RESEARCH COMMUNICATION

HIF-1α siRNA and Cisplatin in Combination SuppressTumor Growth in a Nude Mice Model of Esophageal Squamous Cell Carcinoma

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Abstract

Introduction: The esophagus squamous cell carcinoma (ESCC) is one of the most deadly malignances, and a current challenge is the development of effective therapeutic agents. Our present work addressed the effect of HIF-1α siRNA alone or in combination with cisplatin on the growth of ESCC in nude mice. Materials and Methods: Xenografts were established by inoculating ESCC TE-1 cells in nude mice, and transplanted tumors were treated with HIF-1α siRNA, cisplatin alone or together. Growth was assessed by measuring tumor volume. HIF-1α mRNA and protein expression were detected using RT-PCR and immunohistochemistry, respectively. Apoptosis of ESCC TE-1 cells was analyzed by flow cytometry. Results: In our nude mice model, HIF-1α siRNA effectively inhibited the growth of transplanted ESCC, downregulating HIF-1α mRNA and protein expression, and inducing ESCC TE-1 cell apoptosis. Notably when combinated with cisplatin, HIF-1α siRNA showed synergistic interaction in suppressing tumor growth. Furthermore, the proportion of apoptotic cells in HIF-1α siRNA plus cisplatin group was significantly higher than that in cisplatin or HIF-1α siRNA-treated groups (P<0.05). Conclusions: Down-regulated HIF-1α expression induced by siRNA could effectively suppress the growth of transplanted ESCC *in vivo*. HIF-1α siRNA could enhance the cytotoxicity of cisplatin, which suggests that a combination of these two agents may have potential for therapy of advanced ESCC.

Keywords: RNA interference - HIF-1α - cisplatin - esophageal squamous cell carcinoma

Asian Pacific J Cancer Prev, **13**, 473-477

Introduction

Squamous cell carcinoma is the main histological subtype of esophageal cancers in China. Clinically, cisplatin-based chemotherapy is the main approach for many patients with advanced esophageal cancer. Most patients with advanced esophageal cancer experienced chemotherapy failure, however, mechanisms for chemotherapeutic resistance have not been completely understood (Bazarbashi et al., 2008; Cho et al., 2008; Takashima et al., 2008). The transcription factor hypoxia inducible factor-1 (HIF-1), one heterodimer composed of HIF-1α and HIF-1β, is a critical mediator of the response to hypoxia. Recent studies revealed that hypoxia was associated with poor responses to chemotherapy in esophageal cancers (Koukourakis et al., 2001). More recently, evidences showed that increased expression of HIF-1α was closely correlated with the growth, invasion and resistance to chemotherapeutic agents of human cancer cells including ESCC. In contrast, the loss of HIF-1a activity or suppression of HIF-1α overexpression can effectively inhibit tumor growth, suggesting that HIF-1a is a potent therapeutical target for ESCC (Matsuyama et al., 2005; Tzao et al., 2008).

RNA interference (RNAi), which is a sequence-specific gene-silencing method induced by double-stranded RNA (dsRNA), can precisely regulate or silence the expression of target genes. Numerous studies in animal models of human disease demonstrate the potential application of RNAi therapeutics (Sithanandam et al., 2005; Yonesaka et al., 2006; Angaji et al., 2010). Previous studies have shown that siRNA targeting HIF-1α can induce apoptosis of some humam tumor cells and suppress the tumor growth (Chen and Yu, 2009). More recently, a study reported that HIF-1α siRNA could inhibit the proliferation of ESCC TE-1 cells in vitro (Wu et al., 2007). However, little is known about the effect of HIF-1a siRNA alone or combination with cytotoxic agents on ESCC in vivo. In this study, we demonstrated that down-regulation of HIF-1α expression using HIF-1α siRNA alone or in combination with

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cisplatin induced ESCC TE-1 cell apoptosis and inhibited the growth of transplanted ESCC in nude mice. Our results suggest that HIF-1α-targeting therapy may be a promising strategy to increase the efficacy of chemotherapy drugs for the treatment of esophagus cancers.

Materials and Methods

Cell culture and establishment of animal model

The BALB/c nude mice (28±3d, male and female) were purchased from the Experimental Animal Center of Sun Yat-sen University, and maintained under specific pathogen-free conditions. Human ESCC cell line TE-1 was provided by Shanghai Cell Bank of Chinese Academy of Science, and cultured in RPMI 1640 (Gibco, USA) containing 15% calf serum, 100 U/ml penicillin and 100 mg/L streptomycin at 37°C in a humidified atmosphere containing 5% CO2. About 1×10^7 TE-1 cells were subcutaneously injected into the right upper limb of nude mice. When the tumor size was approximately 0.8 cm in diameter, the inhibition experiments were carried out. All animal experiments were approved by the animal care committee of SUN Yat-sen University and were performed using the criteria for humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Treatment

siRNA targeting HIF-1α was designed and validated by Boya Bio-engineering Company (Boya, China).The antisense sequences of siRNA targeting HIF-1α was 5'-AGTTCACCTGAGCCTAATA -3'. Twenty nude mice were randomly assigned into four groups, and treated with saline, cisplatin (20μg/ml), HIF-1α siRNA (20μmol/L) and HIF-1α siRNA (20μg/ml) plus cisplatin (20μg/ml), respectively. The saline or cisplatin was injected into the center of the tumors, and HIF-1α siRNA was transfected into nude mice with the lipofection according to the manufacturer's instructions of Lipofectamine 2000(Invitrigon, USA). The longest (a) and shortest (b) diameters were measured every week and the volume of tumor was calculated as the formula $V=a^2\times b/2$. Three weeks later, the nude mice were sacrificed, and tumor tissues were removed and weighted. Tumor growth inhibition rate (IR) was calculated according to the following formula: IR (%)=(1-mean weight experimental group/ mean weight control group)×100% (Kim et al., 2011).

Determination of HIF-1α mRNA with RT-PCR

The total RNA in tumor tissues was extracted according to the manufacturer's protocol and stored in -80°C for use. Subsequently, one-step RT-PCR was performed to detect HIF-1α mRNA according to manufacturer's instructions (Wang et al., 2011). The primers for HIF-1α and GAPDH (as internal normalization control) were as follows: HIF-1α: 5'- CAGCAACCAGGTGACCGTG -3' (forward) and 5'-TGCTGCCTTGTATGGGAGCATT -3' (reverse); GAPDH: 5'-CGGAGTCAACGGATTTGGTGGTAT-3' (forward) and 5'- AGCCTTCTCCATGGTGGTGAAGAC -3' (reverse). The reaction mixture contained 3 μl of template RNA, 2 μl of each primer, 17.8μl of DEPC treated water, 25 μl of 2×buffer and 0.2μl of RT-Taq polymerase (5U/μl). Reverse transcription from RNA to cDNA was performed at 50°C for 50min. The DNA was denatured at 94°C for 2 min, following by 30 cycles of amplification.Each cycle consisted of denaturation at 94°C for 45s, annealing at 55°C for 1min, and extension at 72°C for 1min, with an additional extension at 72°C for 7min. The products were stored at 4°C. A total of 5μl of PCR products was added into 1μl 6×loading buffer which was loaded onto 1.5% agarose gel for electrophoresis. The RNA bands were visualized under UV light and the optical density was analyzed which was normalized by the density of GAPDH.

Detection of HIF-1α protein with immunohistochemistry

The immunohistochemistry was performed according to the protocol of S-P kit (Invitrigon, USA). The primary antibody was mouse anti-human polyclonal antibody (Santa, USA), and the secondary antibody was included in the kit. The slides were observed under microscope. Light brown in the cytoplasm suggested weakly positive, brown was regarded as strong positive, and primary color indicated negative (Matsuyama et al., 2005; Tzao et al., 2008). The optical density was analyzed with automatic image analysis system (Kontron IBAS 2.5, Germany) and positive unit (PU) was obtained.

Analysis of apoptosis with flow cytometry

Cell apoptosis assays were done as previously described (Chen et al., 2009; Wang et al., 2011). In brief, the tumor tissues were treated with 0.25% trypsin and 0.02% EDTA. Cell suspension was collected and centrifuged at 1,000 rpm for 5 min. Supernatant was removed and cells were rinsed with PBS thrice. Then, these cells were suspended in 100 μl binding buffer at a final of 1×10^6/ml, and incubated with 10 μl Annexin V-FITC (Invitrigon, USA) for 30 min. Sequently, 5μl PI was added and incubated away from light at room temperature for five minutes. After that, the cells were directly analyzed by FACScan and evaluated by the CellQuest program (Becton Dickinson, USA).

Statistical analysis

The data were presented as mean±SD, and analyzed using SPSS 15.0 software package. One-way analysis of variance (ANOVA) followed by a post hoc LSD-t or Dunnett's test was performed to determine the significance of the differences in multiple comparisons. A value of $P<0.05$ was considered statistically significant.

Results

Combination of HIF-1α siRNA with cisplatin significantly suppressed the growth of transplanted ESCC

To test the ability of HIF-1α siRNA alone or in combination with cisplatin to suppress the growth of ESCC, 1×10^7 TE-1 cells were subcutaneously injected into the right upper limb of nude mice. Two weeks later, the tumor size was approximately 0.6~0.8 cm in diameter. No significant difference in tumor volume was found

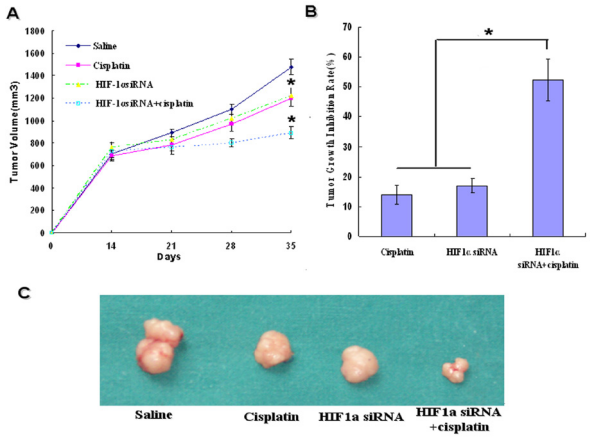

Figure 1. Antitumor Effect of HIF-1α siRNA, Cisplatin Alone or Together Against ESCC in Nude Mice. TE-1 cells (1×10^7) were subcutaneously injected into the right upper limb of BALB/c nude mice.Two weeks later, nude mice were treated with indicated agents. Tumor volume were measured to assess the antitumor effect of indicted agents weekly. (A) HIF-1α siRNA ,or HIF-1α siRNA in combination with cisplatin strongly inhibited the growth of the tumor (B,C). Each bar represents the means±SD (n = 3), *P< 0.05

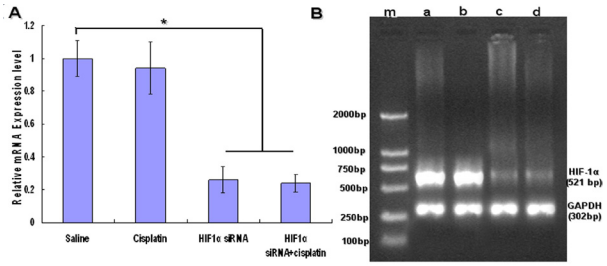

Figure 2. Effect of HIF-1α siRNA or in Combination with Cisplatin on HIF-1α mRNA Expression in ESCC Tissue. HIF-1α mRNA was detected by RT–PCR.Relative mRNA expression of HIF-1α was calculated to evalue the ability of indicated agents to suppress HIF-1α mRNA expression (A). When compared with control group, HIF-1α mRNA expression in HIF-1α siRNA alone or together with cisplatin group was significantly suppressed (*P<0.05). Each bar represents the mean±SD (n=3). PCR products was analyzed and identified with agarose gel electrophoresis(B). m: Marker; a: Saline; b:Cisplatin; c: HIF-1α siRNA;d: Cisplatin+HIF-1α siRNA.

between groups before treatment (p>0.05). After nude mice xenograft were treated with cisplatin, HIF-1α siRNA alone or together, the growth of tumor was inhibited to different extents. As showed in Figure1, a statistically significant reduction in the speed of tumor growth was observed in nude mice treated with HIF-1α siRNA alone or in combination with cisplatin compared with that in control group (Figure 1A,C). When compared with HIF-1α siRNA or cisplatin alone, the combination of HIF-1α siRNA and cisplatin strongly suppressed the growth of transplanted ESCC, the inhibition rate was upto 52.4% which was significantly higher than that of cisplatin- or HIF-1α siRNA-treated group (P <0.05) (Figure 1B).

HIF-1α siRNA downregulated HIF-1α mRNA expresssion

To assess the potency of the HIF-1α siRNA to block HIF-1α expression, mRNA expression level of HIF-1α in transplanted ESCC tissues was analysed by RT-PCR. As shown in Figure 2, when compared to saline or

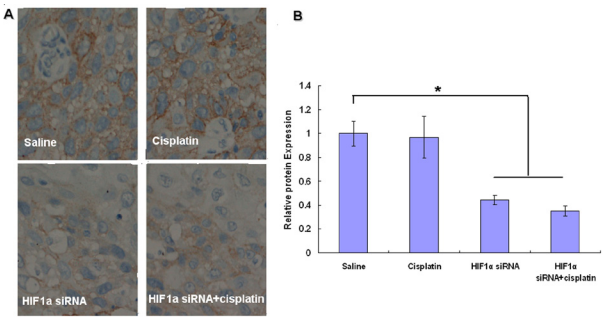

Figure 3. HIF-1α Protein Expression in Tumor Tissue from Transplanted ESCC. HIF-1α protein Expression was determined by immunohistochemistry.The HIF-1α was stained in brown color in the photographs taken at the magnifications of 400× (A) Immunohistochemical staining revealed that HIF-1α siRNA alone or together with cisplatin significantly inhibited HIF-1α expression. (B) Each bar represents the mean±SD (n = 3), *P< 0.05

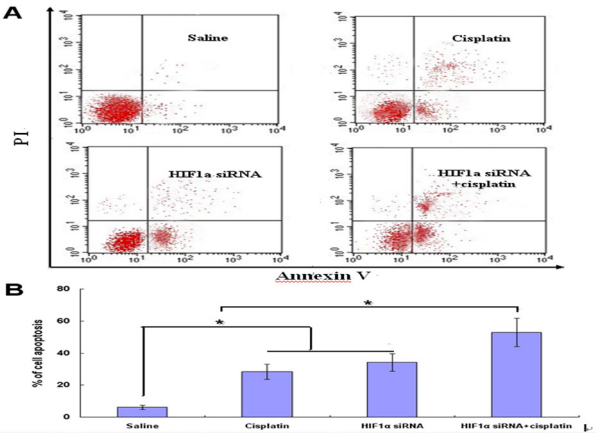

Figure 4. Effect of HIF-1α siRNA or in Combination with Cisplatin on Cell Apoptosis Quantitated Using Annexin-V/PI Detection. The tumor tissues were obtained from ESCC treated by HIF-1α siRNA ,cisplatin alone or together in nude mice. ESCC TE-1 cells were harvested and analyzed by flow cytometry. (A) The combination of HIF-1α siRNA with cisplatin significantly induced the apoptosis of TE1 cells. (B) Each bar represents the means ±SD (n = 3), *P< 0.05

cisplatin treated nude mice, HIF-1α siRNA alone or in combination with cisplatin significantly down-regulated the expression of HIF-1α mRNA (P<0.05). However, no difference in mRNA expression of HIF-1α was observed between HIF-1α siRNA and HIF-1α siRNA combinated with cisplatin group (P>0.05). These results indicated that HIF-1α siRNA could effectively inhibit HIF-1α mRNA expression.

HIF-1α siRNA suppressed HIF-1α protein expression

To investigate the effect of HIF-1α siRNA on the HIF-1α protein expression, the immunohistochemistry analysis was performed. The results were shown in Fig. 3. HIF-1a protein expression was not suppressed in cisplatin group in comparison to saline control (P>0.05). However, HIF-1a protein expression was significantly inhibited in ESCC tissues treated with HIF-1α siRNA or HIF-1α siRNA plus cisplatin when compared with that in saline or cisplatin treated group (P<0.05). But, the reduction of HIF-1a protein expression showed no difference between HIF-1α siRNA and HIF-1α siRNA plus cisplatin group

(P>0.05).

HIF-1α siRNA induced ESCC TE-1 cell apoptosis

Flow cytometric experiments were performed to determine if the growth inhibition of transplanted ESCC treated with HIF-1α siRNA alone or in combination with cisplatin was related with the apoptosis of ESCC TE-1 cell. Annexin-V and PI staining were used to detect apoptotic cells including early-stage apoptosis (FITC+/PI-) and late-stage apoptosis or necrotic cells (FITC+/PI+) (Wang et al., 2011). As shown in Figure 4, 28%~34% of apoptotic cells were observed in cisplatin or HIF-1α siRNA-treated group as compared to about 6% apoptotic cells in saline control (P<0.05). When HIF-1α siRNA combined with cisplatin, the percentage of apoptotic cells (upto 60%) was significantly higher than that in cisplatin or HIF-1α siRNA-treated group (P<0.05).

Discussion

In most human cancers including ESCC, over-expression of HIF-1a promotes tumor growth, angiogenesis and disease progression. Thus, targeting HIF-1a may serve as a viable anticancer strategy to be used in clinics (Kurokawa et al., 2003; Kimura et al., 2004; Matsuyama et al., 2005). Herein, using the strategy of silencing HIF-1a with targeted siRNA, we identified that HIF-1a siRNA could effectively inhibit the growth of transplanted ESCC in nude mice, down-regulate the HIF-1α mRNA and protein expression, and induce ESCC TE-1 cell apoptosis. Interestingly, when combinated with the commonly-prescribed cisplatin, HIF-1α siRNA showed synergistic interaction in inhibiting the tumor growth. These results suggested that combination of siRNA targeting HIF-1α and existing chemotherapeutic agents maybe a promising approach in the treatment of advanced or chemotherapy-resistant ESCC.

A deficiency in apoptosis is a well-known hallmark of many cancers. Many studies validated that HIF-1α is concerned with apoptosis, however HIF-1α possesses dual functions in mediating apoptosis of tumour cells, i.e, anti-apoptotic and pro-apoptotic function (Flamant et al., 2010; Khan et al., 2011). The overall effect of HIF-1α on cell apoptosis is still not fully understood, maybe depend on microenvironment in tumor, especially hypoxic condition. HIF-1α may induce cell apoptosis through enhancing the stabilization of p53 protein under severe hypoxic condition (Hammond and Giaccia, 2005). Pancreatic cancer cell lines with constitutively expression of HIF-1α showed a protective role in limiting hypoxia-induced apoptosis. As a fast-growing cancer, ESCC has a hypoxic microenvironment and metabolic disturbance. Hypoxia suppresses the apoptosis of cancer cells, resulting in the resistance to chemotherapeutic agents and even treatment failure (Matsuyama et al., 2005; Tzao et al., 2008). As shown in our results (Figure 4), HIF-1a siRNA alone or in combination with cisplatin strongly induced apoptosis of ESCC TE-1 cell, which resulted in the growth suppression of transplanted ESCC in nude mice.

Several small molecular inhibitors (e.g. temsirolimus, everolimus, bevacizumab, rencarex, cisplatin, etc) have been identified to decrease the level of HIF-1a mRNA and protein (Rapisarda et al., 2004; Duyndam et al., 2007; Seeber et al., 2010). Moreover, some of these HIF-1 inhibitors are subject of clinical trials at present. Specific siRNA has been showed to effectively reduce HIF-1α expression (Welsh et al., 2004). In the present study, our data revealed that HIF-1a siRNA we designed could effectively suppress the HIF-1a mRNA and protein expression in vivo (Figure 2 and 3). Suppression of tumor growth could be directly caused by the down-regulation of HIF-1a mRNA and protein in ESCC tissues. However, no significant difference in the expression of HIF-1a mRNA and protein was observed between HIF-1a siRNA and HIF-1a siRNA plus cisplatin group, which might be resulted from the effect of two agents combination masked by the cytotoxicity of cispatin.

In conclusion, our study demonstrated that inhibition of HIF-1a is a potential strategy for the treatment of ESCC. Since HIF-1α siRNA enhanced the response of cancer cells to cisplatin, a combinated therapy of HIF-1α siRNA and cisplatin or other chemotherapeutic agents maybe a promising approach for ESCC. However, the specific delivery, the stability and transfection efficiency of siRNA into target tissues remains a challenge (Sithanandam et al., 2005; Seeber et al., 2010). The anti-tumor activity HIF-1α siRNA or in combination with cisplastin in ESCC need to be verified in subsequent trials, subjecting to more in-depth studies.

Acknowledgements

This work was supported by the Natural Science Foundation for Doctoral Initiating Research of Guangdong Province (No. 06300768). We greatly appreciate Dr. Qianglin Duan from Tongji University, for manuscript revision.

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