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ORIGINAL ARTICLE

Tanshinone IIA suppress the proliferation of HNE-1 nasopharyngeal carcinoma an in vitro study

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KEYWORDS

Matrigel;
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Abstract Nasopharyngeal carcinoma (NPC) at present is considered to be one of the fatal diseases detected commonly in the people belonging to Southeast Asia and southern China. According to the WHO reports among the detected cases of NPC worldwide, 80% are from China. The present study investigates the effect of tanshinone IIA on the migration and invasion potential of HNE-1NPC cells and studied the detailed mechanism involved. Effect of the tanshinone IIA on viability of the HNE-1NPC cells was analyzed by MTS assay. Cell matrigel invasion and wound-healing motility assays, respectively were used for the analysis of invasion and migration potential of HNE-1 cells. Tanshinone IIA inhibited the viability of HNE-1cells in a dose dependent manner. Migration and invasion potential of the tanshinone IIA treated cells was reduced significantly ($P < 0.05$) compared to the control cells after 48 h. Analysis of the proteins involved in migration and invasion revealed a significant decrease in the expression of matrix metalloproteinase (MMP)-2 and MMP-9 on treatment with tanshinone IIA. It also inhibited the p65 and p50 expression in the nuclear fractions of HNE-1 cells after 48 h. Thus, tanshinone IIA inhibits migration and invasion potential of the HNE-1NPC cells through reduction in the expression of matrix metalloproteinases. Therefore, tanshinone IIA can be used for the treatment of NPC.

1. Introduction

Nasopharyngeal carcinoma (NPC) at present is considered to be one of the fatal diseases detected commonly in the people belonging to Southeast Asia and southern China (Chang and Adami, 2006). According to the WHO reports among the detected cases of NPC worldwide, 80% are from China. Most of the NPC cases are difficult to be differentiated from the squamous cancer (Guigay, 2008). This malignant tumor can be treated at the early stage by radiotherapy and its progression delayed by chemotherapeutic agents (Kalaiselvi et al., 2016; Serasanambati and Chilakapati, 2016). It is reported that the 5-year survival rate of patients with NPC is very low and the incidence of relapses is high (Vlantis et al., 2007). Studies have demonstrated that NPC metastasizes to the lymphatic nodes present in the neck in around 80% of the cases (Chua

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et al., 2001). NPC metastasis is a big hurdle for the successful inhibition of cancer by various chemotherapeutic agents. Furthermore, NPC penetrates to the distant tissues like lung, liver and bones (Cheng et al., 1998). Carcinoma metastasis involves breakdown of the extracellular matrix (ECM) which is accompanied by cell invasion and migration to other tissues (Raghu et al., 2010; Chen et al., 2013). Breakdown of the ECM and therefore enhancement in the migration and invasion of potential of carcinoma cells is catalyzed by enzymes known as matrix metallo-proteinases (MMPs) (Bjorklund and Koivunen, 2005; Yang et al., 2005).

Metabolites derived from the medicinal plants play an important role in protecting the human body from various diseases (Neelamkavil and Thoppil, 2016; Noorudheen and Chandrasekharan, 2016; Santhosh et al., 2016; Valsan and Raphael, 2016). Tanshinone IIA is isolated from the plant Radix *Salvia miltiorrhiza* and comprises its active constituent (Che et al., 2004; Zhou et al., 2005). Analysis of the biological activity of tanshinone IIA revealed various activities including, anti-inflammatory activities (Jang et al., 2006; Li et al., 2007) and antioxidant properties (Lin et al., 2006; Wang et al., 2003). It has been demonstrated that tanshinone IIA treatment inhibits carcinoma cell growth through suppression of ErbB-2 and promotion of TNF-α expression (Su and Lin, 2008a). In breast carcinoma cells exposure to tanshinone IIA promotes ratio of Bax to Bcl-xL expression (Su and Lin, 2008b). While as in human non-small cell lung cancer cells it induces expression of reactive oxygen species and decreases the mitochondrial membrane potential (Chiu and Su, 2010). In case of lung and breast cancer cells tanshinone IIA treatment inhibits growth through induction of ER stress (Cheng and Su, 2010; Yan et al., 2012). The present study demonstrates the effect of tanshinone IIA on the invasive and migration potential of NPC cells. Tanshinone IIA treatment significantly inhibited the migration and invasion potential of the NPC cells.

2. Materials and methods

2.1. Reagents and chemicals

Tanshinone II and dimethyl sulphoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other common solvents, reagents and chemicals used were of analytical or high-performance liquid chromatography (HPLC) grade from Sigma–Aldrich or Merck (Darmstadt, Germany). All the antibodies used in the study were mouse monoclonal supplied by Cell Signaling Technologies (Danvers, MA, USA).

2.2. Cell culture

The human HNE-1cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured on 75 cm^2 tissue culture flasks with RPMI1640 medium (Gibco BRL). The cell medium with 2 mM L-glutamine was adjusted to contain 10% fetal bovine serum (Gibco BRL), and 1% penicillin–streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) and grown at 37 °C under a humidified 5% CO_2 atmosphere.

2.3. MTS viability assay

HNE-1cells were distributed at a density of 2×10^5 cells per well in 100 μl RPMI with 10% FBS in to the wells of a 96-well plate. The cells were incubated with 10, 20, 30, 40 and 50 μM concentrations of tanshinone II for 48 h. The control cell cultures were treated with medium alone without tanshinone II. For the determination of cell viability 30 μl Cell Titer 96® AQueous One Solution Reagent (MTS; Promega Corporation, Madison, WI, USA) was added to each well of the plate. The plates were incubated for 45 min in an incubator at 37 °C with humid atmosphere of 5% CO_2. For each well absorbance was recorded by ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at a wavelength of 475 nm. The experiment was reported three times for each well and compared with the control cultures.

2.4. Cell matrigel invasion assays

Invasion of the HNE-1NPC cells through a matrigel basement membrane (Sigma–Aldrich, Carlsbad, CA, USA) was analyzed. Briefly, the filters of polycarbonate were coated on the under surface with fibronectin and were placed in 24-well Transwell cell culture chambers (Chemicon, Temecula, CA, USA). The upper surface of the filters was coated with Matrigel and the control wells left uncoated. Dry filters were washed with PBS, rehydrated and then 2×10^6 NPC cells in RPMI-1640 medium were suspended in the chambers. After incubation of the cells with tanshinone II for 48 h, the cells that invaded the lower chamber of the filter were stained with calcein. Quantification of the cells was performed at 489 and 525 nm excitation and emission wavelengths, respectively.

2.5. Migration assay

Migration potential of the HNE-1 cells was analyzed by using wound-healing motility assay. The cells were distributed at a density of 3×10^5 cells into the 6-well culture plates with tanshinone II or without as control and incubated for 48 h. The cells were then scratched using a plastic cell scraper and the cellular monolayer was rinsed in PBS for the removal of detached cells. The cells adhered were incubated for 1 h more at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Decrease in the scraped cellular zone width compared to the control determined the cell migration. The Olympus-CX31 microscope (Olympus Corp.) was used for the measurement of migratory distance covered by the cells.

2.6. Zymography

In order to determine the activity of MMP-2 and MMP-9 NPC cells were incubated with tanshinone II for 48 h. Following incubation, the cell lysates were collected to determine the expression of the proteins. The protein samples (30 μl) were isolated by electrophoresis on 10% SDS–PAGE gel. The gels were treated for 45 min at room temperature with 5% Triton X-100 solution (Sigma–Aldrich) followed by incubation for 12 h with reaction buffer (10 mM $CaCl_2$, 40 mM Tris–HCl and 0.01% NaN3, pH 8.0). Staining and de-staining of the gels was performed using 0.1% Coomassie brilliant blue R-250

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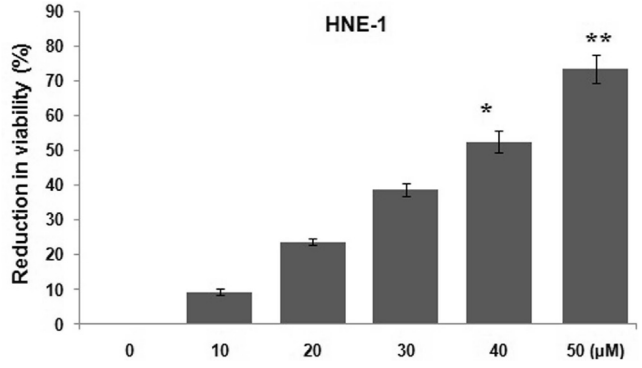

Figure 1 Inhibition of HNE-1NPC cell viability by tanshinone IIA in dose dependent manner. The cells were incubated for 48 h with 10, 20, 30, 40 and 50 μM doses of tanshinone IIA followed by analysis using MTS assay. The experiments were performed in triplicates and the data presented are the mean ± standard deviation. Where, $^{*}P < 0.04$ and $^{**}P < 0.02$, data were compared to the control group at 48 h.

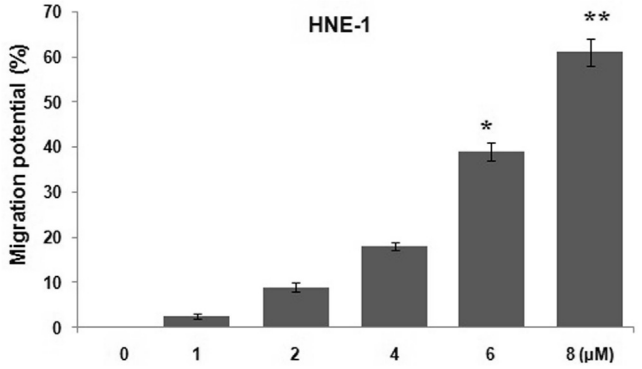

Figure 2 Tanshinone II inhibits the migration potential of the HNE-1NPC cells. The cells were incubated in media containing various concentrations of tanshinone II or in media alone as control. Following incubation the cells were analyzed by matrigel invasion assay for migration potential. The data presented are the mean ± standard deviation of the experiments performed in triplicates. Where $^{*}P < 0.05$ and $^{**}P < 0.01$, data were compared with the control group.

(Sigma–Aldrich) and mixture of 30% methanol and 10% acetic acid, respectively. The image analysis system (Quantity One v4.62; Bio-Rad Laboratories) was used to record the band intensities.

2.7. Western blot analysis

The HNE-1 cells after incubation with tanshinone II were collected and lysed in RIPA buffer (50 mM Tris-base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF). The extracted proteins were quantified using DC protein assay Kit (Bio-Rad). The protein samples were isolated on 10% SDS–PAGE and then transferred to polyvinylidenedifluoride (PVDF) membranes (Amersham Biosciences). The non-specific sites on the membranes were blocked by incubation with PBS supplemented with 0.1% Tween 20 in 5% skimmed milk. The probing of the proteins was performed by incubation with of the membranes with polyclonal antibodies against MMP-2, MMP-9, p50, p65 and β-actin (diluted 1:500, Abcam, UK). Following overnight incubation, the membranes were incubated for 1 h with secondary antibodies conjugated to horseradish peroxidase. The enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, USA) were used for the visualization of the proteins.

2.8. Statistical analysis

The data expressed are the means ± SD. Student's *t*-test was used for the comparing the two groups. One-way ANOVA analysis was also used to analyze the data obtained. Relevance analysis of ordinal data was performed by cross χ^2 test. A statistically significant difference was defined as $p < 0.05$.

3. Results

3.1. Inhibition of HNE-1NPC cell proliferation by tanshinone IIA

Effect of tanshinone IIA on the viability of HNE-1 cells was analyzed by MTS assay. The results revealed a dose dependent decrease in the cell viability on treatment with tanshinone IIA for 48 h. Viability of HNE-1 cells was decreased by 9.37, 23.64, 38.65, 52.52 and 73.55%, respectively on treatment with 10, 20, 30 and 50 μM concentration of tanshinone IIA after 48 h (Fig. 1).

3.2. Inhibition of NPC cell migration by tanshinone II

The effect of tanshinone II on the migration potential of HNE-1 cells was also analyzed. The results showed reduction in the migration potential of HNE-1 cells in a concentration dependent manner after 48 h. Tanshinone II treatment at the doses of 1, 2, 4, 6 and 8 μM concentration reduced the migration potential by 2.5, 9.12, 18.76, 39.45 and 61.23%, respectively after 48 h (Fig. 2).

3.3. Inhibition of HNE-1NPC cell invasion by tanshinone II

Analysis of the invasive behavior revealed a significant decrease on treatment with tanshinone II for 48 h in HNE-1 cells (Fig. 3). The invasive potential at 1, 2, 4, 6 and 8 μM concentration of tanshinone II was decreased by 4.2, 7.43, 21.54, 42.31 and 72.62%, respectively after 48 h.

3.4. Inhibition of MMP-2 and MMP-9 protein expression by tanshinone II

We also analyzed the effect of tanshinone II on the expression level of MMP-2 and MMP-9 protein and their corresponding activity. The results revealed that tanshinone II treatment caused a comparatively significant decrease in the expression of MMP-2 and MMP-9 after 48 h in HNE-1cells (Fig. 4). The reduction in expression was also evident by a significant decrease in the MMP-2 and MMP-9 activity.

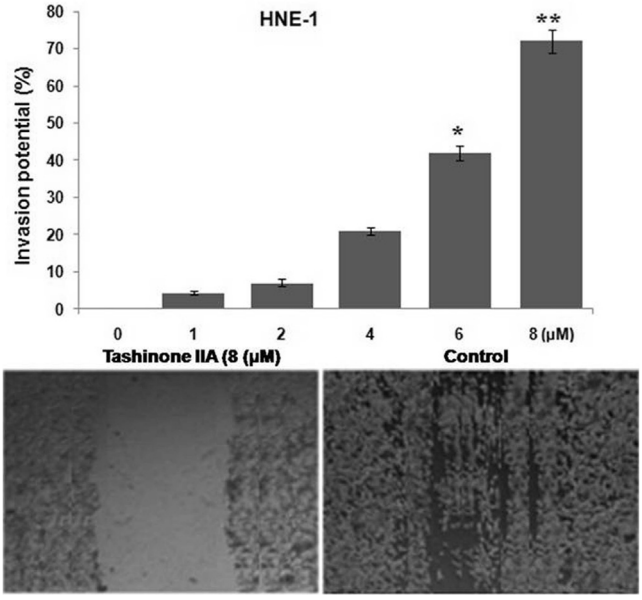

Figure 3 Tanshinone II inhibits the invasion potential of the HNE-1 cells. The cells were incubated in media containing tanshinone II or in media alone as control. Following incubation the cells were analyzed by wound-healing assay for invasion potential. The data presented are the mean ± standard deviation of the experiments performed in triplicates. Where $^{*}P < 0.05$ and $^{**}P < 0.01$, data were compared with the control group.

3.5. Effect of tanshinone IIon nuclear translocation of p50 and p65

Analysis of the level of NF-κB in the tanshinone II treated HNE-1cells revealed a significant decrease in p65 and p50 expression after 48 h (Fig. 5). The reduction in the expression of NF-κB was found to be similar in the cells treated with both henenalin (NF-κB inhibitor) and tanshinone II.

4. Discussion

Nasopharyngeal carcinoma is one of the most commonly detected malignant tumor that can be treated at the early stage by radiotherapy and its progression delayed by chemotherapeutic agents. However, NPC metastasis is a big hurdle for the successful inhibition of cancer by various chemotherapeutic agents. Therefore, inhibition of the NPC metastasis by any method can play a promising role in the treatment of NPC. In the present study effect of tanshinone II on the inhibition of migration and invasion potential of NPC cells and the underlying mechanism was investigated. Treatment of the NPC cells with tanshinone II significantly reduced the migration and invasion potential of NPC cells. Carcinoma cell metastasis to lymph and blood vessels is facilitated by the breakdown of ECM (Chen et al., 2013). The results from the present study revealed that treatment of the NPC cells with tanshinone II significantly reduced the migration potential. Tanshinone II induced reduction in the migration and invasion potential of NPC cells was clearly evident from the wound healing and the Boyden chamber assays.

It is reported that breakdown of the ECM to mediate carcinoma cell metastasis is regulated by MMP-2 and MMP-9 (Yan et al., 2012; Gialeli et al., 2011). Results from the present study revealed that tanshinone II treatment caused a significant decrease in the MMP-2 and MMP-9 expression in NPC-039 cells after 48 h. Tanshinone II treatment also resulted in decrease in the MMP-2 and MMP-9 activity in NPC cells after 48 h. Studies have demonstrated that expression of MMP-2 and MMP-9 is enhanced in the carcinoma cells by NF-κB (Yeh et al., 2012; Yu et al., 2009, 2011). Results from the present study demonstrated that tanshinone II treatment in NPC cells enhanced the p65 and p50 expression. Thus it appears that tanshinone II inhibits migration and invasion potential of the NPC cells through reduction in the NF-κB expression.

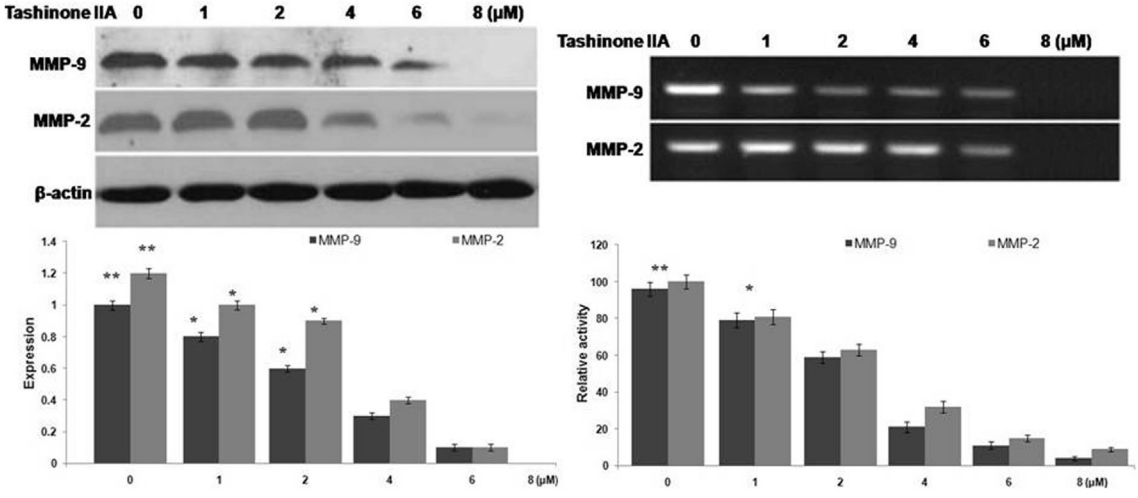

Figure 4 Inhibition of matrix metalloproteinase (MMP)-2 and MMP-9 expression by tanshinone IIA in HNE-1 cells. The cells were treated with tanshinone IIA at different doses for 48 h followed by western blotting analysis to examine the MMP-2 and MMP-9 expression. Inhibition of MMP-2 and MMP-9 activity by tanshinone IIA in NPC cells. The presented are the mean ± standard deviation of the experiments performed in triplicates. Where $^{*}P < 0.03$ and $^{**}P < 0.02$, data were compared with the control group.

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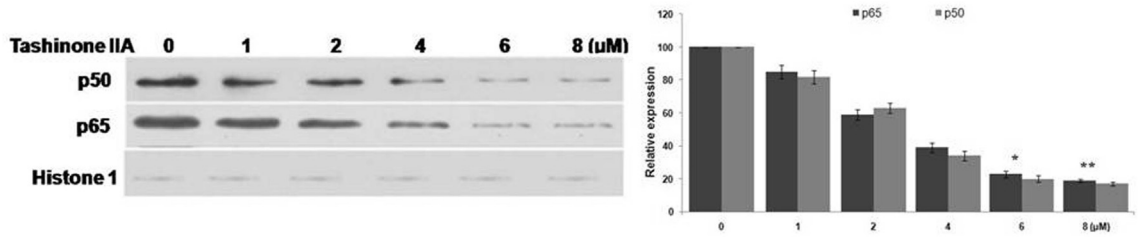

Figure 5 Effect of tanshinone IIA on the nuclear factor (NF)-κB-related protein expression in HNE-1 cells. (A) Inhibition of the p50 and p65 nuclear translocation by the tanshinone IIA after 48 h was analyzed using western blot analysis in the nuclear and whole cell lysates. The expressed data are the mean ± standard deviation of three independent experiments, performed in triplicate. Where $^{*}P < 0.05$ and $^{**}P < 0.01$, data were compared with the control group.

5. Conclusion

In summary, tanshinone II inhibits the migration and invasion potential of NPC cells and decreases the expression of MMP-2 and MMP-9 through down-regulation of the NF-κB pathway. Thus tanshinone II can play an important role in the treatment of NPC.

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