Tanshinone IIA decreases the protein expression of EGFR, and IGFR blocking the PI3K/Akt/mTOR pathway in gastric carcinoma AGS cells both *in vitro* and *in vivo*

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Abstract. Tan-IIA exerts powerful inhibitory effects in gastric cancer AGS cells. The PI3K/AKT/mTOR pathway is one of the most frequently dysregulated kinase cascades in human cancer. In the present study, we investigated the protein expression levels of PI3K, AKT and mTOR in AGS cells treated with Tan-IIA both *in vitro* and *in vivo*. The AGS cells were treated with Tan-IIA for different durations *in vitro*. In the *in vivo* study, AGS cell xerograft SCID mice were treated with Tan-IIA for 8 weeks. Subsequently, the protein expression of EGFR, IGFR, PI3K, AKT and mTOR was measured by western blotting. The results showed that Tan-IIA was able to decrease the protein expression levels of EGFR, IGFR, PI3K, AKT and mTOR significantly and dose-dependently *in vitro* and *in vivo*. In conclusion, these findings indicate Tan-IIA could inhibit AGS cells through decreasing the protein expression of EGFR, IGFR and blocking PI3K/AKT/mTOR pathway both *in vitro* and *in vivo*.

Introduction

According to the International Agency for Research on Cancer, the number of estimated new cases of gastric cancer in worldwide was approximately 989,600, and there were approximately 748,000 estimated deaths due to gastric cancer in the worldwide in 2008 (1). Tanshinone IIA (Tan-IIA; $C^{19}H^{18}O^{3}$), is one of the diterpine quinones extracted from Danshen (*Salviae miltiorrhizae radix*) (2,3), with anti-inflammatory activities (4,5) and antioxidant properties (6,7). It is well documented that Tanshinone IIA can inhibit many human cancer cell lines through different molecular mechanisms (8-15). PI3K can prevent apoptosis and promote cellular survival and proliferation in a wide variety of cells. Akt is a serine/threonine-specific protein kinase, which plays a key role in apoptosis and cell proliferation. Raha *et al* showed that Naringin induces autophagy-mediated growth inhibition by downregulating the PI3K/Akt/mTOR cascade via activation of MAPK pathways in AGS cancer cells (16). Lee *et al* also showed that flavonoids isolated from *Citrus platymamma* induce mitochondrial-dependent apoptosis in AGS cells by modulation of the PI3K/AKT and MAPK pathways (17). Zheng *et al* documented that Paeoniflorin inhibits human gastric carcinoma cell proliferation through suppression of PI3K/Akt and STAT3 signaling (18). These documents showed that agents can inhibit AGS cells through downregulating the PI3K/Akt/mTOR cascade. Tan-IIA could inhibit human gastric cancer SGC7901 cells and MKN-45 cells time- and dose-dependently through inducing apoptosis and cell cycle phase arrest (19-21). Tan-IIA also inhibited human gastric cancer AGS cells; one of the molecular mechanisms may be through MAPK pathways to induce G2/M phase arrest. The other may be through extrinsic apoptotic signaling pathway to induce apoptosis *in vitro* (22,23). Tan-IIA shows potential as an alternative therapeutic agent for human gastric carcinoma. However, the underlying anticancer mechanism still needs to be explored further. In the present study, we investigated the protein expression levels of EGFR, IGFR, PI3K, AKT, mTOR and PTEN in human gastric cancer AGS cells treated with Tan-IIA *in vitro* and *in vivo*.

Materials and methods

The EGFR (#2239, MW 175 kDa), IGFR (#3018, MW 95 kDa), PI3K (#4292, MW 85 kDa), AKT (#3063, MW 60 kDa), mTOR (#2983, MW 289 kDa) and PTEN (#9559, MW 54 kDa) antibodies were all obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Tan-IIA was obtained from Sigma-Aldrich

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(St. Louis, MO, USA; CAS-No 568-72-9). Fetal bovine serum (FBS), F-12K medium, glutamine and penicillin-streptomycin were obtained from Gibco-BRL (Grand Island, NY, USA). Triton X-100, Tris-HCl, ribonuclease-A, sodium deoxycholate, leupeptin, sodium pyruvate, HEPES, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Tween-20, mouse anti-β-actin were obtained from Sigma-Aldrich. Potassium phosphate and 0.2 mm PVFD membranes were purchased from Merck Co. (Darmstadt, Germany); the AGS human gastric adenocarcinoma cell line (BCRC number: 60102) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). BioMax film was obtained from Kodak.

Male SCID mice. Sixty male SCID mice, weighing 10-15 g (3-4 weeks old), were obtained from the Laboratory Animal Center, Tzu Chi University (Hualien, Taiwan).

Cell culture. The human gastric adenocarcinoma AGS cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cell culture procedure was as described (22,23). Briefly, the AGS cells were placed into 75-cm^2 tissue culture flasks and maintained in F-12K contained with 10% heat-inactivated FBS (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

The protein expression of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin in AGS cells treated with various concentrations of Tan-IIA. The AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 μg/ml) for 24 or 48 h and then the protein expression levels of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin were evaluated by western blot analysis.

The protein expressions of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin in AGS cells treated with Tan-IIA for different durations. The AGS cells were treated with Tan-IIA (3.7 μg/ml) for different durations (0, 24 and 48 h) and then the protein expression levels of VEGFR, HER2, Ras, Raf, MEK, ERK, PARP, caspase-3 and β-actin were evaluated by western blot analysis.

Effects of Tan-IIA on the protein expression of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin in AGS cell xenograft tumors. Three-week-old male nude SCID mice (number =60) were xenograft with AGS cells ($2x10^6$/0.2 ml) and maintained in a pathogen-free environment (CCH-AE-102-007; Laboratory Animal Center of Tzu Chi University, Hualien, Taiwan). When the xenograft tumors reached more than 0.5 cm in diameter, the mice were divided randomly into four groups. Tan-IIA was dissolved in corn oil and then administered to the mice at concentrations of 30, 60 and 90 mg/kg, QW1, 3, 5 by intraperitoneum injection for 8 weeks. The control group was treated with an equal volume of corn oil. SCID mice were scarified by CO_2 inhalation and then the xenograft tumors were dissected. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), approval no. CCH-AE-102-007). Subsequently, the protein expressions of EGFR, IGFR, PI3K, AKT, mTOR and PTEN in the tumors were measured by western blotting.

Protein preparation. Proteins were extracted from xenograft tumors. The xenograft tumors were dissected and ground, then the thick liquid (0.06 gm) was lysed in the ice-cold whole cell extract buffer containing the protease inhibitors. The lysate was vibrated for 30 min at 4˚C and centrifuged at 12,281 x g for 10 min. Protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL, USA).

Western blot analysis. The western blot procedures were as described (22,23). Briefly, AGS cells were treated with various concentrations of Tan-IIA for different durations, and then the cells were lysed in the ice-cold whole cell extract buffer containing the protease inhibitors. The lysate was vibrated for 30 min at 4˚C and centrifuged at 12,281 x g for 10 min. Protein concentration was measured by BCA protein assay kit (Pierce). Equal amounts of proteins were subjected to electrophoresis using 12% sodium dodecyl sulfate-polyacrylamide gels. To verify equal protein loading and transfer, proteins were then transferred to polyvinylidene difluoride membranes and the membranes were blocked for 1 h at 4˚C using blocking buffer [5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween-20 and 0.02% sodium azide]. The membranes were then incubated for 2 h at room temperature with specific primary antibody followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times for 10 min with washing solution. Finally, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).

Statistical analysis. Values are presented as the means ± SD. The Student's t-test was used to analyze statistical significance. A p-value of <0.05, was considered to indicate a statistically significant difference for all the tests. $P<0.05$, $P<0.01$, $P<0.001$.

Results

Effects of Tan-IIA on the viability of AGS cells. The results revealed that Tan-IIA can inhibit AGS cells in a time- and dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) was 5.5, 3.7 and 3.5 μg/ml at 24, 48 and 72 h, respectively (data not show). This is agreement with our previous studies (22,23).

Effects of Tan-IIA on the protein expression of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin in AGS cells. The AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 μg/ml) for 24 or 48 h and then the protein expression levels of EGFR, IGFR, PI3K, AKT, mTOR, p-TEN and β-actin were evaluated by western blot analysis. The results showed that Tan-IIA can decrease the protein expression levels of EGFR (Fig. 1A), IGFR (Fig. 1B), PI3K (Fig. 1C), AKT (Fig. 1D), mTOR (Fig. 1E) and PTEN (Fig. 1F) significantly.

Effects of Tan-IIA on the protein expression of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin in AGS cells. The AGS cells were treated with Tan-IIA (3.7 μg/ml) for different

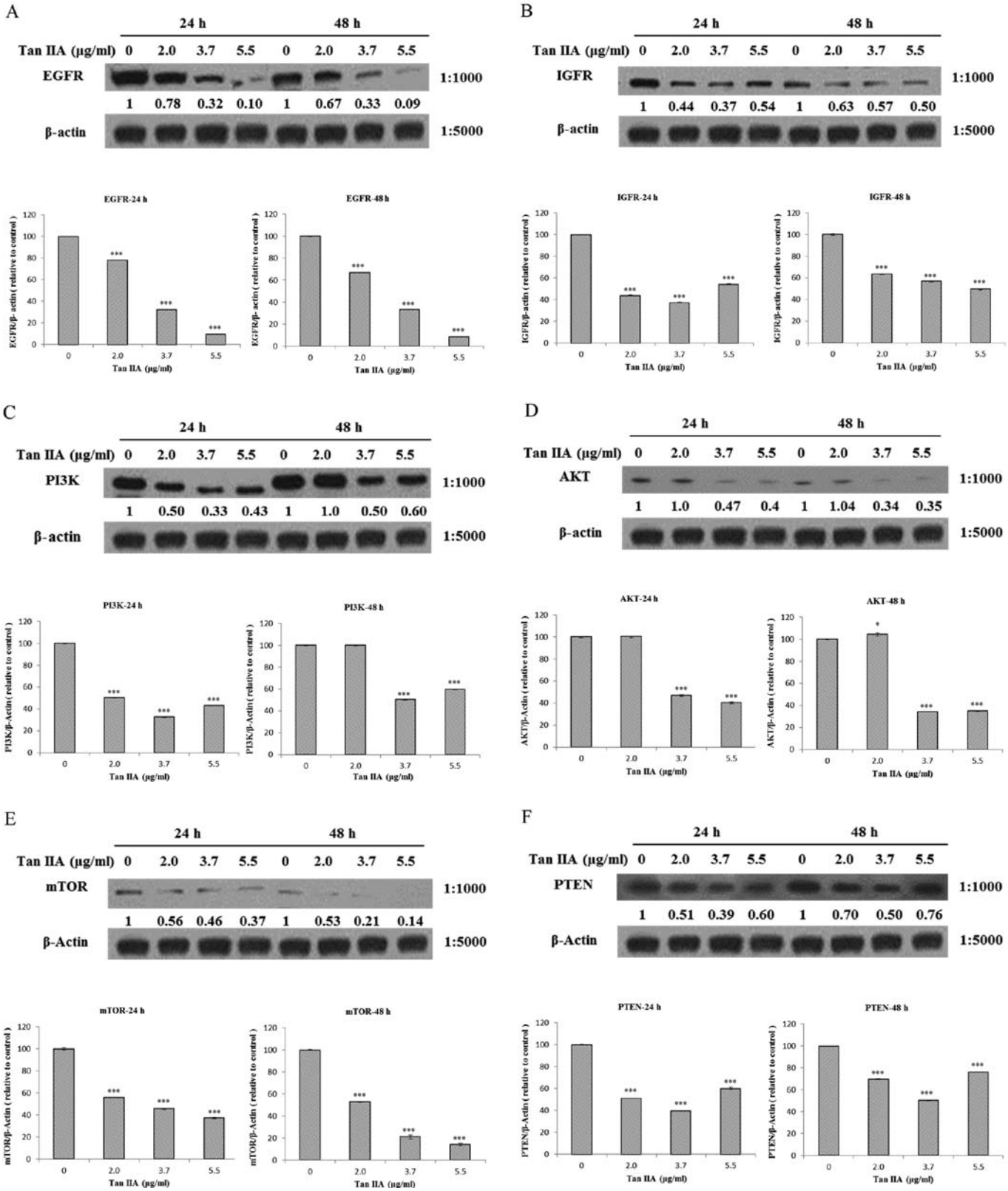

Figure 1. Effects of Tan-IIA on the protein expression of EGFR, IGFR, PI3K, AKT, mTOR, p-TEN and β-actin in AGS cells. The AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 μg/ml) for 24 or 48 h and then the protein expression levels were evaluated by western blot analysis as described in Materials and methods. The results showed that Tan-IIA can decrease the protein expression levels of EGFR (A), IGFR (B), PI3K (C), AKT (D), mTOR (E) and p-TEN (F) significantly and dose-dependently. *P<0.05, was considered to indicate a statistically significant difference for all the tests. *P<0.05, **P<0.01, ***P<0.001.

durations (0, 24 and 48 h) and then the protein expression levels of EGFR, IGFR, PI3K, AKT, mTOR, p-TEN and β-actin were evaluated by western blot analysis. The results showed that Tan-IIA can decrease the protein expression

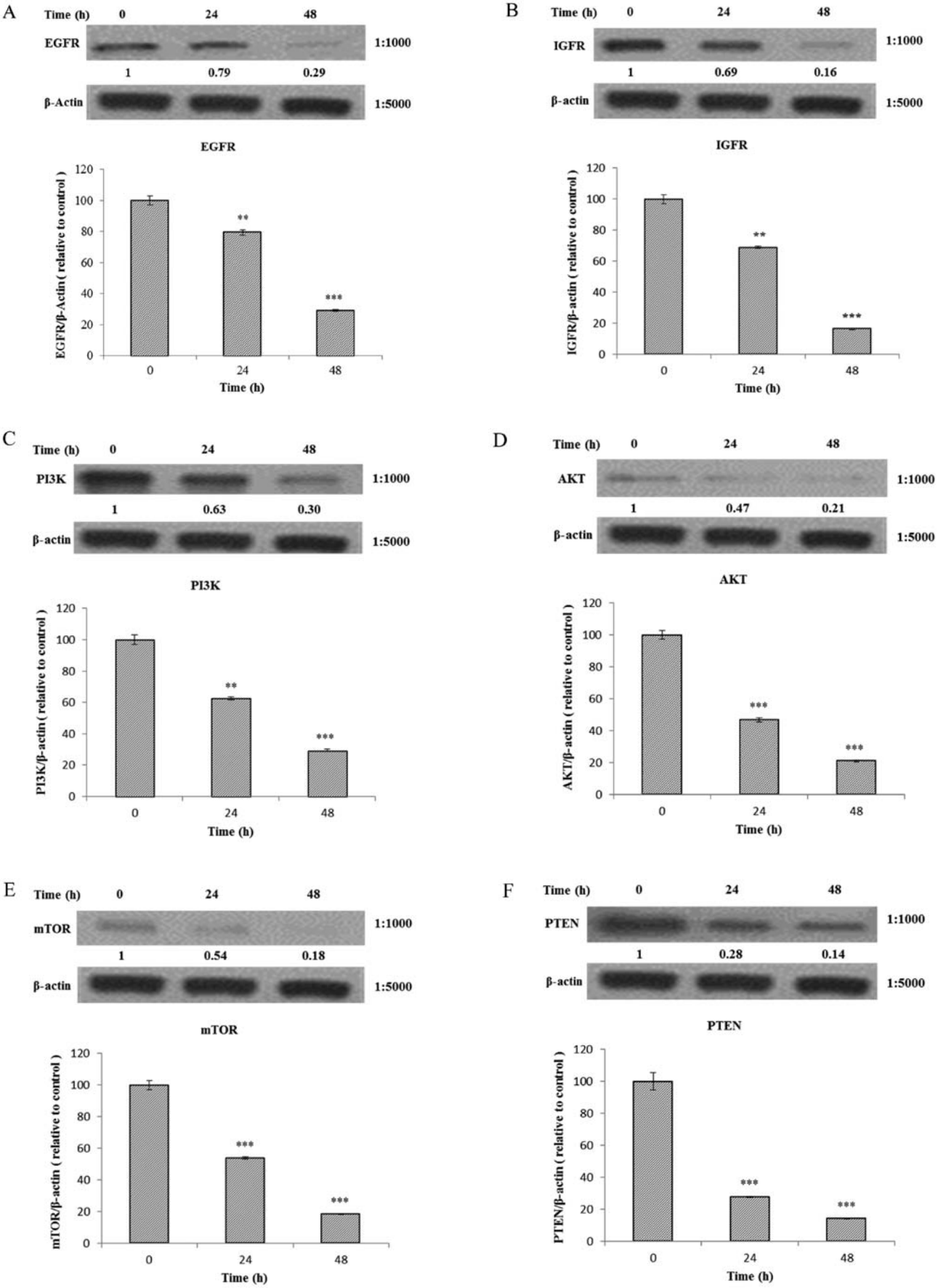

Figure 2. Effects of Tan-IIA on the protein expression of EGFR, IGFR, PI3K, AKT, mTOR, p-TEN and β-actin in AGS cells. The AGS cells were treated with Tan-IIA (3.7 μg/ml) for different durations (0, 24 and 48 h) and then the protein expression levels were evaluated by western blot analysis as described in Materials and methods. The results showed that Tan-IIA can decrease the protein expression levels of EGFR (A), IGFR (B), PI3K (C), AKT (D), mTOR (E) and p-TEN (F) significantly and dose-dependently. *P<0.05 was considered to indicate a statistically significant difference for all the tests. *P<0.05, **P<0.01, ***P<0.001.

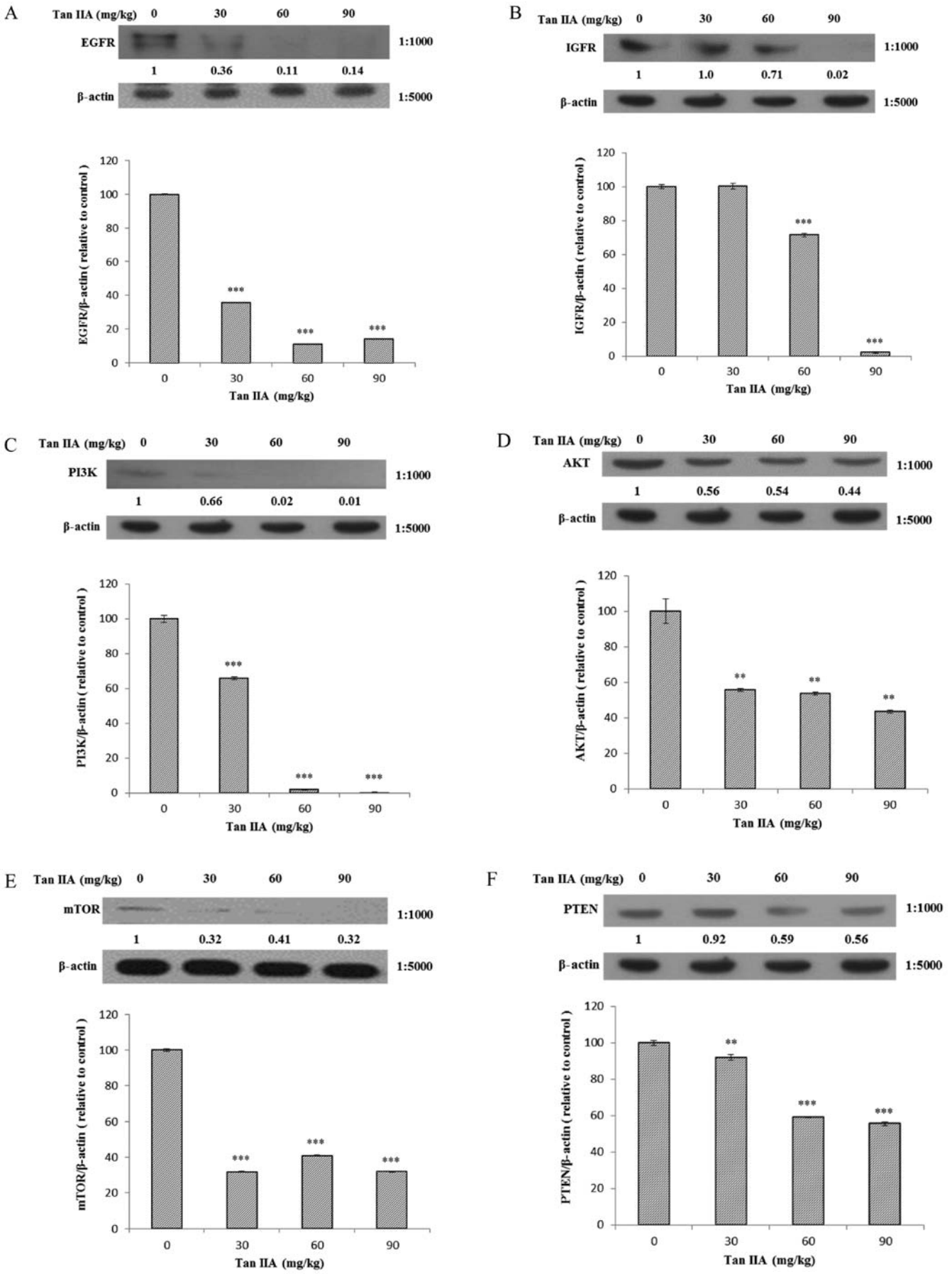

Figure 3. Effects of Tan-IIA on the protein expressions of EGFR, IGFR, PI3K, AKT, mTOR, p-TEN and β-actin in AGS cell xenograft tumors. AGS cell xenograft tumors were treated with different doses of Tan-IIA then the protein expressions were measured by western blotting as described in Materials and methods. The results showed that Tan-IIA can decrease the protein expression levels of EGFR (A), IGFR (B), PI3K (C), AKT (D), mTOR (E) and p-TEN (F) significantly and dose-dependently. $^{*}P<0.05$ was considered to indicate a statistically significant difference for all the tests. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

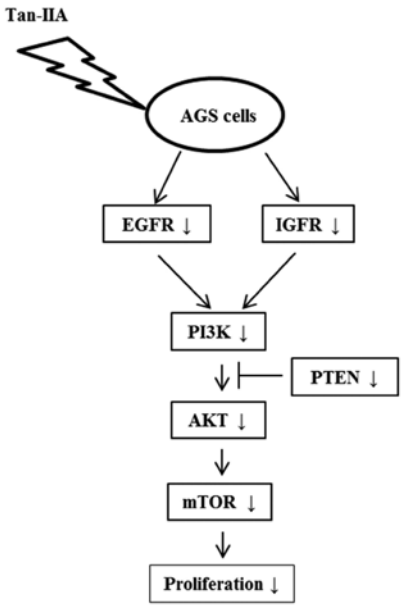

Figure 4. The proposed model for Tan-IIA to inhibit the proliferation of AGS cells. Tan-IIA inhibits human gastric cancer AGS cells through decreasing the protein expression of EGFR, IGFR and blocking PI3K/AKT/mTOR pathway.

levels of EGFR (Fig. 2A), IGFR (Fig. 2B), PI3K (Fig. 2C), AKT (Fig. 2D), mTOR (Fig. 2E) and PTEN (Fig. 2F) significantly.

Effects of Tan-IIA on the protein expression of EGFR, IGFR, PI3K, AKT, mTOR, PTEN and β-actin in AGS cell xenograft tumors. The AGS cell xenograft tumor SCID mice were treated with different doses of Tan-IIA for 8 weeks then sacrificed. The protein expression of EGFR, IGFR, PI3K, AKT, mTOR and PTEN in xenograft tumors were measured by western blotting as described in Materials and methods. The results showed that Tan-IIA can decrease the protein expression levels of EGFR (Fig. 3A), IGFR (Fig. 3B), PI3K (Fig. 3C), AKT (Fig. 3D), mTOR (Fig. 3E) and PTEN (Fig. 3F) significantly and dose-dependently.

Discussion

It is well documented that PI3K/AKT/mTOR pathway is one of the most frequently dysregulated kinase cascades in human cancer (24,25). PI3K/PTEN/Akt/mTOR cascade inhibitors have been investigated in pre-clinical and clinical investigations and reported as having potential (26). The transmembrane tyrosine kinases, such as insulin-like growth factor receptor (IGFR) or epidermal growth factor receptor (EGFR) have been strongly implicated in the growth, survival, and metastasis of a wide variety of human tumors (27,28). PI3K/PTEN/Akt/mTOR pathway represents important signal transduction mechanisms that facilitate the proliferation and survival of cancers driven by growth factor receptors (29).

It has been well documented that Naringin, flavonoids and Paeoniflorin can inhibit AGS cells through downregulating the PI3K/Akt/mTOR cascade (16-18). Our results showed that AGS cells treated with Tan-IIA can downregulate the protein expressions of PI3K/ Akt/mTOR both *in vitro* and *in vivo*.

Protein phosphatase activity is closely associated with tumors. PI3K is necessary for the activation of AKT. PTEN regulates the activity of AKT via activated phosphatidylinositol triphosphate (PIP3) (30). Our results showed that AGS cells treated with Tan-IIA can downregulate the protein expression of PTEN both *in vitro* and *in vivo*. These findings indicate Tan-IIA can inhibit the proliferation of AGS cells, one of the molecular mechanisms may be through downregulating the PI3K/Akt/mTOR cascade. The proposed model is shown in Fig. 4. This is the first report that Tan-IIA could inhibit gastric carcinoma AGS cells by decreasing the protein expression of EGFR, IGFR and blocking the PI3K/Akt/mTOR pathway in a cancer-bearing animal model.

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