IJPSR (2023), Volume 14, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 August 2022; received in revised form, 29 March 2023; accepted 18 April 2023; published 01 May 2023

EFFECT OF QUERCETIN ON HYPOXIA-REGULATED METASTATIC MARKERS IN AGGRESSIVE PANCREATIC CANCER CELLS UNDER HYPOXIC CONDITIONS

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Keywords:

Quercetin, HIF1α, E- cadherins, PC 1, N cadherin, Vimentin, Cytokeratin

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ABSTRACT: Pancreatic cancer is an intractable and rare malignancy ranked 12th in incidence and 7th in mortality. It is the leading cause of cancer deaths in developed countries and is rising in developing countries like India. According to GLOBOCAN 2020 statistics, pancreatic cancer has ranked as the 11th most common malignancy in the world and accounts for 495773 new cases and caused 466003 deaths in 2020. Tumor hypoxia is an important pathological condition that influences several signaling cascades in malignant cells, eventually leading to therapy resistance. Several natural bioflavonoids hold promise as anti-cancer agents. However, the effect of Quercetin on hypoxic pancreatic cancer cells is unknown. We analyze the effect of Quercetin on expression levels of metastatic markers HIF1α and E-cadherin under hypoxic conditions. Quercetin treatment was significantly correlated with reduced HIF1α expression levels and elevated E-cadherin levels. Our results suggest that Quercetin may alter E-cadherin levels by regulating HIF1α expression. Quercetin may inhibit pancreatic cancer cell metastasis by regulating metastasis genes in a hypoxic microenvironment and has pharmacological potential in aggressive pancreatic cancer treatment.

INTRODUCTION: Pancreatic cancer is an intractable and rare malignancy ranked 12th in incidence and 7th in mortality. It is the leading cause of cancer deaths in developed countries and is rising in developing countries like India. According to GLOBOCAN 2020 statistics, pancreatic cancer has ranked the 11th most common malignancy in the world and accounts for 495773 new cases and caused 466003 deaths in 2020 [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(5).2343-46

This article can be accessed online on www.ijpsr.com

DOI link: http://doi.org/10.13040/IJPSR.0975-8232.14(5).2343-46

The incidence and mortality of pancreatic cancer have been associated with age and are slightly more common in men than in females. Several risk factors have been found to be associated with the development of pancreatic cancer. Among lifestyle habits, consumption of alcohol and tobacco abuse are the common causes of malignancy development [2, 3].

Despite advanced therapeutic strategies applied for pancreatic cancer treatment, the outcome of the therapies is moderate, which might be due to the complex histology and tumor microenvironment of the pancreas. It processes extracellular matrix proteins and non-neoplastic cells like fibroblastic, vascular, and immune cells. Recent studies have reported that the stroma of the pancreas supports tumor cell growth, promotes cancer cell

dissemination, and simultaneously acts as a physical barrier to drug delivery. Moreover, a tumor hypoxic microenvironment that arises due to low oxygen supply to a growing tumor may alter the pathophysiological functions of several genes. Hypoxic tumor microenvironment may contribute to tumor progression, metastasis, and chemo/radiotherapy resistance [4]. Several natural plant products have shown anti-cancer properties influencing tumor cell proliferation and metastasis. Among several naturally occurring compounds, flavonoids are thought to be promising therapeutic agents against human malignancies. Quercetin (3, 3′,4′,5,7- pentahydroxy flavone) is one of the important flavonoids shown to exhibit anti-cancer properties by influencing several intracellular pathways [5]. Quercetin has been reported to inhibit several cell signaling components in cancer cells, including PI3K/Akt/mTOR, GSK-3β, NFκB, and heat shock protein 70 (HSP70). However, the effect of Quercetin on the regulation of metastatic markers in a hypoxic microenvironment is obscure [5]. In our study, we aimed to analyze the effect of Quercetin on the expression of key metastatic regulators such as HIF-1α (Hypoxia-inducible factor 1 alpha), a master regulator of the hypoxic microenvironment, and E- Cadherin under the chemical induction of hypoxia and Quercetin treatment.

MATERIALS AND METHODS: To study the effect of chemical induction of hypoxia and Quercetin treatment on expression levels of metastatic markers (HIF1α and E cadherin) we have selected one aggressive pancreatic cell line AsPC1. Cell lines were purchased from NCCS, Pune and cultured using MEBM/DMEM+10%FBS medium. Subcultures and passages were performed as per standard protocols [6].

Optimization of CoCl2 and Quercetin Treatments on Cell Lines: Various concentrations of CoCl2 (100 µM, 200µM and, 300µM) were prepared. Two thousand cells were seeded in each well along with 100 µl of culture media in a 96-well plate. The experiment was performed in triplicates with three different concentrations of CoCl2 at 24 h, 48 h and, 72 h to determine the IC_{50} value of $CoCl_2$ on the cell line. Cells without $CoCl_2$ were used as a negative control. Cells from different exposures of $CoCl_2$ were subjected to an

MTT assay to calculate the rate of proliferation. 200µM $CoCl_2$ concentration conveyed a significantly reduced cell proliferation rate in comparison with control cells. Three different concentrations of Quercetin (3 µM, 12 µM, 48 µM) were prepared. Each concentration was applied on cell lines at different time intervals (0, 24, 48, 72 hours).RNA was isolated from treated cell lines using the Trizol method and subjected to cDNA conversion. Expression analyses of HIF1α and E-Cadherin were assessed using Real-Time PCR (ABI7500) with Sybr green method. Each experiment was carried out in triplicate and Beta-actin was used as an endogenous control.

RESULTS AND DISCUSSION:

HIF1α and E Cadherin Expression in Control and Hypoxia-Induced as PC1 Cell Line: $CoCl_2$ treated cell line differed significantly concerning HIF1α levels (1.08±0.01) compared. To the control (Un treated cell line (0.98±0.004) even before chemical induction of hypoxia. When both cell lines were exposed to hypoxic conditions, HIF1α levels were significantly elevated as the duration of hypoxic exposure increased **Table 1**.

TABLE 1: HIF1A EXPRESSION IN CONTROL AND HYPOXIA-INDUCED AS PC1 CELL LINE

Duration of exposure (Hours)	Control cell line X ±S. D	d	Treated cell line X ±S. D	d	t	p-value
0	0.98±0.004		1.08±0.01		5.59	0..008**
24	1.118±0.06	0.14	1.20±0.002	0.12	6.13	0.009**
48	1.51±0.007	0.44	1.61±0.002	0.41	3.89	0.01*
72	1.69±0.008	0.15	1.85±0.006	0.24	3.12	0.02*
F test two way * p<0.05, **p<0.001	Between cell lines** Between durations**					

However, the elevation was more prominent after 48 hrs of induction. Before hypoxic induction, E cadherin levels were significantly elevated in the control cell line (1.55±0.01) compared to the

treated cell line (1.41±0.01). As the duration of hypoxic exposure increased, the E cadherin levels gradually decreased in both the treated and control cell lines. The comparison between treated and control cell lines indicated that response was maximum after 24 hrs in the normal cell line but was after 72 hrs in tumor cell line **Table 2.**

TABLE 2: E CADHERIN EXPRESSION IN CONTROL AND HYPOXIA-INDUCED AS PC1 CELL LINE

Duration of exposure (Hours)	Control cell line X ±S. D	d	Treated cell line X ±S. D	d	t	p-value
0	1.55±0.01		1.41±0.001		4.74	0..006**
24	1.11±0.002	0.42	1.05±0.006	0.07	3.05	0.005**
48	0.71±0.003	0.31	0.54±0.005	0.30	2.67	0.01*
72	0.79±0.002	0.41	0.41±0.002	0.43	2.09	0.03*
F test two way, * p<0.05, **p<0.001	Between cell lines**, Between durations**					

HIF1α and E Cadherin Expressions in Quercetin are Treated as PC1 Cell Line: HIF1α levels were significantly decreased in both cell lines during 48 hours and 72 hours of quercetin exposure at 3µM concentrations. On 24 hours of treatment, both cell lines did not show a significant decrease regarding HIF1α levels Fig. 1. However, HIF1α levels steeply declined in the normal cell lines compared to the tumor cell lines at 3µM quercetin concentrations during 48 hours, 72 hours of exposure. E cadherin levels were gradually increased as the duration of quercetin exposure increased in cancer cell lines. E cadherin levels were significantly different between the two cell lines. However, E cadherin levels were significantly increased at 3 µM concentration for 24, 48, 72 hours of exposure in normal cell lines. Other concentrations did not show exerted influence concerning E cadherin levels in 24, 48 and 72 hours **Fig. 2.**

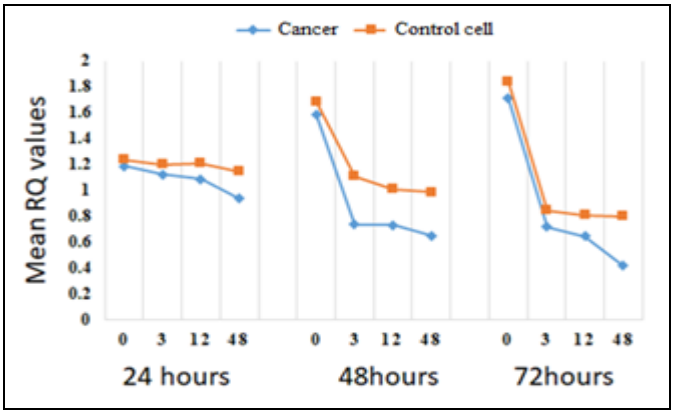

FIG. 1: 3 HIF1A EXPRESSION IN QUERCETIN TREATED AS PC1 CELL LINE

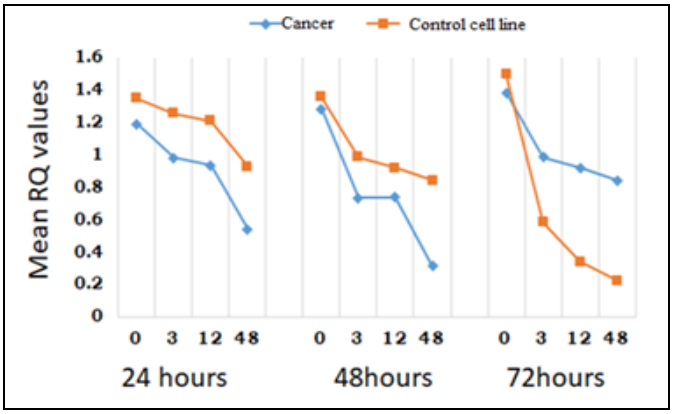

FIG. 2: E CADHERIN EXPRESSION IN QUERCETIN TREATED AS PC1 CELL LINE

CONCLUSION: Hypoxia or low oxygen concentration is a salient feature of solid tumors. Under a hypoxic microenvironment, tumor cells are deficient in oxygen and nutrients due to impaired vasculature which fails to supply adequate oxygen/nutrients to the growing tumor cells. The hypoxic microenvironment regulates several cell signaling pathways by regulating crucial genes that play an important role in angiogenesis, metastasis, and proliferation. HIF-1α (Hypoxia-inducible factor 1 alpha), is a transcription factor that regulates nearly 150 genes that influence tumor cell development and progression. HIF-1 α levels are increased in a hypoxic microenvironment which further promotes angiogenesis. Therefore, tumor cells get oxygen and nutrients for growing cells. Increased expression of HIF-1 α has been associated with elevated microvessel density and aggressive tumor phenotype [7]. HIF-1 α gene mediates the E cadherin gene function and influences cancer cell invasion/ mobility [6]. Hypoxic tumor cells resist chemotherapeutic agents, resulting in poor outcomes for patients [4]. Several natural plant components may potentially

affect cancer cells and a favorable outcome. Quercetin is a versatile molecule with many pharmacological properties including antioxidant, neurological, antiviral, anticancer, cardiovascular, antimicrobial, anti-inflammatory, Hepatoprotective, and anti-obesity agents. Earlier studies reported that quercetin might influence HIF1a activity [8].

Our study observed that HIF-1 α expression levels significantly decreased under Quercetin treatment in chemically induced hypoxic pancreatic cell lines. The levels of HIF-1 α decreased as the concentration and duration of Quercetin increased. Our results followed previous reports where HIF-1 α levels have been shown to be decreased upon Quercetin treatment in LNCaP prostate cancer cells, CX-1 colon cancer cells, and SkBr3 breast cancer cells [8]. Further, expression levels of the E-cadherin gene were significantly elevated in Quercetin-treated pancreatic cells. Previously it was shown that Quercetin can inhibit EMT by increasing E-cadherin expression and decreasing the N-cadherin, Vimentin, and Snail protein family in many cancers [9]. Decreased HIF-1 α levels might result in the accumulation of E-cadherin in chemically induced hypoxic cells. Zhu *et al.* reported that HIF 1α inhibition by HIF 1α Homo 1216 siRNA transfection repressed hypoxia-induced HIF 1α, RGC 32, N cadherin, and vimentin, but elevated the levels of E cadherin and cytokeratin.

In conclusion, Quercetin treatment is significantly associated with decreased levels of HIF-1 α and elevated E-cadherin expression in aggressive pancreatic cell lines, suggesting that Quercetin might have a potential pharmacological application to treat aggressive pancreatic cancer.

ACKNOWLEDGEMENT: This work was supported by Vimta Labs.

CONFLICTS OF INTEREST: All the authors declare no conflicts of interest.

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How to cite this article:
Meka PB, Ahmed H, Unissa H, Tahera H, Waheed MA, Sadiq M, Afreen S and Begum N: Effect of quercetin on hypoxia regulated metastatic markers in aggressive pancreatic cancer cells under hypoxic conditions. Int J Pharm Sci & Res 2023; 14(5): 2343-46. doi: 10.13040/IJPSR.0975-8232.14(5).2343-46.