues and cancer tissues of oropharyngeal carcinoma. D, E. TCGA data and GEPIA platform illustrated elevated expression levels of IFIH1 mRNA in oropharyngeal carcinoma tissues compared with those of normal tissues. F. IHC assays represent the protein expression of IFIH1 in paracancerous tissues and cancer tissues of oropharyngeal carcinoma that cancer tissues carriers are associated with higher expression of IFIH1 proteins. G. The mRNA of IFIH1 detected by qPCR assays in paracancerous tissues and cancer tissues of oropharyngeal carcinoma showed that cancer tissues carriers are associated with higher expression of IFIH1 mRNA. H. The levels of IFIH1 mRNA showed no correlation with the levels of PTTG1 mRNA in oropharyngeal carcinoma tissues. I. The protein expression of IFIH1 exhibited a positive correlation with protein expression of PTTG1 in oropharyngeal carcinoma tissues. ****, $P < 0.0001$.

tion (http://pridb.gdcb.iastate.edu/RPISeq/) tools, we predicted that the binding site of the translation regulatory protein IFIH1 to the translation regulatory region is located in the 42-52 nt region of PTTG1 mRNA. We mutated this sequence from "GGGCTGCGGT" to "AAATCATAAATC" (**Figure 3A**). Both RNA pull-down and RIP assays confirmed the interaction between IFIH1 and the translational regulatory region of PTTG1 (**Figure 3B**, **3C**).

To detect the post-transcriptional regulatory effect of IFIH1 on PTTG1, we constructed the luciferase plasmid pGL3-WT containing the wild-type translation regulatory region of PTTG1 mRNA and the pGL3-Mut plasmid containing a mutated binding site (**Figure 3D**). Moreover, an IFIH1 overexpression plasmid pc-IFIH1 was constructed, and luciferase activity was detected in the IFIH1-overexpressing 293T, Cal27, and FaDu cells. We found that Fluc activity was significantly increased in the IFIH1-treat group compared to that in the NC group ($P < 0.05$); however, there was no significant difference between the mut groups ($P > 0.05$) (**Figure 3E-G**). Moreover, we constructed the IFIH1 silencing plasmids sh-IFIH1-1 and sh-IFIH1-2. The luciferase activity was significantly de-

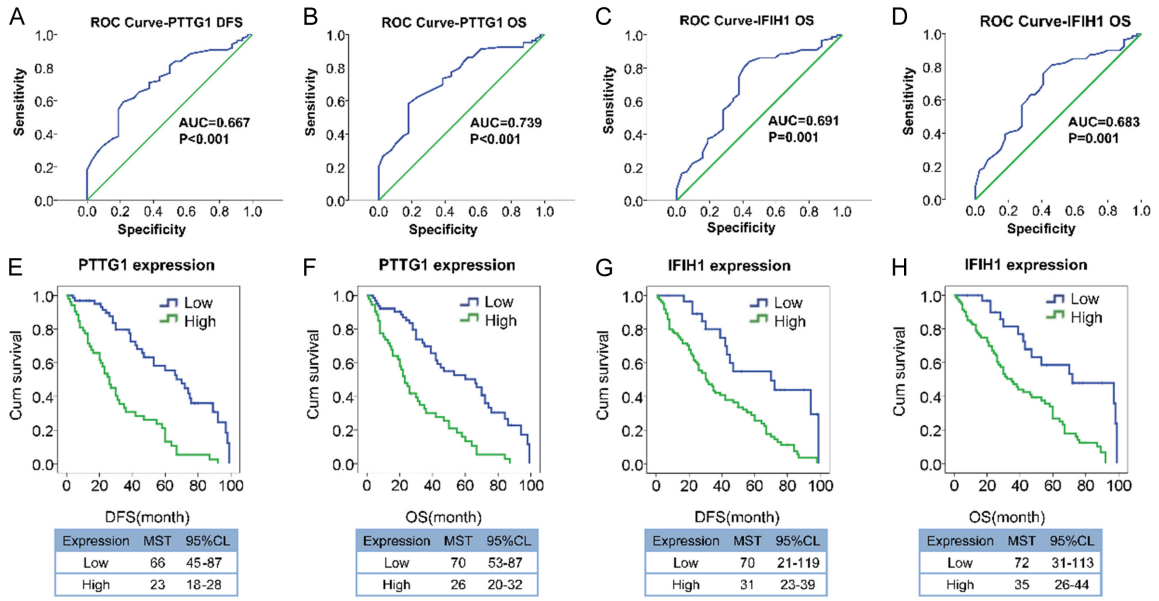


Figure 2. The expression of IFIH1 and PTTG1 is correlated with the prognosis of oropharyngeal carcinoma. A, B. The DFS and OS critical points of low or high expression of PTTG1 protein in 118 oropharyngeal carcinoma patients were determined by the cut-off point values assayed by ROC curve method. C, D. The DFS and OS critical points of low or high expression of IFIH1 protein in 118 oropharyngeal carcinoma patients were determined by the cut-off point values assayed by ROC curve method. E, F. Kaplan-Meier plots of DFS and OS prognostic difference between the difference of PTTG1 protein expression level in 118 patients of oropharyngeal carcinoma. G, H. Kaplan-Meier plots of DFS and OS prognostic difference between the difference of IFIH1 protein expression level in 118 patients of oropharyngeal carcinoma.

creased in the IFIH1 silenced group compared to that in the NC group ($P < 0.05$); however, there was no significant difference between the mut groups ($P > 0.05$) (**Figure 3H-J**).

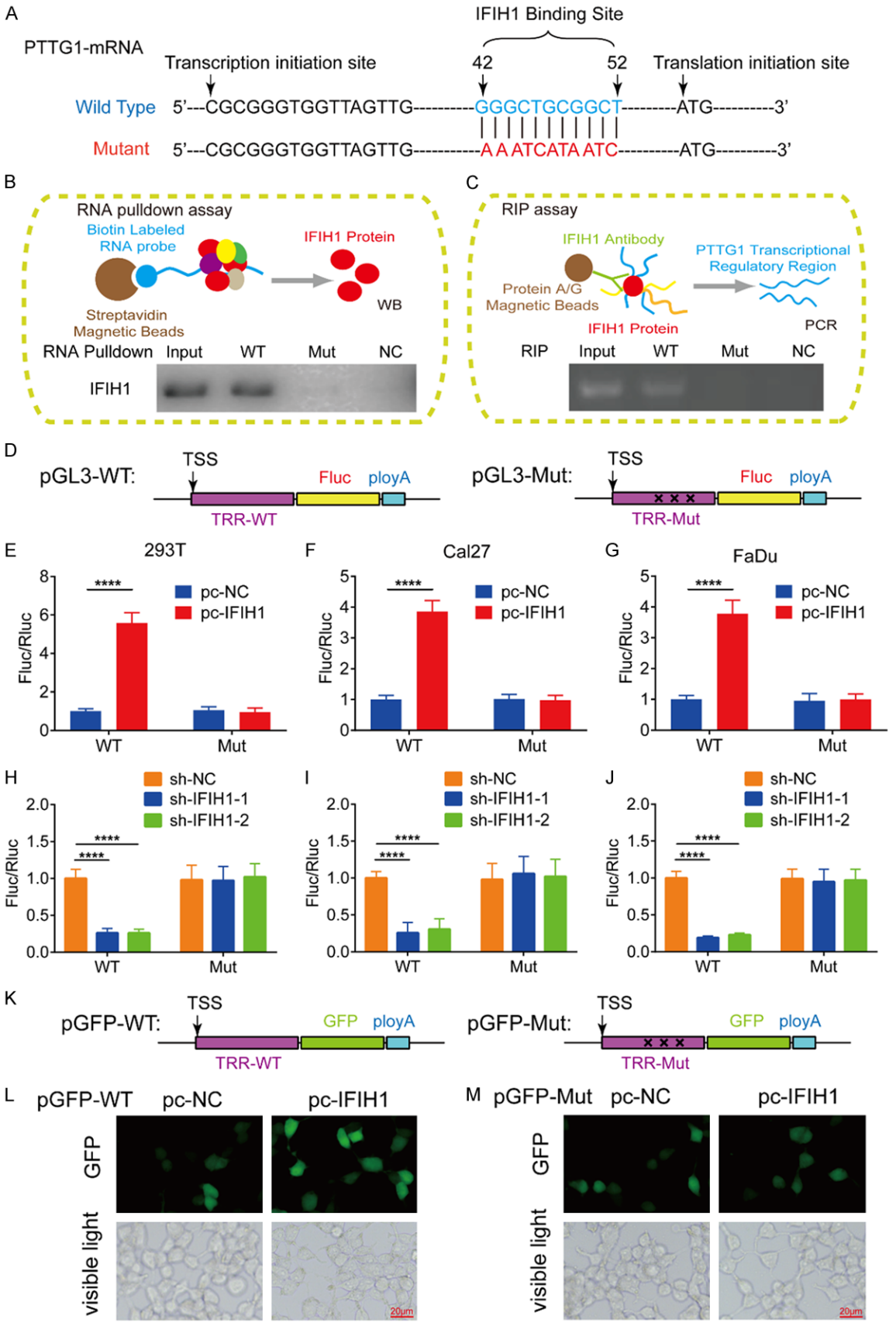
To further confirm the post-transcriptional regulation of IFIH1 on PTTG1, we constructed pGFP-WT and pGFP-Mut plasmids containing the translation regulatory regions of the wild-type and mutant type (**Figure 3K**), respectively. In the pGFP-WT group, compared with that in the NC group, GFP expression in 293T cells was significantly increased by transfection with the IFIH1 overexpression plasmid ($P < 0.05$) (**Figure 3L**). However, in the pGFP-Mut group, there was no significant difference in GFP expression in 293T cells after transfection with the IFIH1 overexpression plasmid ($P > 0.05$) (**Figure 3M**). IFIH1 has been shown to regulate the translation of PTTG1 through the 42-52 nt region of PTTG1 mRNA.

Overexpression of IFIH1 promotes oropharyngeal carcinoma proliferation

The effects of IFIH1 overexpression and PTTG1 silencing on proliferation were examined in Cal27 and FaDu cells. The results showed that, compared with that in the NC group, the proliferation of cells was significantly increased by the overexpression of IFIH1 and silencing of PTTG1 ($P < 0.05$) (**Figure 4A**, **4B**). Simultaneously, we used clone formation experiments to demonstrate that cell proliferation was significantly increased by overexpression of IFIH1 and PTTG1 (**Figure 4C**). We then measured mRNA levels in each group. The mRNA levels of IFIH1 was significantly increased by the overexpression of IFIH1 ($P < 0.05$) compared with that in the NC group, but there was no significant difference in PTTG1 mRNA levles ($P > 0.05$) (**Figure 4D**, **4E**). Protein expression in each group was detected using western blotting. The protein levels of IFIH1 and PTTG1 were significantly increased by IFIH1 overexpression compared with that in the NC group ($P < 0.05$) (**Figure 4F**). Immunofluorescence demonstrated the same results, indicating that IFIH1 promotes the translation of PTTG1 through post-transcriptional regulation (**Figure 4G**, **4H**).

Silencing IFIH1 inhibits oropharyngeal carcinoma proliferation

The effect of silencing IFIH1 and overexpressing PTTG1 on proliferation were examined in


A
IFIH1 Binding Site
PTTG1-mRNA
Transcription initiation site
42
52
Translation initiation site
Wild Type 5'---CGCGGGTGGTTAGTTG----------GGGCTGCGGCT---------ATG----------3'
Mutant 5'---CGCGGGTGGTTAGTTG----------A A ATCATA ATC----------ATG----------3'
B
RNA pulldown assay
Biotin Labeled RNA probe
IFIH1 Protein
WB
Streptavidin Magnetic Beads
RNA Pulldown Input WT Mut NC
IFIH1
C
RIP assay
IFIH1 Antibody
PTTG1 Transcriptional Regulatory Region
Protein A/G Magnetic Beads
IFIH1 Protein
PCR
RIP Input WT Mut NC
D
pGL3-WT:
TSS
Fluc ployA
TRR-WT
pGL3-Mut:
TSS
Fluc ployA
TRR-Mut
E
293T
F
Cal27
G
FaDu
Fluc/Rluc
pc-NC
pc-IFIH1
WT
Mut
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H
I
J
sh-NC
sh-IFIH1-1
sh-IFIH1-2
K
pGFP-WT:
TSS
GFP ployA
TRR-WT
pGFP-Mut:
TSS
GFP ployA
TRR-Mut
L
pGFP-WT pc-NC pc-IFIH1
GFP
visible light
20μm
M
pGFP-Mut pc-NC pc-IFIH1
GFP
visible light
20μm

Figure 3. IFIH1 targeted binding PTTG1 translation regulatory region 42 nt-52 nt sites triggered the translation of PTTG1. A. The wild type and mutant binding site sequence of translation regulatory protein IFIH1 to translation regulatory region of PTTG1. B. RNA pulldown was used to detect the binding of wild and mutant PTTG1 translation regulatory region to translation regulatory protein IFIH1. C. The binding of translation regulatory protein IFIH1 to wild type and mutant sequences of PTTG1 translation regulatory region were detected by RIP. D. Structural diagram of luciferase plasmid containing wild type and mutant PTTG1 translation regulatory region respectively. E-G. The effects of overexpression IFIH1 on pGL3-WT and pGL3-Mut plasmids were detected by luciferase on 293T, Cal27 and FaDu cell lines. H-J. The effects of silencing IFIH1 on pGL3-WT and pGL3-Mut plasmids were detected by luciferase on 293T, Cal27 and FaDu cell lines. K. Structural diagram of GFP plasmids containing wild type and mutant PTTG1 translation regulation region respectively. L, M. Overexpression of IFIH1 showed different effects on the translation regulatory region of wild-type and mutant PTTG1 in 293T cell lines. ****, $P < 0.0001$.

Cal27 and FaDu cells. The results showed that the proliferation of cells in the IFIH1-silenced and PTTG1-overexpressing group was significantly reduced ($P < 0.05$) compared with that in the NC group (**Figure 5A**, **5B**). Simultaneously, we performed clone formation experiments to demonstrate that cell proliferation was significantly reduced by silencing IFIH1 and overexpressing PTTG1 (**Figure 5C**). Next, the mRNA levels in each group were measured. The mRNA levels of IFIH1 was significantly lower after silencing IFIH1 and overexpressing PTTG1 ($P < 0.05$) than those in the NC group, but there was no significant difference in PTTG1 mRNA levels ($P > 0.05$) (**Figure 5D**, **5E**). Protein expression in each group was detected using western blotting. Compared with those in the NC group, the protein levels of IFIH1 and PTTG1 were significantly decreased by silencing IFIH1 and overexpressing PTTG1 ($P < 0.05$) (**Figure 5F**). Immunofluorescence demonstrated the same results, indicating that IFIH1 promotes the translation of PTTG1 via post-transcriptional regulation (**Figure 5G**, **5H**).

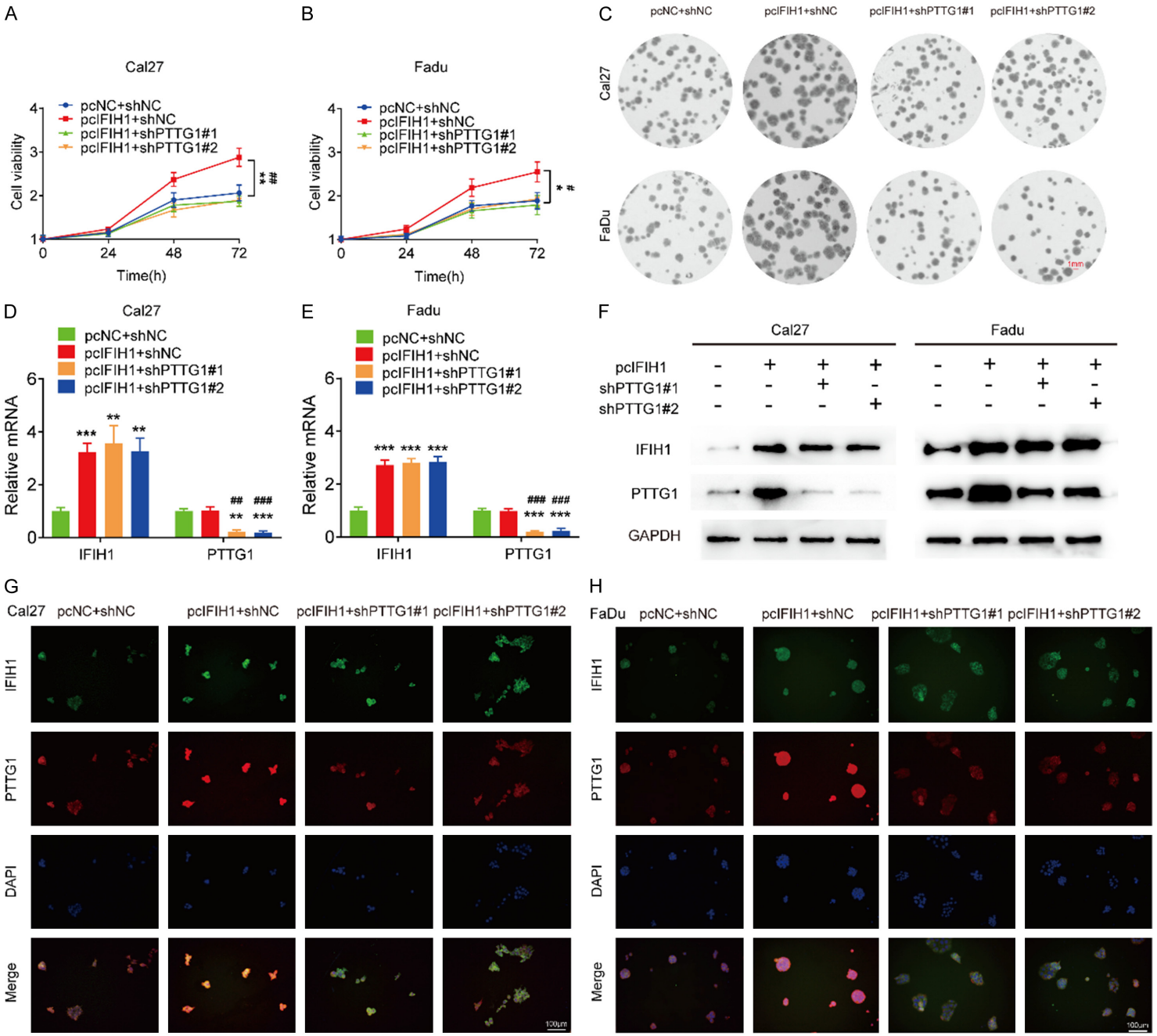
Combination application of PHA-848125 and IFIH1 silencing inhibits the proliferation of xenograft tumors and improves prognosis in nude mice

Cal27 cells (5×10^6) were subcutaneously injected into the scapula of nude mice of age 4-6 weeks. During the experiment, the tumor-bearing mice were weighed and recorded every 5 days until the end of the experiment (**Figure 6A**). The long and short diameters of the tumors were measured every 5 days, and the tumor volumes were calculated (**Figure 6B**). Nude mice in the experimental group were orally administered PHA-848125 (20 $mg \cdot kg^{-1}$) once daily, while the control group was administered normal saline.

The transplanted tumors in the four groups of tumor-bearing mice grew continuously, and the mice were sacrificed on the 40th day. No significant differences were found between the body weights of tumor-bearing mice among the different groups ($P > 0.05$). Compared with those in the NC group, the tumor volumes of the IFIH1-silenced and PHA-848125 groups were dramatically reduced ($P < 0.05$). The tumor volume in the IFIH1-silenced plus PHA-848125-administered group was significantly lower than that in the other three groups ($P < 0.05$) (**Figure 6C-E**).

The protein levels of IFIH1 and PTTG1 in the transplanted tumors from each group were detected using western blotting. Compared with that in the NC group, there was no significant difference in the protein expression of IFIH1 in the PHA-848125 group ($P > 0.05$). However, IFIH1 levels in the IFIH1-silenced group as well as the IFIH1 silenced plus PHA-848125-administered group were significantly decreased ($P < 0.05$). In addition, compared with those in the NC group, the PTTG1 protein levels in the IFIH1-silenced and PHA-848125-adminstered groups were dramatically reduced, whereas that in the IFIH1-silenced plusPHA-848125-administered group was significantly lower than that in the other three groups ($P < 0.05$) (**Figure 6F**). The protein levels of IFIH1 and PTTG1 were detected by IHC. The results showed that compared with those in the NC group, the protein levels of IFIH1 and PTTG1 in the IFIH1-silenced group were significantly decreased ($P < 0.05$) (**Figure 6G**).

The nude mouse experiment was repeated to determine the prognosis of the mice in each group. Compared with that in the NC group, the survival time of mice was significantly prolonged in both the IFIH1-silenced and PHA-


A
Cal27
pcNC+shNC
pcIFIH1+shNC
pcIFIH1+shPTTG1#1
pcIFIH1+shPTTG1#2
Cell viability
Time(h)
B
Fadu
C
pcNC+shNC
pcIFIH1+shNC
pcIFIH1+shPTTG1#1
pcIFIH1+shPTTG1#2
Cal27
FaDu
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Cal27
Relative mRNA
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PTTG1
E
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F
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shPTTG1#1
shPTTG1#2
IFIH1
PTTG1
GAPDH
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Cal27
H
FaDu
DAPI
Merge

Figure 4. Overexpression of IFIH1 and silencing PTTG1 promotes the proliferation of oropharyngeal carcinoma. A, B. The effect of overexpression IFIH1 and silencing PTTG1 on proliferation was detected by CCK8 in Cal27 and FaDu cells. C. The effect of overexpression IFIH1 and silencing PTTG1 on proliferation was detected by cell clone formation experiment in Cal27 and FaDu cells. D, E. The mRNA changes of IFIH1 and PTTG1 by overexpression IFIH1 and silencing PTTG1 were detected by qPCR in Cal27 and FaDu cells. F. WB were used to detect the changes of protein expression by overexpression of IFIH1 and silencing PTTG1 in Cal27 and FaDu cells. G, H. IF were used to detect the changes of protein expression by overexpression of IFIH1 and silencing PTTG1 in Cal27 and FaDu cells. *: The group data compared with pcNC+shNC; #: The group data compared with pcIFIH1+shNC; *, P < 0.05, **, P < 0.01, ***, P < 0.001, ****, P < 0.0001, the same means with #.

848125 groups ($P < 0.05$). When PHA-848125 was administered after silencing IFIH1, the survival time further prolonged and was significantly longer than that of the other three groups ($P < 0.05$) (**Figure 6H**). These in vivo experimental results confirmed that silencing of IFIH1 and administration of PHA-848125 synergistically inhibited the proliferation of oropharyngeal carcinoma cells and improved prognosis.

Discussion

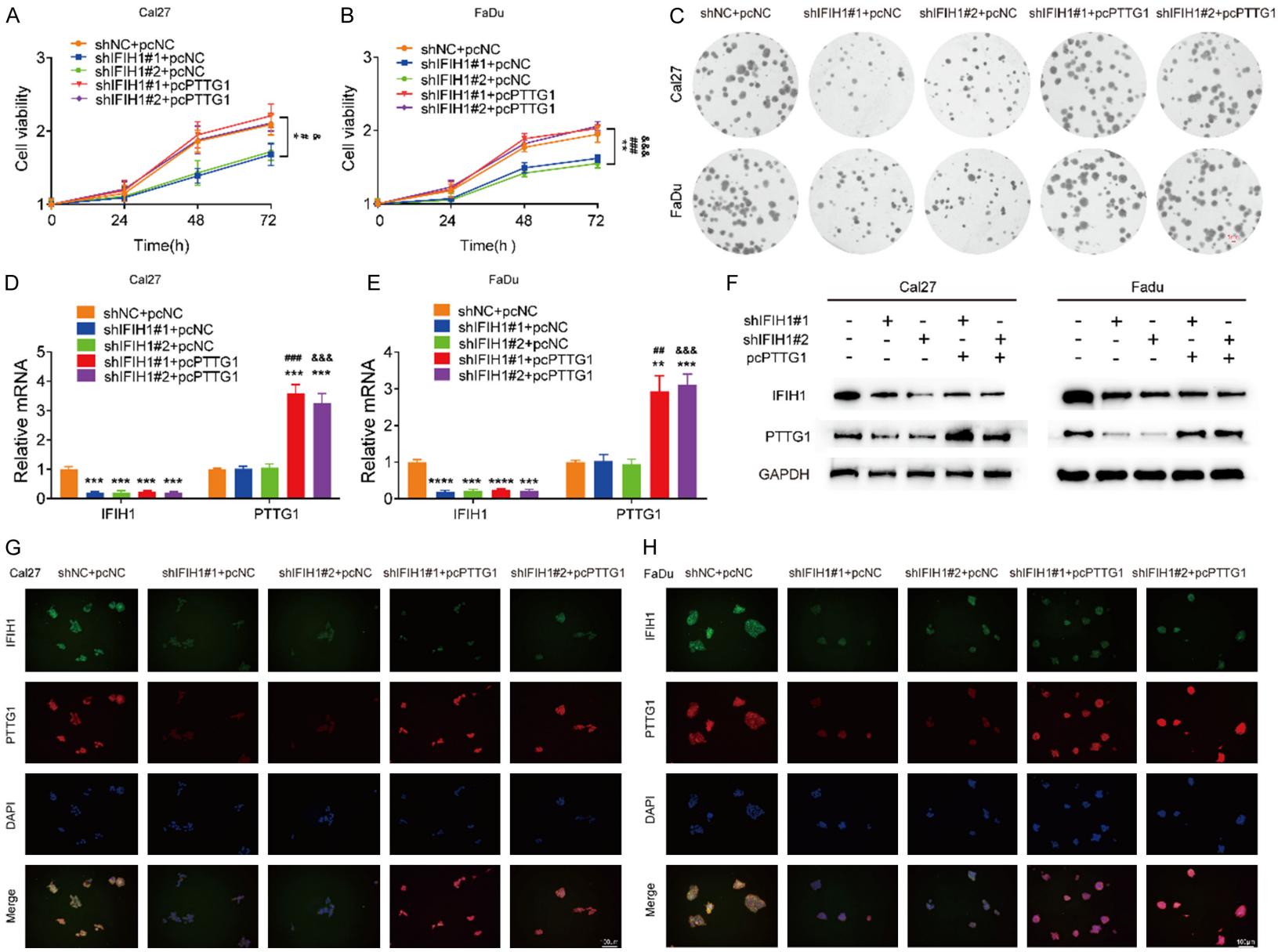
The correlation between mRNA and protein abundance in cells is very poor [20]. The correlation coefficient between mRNA and protein copy numbers was 0.41, and although the translation rate significantly increased to 0.95, the translation rate constants played a dominant role in controlling protein levels. Therefore, protein levels contribute more to gene function than mRNA levels do.

The mRNA abundance of a particular gene does not necessarily have a linear relationship with the expression of its translated protein product because of many levels of gene expression regulation. While regulation at the transcriptional level is only one link, post-transcriptional, translational, and post-translational regulation also play different roles in final protein expression. Factors such as mRNA and protein degradation and protein modification and folding may lead to inconsistencies between mRNA and protein expression levels.

PTTG1 is regulated by various transcription factors. KLF6 inhibits PTTG1 [21]. Rac1, Rasgrf1, and mTOR promotes the expression of PTTG1 [2, 22]. Proteomic studies have shown that PTTG1 increases the expression of multiple downstream proteins involved in hypoxia, cell proliferation, apoptosis, and metastasis [3]. The phosphorylation of PTTG1 may lead to a loss of its cellular transformation function. PTTG1 is distributed in both the nucleus and cytoplasm of cells, and as a nuclear protein, it binds to separase and inhibits its activity. Impairment of this function can result in chromosomal instability and consequently induce tumor formation. PTTG1 activates the expression of angiogenic genes, fibroblast growth factor, vascular endothelial growth factor, apoptosis factor p53, and c-Myc [23-25]. Upregulation of PTTG1 led to the increase of oncogene β-Catenin [26]. PTTG1 deficiency upregulates CDK inhibitor p21 and decreases cell proliferation and Rb phosphorylation [27]. PTTG1 promotes oral squamous cell carcinoma invasion similar to its action in most other cancers, and its expression is directly regulated by miR-186 and miR-655 [28]. However, the reasons for the high expression of PTTG1 in tumors involve the dysregulation of non-coding RNAs such as miRNAs and the aberrant expression of upstream proteins that promote their transcription. No study has demonstrated the relative causes of the high expression of PTTG1 in different tumors or the same reason behind high PTTG1 expression in different tumors. On the contrary, its mechanism of action involves Wnt/β-catenin, MAPK, TGF-β, and PI3K/AKT to promote tumor progression, but the detailed mechanism of action, such as the specific structure or protein modification of PTTG1 is lacking. Therefore, the detailed oncogenic mechanisms of PTTG1 require further investigation. However, the mechanisms underlying tumor progression remain to be explored.

PHA-848125, a cyclin-dependent kinase inhibitor, inhibit CDK1, CDK2, CDK4, and CDK7 [29]. PHA-848125 decreased the levels of retinoblastoma protein and cyclin A and increased the expression of p21cip1, p27kip1, and p53 [30]. PHA-848125 can down-regulate the expression of PTTG1 protein [30]. It also displayed significant antitumor activity both in vitro and in vivo [31, 32].

Studies reported in literature have mainly explored the reasons for the high expression of PTTG1 in tumors and have used siRNAs and


A
Cal27
shNC+pcNC
shIFIH1#1+pcNC
shIFIH1#2+pcNC
shIFIH1#1+pcPTTG1
shIFIH1#2+pcPTTG1
Cell viability
Time(h)
B
FaDu
C
shNC+pcNC
shIFIH1#1+pcNC
shIFIH1#2+pcNC
shIFIH1#1+pcPTTG1
shIFIH1#2+pcPTTG1
Cal27
FaDu
D
Cal27
Relative mRNA
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PTTG1
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FaDu
F
Cal27
Fadu
shIFIH1#1
shIFIH1#2
pcPTTG1
IFIH1
PTTG1
GAPDH
G
Cal27
H
FaDu
DAPI
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Figure 5. Silencing IFIH1 and overexpression PTTG1 inhibits the proliferation of oropharyngeal carcinoma cells. A, B. The effect of silencing IFIH1 and overexpression PTTG1 on proliferation was detected by CCK8 in Cal27 and FaDu cells. C. The effect of silencing IFIH1 and overexpression PTTG1 on proliferation was detected by cell clone formation experiment in Cal27 and FaDu cells. D, E. The mRNA level changes of IFIH1 and PTTG1 after silencing IFIH1 and overexpression PTTG1 were detected by qPCR in Cal27 and FaDu cells. F. WB were used to detect the changes of protein expression by silencing IFIH1 and overexpression PTTG1 in Cal27 and FaDu cells. G, H. IF were used to detect the changes of protein expression by silencing IFIH1 and overexpression PTTG1 in Cal27 and FaDu cells. *: The group data compared with pcNC+shNC; #: The group data compared with shIFIH1#1+pcNC; &: The group data compared with shIFIH1#2+pcNC; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$, the same means with # and &.

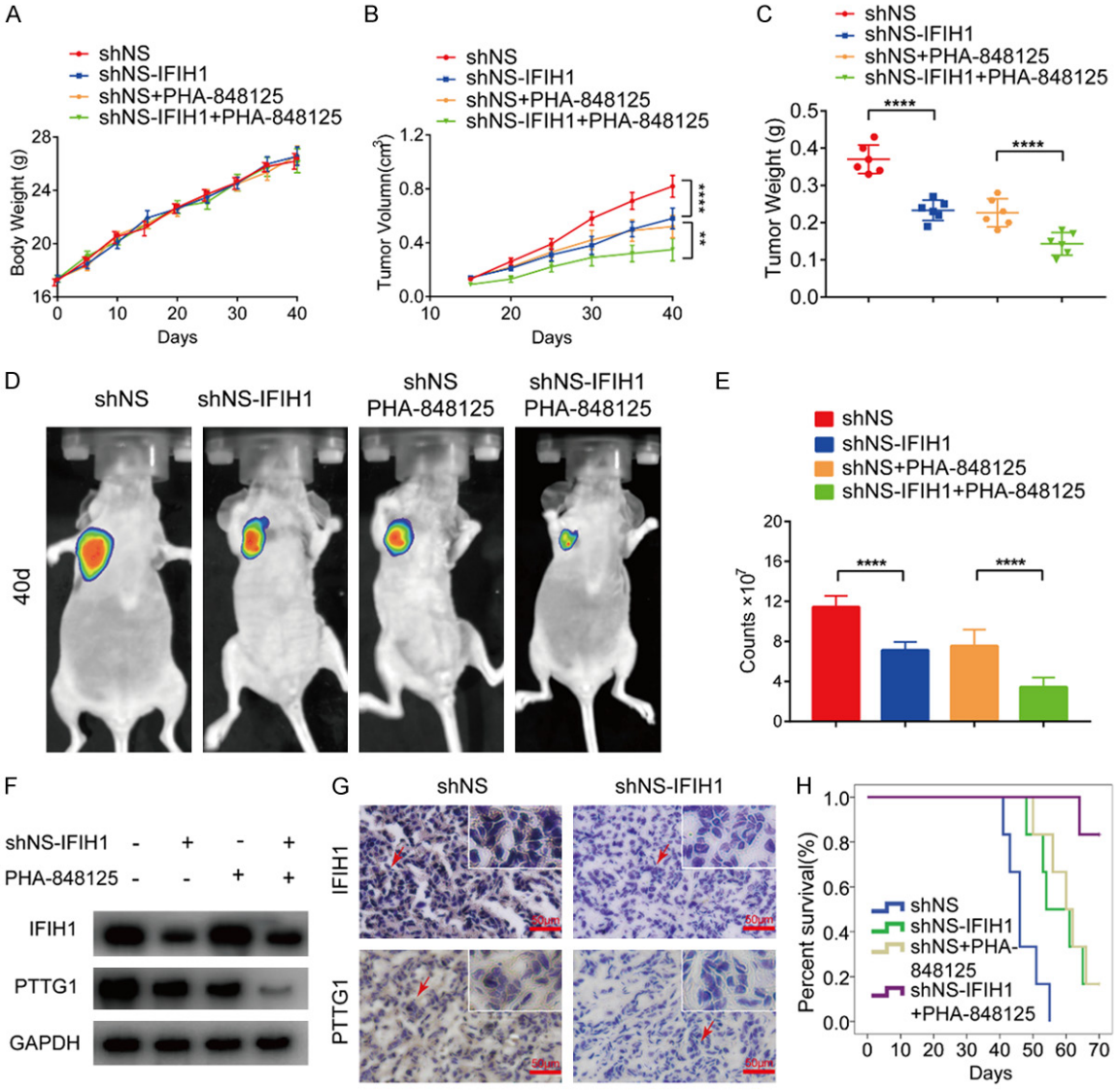

Figure 6. Combined application of IFIH1 and PHA-848125 inhibits the proliferation of transplanted tumor in xenograft mice and improves the prognosis. A. The body weight of xenograft mice in each group. B. Growth curves of tumor volumes in each group (n = 6). C. Tumor weights of xenograft mice at sacrifice (n = 6). D, E. Fluorescence images and statistical analysis of xenograft mice in each group. F. The protein expression levels of IFIH1 and PTTG1 were detected by WB in each group. G. The IFIH1 and PTTG1 expression in xenograft mice by sliencing IFIH1 tested by IHC method. H. Prognosis analysis of nude mice in each group. **, $P < 0.01$, ****, $P < 0.0001$.

other small molecules to regulate the expression of PTTG1 in tumor cells and inhibit tumor progression. However, the nucleoplasmic distribution of PTTG1 in different cells and its role

in the cytoplasm remain unknown. In the future, it will be necessary to study the function of PTTG1 in the cytoplasm and the detailed mechanism of cancer progression in depth, to facilitate effective development of drugs by targeting the specific structural domains or modification sites of PTTG1 and designing small-molecule drugs for certain functional domains. Therefore, targeting PTTG1 for cancer treatment in the clinic needs to be further investigated.

Conclusion

In this study, the mRNA expression of PTTG1 remained unchanged, whereas its protein expression was significantly elevated in tissues affected by oropharyngeal carcinoma. The regulatory protein IFIH1 binds to the translation regulatory region of PTTG1, thereby post-transcriptionally influencing the translation rate and efficiency of PTTG1 This, in turn, facilitates oropharyngeal carcinoma proliferation. Notably, both PTTG1 and IFIH1 have been identified as independent prognostic indicators in patients with oropharyngeal cancer. Furthermore, administration of the PTTG1 inhibitor PHA-848125, in conjunction with silencing of IFIH1, effectively suppressed the expression of PTTG1 and inhibited the proliferation of oropharyngeal cancer cells. These findings offer novel insights into the clinical diagnosis and treatment of oropharyngeal cancer.

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Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

None.

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Table S1. Primers used in qRT-PCR assay

Name	Sequence
PTTG1-F	ACTGTCTGATGAGTGCCAGC
PTTG1-R	AAACAGCGGAACAGTCACGG
IFIH1-F	AGCTTCACCTGGTGTTGGAG
IFIH1-R	TGGCAAACTTCTTGCATGGC

Table S2. Multivariate COX regression analysis of included indicators expression with DFS in oropharyngeal carcinoma patients (n=118)

Virables	Total n	Events n (%)	P^{Π}	Adjusted OR (95% CI)$^{\Pi}$
PTTG1 Protein				
Low	65	39 (60.0)		1.00 (Reference)
High	53	47 (88.7)	< 0.001	3.030 (1.935-4.747)
IFIH1 Protein				
Low	32	14 (43.7)		1.00 (Reference)
High	86	72 (83.7)	< 0.001	3.017 (1.658-5.489)

Abbreviations: 95% CI, 95% confidence interval; DFS, Disease-free survival. *P* values, Adjusted HR (95% CI) were assessed using multivariate Cox regression analysis. $^{\Pi}$*P* values and OR (95% CI) were calculated by unconditional logistic regression.

Table S3. Multivariate COX regression analysis of included indicators expression with OS in oropharyngeal carcinoma patients (n=118)

Virables	Total n	Events n (%)	P^{Π}	Adjusted OR (95% CI)$^{\Pi}$
PTTG1 Protein				
Low	65	33 (50.8)		1.00 (Reference)
High	53	46 (86.8)	< 0.001	3.581 (2.227-5.757)
IFIH1 Protein				
Low	36	15 (41.7)		1.00 (Reference)
High	82	64 (78.0)	< 0.001	3.266 (1.752-6.087)

Abbreviations: 95% CI, 95% confidence interval; OS, Overall survival. *P* values, Adjusted HR (95% CI) were assessed using multivariate Cox regression analysis. $^{\Pi}$*P* values and OR (95% CI) were calculated by unconditional logistic regression.

Table S4. Association of PTTG1 Protein expression with clinicopathological parameters in patients with oropharyngeal carcinoma (n=118)

Virables	*Low Expression*	*High Expression*	P^{Π}	Adjusted OR (95% CI)$^{\Pi}$
Sex				
Man	51 (56.0)	40 (44.0)		1.00 (Reference)
Woman	14 (51.9)	13 (48.1)	0.701	1.184 (0.501-2.800)
Age, yrs[#]				
< 51	32 (54.2)	27 (45.8)		1.00 (Reference)
≥ 51	33 (55.9)	26 (44.1)	0.853	0.934 (0.452-1.929)
Tumor size (cm)				
≤ 2.0	47 (71.2)	19 (28.8)		1.00 (Reference)
> 2.0	18 (34.6)	34 (65.4)	< 0.001	4.673 (2.139-10.205)
Family Histology				
Negative	45 (68.2)	21 (31.8)		1.00 (Reference)
Positive	20 (38.5)	32 (61.5)	0.002	3.429 (1.600-7.345)
Lymph node metasttasis				
Negative	27 (52.9)	24 (47.1)		1.00 (Reference)
Positive	38 (56.7)	29 (43.3)	0.683	0.859 (0.413-1.785)
Vascular invasion				
Negative	48 (57.1)	36 (42.9)		1.00 (Reference)
Positive	17 (50.0)	17 (50.0)	0.480	1.333 (0.600-2.965)
Differentiation				
Media-poorly	39 (52.0)	36 (48.0)		1.00 (Reference)
Well	26 (60.5)	17 (39.5)	0.374	0.708 (0.331-1.516)
Clinnical stage				
I II	48 (54.5)	40 (45.5)		1.00 (Reference)
III IV	17 (56.7)	13 (43.3)	0.840	0.918 (0.398-2.115)

[#]The median age was 51 years. $^{\Pi}P$ values and OR (95% CI) were calculated by unconditional logistic regression.

Table S5. Association of IFIH1 Protein expression with clinicopathological parameters in patients with oropharyngeal carcinoma (n=118)

Virables	*Low Expression*	*High Expression*	P^{Π}	Adjusted OR (95% CI)$^{\Pi}$
Sex				
Man	28 (30.8)	63 (69.2)		1.00 (Reference)
Woman	8 (29.6)	19 (70.4)	0.910	1.056 (0.413-2.698)
Age, yrs[#]				
< 51	19 (32.2)	40 (67.8)		1.00 (Reference)
≥ 51	17 (28.8)	42 (71.2)	0.689	1.174 (0.535-2.572)
Tumor size (cm)				
≤ 2.0	28 (42.4)	38 (57.6)		1.00 (Reference)
> 2.0	8 (15.4)	44 (84.6)	0.002	4.053 (1.652-9.944)
Family Histology				
Negative	27 (40.9)	39 (59.1)		1.00 (Reference)
Positive	9 (17.3)	43 (82.7)	0.007	3.308 (1.386-7.895)
Lymph node metasttasis				
Negative	13 (25.5)	38 (74.5)		1.00 (Reference)
Positive	23 (34.3)	44 (65.7)	0.303	0.654 (0.292-1.466)
Vascular invasion				
Negative	24 (28.6)	60 (71.4)		1.00 (Reference)
Positive	12 (35.3)	22 (64.7)	0.473	0.733 (0.314-1.712)
Differentiation				
Media-poorly	24 (32.0)	51 (68.0)		1.00 (Reference)
Well	12 (27.9)	31 (72.1)	0.642	1.216 (0.533-2.772)
Clinnical stage				
I II	24 (27.3)	64 (72.7)		1.00 (Reference)
III IV	12 (40.0)	18 (60.0)	0.194	0.563 (0.236-1.340)

[#]The median age was 51 years. $^{\Pi}P$ values and OR (95% CI) were calculated by unconditional logistic regression.