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Hypoxia-inducible Factor 1-α: A Key Regulator of Immune Checkpoint Receptors in Acute Myeloid Leukemia Cell Lines

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ABSTRACT

The Warburg effect is one of the most important metabolic alterations in tumor cells. Hypoxia-inducible factor 1-alpha (HIF-1α) targets a broad range of gene promoters in normoxic and hypoxic conditions in cancers. Herein, we investigate the effects of HIF-1α inhibition on cell viability and messenger RNA (mRNA) expression of immune checkpoint receptors (ICRs) in acute myeloid leukemia cell lines.

K-562 and HL-60 cells were treated with silibinin as an HIF-1α inhibitor. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, followed by quantification of V-domain immunoglobulin suppressor of T-cell activation (VISTA), T-cell immunoglobulin and mucin domain 3 (TIM3), and Galectin-9 mRNA expression via quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

The expression levels of VISTA, TIM3, and Galectin-9 decreased after silibinin treatment within both K-562 and HL-60 cells; however, there were some disparities in gene expression levels between the two cell lines. VISTA and TIM3 expression were reduced by approximately 70% in K-562 at the 40% inhibitory concentration (IC40), while no significant changes were observed in HL-60 cells. Conversely, Galectin-9 expression was decreased significantly at both the IC30 and IC40 in HL-60, whereas it was almost consistent in K-562 cells.

Collectively, we have shown that silibinin could serve as a cytotoxic small-molecule inhibitor and regulate the expression of ICRs, potentially counteracting T-cell exhaustion.

Keywords: Acute myeloid leukemia; Immune checkpoint proteins; Hypoxia-inducible factor 1 alpha subunit; Silymarin

INTRODUCTION

Acute Myeloid Leukemia (AML) is a hematopoietic malignancy originating from myeloid cells. It predominantly affects adults and is associated with significant health risks and a high mortality rate.[1] Although immunotherapy has made its way into clinical practices, chemotherapy remains the primary treatment option. However, cancer cells have developed various strategies to evade the immune system and weaken its

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ability to respond effectively.[2] A primary mechanism of immune evasion in cancer cells is the Warburg effect, also known as aerobic glycolysis. This process causes cancer cells to take up large amounts of glucose and produce high levels of lactate, regardless of whether oxygen is available.[3] It would cause extracellular acidification, leading to adverse effects on immune cells, including T-cell exhaustion.[4] Hypoxia-inducible factor 1-alpha (HIF-1α) is a transcription factor that influences a wide array of genes, driving changes in metabolism, immune evasion, tumor metastasis, and angiogenesis in solid tumors.[5] Through various pathways, HIF-1α also plays a role in causing T-cell exhaustion, which undermines effective immune responses. Firstly, HIF-1α can initiate the Warburg effect by activating and increasing the expression of the lactate dehydrogenase A (*LDH-A*) gene. This gene plays a key role in lactate production, contributing to the metabolic reprogramming observed in cancer cells.[6] Lactate is an oncometabolite secreted from cancer cells into the tumor microenvironment (TME), which leads to extracellular acidification.[7] Secondly, HIF-1α can enhance the expression of immune checkpoints on cancer cells, allowing them to achieve immune evasion. Specifically, HIF-1α can directly overexpress V-domain immunoglobulin suppressor of T cell activation (VISTA) and T-cell immunoglobulin and mucin domain 3 (TIM3), the two main immune checkpoint receptors (ICRs), by binding to the conserved hypoxia response element in its promoter region within myeloid cells, further contributing to immune suppression.[8,9] Furthermore, it could indirectly overexpress another important ICR, Galectin-9, via transforming growth factor-β1 (TGF-β1) and Smad signaling pathways in human cancer cells.[10] All mentioned ICRs are strongly involved in the induction of T-cell exhaustion.[11]

Several studies have shown the roles of HIF-1α in the metabolism and downstream genes of solid tumors,[12,13] but further research is needed to elucidate their roles in hematological malignancies. Therefore, this study aimed to investigate the effect of HIF-1α on gene expression to shed some light on potential remarking mechanisms.

MATERIALS AND METHODS

Cell lines and reagents

K-562 and HL-60 cells, as AML cells, were purchased from the Pasteur Institute of Iran (Tehran, Iran). Cells were cultured in RPMI-1640 medium (Biosera, France) supplemented with 10% heat-inactivated fetal bovine serum (Biosera, France), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Biosera, France). Silibinin, as an HIF-1α inhibitor, was purchased from Sigma-Aldrich (Massachusetts, the US) and dissolved in dimethyl sulfoxide (DMSO) and stored frozen in aliquots. Doxorubicin, as an approved chemotherapy drug, was purchased from Sobhan Oncology (Tehran, Iran).

Cell Viability Assay

The cell viability assay and 50% inhibitory concentration (IC50) value measurements were done in our previous study,[14] and a summary of them is presented below. The viability of leukemic cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. K-562 (5×10^3/well) and HL-60 (1×10^4/well) were seeded in 96-well culture plates and treated with a serial dilution of different concentrations of silibinin and then incubated at 37 °C for 24, 48, and 72 hours, and then the most suitable one, 48 hours, was selected. Silibinin was used at concentrations of 400–1600 µM for both cell lines. Also, doxorubicin was used as a chemotherapy drug, at concentrations of 156.2–10 000 nM and 4.68–150 nM for K-562 and HL-60 cells, respectively. All experiments were done in triplicate. Following incubation, the MTT reagent (Sigma-Aldrich, Burlington, Massachusetts, the US) was added to each well at a final concentration of 0.5 mg/mL. After incubation for 4 hours at 37°C, the microplates were centrifuged at 300*g* for 10 minutes, the supernatants were discarded, and 150 µL of DMSO (Merck, Darmstadt, Germany) was added to each well. Crystals of formazan were dissolved through shaking of microplates, and absorbance was measured using a microplate spectrophotometer (Synergy H1 BioTek, Winooski, US) at 570 nm in a 720 nm. Determination of cell viability was calculated as follows: percentage of cell viability=(absorbance Sample – absorbance blank)/(absorbance control–absorbance blank)×100%. Culture medium without cells was considered as blank, and HL-60 cells cultured without any drugs (untreated cells) were defined as control. Furthermore, IC50 values were measured using GraphPad Prism 10 software. IC50 values were 1127 and 811.1 µM for K-562 and HL-60 cells, respectively. For K-562 cells, we utilized concentrations of 901.6 µM and 676.2 µM as the 40%

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inhibitory concentration (IC40) and 30% inhibitory concentration (IC30), respectively. For HL-60 cells, the IC40 and IC30 values were 648.8 μM and 486.6 μM, respectively.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

K-562 and HL-60 cells were seeded in 6-well cell culture plates at 5×10^5/well and then treated with IC30 and IC40 concentrations of silibinin for 48 hours. Treatments were done in triplicate. Total RNA was isolated using an RNA Isolation Kit (FAVORGEN Biotech Corp, Ping-Tung, Taiwan) based on the manufacturer's protocol. The quantity and quality of extracted RNA were checked and affirmed using spectrophotometry and RNA electrophoresis, respectively. Then, the complementary DNA (cDNA) was synthesized using the RNA reverse transcription using a cDNA Synthesis Kit (Yekta Tajhiz Azma, Tehran, Iran) based on the manufacturer's protocol. To evaluate relative messenger RNA (mRNA) expression of *VISTA*, *TIM3*, and *Galectin-9*, primers were designed using the CLC Genomics workbench and AlleleID software (Table 1, Supplementary Materials), and their efficiencies were confirmed using the LinregPCR software. qRT-PCR was carried out using the Real Q Plus 2x Master Mix (High Rox, Ampliqon, Denmark) on a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, US). After being normalized to hypoxanthine phosphoribosyl transferase 1 (*HPRT*),[15] relative expression levels of *VISTA*, *TIM3*, and *Galectin-9* were determined using the Pfaffl method.[16]

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 10 software. Quantitative data are expressed as mean ± standard error of the mean (SEM). Analysis was performed using the Shapiro-Wilk test to determine the normal distribution of the obtained data, and one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test for multiple comparisons. *p* values less than 0.05 were considered statistically significant.

RESULTS

Silibinin causes inhibitory effects on viability of K-562 and HL-60 cells

The effect of HIF-1α inhibition on the viability of the AML cells was determined using the MTT assay after 48 hours of treatment with various concentrations of silibinin. Subsequently, IC50 concentrations were determined based on the results. As illustrated in Figure 1, the cells exhibited a dose-dependent decrease in viability when treated with silibinin and doxorubicin.[14]

AML cell lines exhibit distinct responses to HIF-1α inhibition in mRNA expression level

ICRs play an important role in cancer evasion and T-cell exhaustion. To reveal the effects of HIF-1α inhibition on the expression of ICRs, we treated the cells with IC30 and IC40 concentrations of silibinin and then evaluated the relative mRNA expression of *VISTA*, *TIM3*, and *Galectin-9* using qRT-PCR. We used IC30 and IC40 rather than IC50 concentrations for gene expression evaluation, as both our previous study[14] and others' work[17] suggested that while IC50 concentrations can kill 50% of cells, they may potentially introduce confounding effects on gene expression. As shown in Figure 1A, both IC30 and IC40 concentrations of silibinin significantly decreased *VISTA* mRNA expression in K-562 cells compared to control cells. Although the IC40 concentration of silibinin reduced *TIM3* mRNA expression, no significant effect was observed at the IC30 concentration. Results also showed that silibinin had no significant effect on *Galectin-9* mRNA expression in K-562 compared to control cells (Figure 1A). In HL-60 cells, both IC30 and IC40 concentrations of silibinin significantly reduced *Galectin-9* expression while having no significant effect on *VISTA* or *TIM3* expression (Figure 1B).

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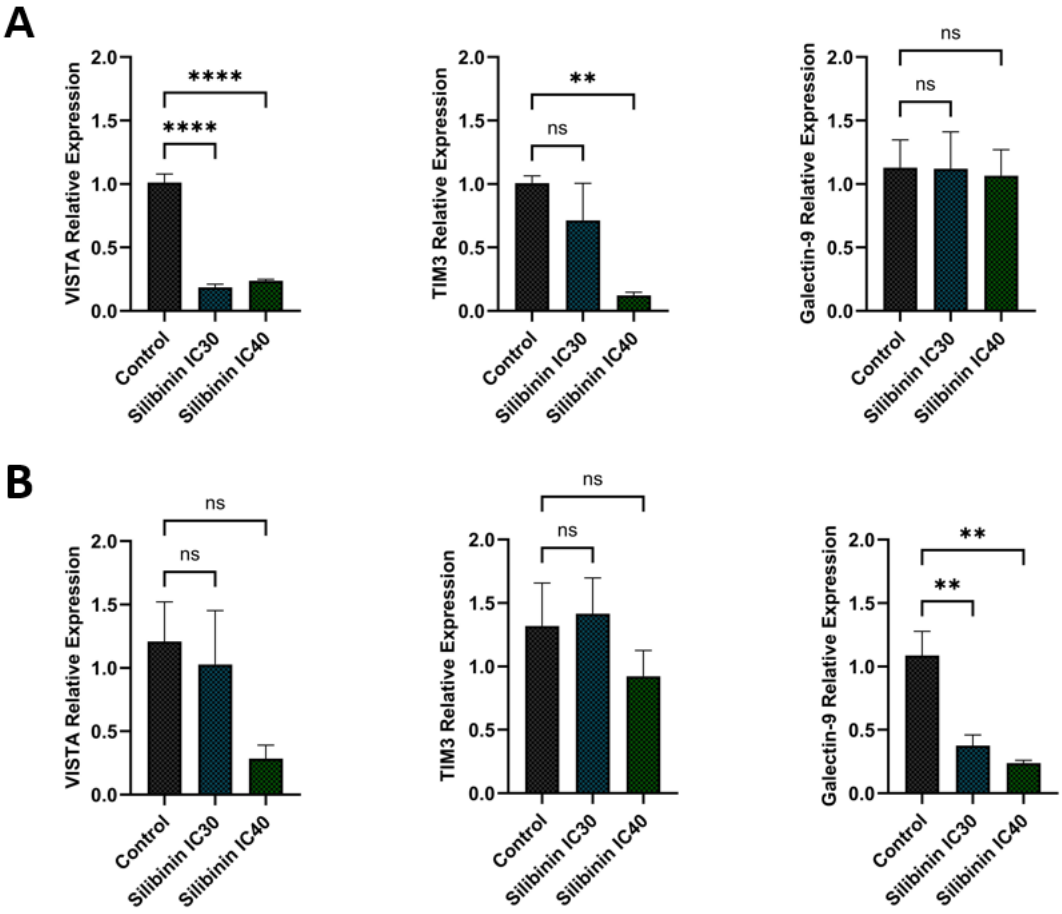

Figure 1. Effects of silibinin on *VISTA*, *TIM3*, and *Galectin-9* mRNA expression levels in treated acute myeloid leukemia cell lines. A. K-562 cells. B. HL-60 cells were treated with IC30 and IC40 concentrations of silibinin for 48 hours. Relative mRNA expression of *VISTA*, *TIM3*, and *Galectin-9* was then measured using qRT-PCR. Treatments at IC30 and IC40 concentrations were performed in triplicate, while qRT-PCR was conducted in duplicate. Untreated cells were used as a control for comparison. Differences between groups were analyzed using the one-way ANOVA test (Dunnett post hoc). Quantitative data are expressed as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Abbreviations: ANOVA: analysis of variance; IC: inhibitory concentration; ns: non-significant; qRT-PCR: quantitative reverse-transcription polymerase chain reaction; SEM: standard error of the mean; TIM3: T-cell immunoglobulin and mucin domain 3; VISTA: V-domain immunoglobulin suppressor of T cell activation.

DISCUSSION

ICRs expressed on T-cells, along with their ligands on cancer cells, contribute to immune evasion and T-cell exhaustion.[18] In recent years, immunotherapy employing monoclonal antibodies targeting ICRs, such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), has shown success. However, certain cancers remain unresponsive to this treatment, posing a significant challenge.[19] Some ICRs, such as VISTA, TIM3, and Galectin-9, are expressed on both cancer and T-cells, making them potential immunotherapy targets.

The activation of HIF-1α under hypoxic conditions is strongly associated with the development of chemoresistance in AML. HIF-1α facilitates leukemic cell survival by upregulating anti-apoptotic factors and promoting DNA repair processes, thereby enabling resistance to chemotherapy-induced cytotoxicity. Targeting HIF-1α can disrupt these protective mechanisms, thereby enhancing the susceptibility of AML cells to chemotherapeutic drugs, such as cytarabine and daunorubicin.[20]

VISTA and TIM3 act as receptors of Galectin-9 on T-cells, suggesting an autocrine mechanism in T-cell exhaustion.[21] It has been shown that TIM3/Galectin-9

interaction enhances the self-renewal of AML cells, driving the progression of the disease.[22] Moreover, HIF-1α is activated in cancer cells under both normoxic and hypoxic conditions, leading to increased mRNA expression of ICRs through direct binding to their gene promoters.[8-10] Therefore, in this study, we explored the association between HIF-1α and the changes in the mRNA expression of ICRs in human AML cell lines.

Silibinin is a highly-specific small molecule inhibitor that inhibits HIF-1α through suppression of protein translation. We first demonstrated that HIF-1α inhibition could remarkably decrease the viability of K-562 and HL-60 cell lines in a dose-dependent manner following a 48-hour treatment.[14]

Likewise, silibinin has been reported to reduce the viability of the HT-29 cell line, a colorectal cancer cell line, in a dose-dependent manner after a 24-hour treatment.[23] Moreover, the viability of MCF-7, MDA-MB-231, and MDA-MB-468 mammospheres was reduced following a 72-hour treatment with silibinin.[24] These results indicate that HIF-1α inhibition would impair cell proliferation and viability in both solid and hematopoietic cancers, emphasizing the critical role of HIF-1α in cancer progression.

Having understood the significant impact of HIF-1α on cancer progression, the next step was to evaluate its effect on targeting the mRNA expression of ICRs. In the K-562 cell line, a 48-hour treatment with silibinin at both IC30 and IC40 concentrations significantly reduced *VISTA* mRNA expression. Our findings align well with another study, which demonstrated that HIF-1α enhances *VISTA* expression through binding to a conserved hypoxia response element in the *VISTA* promoter in human myeloid cells.[8] However, in the HL-60 cell line, a nonsignificant decrease in mRNA expression was observed. Regarding *TIM3* expression, while the IC30 concentration of silibinin had no significant effect, the IC40 concentration significantly reduced *TIM3* mRNA levels in the K-562 cell line. Similarly, it has been shown that HIF-1α could distinctively increase the expression of TIM3 in hypoxic brain regions of a mouse cerebral hypoxia-ischaemia (H/I) model.[9] Conversely, silibinin could not alter the mRNA expression of *TIM3* in the HL-60 cell line. Finally, although silibinin treatment at both IC30 and IC40 concentrations did not alter *Galectin-9* mRNA expression in K-562 cells, both concentrations significantly reduced *Galectin-9* expression in HL-60 cells. Concurrently, a previous study showed increased expression of TIM3 and Galectin-9 in human AML cells, with TIM3 facilitating Galectin-9 secretion through a trafficking mechanism.[25]

Studies have demonstrated that the transcription factors HIF-1 and the activator protein 1 (AP-1) increase TGF-β1 expression. Through autocrine signaling, elevated TGF-β1 activates Smad3, which in turn induces the expression of Galectin-9.[10] This regulatory axis has been observed in both embryonic cells and malignant cells, particularly in breast cancer, colorectal cancer, and AML. The results of our study were in line with those of previous studies, with HIF-1α inhibition significantly reducing mRNA expression of *Galectin-9* in HL-60 cells, although the results were not significant in K-562 cells.

The results of this study show the inconsistency of gene expression between the two cell lines. The inconsistency of the gene expression pattern could be due to the differences in the genetic cell line background. The protein expression of VISTA, Galectin-9, and TIM3 was checked in the Human Protein Atlas database. Data showed that there are disparities in immune checkpoints' gene and protein expression in different AML cell lines, including K-562 and HL-60 cells. This might be due to the genetic background of the cell lines.

This study has some limitations, including in vitro mRNA-level data, and not assessing functional protein assays. It is hoped that future studies will continue research, conducting validation in primary AML samples, in vivo studies, and coculture assays with T cells.

In conclusion, our findings demonstrate that treatment with silibinin, a highly specific inhibitor of HIF-1α, significantly reduced *VISTA* and *TIM3* mRNA expression in the K-562 cell line. Additionally, *Galectin-9* mRNA expression was remarkably reduced in the HL-60 cell line. These results suggest VISTA, TIM3, and Galectin-9 as promising targets for immune checkpoint blockade therapy in AML; however, further studies in animal models are needed to confirm these results.

STATEMENT OF ETHICS

Ethics code: IR.MAZUMS.REC.1403.19943.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

Upon reasonable request from the corresponding author.

AI ASSISTANCE DISCLOSURE

Microsoft Copilot was used to improve clarity and correct grammar in some sentences. The authors retained full control over the content, ensuring that the original ideas and arguments were preserved without alteration.

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