Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Anti-hepatocarcinoma activity of TT-1, an analog of melittin, combined with interferon-α via promoting the interaction of NKG2D and MICA*

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Received Aug. 23, 2016; Revision accepted Dec. 4, 2016; Crosschecked May 10, 2017

Abstract: Hepatocarcinoma is one of the malignant cancers with significant morbidity and mortality. Immunotherapy has emerged in clinical treatment, owing to the limitation and severe side effects of chemotherapy. In the immune system, natural killer (NK) cells are important effectors required to eliminate malignant tumor cells without the limitation of major histocompatibility complex (MHC) molecule issues. Hence, treatment which could stimulate NK cells is of great interest. Here, we investigated the efficacy of the combined therapy of TT-1 (a mutant of melittin) and interferon-α (IFN-α) on NK cells and human liver cancer HepG-2/Huh7 cells in vitro and in vivo, as well as the mechanism involved. The combination therapy significantly inhibited the growth of HepG-2/Huh7 cells in vivo, but this effect was impaired after depleting NK cells. TT-1 not only up-regulated MHC class I-related chain molecules A (MICA) expression, but also prevented the secretion of soluble MICA (sMICA). Both the mRNA and protein of a disintegrin and metallopeptidase 10 (ADAM 10) in HepG-2/Huh7 cells were decreased after TT-1 treatment. The combined therapy of TT-1 and IFN-α could suppress the growth of HepG-2/Huh7 xenografted tumor effectively via promoting the interaction of NK group 2, member D (NKG2D) and MICA, indicating that TT-1+IFN-α would be a potential approach in treating liver cancer.

Key words: TT-1; Interferon-α (IFN-α); Natural killer (NK) cells; Hepatocarcinoma; Immunotherapy
http://dx.doi.org/10.1631/jzus.B1600369 **CLC number:** R96

1 Introduction

Hepatocellular carcinoma (HCC), one of the fatal malignant tumors, is a leading cause of death among cirrhotic patients (Taïeb *et al*., 2003). Surgical resection, liver transplantation, ablation, and chemotherapy are common therapeutic methods for HCC (Liu *et al*., 2016). In most cases, the disease is found in the intermediate or advanced stage rather than at a more treatable stage. Unfortunately, fewer than 20% of patients with late-stage HCC can be treated by surgical resection (Hung, 2005; Yang *et al*., 2015). We found that the objective response rate (ORR) to a single cytotoxic regimen was merely 0%–10%, with no survival benefit after evaluating different cytotoxic agents for HCC (Guan *et al*., 2003; Boige *et al*., 2006; Hebbar *et al*., 2006; Liu *et al*., 2015). Immunotherapy, which is a novel therapeutic regimen for malignancy, aims at overcoming the limitations of conventional treatments.

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The natural killer (NK) cell, one of the essential effector cells in the immune response, has been adopted in cellular immunotherapy for different cancers (Morisaki *et al*., 2011). NK cells express a variety of activating receptors, among which NK group 2, member D (NKG2D) has been shown to play a key role in tumor cell rejection and tumor immunosurveillance through binding to ligands such as major histocompatibility complex (MHC) class I-related chain molecules A (MICA) (Cerwenka *et al*., 2001; Xie *et al*., 2016). However, the shedding of MICA by tumor cells increases the serum level of soluble MICA (sMICA) and hinders the recognition of HCC by immune cells, resulting in tumor immune escape (Groh *et al*., 2002; Wang *et al*., 2016). In vitro studies have shown that sMICA could clearly reduce the expression of NKG2D on NK cells (Wu *et al*., 2004; 2009). Thus, sMICA is believed to cause the functional impairment of NK cells in $MICA^+$ patients.

Cytokine therapy has been established as one of the main pillars of human cancer immunotherapy (Floros and Tarhini, 2015). Interferon-α (IFN-α), one of the cytokines, mediates immune responses towards Th1 cell, and enhances cytotoxicity and survival of NK cells, resulting in remarkable immunomodulatory effects. IFN-α is approved as a first line treatment for metastatic renal cell carcinoma (RCC), hairy cell leukemia, follicular lymphoma (in combination with Avastin), and as adjuvant treatment for high-risk melanoma (Thompson and Allison, 1997; Parlato *et al*., 2001). Nevertheless, there are some problems with IFN-α therapy, such as dose-limiting adverse effects and poor tolerability (Ueda *et al*., 2016). Thus, combination drug therapy of IFN-α and other antitumor drugs has emerged to foil resistance development.

Melittin is a 26-amino acid residue antimicrobial peptide with known antitumor activity. TT-1 (amino acid sequence: KIKAVLKVLTT), a mutant of melittin, was generated by a reduction of the peptide chain length and replacing glycines with lysines (Son *et al*., 2007; Oršolić, 2012; Sommer *et al*., 2012). The TT-1 retains the amino-terminal active site region of melittin, and has an increased hydrophobicity but a decreased net charge, which indicates a higher stability and lower toxicity than melittin. In our previous study, TT-1 exhibited a significant inhibitory effect on thyroid cancer cells in vitro, and showed significant anti-tumor activity on thyroid cancer cells in vivo, which suggested a potential inhibitory activity towards HCC cells. Here, we investigated the inhibiting effect of TT-1 on HepG-2/Huh7 cells and the regulatory effects to the secretion of sMICA and the expression of MICA on HepG-2/Huh7 cells. We also evaluated the anticancer efficacy of TT-1+IFN-α in a HepG-2/Huh7 xenografted tumor model. Further, the synergistic immunostimulatory activity of TT-1+IFN-α was verified through cytotoxicity assay.

2 Materials and methods

2.1 Cell culture and reagents

HepG-2/Huh7 and NK cells were obtained from American Type Culture Collection (ATCC, Manassas, USA). HepG-2/Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA). The NK cell line NK92 was cultured in supplemented alpha minimum essential medium (α-MEM), 12.5% (v/v) FBS (Hyclone, Mordlalloc, Australia), 12.5% (v/v) horse serum (Hyclone, Logan, USA), 0.2 mmol/L myo-inositol, 0.02 mmol/L folic acid, 0.1 mmol/L 2-mercaptoethanol (Sigma-Aldrich, St. Louis/MO, USA), and 200 U/ml hIL-2 (Millipore, Temecula, CA, USA). Nude mice (Academy of Military Medical Science, Beijing, China) were housed in a rodent facility at 22 °C and provided with continuous rodent food and water. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. Monoclonal antibodies against a disintegrin and metallopeptidase 10 (ADAM 10) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The anti-MICA-PE antibody was obtained from Miltenyi Biotec (Teterow, Germany) and the anti-asialo GM-1 was purchased from Abcam (Cambridge, UK). The MICA enzyme-linked immunosorbent assay (ELISA) kit was purchased from KeyGen (Nanjing, China).

2.2 Cell proliferation assay

The effects of TT-1 on HepG-2/Huh7 cells were detected by MTT assay. A total of 5×10^3 HepG-2/Huh7 cells were inoculated to 96-well plates per well, and treated with various concentrations (ranging from 0 to 32 μg/ml) of TT-1 for 48 h. Subsequently, cells

were incubated with the MTT solution (5 μg/ml) for 4 h at 37 °C followed by the addition of 150 μl dimethyl sulfoxide (DMSO) per well, and then shaken for 5 min before the absorbance was measured at 490 nm.

2.3 HepG-2/Huh7 xenograft mouse model and administration

HepG-2/Huh7 cells (1×10^7) were collected and then inoculated intradermally. Twelve days later nude mice were randomly (n=10) divided into four groups, which were the control group (normal saline), the TT-1 (1 mg/kg) group, the IFN-α (1 mg/kg) group, and the TT-1 (1 mg/kg)+IFN-α (1 mg/kg) group. Intra-tumor injection three times a week was adopted. At the indicated time points (the end of the fourth week after the treatment), the mice were sacrificed 24 h after the final administration. Tumor volume (V) was measured every 3 d during the treatment and calculated using the formula: V=(length×width^2)/2. Intravenous injection of anti-asialo GM-1 has been shown to eliminate NK cell activity (Habu *et al.*, 1981). To demonstrate that the antitumor efficacy of TT-1+IFN-α was mediated by NK cells via MICA-NKG2D pathway specifically, depletion of NK cells together with TT-1+IFN-α treatment was executed.

2.4 Flow cytometry

The expression of MICA on HepG-2/Huh7 cells after the incubation of TT-1 was detected by flow cytometry. Basically, HepG-2/Huh7 cells were incubated with various concentrations of TT-1 (0.5, 1, and 2 μg/ml), followed by species-specific (1:100) anti-MICA-PE antibody. Finally, the cells were detected by FACSCalibur.

2.5 ELISA

The secretion of MICA from HepG-2/Huh7 cells after the incubation of TT-1 was detected using the commercial ELISA kit. Briefly, HepG-2/Huh7 cells in 96-well plates were treated with various concentrations of TT-1 (from 0 to 16 μg/ml) for 48 h, then the cell culture supernatant was collected and the sMICA level was detected by the ELISA kit.

2.6 RT-PCR

The shedding of MICA by tumor cells was inhibited by silencing ADAM 10 protease (Waldhauer *et al.*, 2008). The effect of TT-1 on the ADAM 10 RNA expression was examined by real-time polymerase chain reaction (RT-PCR). HepG-2/Huh7 cells were treated with TT-1 (ranging from 0 to 2 μg/ml) for 48 h, then the total RNA was extracted using TRIzol reagent and purified with the RNeasy Mini Kit (QIAGEN, CA, USA). Subsequently, RT-PCR was conducted with an ABI PRISM 7300 sequence detection system. The comparative C_T ($2^{-\Delta\Delta C_T}$) method was used to calculate the relative mRNA expression.

2.7 Immunoblotting assay

The effect of TT-1 on ADAM 10 expression was detected by Western blot. HepG-2/Huh7 cells were treated with TT-1 (ranging from 0 to 2 μg/ml) for 48 h, and then homogenized in a lysis buffer (0.5% (v/v) Trition X-100, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 50 mmol/L Hepes, pH 7.5). Proteins were resolved by electrophoresis then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with the anti-ADAM 10 antibody after being blocked, then the membranes were incubated by the secondary antibody. Finally, the membranes were reacted with the electrochemiluminescence (ECL) reagent and exposed to the Bio-Rad detection system. The β-actin was detected to ensure that the proteins were equally loaded.

2.8 Lactate dehydrogenase release cytotoxicity assay

The lactate dehydrogenase (LDH) released from the HepG-2/Huh7 cells was detected by the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, USA). A total of 5×10^3 HepG-2/Huh7 cells (target cells) were co-cultured with various amounts of NK92 cells (effector cells) in the presence or absence of TT-1 (2 μg/ml) and IFN-α (2 μg/ml) for 4 h at 37 °C. Controls for spontaneous LDH release in effector and target cells, as well as target maximum release, were prepared. The calculation of cytotoxicity percentage was as follows: cytotoxicity (%)= (experimental release−effector spontaneous−target spontaneous)/(target maximum−target spontaneous)× 100%.

2.9 Immunohistochemical analysis

We cut the paraffin sections into 5-μm sections and fixed them with 4% paraformaldehyde. For immunohistochemical (IHC) staining, the sections were

incubated with MICA/NKG2D antibody (Abcam, Cambridge, UK), followed by the horseradish peroxidase-labeled secondary antibody, and then analyzed with the Vectastain ABC kit (Dako, Copenhagen, Denmark).

2.10 Statistical analysis

All experiments were performed in triplicate. The data were presented as the mean±standard deviation (SD). Statistical analysis was executed by one-way analysis of variance (ANOVA), Dunnett's multiple comparison, or Student's *t*-test, where $P<0.05$ was considered as a statistically significant result.

3 Results

3.1 Inhibitory effect of TT-1 on proliferation of HepG-2/ Huh7 cells

As shown in Fig. 1, the inhibitory effect of TT-1 on HepG-2/Huh7 cells was in a dose-dependent manner with the 50% inhibitory concentrations (IC_{50}) of (3.762±0.285) μg/ml/(5.823±0.138) μg/ml at 72 h. Especially, at 32 μg/ml TT-1, the cell viability of HepG-2/Huh7 cells decreased to 14.8%/26.9%, which showed that TT-1 exhibited potent cytotoxicity to HepG-2/Huh7 cells.

3.2 Inhibitory effect of TT-1+IFN-α on tumor growth in HepG-2/Huh7-bearing mice

The HepG-2/Huh7 xenograft model was established successfully. After the treatment, the tumor volumes and weights were measured. As shown in Fig. 2, TT-1 could inhibit the growth of the tumor effectively. IFN-α could suppress the tumor growth slightly, but the tumor volumes and weights were not significantly different from those of other groups. It is noteworthy that the combination of TT-1 and IFN-α could enhance the antitumor activity significantly compared to TT-1. Moreover, the data of tumor volumes and weights indicated that the tumor growth inhibitory activity of TT-1+IFN-α was suppressed remarkably after depleting NK cells using anti-asialo GM-1 antibody (Fig. 3). The control group, in which anti-asialo GM-1 was replaced by an isotype antibody, exhibited a similar tumor inhibitory rate to the TT-1+IFN-α group, which suggested that NK cells played a key role in the immune response. The results above demonstrated that the antitumor efficacy of TT-1+IFN-α was mediated by NK cells specifically via MICA-NKG2D pathway.

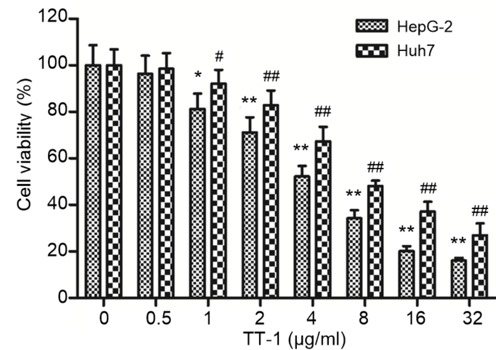

Fig. 1 Inhibitory effect of TT-1 on proliferation of HepG-2/Huh7 cells

HepG-2/Huh7 cells were treated with a series of concentrations of TT-1 for 48 h. Cell viability was measured by MTT assay. Values are presented as mean±SD for three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P<0.05$, $^{\#\#}P<0.01$, versus TT-1-untreated group

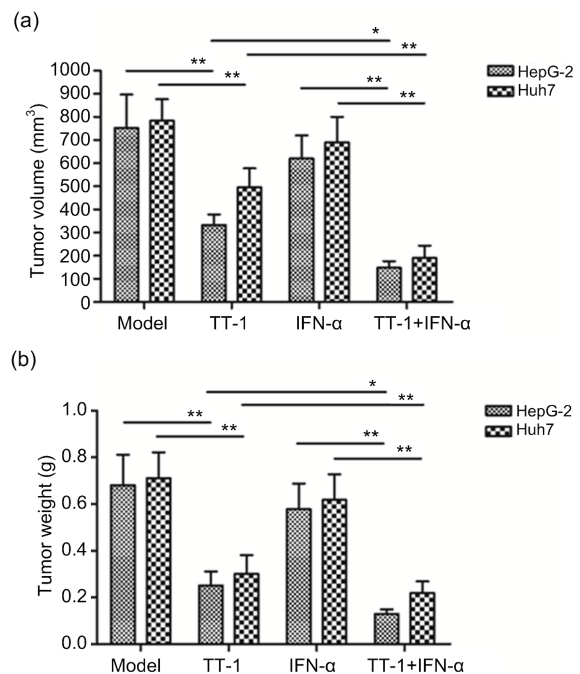

Fig. 2 In vivo TT-1+IFN-α efficacy against a HepG-2/Huh7 xenograft

(a) Tumor volumes in HepG-2/Huh7-bearing nude mice. (b) Tumor weight of each group. Each BALB/c nude mouse was subcutaneously injected 1×10^7 HepG-2/Huh7 cells for different treatments. Treatment began following tumor development, when the measurement of tumor volume also started. Data are presented as mean±SD (n=10). $^{*}P<0.05$, $^{**}P<0.01$

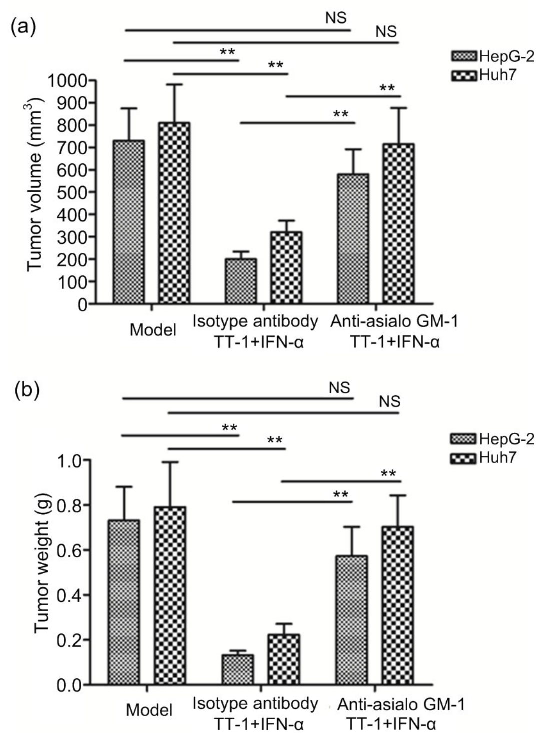

Fig. 3 Antitumor activity of TT-1+IFN-α impaired significantly by anti-asialo GM-1

Intravenous injection of anti-asialo GM-1 was carried out to eliminate NK cell activity before the treatment with TT-1+ IFN-α, and an isotype antibody was used as control. (a) Tumor volume of each group. (b) Tumor weight of each group. Data are presented as mean±SD (n=10). ** P<0.01. N.S.: no significance

3.3 Effect of TT-1 on the expression of MICA on HepG-2/Huh7 cells

In vivo activity study showed that TT-1 exhibited remarkable antitumor activity when combined with IFN-α which could stimulate the NK cells, implying that TT-1 was likely to up-regulate the MICA expression on HepG-2/Huh7 cells to assist the function of IFN-α. Hence, the flow cytometry was conducted to evaluate the MICA expression. As shown in Fig. 4, the MICA positive rate of HepG-2/Huh7 cells increased with increasing TT-1 concentration. The mean fluorescence intensity (MFI) could reach 40% approximately when the TT-1 concentration was 2 μg/ml. In conclusion, TT-1 treatment could increase the expression of MICA on HepG-2/Huh7 cells as anticipated.

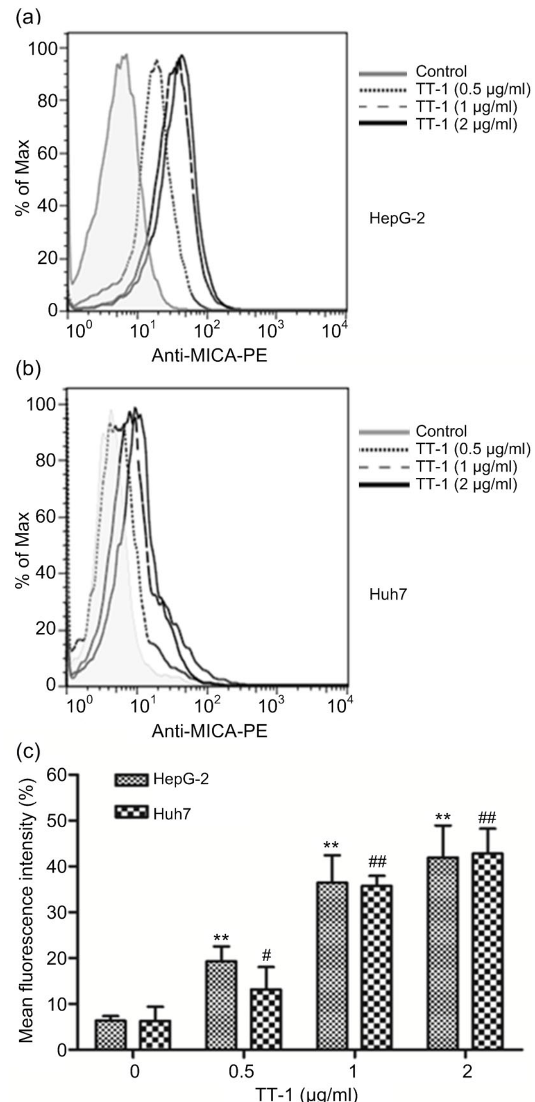

Fig. 4 Expression of MICA on HepG-2/Huh7 cells

HepG-2/Huh7 cells were treated with TT-1 (ranging from 0 to 2 μg/ml) for 48 h, and MICA expression was quantified by flow cytometry. (a) Representative histogram of HepG-2 cells. (b) Representative histogram of Huh7 cells. (c) Representative normalized mean fluorescence (mean fluorescence intensity (MFI) of TT-1-treated cells/MFI of control cells). Values are presented as mean±SD for three independent experiments. ** P<0.01, # P<0.05, ## P<0.01, versus TT-1-untreated group

3.4 Effect of TT-1 on the secretion of MICA by HepG-2/Huh7 cells

ELISA assay was executed to demonstrate whether TT-1 could decrease the expression of sMICA at the same time as increasing the expression

of MICA on HepG-2/Huh7 cells. The results of ELISA (Fig. 5a) showed that TT-1 inhibited the expression of sMICA in a dose-dependent manner. In order to further confirm this effect of TT-1 on HepG-2/Huh7 cells, the expression of the related ADAM 10 protease was detected on the levels of RNA and protein after TT-1 treatment. As shown in Fig. 5b, the mRNA level of ADAM 10 in HepG-2/Huh7 cells was dose-dependently down-regulated by TT-1. Analysis by *t*-test showed that a significant difference of ADAM 10 mRNA appeared when the concentration of TT-1 reached 1 μg/ml. Similarly, the Western blot results showed that TT-1 decreased the expression of ADAM 10. Specifically, at 2 μg/ml TT-1, the ratio (ADAM 10:β-actin) of HepG-2/Huh7 was 0.35/0.46 in the group without TT-1. The data above demonstrated that TT-1 could reduce the secretion of MICA by HepG-2/Huh7 cells.

3.5 Effect of TT-1+IFN-α on the cytotoxicity of NK92 cells

The immunological enhancement mediated by the MICA-NKG2D pathway was evaluated through cytotoxicity assay in vitro. As shown in Fig. 6, the NK cytotoxicity to HepG-2/Huh7 cells increased with increasing effector-target (E:T) ratios. The addition of IFN-α (2 μg/ml) could strengthen the cytotoxicity of NK cells, but there were no obvious changes of the cytotoxicity found when treated with TT-1 (2 μg/ml). It is noteworthy that the cytotoxicity of NK cells increased significantly when treated with the combination of TT-1 (2 μg/ml) and IFN-α (2 μg/ml). Fig. 6a showed that the NK cytotoxicity on HepG-2 cells approached 45% at the 10:1 E:T ratio and 35% at the 5:1 E:T ratio, which were levels considerably higher than those of the control group (where there was neither TT-1 nor IFN-α); Fig. 6b exhibited a similar result in Huh7 cells. The results of cytotoxicity assay were consistent with those of the tumor inhibition experiments in vivo.

3.6 Effect of TT-1+IFN-α on the expression of MICA and NKG2D in tumor tissues

IHC analysis was undertaken to examine additional evidence for the conclusions that the antitumor efficacy of TT-1+IFN-α was mediated by NK cells via MICA-NKG2D pathway and that TT-1 could up-regulate the MICA expression on tumor cells to

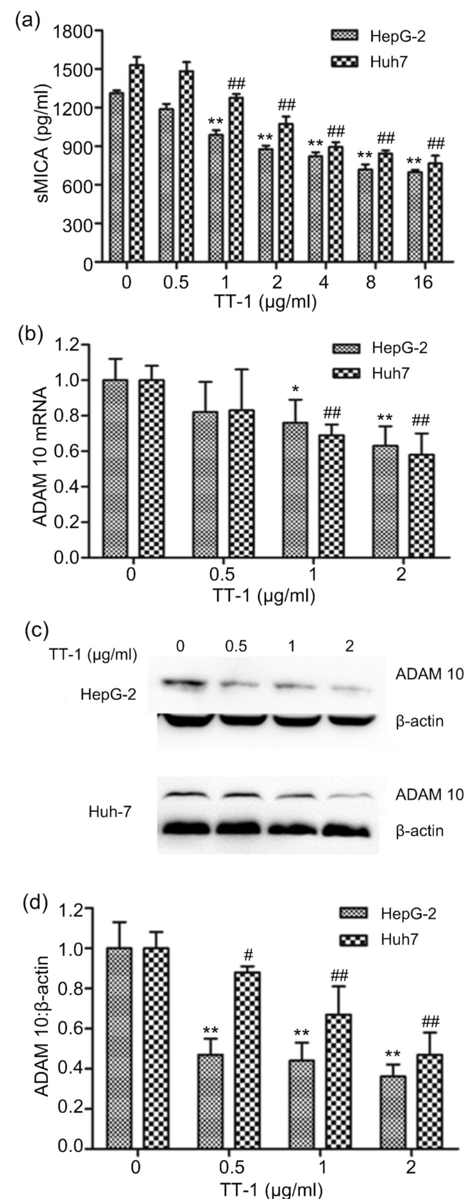

Fig. 5 Secretion of MICA by HepG-2/Huh7 cells
(a) ELISA detected the sMICA level in the culture supernatant. HepG-2/Huh7 cells were treated with various concentrations of TT-1 (from 0 to 16 μg/ml) for 48 h, then the sMICA level of the culture supernatant was detected using the ELISA kit. (b) Quantitative PCR analysis of ADAM 10 genes in HepG-2/Huh7 cells. HepG-2/Huh7 cells were treated with various concentrations of TT-1 (from 0 to 2 μg/ml) for 48 h, then total RNA was extracted from HepG-2/Huh7 cells, and real-time PCR was conducted. Data are expressed as the fold increase of mRNA expression in TT-1 treated cells relative to untreated cells. (c) Western blot analysis confirmed the down-regulation of ADAM 10 at the protein level. (d) Gray scanning and data statistics of (c). Values are presented as mean±SD for three independent experiments. * $P<0.05$, ** $P<0.01$, # $P<0.05$, ## $P<0.01$, versus TT-1-untreated group

assist the function of IFN-α. Fig. 7a demonstrates that there was a significant augmentation in the numbers and intensity of MICA expression in TT-1+IFN-α-treated tumors compared to untreated groups. IHC analysis revealed that the expression level of NKG2D on infiltrating NK cells was considerably higher in tumor tissues of mice treated with TT-1+IFN-α than in tumors from the untreated mice (Fig. 7b).

4 Discussion

The ORR of HCC to chemotherapy has been very frustrating, putting the oncologists in a predicament in the long-term (Dusheiko *et al*., 1992; Lee *et al*., 2003). Moreover, conventional radiotherapy and chemotherapy often have severe side effects (Leung *et al*., 2007). Thus, it is essential to find new therapeutic strategies with low cytotoxicity, but with specificity and availability for chronic treatment.

Antimicrobial peptides with low intrinsic cytotoxicity which could reduce drug resistance have recently attracted significant attention as new antitumor agents. It has been demonstrated previously that melittin (a cationic, hemolytic peptide isolated from honeybee venom) has anti-arthritic, antibacterial, and anti-inflammatory activity (Brown and Kirkwood, 2003). In addition, it has been confirmed that melittin can selectively destroy several tumor cells, such as breast, renal, and lung cells in vitro. To make it more effective in treating cancer cells, we designed a novel peptide, TT-1, based on the amphipathic structure of melittin. In this study, the cytotoxic effect of TT-1 in vitro revealed that TT-1 could dose-dependently suppress the proliferation of HepG-2/Huh7 cells. The antitumor study in vivo also implied the potential inhibition activity of TT-1 on a HepG-2/Huh7 xenografted tumor.

Further, we found that MICA expression on the surface of HepG2/Huh7 cells was increased after

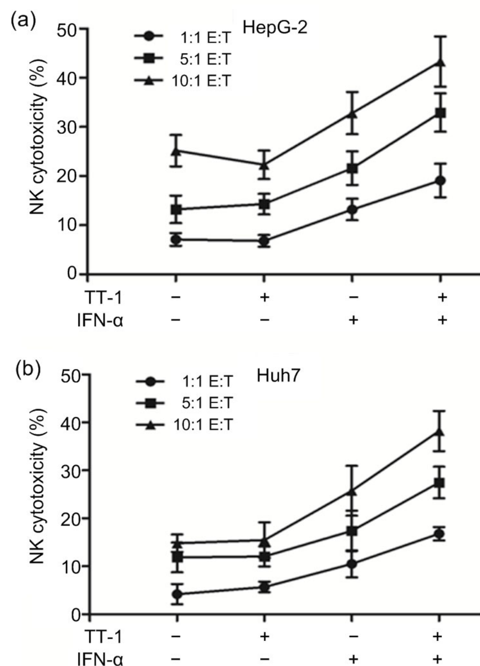

Fig. 6 Enhancement of TT-1+IFN-α on the cytotoxicity of NK92 cells

HepG-2 (a)/Huh7 (b) cells were co-cultured with various amounts of NK92 cells in the presence or absence TT-1/IFN-α, and cytotoxicity was determined by measuring the released LDH. The 1:1, 5:1, or 10:1 E:T ratio was set respectively. Values are presented as mean±SD for three independent experiments

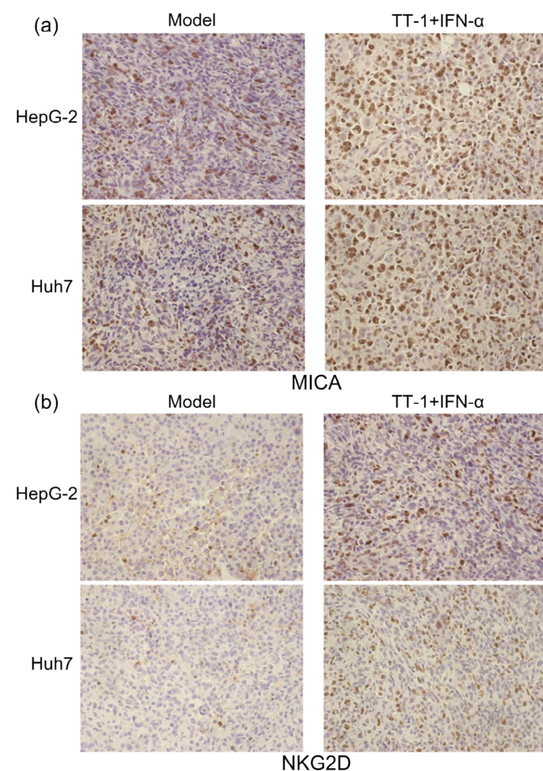

Fig. 7 IHC analysis of the expression of MICA and NKG2D in tumor tissues

(a) IHC staining of MICA on paraffin sections of a xenografted tumor. $MICA^+$ cells were identified with an anti-MICA antibody (brown staining). (b) Infiltrated $NKG2D^+$ cells were detected by IHC staining (brown staining) on serial sections, demonstrating more expression of NKG2D with TT-1+IFN-α treatment (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

exposure of HepG2/Huh7 cells to TT-1. Also TT-1 could reduce the shedding of MICA from HepG2/Huh7 cells. Previous study had demonstrated that the release of sMICA from tumor cells is impaired by the metalloproteinase inhibitors, such as members of matrix metalloproteinase (MMP) families and ADAM proteins (Salih *et al*., 2002; Waldhauer and Steinle, 2006). Whereas MMPs are mainly involved in the destruction of the extracellular matrix, many ADAMs are membrane-tethered proteases and one of their major functions is the proteolytic release of the extracellular domain of transmembranous proteins from the cell surface (Reiss *et al*., 2006). Thus, the reduction of ADAM 10 protein expression and ADAM 10 mRNA after TT-1 treatment confirmed the effect of TT-1 on sMICA. The immunomodulatory effects of TT-1 on cancer cells were found for the first time, suggesting that immune cell-activated cytokines could therefore be used combined with TT-1. The cytokine IFN-α with significant immunomodulatory effects selected in this study, combined with TT-1, could really enhance the cytotoxicity and survival of NK cells, and this was confirmed by the NK cytotoxicity assay in vitro, the tumor inhibition experiments in vivo, and the IHC analysis.

Treatment with TT-1 enhanced the susceptibility of HepG-2/Huh7 cells to NK cells treated by IFN-α, which indicated that the interaction between NKG2D and MICA played a key role in NK-mediated lysis of HepG-2/Huh7 cells, and the susceptibility of tumor cells (treated by TT-1) to NK cells might be upregulated by the increase of MICA. Given the extensive distribution of NKG2D on immune cells and the anticancer significance of interactions between NKG2D and MICA, it is reasonable to speculate that IFN-α may thus enhance NK cell immunotherapies (Zwirner *et al*., 2007). It is noteworthy that IFN-α is a potent inhibitor of myeloid-derived suppressor cells (MDSCs) which could interfere with the host immune response to tumors. Hence, whether the potent antitumor activity of TT-1+IFN-α partially benefited from the host immune response enhanced by the interaction of IFN-α and MDSCs needs further research (Mundy-Bosse *et al*., 2011).

In conclusion, we have shown that TT-1 could inhibit the proliferation of HepG-2/Huh7 cells and induce MICA expression in HepG-2/Huh7 cells, resulting in the enhancement of the cytotoxic effects of NK cells and a synergistic antitumor effect when combined with IFN-α. Therefore, the combination of TT-1 with IFN-α immunotherapy may have clinical significance for the therapy of HCC.

Compliance with ethics guidelines

Lan-lan WAN, Da-qi ZHANG, Jin-nan ZHANG, and Li-qun REN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：联合使用 IFN-α 和 TT-1 通过增强 NKG2D 和 MICA 的相互作用达到抗肝癌效果的研究

目　的： 评估干扰素 α（IFN-α）和 TT-1（一种蜂毒肽的类似物）联合用药的抗肿瘤效果，并初步研究联合用药的抗肿瘤及免疫调节机制。

创新点： 为了增强蜂毒肽的抗肿瘤效果，本课题组在其基础上进行改造，合成了一种新的化合物 TT-1。该研究第一次将蜂毒肽类似物和免疫细胞因子 IFN-α 联合使用，并通过实验证实联合用药可以通过激活免疫调节来增强 TT-1 的抗肿瘤效果。

方　法： 首先通过 MTT 实验验证 TT-1 对 HepG-2/Huh7 细胞的增殖抑制作用。接着建立 HepG-2/Huh7 小鼠移植瘤模型，考察 TT-1+IFN-α 的体内抗肿瘤效果；使用 anti-asialo GM-1 抗体消除自然杀伤（NK）细胞，验证 NK 细胞在联合用药中的关键作用。使用流式细胞术和酶联免疫吸附法（ELISA）验证 TT-1 对 HepG-2/Huh7 细胞 MHC I 链相关分子 A（MICA）表达的影响，并用实时聚合酶联反应（RT-PCR）和蛋白质印迹（Western blot）对其机制进行探究；通过细胞毒性实验考察 TT-1+IFN-α 是否可以增强 NK 细胞对 HepG-2/Huh7 细胞的特异性杀伤作用。最后使用免疫组化的方法考察 TT-1+IFN-α 联合用药对肿瘤组织中 MICA 和 NKG2D 的表达量的影响。

结　论： MTT 实验表明 TT-1 可以在体外有效地抑制 HepG-2/Huh7 细胞的增殖。小鼠移植瘤模型实验结果显示 TT-1+IFN-α 联合用药比 TT-1 单独给药更能有效地抑制 HepG-2/Huh7 移植瘤的生长，但是在消除 NK 细胞之后该效应明显减弱，说明 TT-1+IFN-α 的抗肿瘤效应是通过 NK 细胞特异性介导的。TT-1 不仅可以上调肿瘤细胞表面 MICA 的表达量，而且可以减少可溶性 MICA 的分泌；进一步研究表明，TT-1 通过抑制去整合素金属蛋白酶 10（ADAM 10）的表达来阻止 MICA 从肿瘤细胞表面脱落。细胞毒性实验表明，TT-1+IFN-α 可以显著增强 NK 细胞对 HepG-2/Huh7 细胞的杀伤作用。免疫组化实验结果显示，TT-1+IFN-α 联合用药可以明显增加肿瘤组织中肿瘤细胞表面 MICA 和 NK 细胞 NKG2D 的表达量。综上所述，TT-1+IFN-α 联合用药可以通过增强 MICA 和 NKG2D 的相互作用达到显著的抗肿瘤效果。

关键词： TT-1；干扰素 α；自然杀伤细胞；肝癌；免疫治疗