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Original Article

IGF1R and HIF-1α Gene Silencing Inhibits Cancer Cell Growth

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Abstract

Background: According to several studies, hypoxia-inducible factor-1α (HIF-1α) and insulin-like growth factor 1 receptor (IGF1R) promote cancer progression and drug resistance. The overexpression of IGF1R and HIF-1 α is observed in various cancers, including breast and colon cancers. Thus, we tried to inhibit the progression of tumor cells by blocking IGF1R and HIF-1α.

Methods: This study used Lipofectamine to transfect small interfering RNAs (siRNAs) into tumor cells. We treated murine mammary carcinoma (4T1) and murine colon carcinoma (CT26) cells with anti-IGF1R and anti-HIF-1 siRNAs. The real-time polymerase chain reaction (PCR) assay studied the impact of siRNA transfection on the mRNA levels of affected factors. Moreover, the viability of cancer cells was investigated by the MTT assay.

Results: The investigations demonstrated that the mRNA levels of IGF1R and HIF-1α strongly reduced in tumor cell lines following siRNA transfection. Moreover, silencing IGF1R and HIF-1 additively downregulated cell viability in cancer cell lines.

Conclusion: These findings imply that cancer combination therapy based on IGF1R and HIF-1α targeting may be a novel promising approach for the immunotherapy of breast and colorectal cancers; however, more study is required.

Keywords: IGF1R, HIF-1α, siRNA, Cancer therapy

Introduction

Cancer is one of our most critical healthcare problems, so 19.3 million new cases and about 10 million cancer-mediated deaths were recorded worldwide in 2020.[1] Cancer cells have various features, including replicating indefinitely, escaping from the host's immune system, and resisting cell death.[2-5] Conventional cancer therapies include chemotherapy, radiotherapy, and surgery. These treatments focus on killing cells with low specificity, which can cause serious side effects and toxicities in normal tissues.[6,7] Therefore, new treatment strategies are needed to eliminate tumors and minimize side effects. Accordingly, several investigators have tried to target tumor-promoting factors in the tumor microenvironment to enhance anti-tumor immune responses. Cancer cells overexpress several factors that help to progress cancer growth and suppress anti-tumor immune responses. Therefore, blocking these cancer-promoting overexpressed factors can be considered an important therapeutic approach. The insulin-like growth factor (IGF) and hypoxia-inducible factor-1 (HIF-1) are two critical tumor-promoting factors that are overexpressed in the tumor microenvironment. The reciprocal impact of these factors on each other has made them attractive candidates for the simultaneous blockade.

The IGF signaling pathway regulates the growth and development of many tissues by inhibiting apoptosis and stimulating cellular proliferation. The IGF family includes insulin and two ligands, IGF1 and IGF2, that affect cellular activities by binding to cell membrane receptors such as the IGF-1 receptor (IGF1R), the IGF2R, and the insulin receptor, all of which are of the tyrosine kinase receptor family.[8] IGF participates in the formation and growth of cancer cells, and it has been related to

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developing resistance to some standard treatments.[9] The overexpression of IGF1R and IGF1 in the blood has been found in several cancer types, including colon, melanoma, prostate, pancreatic, and breast cancers. Studies have displayed that IGF pathway modulators effectively treat several cancers.[10] Therefore, targeting the IGF axis could represent a promising strategy for developing anti-cancer therapies.

On the other hand, hypoxia is a common hallmark of cancers and is responsible for the mechanisms of resistance to chemotherapy, immunotherapy, and radiotherapy.[11-13] HIF-1 transcription factor plays a pivotal role in the modulation of hypoxia-related factors.[14] The α and β subunits form a HIF-1 complex, which is a pivotal factor in tumorigenesis process. HIF-1 regulates the expression and function of several tumor-promoting factors, affecting the cancer cell development and metastasis of tumors.[15,16] The increased expression of HIF-1α has been found in various cancers such as breast, colorectal, melanoma, and prostate.[17] Interestingly, suppressing HIF-1α with small interfering RNAs (siRNAs) markedly improved anti-tumor effects.[18]

We hypothesized that suppressing IGF1R and HIF-1 could inhibit tumor cell growth and progression. In the following study, IGF1R and HIF-1α were inhibited by siRNA in murine colon carcinoma (CT26) and murine mammary carcinoma (4T1) cells. Moreover, the impact of silencing IGF1R and HIF-1α on cell viability was also studied.

Methods

Materials

Anti-murine siRNA molecules targeting HIF-1α, IGF1R, and irrelevant control siRNA molecules were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the MTT Assay Kit was purchased from Merck (Germany).

Cell Lines

The 4T1 and CT26 cell lines were purchased from the Pasteur Institute (Tehran, Iran). Both cell types were cultivated in RPMI-1640 medium with 10% fetal bovine serum, 2% L-glutamine, and pen/strep cocktails. These cell lines were maintained in the incubator at 5% CO_2, 37°C, and 95% humidity.

Cell Transfection

In this study, 1×10^4 cells were seeded in a 96-well plate and incubated for 24 hours. According to the manufacturer protocol, the siRNA transfection was done using 60 pM siRNA by Lipofectamine RNAiMax (Invitrogen).

Transfection Efficiency

To evaluate the transfection efficiency of nanoparticle (NP)-encapsulated siRNA, NPs were loaded with phycoerythrin (PE) -conjugated siRNAs and incubated with cancer cells as described above. Subsequently, cells were washed twice and scanned by flow cytometry to evaluate the frequency of cancer cells transfected with NPs.

Cytotoxicity Assay

The MTT test was performed to measure the impact of blocking IGF1R and HIF-1α in 4T1 and CT26 tumor cells. In a 96-well plate, 10^4 cells were seeded and incubated for 24 hours. The cells were then incubated with Naked siRNA, Lipofectamine, IGF1R siRNA, HIF-1α siRNA, Lipofectamine IGF1R siRNA, Lipofectamine HIF-1α siRNA, and Lipofectamine IGF1R/HIF-1α siRNAs for 24 and 48 hours. The cells' supernatant was then removed and substituted with Roswell Park Memorial Institute medium enclosing 50 µL MTT mixture (5 mg/mL). After four hours, the supernatant was removed, and 150 µL of dimethyl sulfoxide was added and incubated for 30 minutes. Then, the absorbance of each well was analyzed using spectrophotometers (BioTek, USA) at 570 nm.

Gene Expression Analysis

Following the treatment of cells with different therapeutic groups, total RNAs were purified using the Trizol reagent (Bioneer, South Korea), according to the company's guidelines. Afterward, cDNA synthesis was done using the RNA samples via a cDNA synthesis kit (Qiagen). The real-time reverse transcription-PCR (qRT-PCR) test used a Light-Cycler 480 RT-PCR system (Roche) and SYBR Green Master Mix (Thermo Fisher Scientific). Finally, the relative mRNA expression of IGF1R and HIF-1α was quantified using the $2^{-\Delta\Delta CT}$ formula. Primer sequences are demonstrated in Table 1.[19,20]

Statistical Analysis

Statistical analyses were done by GraphPad Prism V8 software and the one-way ANOVA test.
P value < 0.05 was also considered significant.

Results

Nanoparticles Markedly Transfected Cancer Cells

To evaluate the transfection potential of NPs, we incubated cancer cells with NPs loaded with PE-labeled siRNAs and assessed the transfection efficiency using the flow cytometry assay. As shown in Figure 1, about 70% of cells were successfully transfected with NPs, which is an acceptable transfection rate.

The siRNA Transfection Meaningfully Silenced the Expression of Insulin-like Growth Factor 1 Receptor and Hypoxia-inducible Factor-1α in Cancer Cell Lines

Following the treatment of cells with different therapeutic groups, the mRNA levels of IGF1R and HIF-1α were evaluated using qPCR to investigate the efficiency of gene silencing. Cells treated with Naked siRNA and Lipofectamine alone did not exhibit significant changes in the mRNA levels. Moreover, mRNA levels did not change when IGF1R siRNA and HIF-1α siRNA were used alone, which indicates that cells need a carrier to transfect

Table 1. Primer sequences

Gene Name	Forward	Reverse
β-actin	5′-GGTCATCACTATTGGCAACG-3′	5′-ACGGATGTCAACGTCACACT-3′
IGF1R	5′-GTGGGGGCTCGTGTTTCTC-3′	5′-TGATCACCGTGCAGTTTTCCA-3′
HIF-1α	5′-AGCAGGAATTGGAACATTATTGCAG-3′	5′-TGTGGTAATCCACTCTCATCCATTG-3′

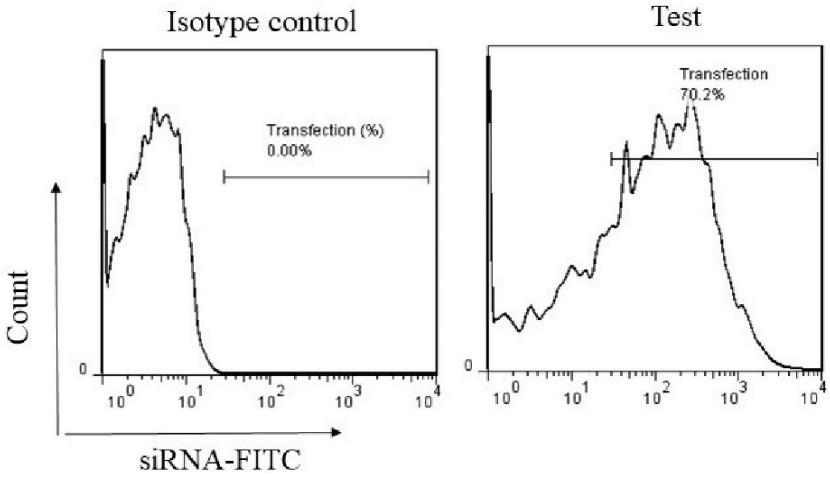

Figure 1. NPs Encapsulating siRNA Molecules Efficiently Transfected Cancer Cells. Flow cytometry analysis was used to investigate the transfection efficiency of NPs. Cancer cells were treated with PE-labeled siRNAs and incubated for 24 hours. Following cell washing, cancer cells were scanned by flow cytometry to assess the percentage of cells transfected with NPs containing siRNAs. *Note.* NPs: Nanoparticles; siRNAs: Small interfering RNAs

siRNA. However, treatment of cells with Lipofectamine IGF1R siRNA and Lipofectamine HIF-1α siRNA markedly decreased the mRNA levels of IGF1R and HIF-1α compared to other treatment groups. As depicted in Figure 2, the most significant decrease in mRNA expression levels was detected in cells incubated with Lipofectamine IGF1R/HIF-1α siRNAs. This indicates a reciprocal relationship between the molecules, the blockade of which one affects the expression of another.

Silencing of Insulin-like Growth Factor 1 Receptor and Hypoxia-inducible Factor-1α Expression Induced Apoptosis in Cancer Cell Lines

The MTT assay was used to analyze the cytotoxic impact of various therapeutic groups on the viability of tumor cells after 24 and 48 hours of incubation with the therapeutic groups. The results are presented in Figure 3.

Cells treated with Naked siRNA and Lipofectamine alone did not affect the viability of both cell lines, which shows their safety. Furthermore, IGF1R siRNA and HIF-1α siRNA had no effect on viability, indicating the carrier requirement for cellular transfection. On the other hand, Lipofectamine IGF1R siRNA and Lipofectamine HIF-1α siRNA significantly decreased cell viability. In addition, the highest apoptosis rate was found in cells treated with the Lipofectamine IGF1R/HIF-1α siRNAs group compared to the control group, and the apoptotic effects were additive.

Discussion

Identifying the pathways in tumor progression is critical for developing effective therapeutic strategies. IGF is an essential factor in the oncogenesis of most solid cancers. This pathway has a role in cancer cell proliferation and metastasis, which makes it an attractive therapeutic target.[21] Hypoxia serves as a selective pressure, leading tumor biology to become aggressive. HIF-1α promotes cancer progression by increasing the expression of factors that modulate glucose metabolism, stem cell maintenance, cell proliferation, cell invasion and metastasis, angiogenesis, and drug resistance.[22,23] Therefore, inhibiting HIF-1α may be an effective strategy for cancer treatment.

Evidence shows that IGF-1 synergizes with HIF-1α to promote tumor growth.[24,25] IGF1R and HIF-1 may also upregulate vascular endothelial growth factor, provoking angiogenesis.[26,27]

The current research investigated the effects of inhibiting IGF1R and HIF-1α. Lipofectamine was utilized to transfect IGF1R and HIF-1α siRNA into tumor cells. The qRT-PCR findings showed that siRNAs targeting IGF1R and HIF-1α were effectively transfected to tumor cells as IGF1R and HIF-1α mRNA levels were markedly suppressed. Moreover, treatment with Naked siRNA, IGF1R siRNA, and HIF-1α alone did not affect IGF1R and HIF-1α.

After treating cancer cell lines with different therapeutic groups for 24 or 48 hours, MTT assays were conducted (Figure 2). The results suggested that cells given Lipofectamine IGF1R siRNA and Lipofectamine HIF-1α siRNA alone show significant apoptosis rates. Moreover, cells treated with Lipofectamine IGF1R/HIF-1α siRNAs combination therapy exhibited the highest apoptosis rate.

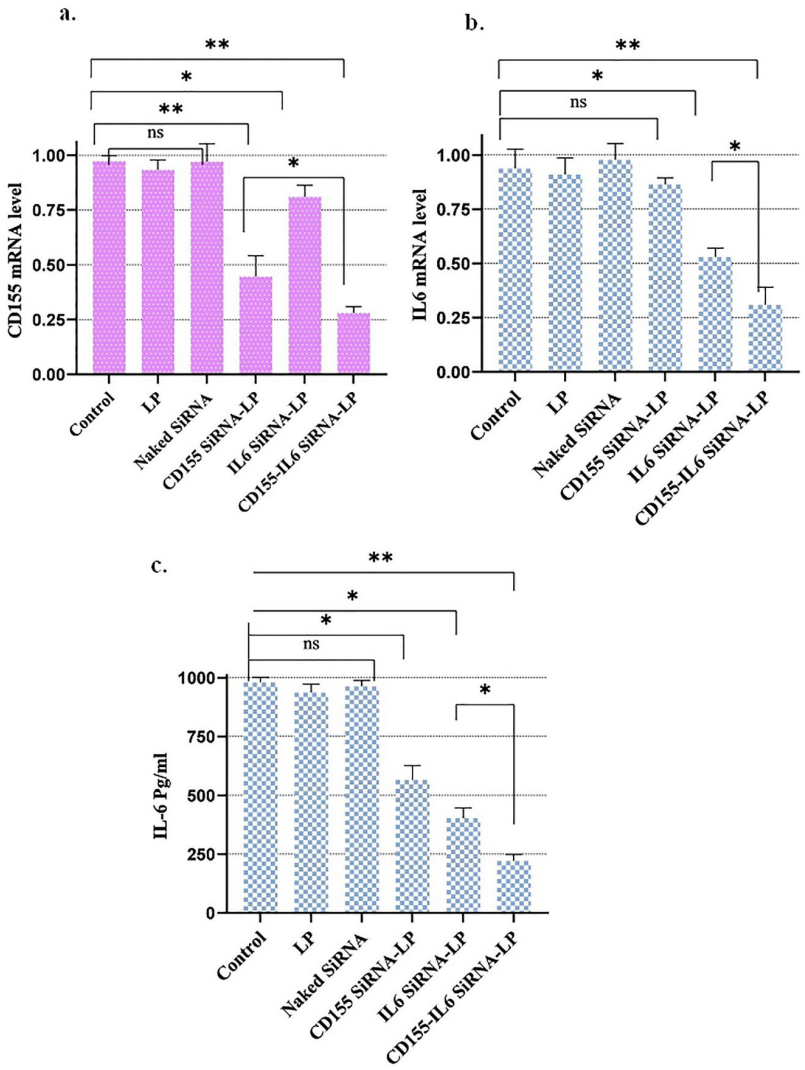

Figure 2. The mRNA and protein expression levels of CD155 and IL-6. As measured by qPCR assay, the transformation of cancer cell lines by siRNA downregulated HIF-1 and IGF1R mRNA levels . Bar charts demonstrate the mean values of gene expression ± SEM. Note. qPCR: Real-time polymerase chain reaction; HIF-1: Hypoxia-inducible factor-1; IGF1R: Insulin-like growth factor-1 receptor; SEM: Standard error of measurement; * $P<0.05$; ** $P<0.01$.

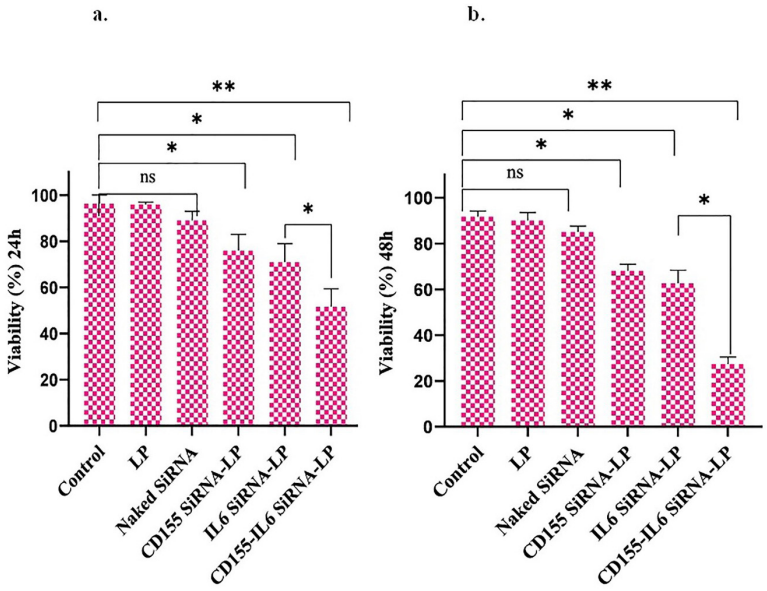

Figure 3. Viability assay in cancer cells. The downregulation of HIF-1 and IGF1R induces cell death in cancer cells . Bar charts demonstrate the mean values of cell viability ± SEM. Note. HIF-1 α: Hypoxia-inducible factor-1α; IGF1R: Insulin-like growth factor-1 receptor; SEM: Standard error of measurement; * $P<0.05$; ** $P<0.01$.

Furthermore, cell viability was time-dependent since 48-hour treatment induced more apoptosis than 24-hour treatment. However, it should be noted that finding the mechanism by which this therapeutic strategy causes cell death is significant, which was not investigated in this study and should be explored in future studies.

Similarly, our findings were consistent with previous studies on the role of IGF1R and HIF-1α inhibition. For example, Durfort et al found that silencing IGF1R using 2′-O-methyl-modified siRNAs blocks the cell cycle and reduces cell proliferation in murine breast cancer cell lines.[28] Subramani et al showed that blocking IGF1R inhibits growth and metastasis in pancreatic cancer cell lines.[29] Moreover, Hajizadeh et al demonstrated that silencing of HIF-1α /CD73 markedly decreases the expression of CD73 and HIF-1α levels, cell growth, and angiogenesis in cancer cells.[20] The inhibition of HIF-1α by cationic mixed micellar nanoparticles containing anti-HIF-1α siRNA also suppressed proliferation, angiogenesis, and migration in PC3 prostate cancer cells. It also makes cancer cells more sensitive to doxorubicin *in vitro* and *in vivo*.[30]

Conclusion

In sum, our results indicate that blocking IGF1R and HIF-1 causes an additive anti-tumor response and inhibits tumor cell growth and progression of breast and colon cancer cells.

Ethics statement

The Ethics Committee of Tabriz University of Medical Sciences has approved this study (Ethics No. IR.TBZMED.VCR.REC.1401.256).

Disclosure of funding source

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Conflict of interests declaration

There is nothing to declare.

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Data availability statement

The data supporting this study's findings are available on request from the corresponding author following the reasonable request.

Author contributions

Conceptualization: Shiva Abolhasani, Sepideh Javadi, Hadi Hassannia, Farhad Jadidi-Niaragh
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Writing–review & editing: Ghasem Ghalamfarsa, Mohammad Hojjat-Farsangi, Farhad Jadidi-Niaragh.

Consent for publication

I hereby provide consent for the publication of this article, including any accompanying images or data contained within the manuscript.

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