Modulation of Tumor Microenvironment by Targeting HIF-1α, Enhances the Therapeutic Efficacy of Chemoimmunotherapy in Mice Model of Colon Cancer

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Abstract

Recently, combination therapies have become a promising approach with hopeful therapeutic outcomes due to their strong antitumor effects. Among them, despite the great success of cancer chemoimmunotherapy, it has not been able to improve the outcome of patients. Immunosuppressive tumor microenvironment (TME) has been known as the main barrier to therapy. It has been assumed that targeting HIF-1α as a reshaping of TME combined with chemoimmunotherapy can capably enhance the antitumor response of therapy.

Methods

We established CT26 mouse models to assess the synergistic effect of genetic silencing of HIF-1α combined with oxaliplatin (OXA) and imiquimod (IMQ) on tumor growth and TME.

Results

We showed that cotreatment of HIF-1α siRNA with OXA + IMQ exhibited a significant delay in tumor growth, which was correlated with high levels of cellular immune-related cytokines. Besides, mice without HIF-1α siRNA treatment exhibited high tumor growth and high levels of immunosuppressive factors, indicating an immunosuppressive phenotype. Briefly, we found that HIF-1α inhibition could synergize with OXA and IMQ to inhibit tumor growth in vivo.

Conclusions

Our data suggest that targeting HIF-1α represents a promising option to augment the antitumor response of chemoimmunotherapy.

1. Introduction

Due to the heterogeneity of cancer cells, combination therapies are preferred over monotherapy for many types of cancers [1]. Combination therapy enhances anticancer drug efficacy and reduces drug resistance, leading to inhibiting tumor growth and metastatic potential [2]. Among several mixtures of combination therapy, chemoimmunotherapy has shown promising results as a therapeutic option rather than other combination therapies. While immunotherapy stimulates and restores the patient's immune system, chemo agents upon providing tumor-specific antigens trigger further antitumor immune responses [3]. Despite the great success of cancer chemoimmunotherapy, it has not been able to achieve long-lasting therapeutic benefits for patients [4, 5]; so, supplementary strategies are still required for the majority of cancer patients.
Immunosuppression in the tumor microenvironment (TME) has been a key barrier to cancer immunotherapy's success [6, 7]. Increasing evidence has shown the central role of hypoxic TME in the development of immunosuppressive TME [8]. At the molecular level, the growth and survival of cancer cells in hypoxic TME are largely facilitated by the hypoxia-inducible factor (HIF) family of transcription factors [9, 10]. Several studies have shown that a high level of HIF-1α is associated with tumor metastasis, angiogenesis, poor prognosis, and tumor resistance therapy [11, 12]. Therefore, targeting HIF-1α combined with other therapy may enhance antitumor effects and improve patient outcomes [13, 14]. Li, Q. et al. reported that HIF-1α overexpression induces resistance to oxaliplatin (OXA) and it is a potential therapeutic target for reversing resistance to OXA in the management of CRC (colorectal cancer) [15]. Among several strategies for targeting HIF-1α, RNA interference (RNAi) technology has great potential in blocking critical genes involved in this pathway [16]. Chitosan (CH) as a natural polymer is a very attractive vector for nucleic acids delivery in biological applications due to its biocompatibility characteristics [17]. Several preclinical studies have demonstrated that the CH nanoparticles are a safe and effective strategy for siRNA delivery into tumor cells in vivo [18].

It has been reported that TLR (Toll-like receptor) agonists induce a robust innate and adaptive immunity against tumor cells [19]. Immune cells (such as DCs; Dendritic cells) in the TME are functionally immature due to the immunosuppressive actions of HIF-1α overexpression. Activation of DCs via TLR agonists leads to upregulation of costimulatory molecules and secretion of inflammatory cytokines, which can remodel the TME [20]. Imiquimod (IMQ), a TLR7 agonist, represents direct and indirect anticancer effects through apoptosis stimulation in tumor cells and induction of effective immune responses [21]. Several preclinical studies have shown that the intratumoral (i.t.) application of IMQ modulates TME through increasing effector immune cells (CTL; cytotoxic T lymphocyte and mDCs; mature DCs) and decreasing suppressor immune cells (MDSC; Myeloid-derived suppressor cells and Treg; T regulatory cells) [22]. Huang S.W. et al. demonstrated that IMQ up-regulates HIF-1α expression and genetic silencing of HIF-1α sensitized cells to IMQ. Thus, co-treatment of IMQ with HIF-1α inhibition may be a useful therapeutic option to augment the antitumor effects of IMQ in cancer therapy [23].

Several reports support the concept that coadministration of TLR agonists and chemotherapy can have greater therapeutic effects compared to each treatment alone in mouse tumor models [24, 25]. Chemo agents through immunogenic tumor cell death provide a microenvironment with an abundance of available tumor-specific antigens as a ligand for TLR stimulation, leading to activation of immune cells (DCs) and antitumor immune responses [26]. It has been shown that a combination of IMQ and chemo agents generates robust synergic immune responses and improves survival in mice model studies [25, 27].

Here, we studied the synergistic antitumor effect of a novel combination of HIF-1α siRNA with chemoimmunotherapy (OXA+IMQ) in the mice model of CRC. This regime guarantees that sufficient levels of tumor-specific antigens are presented to immune cells in a favorable TME. In addition, this strategy allows DCs to access tumor antigens and TLR agonists in situ to overcome tumor tolerance, which leads to potent antitumor immunity (Scheme 1). We hypothesized that remodeling of TME through
HIF-1α inhibition could increase the therapeutic efficacy of OXA and IMQ, which can suppress the growth of murine CRC.

Scheme 1. Schematic illustration of the triple combination therapy. Remodeling of TME through HIF-1α siRNA combined with abundant TAA released by OXA could increase the therapeutic efficacy of IMQ, which can suppress the growth of murine CRC.

Abbreviations: HIF-1α, Hypoxia-inducible factor 1α; siRNA, small interfering RNA; OXA, Oxaliplatin; DC, Dendritic cell; TME, Tumor microenvironment; TAA, Tumor specific antigen; CRC, Colorectal cancer.

2. Material And Methods

2.1 Materials

The low molecular weight of CH (Chitosan; 114 kDa, 84% deacetylation; Sigma-Aldrich) was used for nanoparticle preparation. The 22-mer, HIF-1α-specific siRNA duplex was supplied by Bioneer Corporation (Korea) containing the sequences: murine HIF-1α sense, 5′-CAGUUACGAUUGUGAAGUU-3′, antisense 3′-AACUUCACAAUCGUAAUCUG-5′. IMQ powder was purchased by InvivoGen (San Diego, United States). Mice BALB/c and CT26 cell line were supplied from the Pasteur Institute (Tehran, Iran). All animal studies were performed by guidelines approved by the Tabriz University of Medical Science for the care and use of laboratory animals.

2.2 Preparation of CH nanoparticles

The CH nanoparticles are generated based on the ionic gelation of the tripolyphosphate (TPP) with cationic CH. The CH working solution (2.1 mg/mL) was prepared by dissolving 10.5 mg of CH in 5 ml acetic acid buffer (0.25%, pH 4.5) while stirring. For more dissolution of CH, dry ultrasonic was used for 3 minutes. The nanoparticles were created after slowly adding TPP (0.3%) into the CH solution under continuous stirring at room temperature. The nanoparticle's characters (size, surface charge (zeta potential), and polydispersity index (PDI)) were evaluated by dynamic light scattering (DLS) using Malvern zeta sizer Nano-ZS ZEN.

2.3 Preparation of the CH/siRNA nanoplexes

The following formula was used to calculate specific N/P ratios (the molar ratio of CH amino groups/RNA phosphate groups) ranging from 20 to 60. To prepare the CH/siRNA nanoplexes in a specific N/P ratio, different amounts of the CH were added to the siRNA solution and incubated at room temperature for 30 min.

\[
\text{CH(\mu g)} = \frac{\text{siRNA(\mu g)} \times 3 \times \text{N/P}}{\text{CH(m.w.)}}
\]

2.4 The CH/siRNA nanoplexes gel retardation assay
The loading ability of siRNA into the nanoparticles was evaluated by gel retardation analysis. The CH/siRNA nanoplexes solution at different N/P ratios was prepared. Then free siRNA and CH/siRNA solutions were loaded into 2% agarose gel and electrophoresed in 1× Tris- Boric acid- EDTA (TBE) buffer (PH:7.2) at 80 V for 45 min. Finally, CH/siRNA nanoplexes were visualized on a gel documentation system (Qiagen) following gel stain by gel red.

2.5 Cytotoxicity study of the CH/siRNA nanoplexes

The MTT assay was carried out to study the cytotoxicity of the nanoparticles. CT26 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate with a total well volume of 100 µl of RPMI media (Gibco®) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and kept at 37°C with 5% CO₂. After 24 h, the cells were treated with serial dilution of the nanoparticles. Tumor cells without the nanoparticles were used as control. Following 48 h incubation, MTT reagent (5 mg/ml, Sigma) was added to each well at 1:3 dilution and incubated for 4 h. Finally, the media was replaced by DMSO (Sigma) and the relative absorbance (at 570 nm) was detected using an ELISA plate reader.

2.6 Hypoxic induction of CT26 cells with CoCl₂

The CoCl₂ (Cobalt (II) chloride) was used to simulate hypoxic TME in the cell culture. Hypoxic conditions were simulated by incubating the cells at different concentrations of CoCl₂ (25, 50, 100, and 150 µM) for 24 h to optimize the appropriate dosage of CoCl₂. Then the expression level of HIF-1α was assessed by real-time PCR (qPCR).

2.7 HIF-1α gene silencing into CT26 cell line

The silencing activity of CH/HIF-1α siRNA nanoplexes on the CT26 tumor cell line was investigated using qPCR. Murine CRC cell line (CT26) (1.5 ×10⁵) were seeded into a 6-well culture plate in RPMI media (containing 10% FBS and 1% penicillin/streptomycin) and incubated at 37°C in 5% CO₂ for 24 h. To induce a model hypoxic response, 24 h before CH/HIF-1α siRNA transfection, the media was replaced by 2 ml fresh media containing 100µM CoCl₂ and 5% FBS, without antibiotics. Transfections were performed on the cells that were approximately 70% confluent. On the day of transfection, the CH/ HIF-1α siRNA (N/P=60) complex at various HIF-1α siRNA molarities (25, 50, 75, and 100 nM) in 500 µl FBS free media was added into each well and incubated at 37°C. Four hours after transfection, 500 µl fresh media containing 20% FBS and 100 µM CoCl₂ was added to the transfected cells in each well. The cells were further incubated for 48 h. The cellular levels of HIF-1α mRNA were measured using qPCR. Transfection with PBS and HIF-1α siRNA alone was used as the control.

2.8 Establishment of CT26 syngeneic mice model of CRC

Female BALB/c mice 5-6 weeks old were maintained under pathogen-free conditions. Syngeneic tumor-bearing mice were established by subcutaneous (s.c.) implantation of 1×10⁶ CT26 cells in 100 µl of PBS into the right flank of mice. On days 12-16 post-implantation of cells, when tumors became palpable, in
the size range of 50–65 mm³, mice were randomly assigned to the following treatment groups (n=4): (1) PBS served as control, (2) the CH nanoparticles, (3) OXA +IMQ, (4) HIF-1α siRNA +OXA, (5) HIF-1α siRNA +IMQ, and (6) HIF-1α siRNA combined with IMQ and OXA. In the groups with OXA treatment, it was injected intraperitoneal (i.p.) at a dose of 5 mg/kg every other day. IMQ and siRNA-HIF-1α were administered i.t. injection at a dose of 25 µg/mouse and 50 pmol/mouse every other day respectively. The CH nanoparticles and PBS were injected i.t. at a dose of 0.1 ml per mouse every day. Treatment was continued for up to 10 days and the tumor size was recorded every two-day using a caliper. Tumor volume was calculated using the following formula: tumor volume (mm³) = A²xB/2 where A and B represent the short and long diameter of tumors, respectively. Following 10 days of therapy, all animals were euthanized and tumor samples were removed. Tumor samples were photographed and stored at -70 °C for further analysis.

2.9 Quantitative real-time PCR

Total RNA was extracted using the chloroform and isopropanol isolation method. A spectrophotometer (Nano-Drop 2000C, USA) was used for the detection quality and quantity of RNA. The qPCR reaction was performed using SYBR Green I master mix in the qPCR thermal cycler (Roche Germany) to the evaluation of the relative expression level of interested genes. The β-actin gene was used to normalize the cycle threshold (Ct) values of the target genes.

2.10 Western blot analysis

Following completing treatment and tumor excision, samples were lysed on ice. The extraction buffer was centrifuged at 12,000 rpm for 10 min at 4 °C and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel. Immunoblotting was carried out using specific antibodies to assess the relative expression of desired proteins. The relative intensity of the interest bands was normalized with β-actin intensity in the same sample with Image J.

2.11 Statistical analysis

All data are expressed as mean ± SD. For comparison of means, ANOVA tests were used. All analyses were made using GraphPad Prism v.9 software. Significance is shown as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. Results

3.1 Preparation and characterization of the nanoparticles

The CH/HIF-1α siRNA nanoplexes were prepared by incubation of the nanoparticles with siRNA. The physical structure of the nanoparticles and the CH/siRNA nanoplexes is shown in Fig1a. The mean diameter of the CH/siRNA nanoparticles was 243±6 nm with the size distribution being relatively narrow (0.3±0.04). The siRNA binding to the nanoparticles and formation of the CH/siRNA nanoplexes was also
evident as the zeta potential of nanoparticles changed to 12.2±0.4 mV. These results have been reported in previous studies [28]. To find the proper ratio of the N/P reaching the maximum encapsulation, the gel retardation assay with CH/siRNA nanoplexes at the different ratios of N/P (20 to 60) was performed. The results indicated that full complexation was conformed at an N/P=60 ratio (Fig. 1b).

3.2 Cytotoxicity of the nanoparticles in CT26 cells

The MTT assay was performed to evaluate the cytotoxicity of the nanoparticles against CT26 cells. Fig. 1c shows cell viability after incubation with various concentrations of the nanoparticles in the range of 10-100 μg/ml. Compared to the non-treated cells, the viability of cells incubated with the nanoparticles was 85%, indicating good biocompatibility and low cytotoxicity of the nanoparticles.

3.3 In vitro HIF-1α gene-silencing by CH/HIF-1α siRNA nanoplexes

The gene silencing efficiency of CH/HIF-1α siRNA nanoplexes was initially evaluated in the cell culture. The relative HIF-1α mRNA levels were evaluated in CT26 cells incubated with CH/HIF-1α siRNA nanoplexes in different molarities of HIF-1α siRNA (25, 50, 75, and 100 nM) at an N/P=60 ratio. Gene expression was analyzed after 48 h by qPCR. As shown in Fig. 1d, the HIF-1α mRNA levels in 100nM siRNA significantly reduced in comparison with other concentrations and the control group (P<0.0001).

Fig. 1 Characterization of CH/HIF-1α siRNA nanoparticles. Size, zeta potential, and PDI of CH and CH/HIF-1α siRNA nanoplexes (a). Gel retardation assay of CH/siRNA nanoparticles with different N/P ratios (b). Cell viability of CT26 cells incubated with different concentrations of CH (c). Gene silencing efficiency of CH/HIF-1α siRNA in CT26 cells. Tumor cells were cultured and transfected with CH/HIF-1α siRNA nanoplexes in different molarities of HIF-1α siRNA (25, 50, 75, and 100 nM) at an N/P=60 ratio. Following 48h incubation, the levels of HIF-1α mRNA were measured by qPCR. β-actin was used as a housekeeping gene (d). Each value represents the mean ± SD of three measurements.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PDI, polydispersity; qPCR, Real-time PCR.

3.4 Animal model study

3.4.1 Synergistic effects of combination therapy on tumor growth inhibition

The therapeutic effects of combination therapies were assessed by s.c. challenging BALB/c mice with CT26 cell line, followed by the drug administration of OXA, IMQ, and HIF-1α siRNA based on the treatment protocol (Fig. 2a). All mice tolerated treatment with OXA, IMQ, and HIF-1α siRNA in the administrated dose and scheduled treatment (established by literature review). To evaluate the effect of combination therapies on tumor growth inhibition, we initially investigated their effects on tumor phenotypes. Fig. 2b shows the inhibitory effects of combination therapies on tumor size and volume.

While tumor volume was strikingly increasing in untreated mice, treatment of a dual or complete mixture of HIF-1α siRNA, OXA, and IMQ significantly suppressed tumor growth. The co-administration of HIF-1α
siRNA +OXA significantly inhibited tumor growth. The simultaneous administration of IMQ with HIF-1α siRNA or OXA gradually induced significant tumor shrinkage from day 1 through day 10 compared with PBS or CH groups. The triple combination therapy with HIF-1α siRNA +OXA +IMQ significantly inhibited the growth of CT26 tumor-bearing mice (Fig. 2c) (P < 0.0001) but did not induce any complete tumor regression. The comparison between triple and dual combination treatment showed that the tumor volume in mice treated with IMQ +OXA was statistically significant (p = 0.004). Our combination therapies gradually decreased tumor growth from day 6 to the final observation day (day 10), and the mean tumor volume was significantly reduced (Fig. 2e). Overall, triple compared with dual therapy further reduced tumor volume.

Combination therapies diminished the average tumor weight in all treatment groups. The average tumor weights decreased in mice exposed to IMQ with HIF-1α siRNA or OXA compared with PBS and the nanoparticles groups (Fig. 2d). Notably, the combination of HIF-1α siRNA +OXA, as well as triple combination therapy, resulted in a further shrinkage of tumor weight compared with the control group (p<0.0001). In total, the tumor weights in the combination treatment groups were 52% lower than those in the control one, which was much lower than those in the HIF-1α siRNA +OXA +IMQ (12.3%) and HIF-1α siRNA +OXA (17.7%) groups. The tumor weight in the triple therapy group decreased more and showed a significant reduction compared to the dual therapy groups (p<0.01).

**Fig. 2** The effects of combination therapies on tumor weight and volume in mice model of CRC.

Treatment protocol; Scheme of therapy protocol developed to treat CT26 tumors. Treatment was initiated at d12-16 following tumor challenge and was administered every other day (a). Images of tumor harvested from the untreated and treated groups after 10 days. Resected tumors showed that triple combination therapy reduced tumor size greater than dual therapies (b). Effects of combination treatments on tumor volume. On day 1, the tumor volume was approximately 50– 60 mm³. Tumor diameters were measured with a caliper every two days (c). All mixture of combination therapies significantly reduced tumor weight compared with the control (d). Tumor volume over time indicated that triple combination therapy was more effective than dual treatments. Each line represents a specific treatment group, plotted to show primary tumor size vs day post tumor challenge (e). Significance is shown as follows *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR.

### 3.4.2 The effects of the CH/HIF-1α siRNA nanoplexes on HIF-1α down-regulation in CT26 syngeneic mice model

We investigated the inhibition effects of i.t. injection of the CH/HIF-1α siRNA nanoplexes on the mRNA and protein expression levels of HIF-1 on tumor compared to control samples. As shown in Fig. 3a, the relative HIF-1α mRNA levels in tumor tissue with an injection of the CH/HIF-1α siRNA nanoplexes were markedly reduced in comparison to uninjected groups (p=0.01). The results of western blot analysis
revealed a significant decrease in HIF-1α protein levels in HIF-1α siRNA-treated tumor tissue compared with untreated groups (Fig. 3e).

### 3.4.3 The effect of combination therapies on apoptosis stimulation

To further elucidate the impact of the combination therapies on tumor cells, we studied mRNA and protein expression levels of apoptosis-related genes in CT26 tumor samples. The mRNA expression levels of pro-apoptotic genes such as BAD and BAX significantly increased in mice treated with HIF-1α siRNA +OXA ($p<0.01$). While the mRNA expression levels of Bcl-2 as an anti-apoptotic gene increased in the control groups, in mice treated with triple combination and OXA+ IMQ significantly decreased (Fig. 3b-d). The BAX protein expression levels significantly increased in mice treated with a triple combination therapy group in comparison with other groups.

Fig. 3 Changes in HIF-1α and apoptosis-related genes expression during combination therapies. Twelve to sixteen days following CT26 cell implantation, mice were randomly divided into six groups and treatment was initiated. Mice were treated with i.t. injection of HIF-1α siRNA and IMQ. OXA was injected i.p. At the end of treatment, resected tumor samples were subjected to qPCR to evaluate mRNA expression. Data were normalized with the housekeeping gene β-actin.

The effects of the CH/HIF-1α siRNA on the expression levels of HIF-1α in mice model of CRC (a). The Effects of combination therapies on the mRNA levels of BAX (b), BAD (c), and BCL2 (d) genes. The protein expression levels of HIF-1α (e) and BAX (f) were determined by western blot. Data are shown as mean ± S.D. of the gene expression levels. Significance is shown as follows *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR; BAD, BCL2 associated agonist of cell death; BAX, Bcl-2 associated X-protein; BCL2, B-cell lymphoma 2.

### 3.4.4 The effect of combination therapies on CT26 tumor cell proliferation

The effect of the combination treatments was also evaluated on cell proliferation. For the initial assessment, we investigated signal transducer and activator of transcription 3 (STAT3) and vascular endothelial growth factor (VEGF) mRNA expression profiles in the tumor tissue specimens after treatment based on the protocol. The mRNA expression levels of STAT3 and VEGF genes significantly decreased in all mice-treated groups compared with PBS and the nanoparticles groups (Fig. 4a and b). Analysis of protein expression levels showed that the STAT3 protein in mice treated with OXA +IMQ significantly decreased ($p=0.03$) (Fig. 4c). Our data showed that the combination therapies effectively down-regulate proliferation genes leading to inhibition of tumor growth.

Fig. 4 Combination therapies change the expression level of genes related to proliferation. Twelve to sixteen days following CT26 cell implantation, mice were randomly divided into six groups and treatment was initiated. Mice were treated with i.t. injection of HIF-1α siRNA and IMQ. OXA was injected i.p. After 10
days tumors were resected and mRNA expression levels of VEGF (a) and STAT3 (b) were quantified by qPCR. The protein expression levels of STAT3 (c) were determined by western blot. Data were normalized with the housekeeping gene β-actin. Data are shown as mean ± S.D. of the gene expression levels. Statistical analysis was performed using one-way ANOVA. Significance is shown as follows *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR.

3.4.5 Combination therapies induce immune cell-related cytokines and Th1 polarization in the TME

The immune-related effects of the combination treatments were assessed through analysis of the mRNA and protein levels of cytokines between treated and untreated tumor samples. In comparison with dual combination therapies, triple combination treatment strongly upregulated Th1(T helper1)-type cytokines including IL-12 (Interleukin-12) and IFN-γ (Interferon-gamma), but not Th2-type cytokines such as IL-4 and IL10 (Fig. 5a-d). These results showed that the co-administration of HIF-1α siRNA+ OXA +IMQ induces cellular immune responses in tumor-bearing mice. In the dual treatment groups, no significant differences were observed in the mRNA expression levels of IL12 and IL4 cytokines compared to the control group. Analysis of the protein expression levels showed that the IFN-γ in mice treated with the triple combination therapy significantly increased compared with the control group (p=0.009) (Fig. 5e). Altogether, triple combination therapy had a strong local effect on the TME and polarizes it towards an inflammatory, Th1 cytotoxic immune contexture which may directly inhibit tumor growth.

Fig. 5 Combination therapies change the mRNA and protein expression levels of genes related to cytokines. Twelve to sixteen days following CT26 cell implantation, mice were randomly divided into six groups, and treatment was initiated. Mice were treated with i.t. injection of HIF-1α siRNA and IMQ. OXA was injected i.p. After 10 days the tumor was extracted and the mRNA expression levels of IL12 (a), IL10 (b), IL4 (c), INFγ (d) were quantified by qPCR. The protein expression levels of INFγ (e) were determined by western blot. Data were normalized with the housekeeping gene β-actin. Data are shown as mean ± S.D. of the gene expression levels. Statistical analysis was performed using one-way ANOVA. Significance is shown as follows *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR

3.4.6 Correlation of HIF-1α gene expression with CT26 tumor growth phenotypes

After the preliminary analysis, we tested the correlation between HIF-1α gene expression and tumor size in all tumor sample groups. The pattern of the genetic correlation coefficient is shown in Fig. 6. The positive correlation coefficient (r=0.68) was obtained between tumor size and HIF-1α expression level in all groups (p= 0.003) (Fig. 6a). In the triple combination therapy group, HIF-1α had positive correlations with VEGF although it was not statistically significant (r=0.8) (Fig. 6b). The expression level of BCL2 and IL-12 genes
had negative correlations with HIF-1α expression (Fig. 6c and d). The most significant genetic correlation (p=0.09) was between HIF-1α and BCL2 genes. The relationships between the HIF-1α gene and other studied genes were simple because a low level of HIF-1α in TME is often associated with a strong immune response and tumor growth inhibition.

**Fig. 6** Correlation of HIF-1α expression with tumor size and gene expression. Correlation of HIF-1α expression with tumor size in all mice treated and control groups (a). There was a positive correlation between HIF-1α mRNA expression and tumor size in all mice. Higher expression of HIF-1α is associated with larger tumor size. Correlation of HIF-1α expression with VEGF(b), BCL2(c), and IL12(d) gene expression in the triple combination therapy group. Positive correlation was detected between HIF-1α and VEGF gene expression level and there was negative correlation between HIF-1α and BCL2 and IL12.

### 4. Discussion

Cancer is characterized by the dysfunction of multiple signaling pathways that facilitate the growth and development of cancerous cells. Therefore, targeting several processes in cancer cells may increase the therapeutic efficiency of therapy. For this reason, combination therapy has been shown an effective antitumor response rather than monotherapy [29]. Currently, the reports of various researches show that despite the robust results of chemoimmunotherapy in comparison with chemotherapy alone, it has not improved outcomes in patients [30]. It has been shown that immunosuppressive TME is the main cause of the failure of immunotherapy, so remodeling of TME may increase the response rate of chemoimmunotherapy. In the present study, on the one hand, we stimulated the immune system against cancer cells by TLR7 agonist (IMQ), and on the other hand, by targeting HIF-1α, we facilitated the TME to function of immune cells. In addition, by inducing immunogenic cell death by OXA, we provided the tumor-specific antigens for further stimulation of the immune system. In this study, we showed that genetic silencing of HIF-1α increases the response rate of chemoimmunotherapy (OXA and IMQ), leading to effective antitumor response and tumor growth inhibition.

Several studies have reported that the CH nanoparticle is an ideal carrier to deliver siRNA into target cells *in vivo* [31]. We found that the HIF-1α siRNA could be efficiently encapsulated into the CH nanoparticles and delivered-siRNA potentially reduces the mRNA and protein expression levels of HIF-1α *in vitro* and *in vivo*.

Increasing evidence from experimental and clinical studies has revealed that elevated levels of HIF-1α are associated with the development of immunosuppressive TME consequently, therapy resistance and progression of cancer [32]. So, targeting HIF-1α has been considered an attractive therapeutic target to increase chemosensitivity and block the aggressive phenotype of tumor-induced by hypoxic TME [33, 34]. Herein, we showed that i.t. injection of HIF-1α siRNA combined with IMQ and/or OXA could effectively down-regulate the HIF-1α mRNA and protein expression levels, induces tumor cell apoptosis, and inhibits the growth of the syngeneic mice model of CRC as reported in previous studies [35, 36]. Our data showed that tumor growth inhibition in the groups treated with HIF-1α siRNA was greater than in those without
HIF-1α siRNA treating groups indicating downregulation of HIF-1α by siRNA enhances the efficiency of IMQ and OXA in mice models of CRC, which is in agreement with previous studies [37, 38].

OXA as an immunogenic cell death inducer agent is commonly used in the clinical treatment of advanced CRC. Despite the remarkable therapeutic effects of OXA, the occurrence of cytotoxicity has limited its application [39]. Thus, the combination of OXA with other therapies can enhance efficiency and reduces its side effects. In this study, we demonstrated that co-administration of OXA with HIF-1α siRNA inhibited cell proliferation, induced tumor cell apoptosis, and resulted in slowing down tumor growth, which is in agreement with previous studies [34, 40]. Our findings showed that targeting HIF-1α plus OXA inhibited tumor cell growth by direct cytotoxicity rather than immunoregulation. These data suggest that the existence of a favorable tumor environment and a sufficient amount of tumor antigens cannot be effective in stimulating the effective antitumor immunity and modification of the suppressed-immune system. So, combining an immune stimulant may induce a further antitumor immune response.

Immunotherapy with TLR agonists alone or in combination with conventional treatment is widely studied in cancer therapy. Several studies have shown that targeting TLR7 enhances recruitment and activation of immune cells in the TME and polarizes antitumor immunity towards a Th1 response which caused inhibition of tumor growth in multiple syngeneic mice models [37, 41]. As, a TLR7 agonist, IMQ, is an FDA-approved agent recently used in the therapy of dermatological cancers such as basal cell carcinoma (BCC) and viral lesions such as human papillomavirus (HPV). The efficacy of IMQ alone or plus other treatment was evaluated in many types of cancers [25, 42]. Our results showed that in mice treated with IMQ plus HIF-1α siRNA, there was a significant increase in apoptotic genes and a decrease in proliferative genes (VEGF and STAT3), leading to tumor growth inhibition. The antitumor effects of HIF-1α siRNA and IMQ were associated with inhibition of cell proliferation rather than immune response. However, remodeling of the immunosuppressive TME by HIF-1α siRNA improved the efficacy of IMQ in stimulating the immune system for tumoricidal activity but showed fewer immunomodulatory effects compared to triple therapy.

Co-treatment of OXA plus IMQ showed significant inhibition of tumor growth although this group showed the lowest rate of inhibition among the studied groups. In this group, inhibition of tumor growth was triggered upon induction of apoptosis and inhibition of proliferation, as well as decreasing immunosuppressive cytokines such as IL10 and IL4. This data suggested that the combination of OXA with an immunomodulator is associated with a shift in the TME towards a more pro-immunogenic phenotype and antitumor immune response as reported in previous studies [43].

A triple combination of OXA + IMQ + HIF-1α siRNA exerts a more inhibitory tumor growth effect on the syngeneic mice model of CRC. The use of the complete therapeutic mixture exhibited the highest antitumor efficacy among the studied groups. Combination therapy with HIF-1α siRNA + OXA + IMQ potentially inhibited tumor growth through a variety of mechanisms. This regime induced potent immune responses against tumor cells as well as inhibition of cell proliferation and induction of apoptosis. Triple combination therapy significantly increased cellular immune cytokines such as IL12 and IFN-γ compared
to that resulting from dual combination therapies. In this group, the cellular immune response induced by IMQ associated with apoptosis induced by OXA caused further tumor growth inhibition. Moreover, triple combination therapy showed low levels of proliferation and angiogenesis factors as well as the anti-apoptotic gene (Bcl-2). Interestingly, administration of HIF-1α siRNA and OXA before the start of IMQ treatment provides a favorable TME and abundant tumor-specific antigens to further immune cell activation. Here, we found that the reduced-HIF-1α by siRNA-loaded CH nanoparticles was associated with increased sensitivity of CRC cells to OXA and IMQ. Our results point to the treatment potential and advantageous effects of this combination therapy in the mice model of CRC. Therefore, novel combination therapy capable of decreasing the HIF-1α level as well as stimulating the immune system may be a potential therapy for CRC treatment. However, the clinical application of this strategy still needs more support in vivo experiments.

Declarations

Conflict of Interest:

Leila Rostamizadeh, Mina Ramezani, Hannaneh Monirinasab, Kobra Rostamizadeh, Ommoleila Molavi, Behzad Baradaran, Seied Rafi Bahavarnia, and Fatemeh Ramezani declare that they have no conflict of interest.

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Ethics approval:

Ethical approval for this study was obtained from Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1399.056).

Authors’ contribution statements:

Leila Rostamizadeh, Conceived and designed of the presented idea, carried out the experiment collected the data, performed the analysis, and wrote the paper.

Mina Ramezani, carried out the mice experiment and verified the experimental.

Hannaneh Monirinasab, performed nanoparticle synthesis.

Kobra Rostamizadeh, contributed in development of nanoparticle synthesis.

Ommoleila Molavi, verified the experimental and analytical methods.

Behzad Baradaran, verified the experimental and analytical methods.
Seied Rafi Bahavarnia, developed the theory and performed the literature reviews.

Fatemeh Ramezani carried out the experiment and wrote the manuscript.

All authors discussed the results and contributed to the final manuscript.

**Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**References**


Figures
Characterization of CH/HIF-1α siRNA nanoparticles. Size, zeta potential, and PDI of CH and CH/ HIF-1α siRNA nanoplexes (a). Gel retardation assay of CH/siRNA nanoparticles with different N/P ratios (b). Cell viability of CT26 cells incubated with different concentrations of CH (c). Gene silencing efficiency of CH/HIF-1α siRNA in CT26 cells. Tumor cells were cultured and transfected with CH/HIF-1α siRNA nanoplexes in different molarities of HIF-1α siRNA (25, 50, 75, and 100 nM) at an N/P=60 ratio. Following 48h incubation, the levels of HIF-1α mRNA were measured by qPCR. β-actin was used as a housekeeping gene (d). Each value represents the mean ± SD of three measurements.
Abbreviations: CH, chitosan; siRNA, small interfering RNA; PDI, polydispersity; qPCR, Real-time PCR.

Figure 2

The effects of combination therapies on tumor weight and volume in mice model of CRC.
Treatment protocol; Scheme of therapy protocol developed to treat CT26 tumors. Treatment was initiated at d12-16 following tumor challenge and was administered every other day (a). Images of tumor harvested from the untreated and treated groups after 10 days. Resected tumors showed that triple combination therapy reduced tumor size greater than dual therapies (b). Effects of combination treatments on tumor volume. On day 1, the tumor volume was approximately 50– 60 mm³. Tumor diameters were measured with a caliper every two days (c). All mixture of combination therapies significantly reduced tumor weight compared with the control (d). Tumor volume over time indicated that triple combination therapy was more effective than dual treatments. Each line represents a specific treatment group, plotted to show primary tumor size vs day post tumor challenge (e). Significance is shown as follows *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR.
Figure 3

Changes in HIF-1α and apoptosis-related genes expression during combination therapies. Twelve to sixteen days following CT26 cell implantation, mice were randomly divided into six groups and treatment was initiated. Mice were treated with i.t. injection of HIF-1α siRNA and IMQ. OXA was injected i.p. At the end of treatment, resected tumor samples were subjected to qPCR to evaluate mRNA expression. Data were normalized with the housekeeping gene β-actin.
The effects of the CH/HIF-1α siRNA on the expression levels of HIF-1α in mice model of CRC (a). The Effects of combination therapies on the mRNA levels of BAX (b), BAD (c), and BCL2 (d) genes. The protein expression levels of HIF-1α (e) and BAX (f) were determined by western blot. Data are shown as mean ± S.D. of the gene expression levels. Significance is shown as follows *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR; BAD, BCL2 associated agonist of cell death; BAX, Bcl-2 associated X-protein; BCL2, B-cell lymphoma 2.

Figure 4

Combination therapies change the expression level of genes related to proliferation. Twelve to sixteen days following CT26 cell implantation, mice were randomly divided into six groups and treatment was initiated. Mice were treated with i.t. injection of HIF-1α siRNA and IMQ. OXA was injected i.p. After 10 days tumors were resected and mRNA expression levels of VEGF (a) and STAT3 (b) were quantified by qPCR. The protein expression levels of STAT3 (c) were determined by western blot. Data were normalized with the housekeeping gene β-actin. Data are shown as mean ±S.D. of the gene expression levels. Statistical
analysis was performed using one-way ANOVA. Significance is shown as follows *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, *Phosphate-buffered saline*; OXA, oxaliplatin; IMQ, imiquimod; qPCR, *Real-time PCR*.

Figure 5
Combination therapies change the mRNA and protein expression levels of genes related to cytokines. Twelve to sixteen days following CT26 cell implantation, mice were randomly divided into six groups, and treatment was initiated. Mice were treated with i.t. injection of HIF-1α siRNA and IMQ. OXA was injected i.p. After 10 days the tumor was extracted and the mRNA expression levels of IL12 (a), IL10 (b), IL4 (c), INFγ (d) were quantified by qPCR. The protein expression levels of INFγ (e) were determined by western blot. Data were normalized with the housekeeping gene β-actin. Data are shown as mean ± S.D. of the gene expression levels. Statistical analysis was performed using one-way ANOVA. Significance is shown as follows *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CH, chitosan; siRNA, small interfering RNA; PBS, Phosphate-buffered saline; OXA, oxaliplatin; IMQ, imiquimod; qPCR, Real-time PCR.

Figure 6
Correlation of HIF-1α expression with tumor size and gene expression. Correlation of HIF-1α expression with tumor size in all mice treated and control groups (a). There was a positive correlation between HIF-1α mRNA expression and tumor size in all mice. Higher expression of HIF-1α is associated with larger tumor size. Correlation of HIF-1α expression with VEGF(b), BCL2(c), and IL12(d) gene expression in the triple combination therapy group. Positive correlation was detected between HIF-1α and VEGF gene expression level and there was negative correlation between HIF-1α and BCL2 and IL12.