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The combined effect of IL-6 and hypoxia increases KLK4 gene expression in colon cancer cells via STAT-3 activation

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Received: 28 January 2025 / Accepted: 27 October 2025 / Published online: 07 November 2025

Abstract
The aggressive phenotype of colorectal cancer (CRC) is largely driven by interactions within the tumor microenvironment, specifically the co-occurrence of inflammation and hypoxic stress. While the pro-metastatic enzyme Kallikrein-related Peptidase 4 (KLK4) is known to contribute to dissemination, the precise molecular mechanism by which IL-6 and hypoxia converge to regulate KLK4 expression and subsequent metastatic potential remains to be elucidated. This study investigated the influence of the IL-6 cytokine on KLK4 gene expression and metastatic potential in the HT-29 colon cancer cell line under both normal and hypoxic conditions. Healthy non-cancerous endothelial cells (HUVEC) served as a comparative control. Expression was assessed via Real-Time PCR (mRNA) and Western blot (protein), while metastatic potential was determined by the scratch assay. Our findings demonstrate a substantial and marked upregulation in KLK4 gene and protein expression in HT-29 cells over a 48-hour period in response to IL-6, hypoxia, and the combined treatments. This increase in KLK4 was found to be associated with simultaneous upregulation of STAT-3 and p-STAT-3 proteins, strongly suggesting that the STAT-3 signaling pathway mediates this induction. The effects observed were tumor-specific: the non-cancerous HUVEC line showed only transient KLK4 changes and decreased proliferation in individual treatments. In sharp contrast, the combined IL-6 and hypoxia treatments significantly enhanced proliferative activity and metastatic potential in HT-29 cells. Western blot analysis collectively indicates that the augmented KLK4 expression in CRC cells is likely mediated through IL-6 and hypoxia-induced STAT-3 activation. These findings establish KLK4 as a potential downstream effector of the IL-6/STAT-3 pathway, offering a novel therapeutic target for mitigating metastatic potential in colon cancer.

Keywords KLK4 · IL-6 · Hypoxia · Colon cancer · HUVEC · STAT-3

Introduction

The second most frequent cancer in women and the third most prevalent in men is colon cancer (Katsaounou et al. 2022). The burden of colorectal cancer is expected to rise to 3.2 million new cases year (63% increase) and 1.6 million deaths annually (73% increase) by 2040, according to the World Health Organization (Morgan et al. 2023). Examining circulating cytokines and cytokine receptors, which are of significant interest as possible

diagnostic and prognostic markers in cancer patients, is one way to better understand the biology of colon cancer. An important cytokine in colon cancer is interleukin-6 (IL-6), with its pleiotropic properties. Recent studies have shown that colorectal cancer (CRC) is regulated by IL-6, that serum IL-6 levels of CRC patients are increased, and that this increase is correlated with tumor size. Serum levels of tumor necrosis factor (TNF), ınterleukin-8 (IL-8), ınterleukin-6 (IL-6), and vascular endothelial growth factor (VEGF) provide important findings about the clinical prognosis of CRC (Kaminska et al. 2005). IL-6 induces anchorage-independent growth of some colorectal carcinoma cells, and in addition, IL-6 has been shown to prevent Fas-mediated apoptosis of CRC cells through activation of Bcl-xL expression (Schneider et al. 2000).

IL-6, known as B cell differentiation factor, is a cytokine with multiple functions regulating hematopoiesis, immune response, and inflammation. It also plays a role in regulating cellular functions such as proliferation, angiogenesis, apoptosis, and differentiation (Azevedo et al. 2011). It is produced by many different cells and has a receptor system that affects many different cells. Many cells in the tumor microenvironment, such as stromal cells, epithelial oncogenic cells and immune cells can secrete IL-6 (Unver and McAllister 2018). While it plays a role in the proliferation and differentiation of malignant cells, it is expressed at high levels in many types of cancer, especially colorectal, breast, prostate, ovarian, pancreatic, lung and cervical cancers, renal cell carcinoma, and multiple myeloma (Babacan et al. 2023; Singh et al. 2015). Studies on lung cancer have shown that IL-6 levels are higher in human non-small cell lung cancer (NSCLC) cells, particularly those with squamous cell histology, compared to adenocarcinoma histology (Song et al. 2014). Another study assessing serum interleukin-6 (IL-6) levels using an enzyme-linked immunosorbent assay in a group of patients with advanced non-small cell lung cancer (NSCLC) found higher IL-6 concentrations than in controls. Tumor progression was associated with increased IL-6 levels (Ferdinando et al. 1998). In a study of 164 colorectal cancer patients, IL-6 levels were significantly higher in colorectal cancer patients compared with normal controls. High serum IL-6 levels (> 12 pg/ml) were associated with larger tumor size, higher serum CRP levels, and liver metastases. IL-6 levels were also reported to increase with stage (Chung and Chang 2003). In a study pooling 50 colorectal cancer tissue samples and 50 matched adjacent mucosa samples, IL-6 expression was found to be significantly higher in CRC tissues compared with non-cancerous tissues, and IL-6 expression was found to be positively correlated with tumor stage (Zeng et al. 2017).

Colon cancer lesions are composed of cancerous and stromal cells; the later includes inflammatory cells, endothelial cells, and bone marrow-derived myofibroblasts (BMFs) and bone marrow-derived myeloid cells, such as myofibroblasts (MFs). All these components create a microenvironment suitable for tumor cell survival and tumor growth in the primary and metastatic areas (Hong et al. 2022; Huang et al. 2022; Liang et al. 2009). Most solid tumors have a hypoxic environment. Under hypoxia, hypoxia inducible factor (HIF) has been shown to be overexpressed in various cancer cells and has been associated with tumor progression and adverse clinical outcome in a large number of different tumoral tissues such as CRC (Xu et al. 2019). Hypoxia plays an important role in cancer by inducing changes in the microenvironment, altering oncogenic genes and metabolism, and inducing blood vessel formation and metastasis (Vadde et al. 2017). Under hypoxic conditions, cancer cells develop escape mechanisms to survive. This feature in tumors is an important factor as it stimulates cancer development, progression, and drug resistance. Given its significant significance in hypoxia-induced drug resistance, angiogenesis, invasiveness, metastasis, cell death resistance, metabolic modification, and genome instability, hypoxia might be regarded as the most validated target (Vadde et al. 2017).

KLK4 (Kallikrein-related peptidase 4), a member of the 15-member serine protease family, shows prostate-specific expression. The KLK4 gene was first cloned from developing dental enamel epithelial cells in pigs using degenerate primers encoding the EMPS1 (Enamel matrix serine protease 1) protein. This gene, regulated by androgens, is expressed in breast cancer, endometrial carcinoma, ovarian carcinoma cell lines, salivary gland, lung, adrenal gland, colon and trachea tissues (Lai et al. 2009) is the expression. The expression of the KLK gene and protein is strongly linked to the prognosis of cancer (Srinivasan et al. 2022). KLK4 mRNA was found to be significant in Dukes stage, histological grade, tumor invasion and size in a study that examined the expression of KLK4 mRNA from the primary tumors of 81 individuals with colorectal adenocarcinoma. According to

reports, KLK4 enhances the capacity of colorectal cancer cells to disseminate and promotes the dissemination of tumor cells (Kontos et al. 2013). Numerous tumors have been discovered to have high expression of HIF, which is linked to tumor development and worse clinical outcomes. Hypoxia has also been shown to induce IL-6 expression and contribute to drug resistance through its effect on the IL-6/STAT-3 signaling pathway. IL-6 levels increase in hypoxic conditions, which has been shown to reduce cleaved caspase-3 levels compared to normoxia by activation of the IL-6/STAT-3/Bcl2 signaling pathway (Xu et al. 2019).

In this study, we aimed to understand the regulatory effect of IL-6, a cytokine associated with drug resistance in colon cancer, on the KLK4 gene which is a potential marker in some cancers. The effect of IL-6 cytokine on the KLK4 gene under normal and hypoxic conditions was evaluated in terms of mRNA, protein, and metastatic potential. Additionally, in our study, the effects of both hypoxia and IL-6 were evaluated in healthy non-cancerous endothelial cells. IL-6 signaling pathway studies were also performed in both cell lines.

Materials and methods

Culturing of cells

HT-29 (colon cancer cell line) and HUVEC (human umbilical vein endothelial cell) used in the study were provided by Prof. Dr. Tuğba Boyueğmez from Çanakkale 18 Mart University, Department of Molecular Biology and Genetics. HT-29 and HUVEC cell lines were grown in DMEM high glucose (EuroClone) medium, 1X antibiotic-antimycotic (EuroClone) medium containing 2mM L-Glutamine and 10% FCS (Gibco) in 75 cm^2 flasks with 5% CO_2 at 37 °C in a humidified environment. IL-6 cytokine was obtained from PeproTech (#200–06). Cells were passaged 2–3 times a week. The grown cells were removed and prepared with trypan blue for cell counting, and live cell counting was performed with a hemocytometer.

MTT assay

MTT (3-(4,5 Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide) method was used to determine the cytotoxic effect of IL-6 cytokine and $CoCl_2$, a chemical hypoxia-inducing agent. Live HT-29 and HUVEC cells identified by trypan blue were transferred to plates containing 96 wells. HT-29 cells were transferred as 15×10^3 cells per well and HUVEC cells were transferred as 5×10^3 cells per well. Experimental groups were created as separate plates for 24 and 48-hour periods. The next day, the medium of the groups to be administered IL-6 cytokine was changed and 0.1% Bovine serum albumin (BSA) was added together with DMEM. Based on prior experiments (Alper et al. 2025; Babacan et al. 2023; Onat et al. 2024), a non-cytotoxic concentration of IL-6 20ng/mL was established across the cell lines and subsequently employed for this study. 20 ng/mL IL-6 cytokine was applied to each well of cells ad-hering to the plate surface in all experimental groups (plates formed for 24 and 48 h), except for the control groups, and 1 h later, 150 µM $CoCl_2$ was applied to the relevant wells (Turkoglu and Kockar 2016). At the end of 24 and 48 h time periods, the plates were coated with 0.5 mg/mL MTT solution (SIGMA M56655-1G) and the cells were kept at 37 °C in an incubator with 5% CO_2. Cells kept in the incubator for 4 h were dissolved with HCl-containing isopropanol. Using a Thermo spectrophotometer, absorbance values were determined at a wavelength of 550 nm. This experiment was carried out in 8 repetitions within itself and 3 independently in each other.

Quantitative RT-PCR (qRT PCR)

After counting the live cells (HT-29 and HUVEC) from the grown cells, 2×10^6 cells were transferred to each 25cm^2 flask. Then, one day later, 20 ng/mL IL-6 and 150 µM $CoCl_2$ were applied to the relevant flasks. At the end of 24 and 48-hour periods, the cells were removed with trypsin EDTA and placed in separate falcons, and the

pellets were isolated following the Thermo Scientific Gene Jet RNA Purification Kit instructions. First, cDNA synthesis was performed from the RNAs obtained. For this purpose, transcriptase (RT) enzyme (Thermo Scientific) was used. Hβ2 (human beta-2-microglobulin) was used as an internal control for PCR amplification. After checking the cDNAs, real-time PCR (Roche) was performed using HIF-1α spe-cific primers for both HIF-1α levels. In our study, to control the chemical hypoxia model, the level of HIF-1α mRNA, which is the main regulator of the hypoxic condi-tion, was controlled with Real-time PCR in the groups with and without $CoCl_2$ cells were used as a control. Thus, it was confirmed whether the chemical hypoxia model was successfully created.In order to determine the effect of $CoCl_2$ and IL-6 effective conditions on KLK4 mRNA expression, qRT-PCR was performed. For this, KLK4-specific primers were used. Hβ2 primers were used for normalization. Primer sequences used; KLK4 forward primer 5'-CACTGGTCATGGAAAACGAATT-3', reverse primer 5'-ATGAGGTCGTTA GCGAGCAAG-3' HIF-1α forward primer 5'-CCACCTATGACCTGCTTGGT-3' reverse primer 5'-TGTCCTG TGGTGACTTGTCC-3' Hβ2 (human beta-2-microglobulin) forward primer 5'-TTTCTGGCCTGGAGGCTAT C-'3 reverse primer 5'-CATGTCTCCATCCCACTTAACT-'3. Real-time PCR was performed on 96-well plates. To create a total volume of 12.5 µL in each well, 6.25 µL SYBR Green PCR Master Mix (LightCycler 480 SSYBR Green I Master Mix), 0.5 µL reverse and forward primer (50 ng/µL), 1 µL cDNA, and 4.25 µL dH_2O was added (Poyrazlı et al. 2024). The LIVAC approach was used to assess the PCR results that acquired (Livak and Schmittgen 2001). KLK4 Ct values obtained as a result of real-time PCR to determine the KLK4 mRNA level were normalized by subtracting the Ct average of Hβ2, which was used as the internal control gene. Study results were confirmed by melting curve analysis. Each value in this study was derived from three independent studies.

Western blot analysis

Western blotting technique was used to determine the effects of IL-6 and $CoCl_2$ on KLK4 protein levels. After the live cells from the grown HT-29 and HUVEC cells were counted with a hemocytometer, they were transferred to 6-well plates as 5×10^5 cells. The next day, the medium of the groups to be administered IL-6 cytokine was changed and 0.1% Bovine serum albumin (BSA) was added together with DMEM. Cells attached to the plates were treated with 20 ng/mL IL-6 cytokine and 150 µM $CoCl_2$. At the end of 24 and 48 h, the experimental groups were collected and isolated by scraping the wells on ice with Ripa Buffer. The Bradford method was used to measure the concentrations of proteins. Proteins were loaded at 35 µg into gels prepared with 10% polyacrylamide. The completed proteins were left to transfer overnight to PVDF (Millipore) membranes. On the following day, membranes were incubated for 1 h in a blocking solution composed of Tris Buffered Saline (TBS) and 5% (w/v) skim milk powder in 0.1% (v/v) Tween-20 to inhibit nonspecific protein binding. Then, it was washed 3 times for 5 minutes each with 1X TBS buffer containing Tween20. After washing, membranes were treated with KLK4 primary antibody (Invitrogen, PA5-72768) overnight at +4 °C. Following three rounds of washing with 1X TBS buffer the following day, they were treated with a secondary antibody (Goat anti-Rabit IgG HRP-conjugated, abcam, AB97051) for 1.5 h at room temperature before being cleaned three times more. Finally, the membranes were injected with Pierce ECL to observe the KLK4 protein (Western Blotting Substrate-Thermo) and visualized on the Fusion FX VILBER LOURMAT device. By stripping on the same membranes, β-actin (A2228- Monoclonal Anti β-actin Mouse, Sc-516102 m-IgGk BP-HRP, sigma-aldrich) protein was also imaged. The amount of KLK4 protein was normalized with the amount of β-Actin protein. Bands were evaluated using the Image J software program, and all values were normalized by dividing by the β-actin values (Poyrazlı et al. 2024). Control groups were divided by themselves to obtain "1" unit. Other groups were divided by the control groups to obtain fold values. Proteins determined using KLK4 and β-actin primary antibodies were analyzed densitometrically in groups treated and not treated with IL-6 and $CoCl_2$. This experiment was performed in duplicate. All these processes were repeated for signaling pathways. Primary antibodies used in signaling pathways: STAT-3 (H-190 Santa Cruz sc-7179), Phospho-STAT-3 (Tyr705, Cell signaling #9145 Rabbit mAb).

Scratch assay

HT-29 and HUVEC cells, for which live cell counting was performed, were transferred to each well of 12-well plates as 2×10^5 cells. The next day, the medium of the groups to be administered IL-6 cytokine was changed and 0.1% Bovine serum albumin (BSA) was added together with DMEM. Cells attached to the plates were treated with 20 ng/mL IL-6 cytokine and 150 µM $CoCl_2$. Then, using a standard 10 µL pipette tip, a cross-shaped scratch was created in the wells. Images were taken from the plates at 0, 24 and 48 h and analyzed with the Image J program (MRI Wound HealingTool). This experiment was carried out in three repetitions.

Statistical analysis

The LIVAC method was used to analyze real-time PCR results. Minitab 14 was used for statistical analysis. $*p \leq 0.05$ was obtained using One Way ANOVA and was considered statistically significant. Western Blot and Scratch Assay analyses were analyzed densitometrically in the ImageJ program.

Results

IL-6 and hypoxia effects on KLK4 gene expression at mRNA level

The effect of IL-6 and $CoCl_2$ on cell viability was determined by MTT test in in colon cancer cell HT-29 and non-cancerous HUVEC cells.The cell viability increased in the IL-6 and hypoxia groups compared to the control group (normoxia group) at 24 and 48 h. However, a decrease in cell viability was observed in the combined groups where IL-6 and hypoxia were applied together compared to the control group (compared to the hypoxia group) in HT-29 cells (Fig. 1A). In HUVEC cells, a statistically significant decrease in cell viability was observed in the IL-6, hypoxia, and combined groups compared to the control group at both 24 and 48 h (Fig. 1B).

HIF-1 α levels were controlled in a chemical hypoxia model. This model has been successfully applied in many of our studies (Türkoğlu and Kockar 2016). Hypoxic conditions were examined throughout the study. There are 24-hour and 48-hour data in our study. Both HIF-1α and KLK4 levels were determined by obtaining cDNA with reverse transcriptase enzyme from RNAs obtained from the same time period. When the HIF-1α mRNA levels in the applied groups were examined in HT-29, a colon cancer cell, and HUVEC cells, our healthy endothelial cell model (non-cancer cell), compared to the control group, the HIF-1α level increased statistically significantly compared to the control group at 24 and 48 h (Fig. 1C, D). Thus, it was confirmed that the chemical hypoxia model was successfully formed.

In colon cancer cell line, IL-6 increased KLK4 mRNA level at both 24 and 48 h compared to the control group. KLK4 expression was increased by IL-6 treatment at 24 and 48 h. Especially at 48 h, KLK4 mRNA level increased statistically significantly in both IL-6, hypoxia and combined groups compared to the control group (Fig. 1E). In the HUVEC cell line, similar to HT-29 cells, IL-6 increased KLK4 mRNA levels at both 24 and 48 h. When hypoxia treatments were examined, KLK4 was upregulated at 24 h in both separate and combined treatments, but at 48 h, only hypoxia and IL-6 were upregulated in separate treatments, and a decrease was observed in the combined 48-hour group (Fig. 1F).

IL-6 and hypoxia effects on KLK4 gene expression at protein level

When the effect of IL-6 on KLK4 protein level in colon cancer was examined, it was seen that it increased similarly to the mRNA level in the 24 and 48 h IL-6 applied groups. In the 48-hour period, a more effective increase was observed in both the separate groups receiving IL-6 and hypoxia and in the combined groups (Fig. 2A, B)

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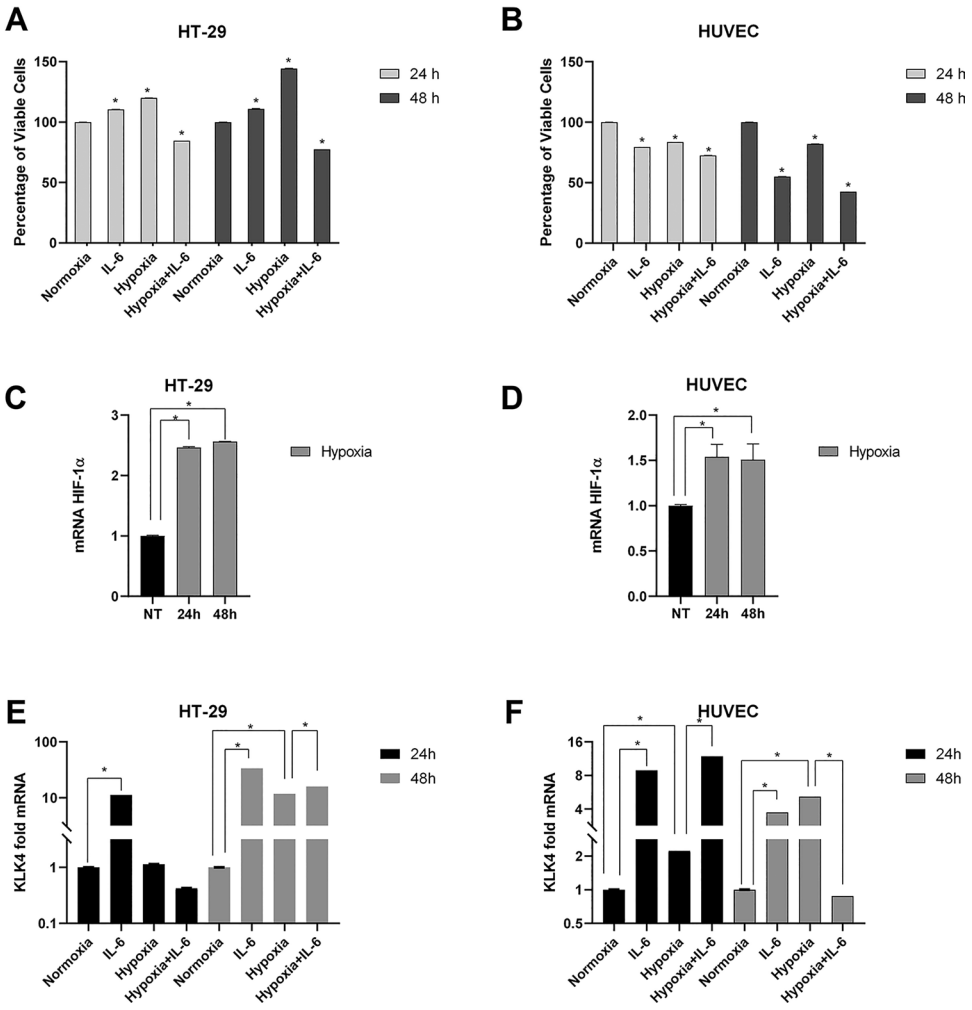

Fig. 1 Effect of IL-6 and $CoCl_2$ on KLK4. **(A)** Effect of IL-6 cytokine and $CoCl_2$ on HT-29% of viable cells. **(B)** Effect of IL-6 cytokine and $CoCl_2$ on HUVEC percentage of viable cells. **(C)** HIF-1α mRNA level in HT-29 cell line. **(D)** HIF-1α mRNA level in HUVEC cell line. **(E)** qRT-PCR result of KLK4 gene in HT-29 cell line. **(F)** qRT-PCR result of KLK4 gene in HUVEC cell line. (* $p \leq 0.05$). In experiments with HT-29 cells, untreated groups of HT-29 cells were used as the control group. In experiments with HUVEC cells, untreated groups of HUVEC cells were used as the control group. (The experiment was performed with 3 repetitions within itself and 3 independent repetitions)

(* $p \leq 0.05$). When the KLK4 protein level was examined in the HUVEC cell line, the highest up-regulation was obtained in the IL-6 and combined IL-6 hypoxia-treated group at 24 h (Fig. 2C, D) (* $p \leq 0.05$).

IL-6 and Hypoxia-Induced KLK4 effective wound healing

The wound healing analyses were examined in HT-29 cells and no significant change was observed in the migration ability of treated cells in the gap created in the groups where IL-6 and hypoxia were applied separately at 24 and 48 h compared to the control group (normoxia). In the combined groups where IL-6 and hypoxia were applied together, a decrease in the gap and an increase in cell migration ability were observed compared to the hypoxia control group at both 24 and 48 h (Fig. 3A, B) (* $p \leq 0.05$).

Since the cell gaps in the HUVEC cell line were completely closed within 48 h, only 24-hour results could be evaluated. Accordingly, it was determined that IL-6 and hypoxia applications, which caused up-regulation at the KLK4 mRNA and protein levels in the HUVEC cell line within 24 h, also caused a decrease in cell proliferation (Fig. 3C, D) (* $p \leq 0.05$).

IL-6 and Hypoxia-Induced KLK4 gene expression via STAT-3 activation

Western blot was used to analyze the STAT-3 pathway in HT-29 cells p-STAT-3 protein expression increased in the IL-6 and hypoxia groups compared to cells under normal conditions at both 24 and 48 h (Fig. 4A, D). STAT-3,

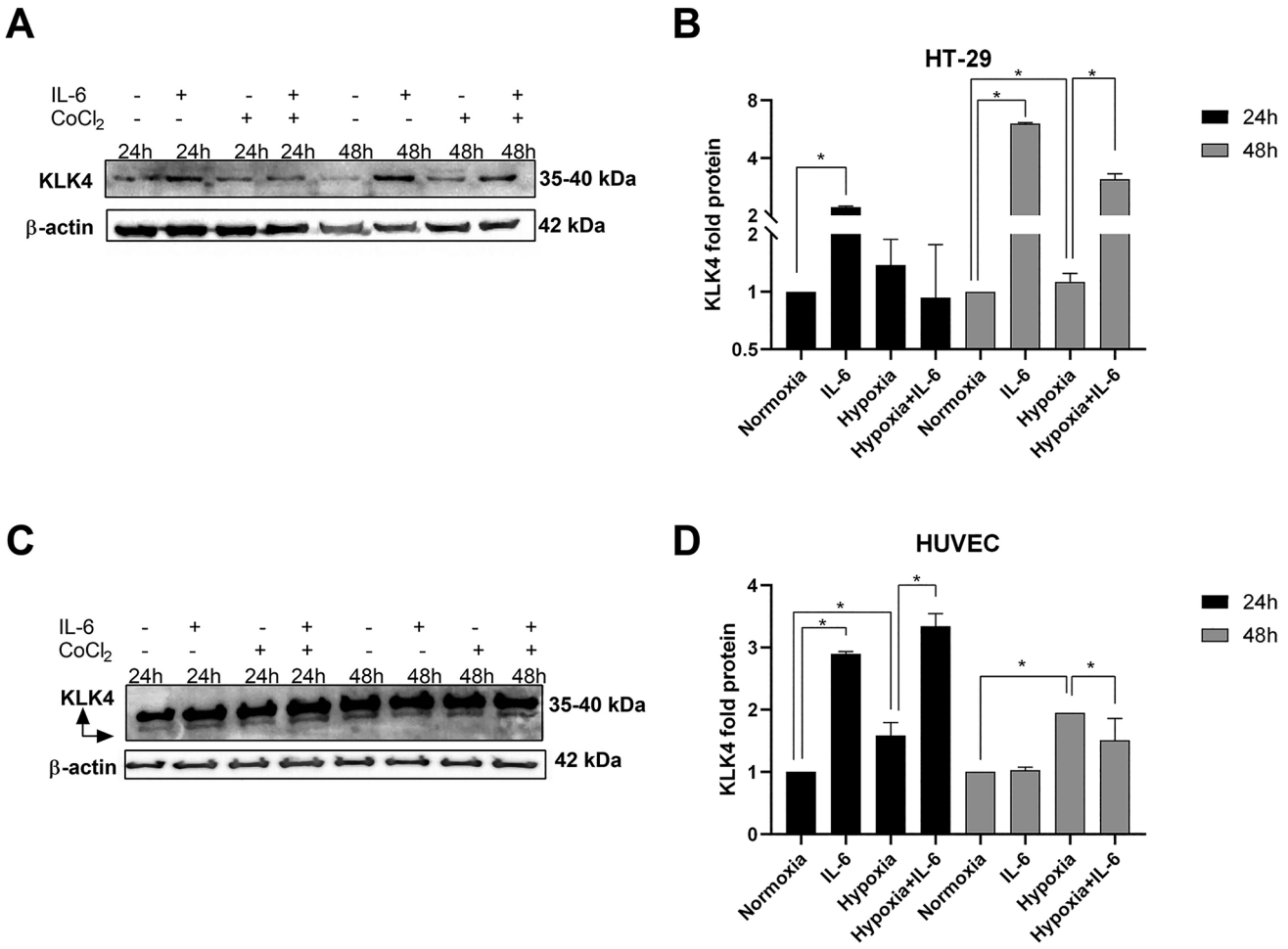

Fig. 2 Effect of IL-6 and $CoCl_2$ on KLK4 protein expression level. **(A)** Western blot image in HT-29 cell **(B)** Western blot result of proteins obtained with 20 ng/mL IL-6 and 150µM $CoCl_2$ applied to HT-29 cells with anti-KLK4 antibody treatment. **(C)** Western blot image in HUVEC cells. **(D)** Western blot result of proteins obtained with 20 ng/mL IL-6 and 150µM $CoCl_2$ applied to HUVEC cells with anti-KLK4 antibody treatment. (* $p \leq 0.05$). In experiments with HT-29 cells, untreated groups of HT-29 cells were used as the control group. In experiments with HUVEC cells, untreated groups of HUVEC cells were used as the control group

on the other hand, increased only at 48 h in groups treated separately with IL-6 and hypoxia compared to cells under normal conditions (Fig. 4A-C).

According to the pathway analysis performed on healthy endothelial cells, both STAT-3 and phospho STAT-3 increased in hypoxia, IL-6 and co-treatment groups. Especially p-STAT-3 showed a significant increase in protein level in HUVEC cells at 24 h in hypoxia and IL-6 groups compared to control group cells under normal conditions (Fig. 4D-F).

Discussion

One of the proinflammatory cytokines that influences the tumor's initiation, growth, progression, and metastasis is IL-6. It carries out its IL-6 activity through IL-6 receptor (IL-6R) and glycoprotein 130 kDa (gp130) molecules (Rose-John 2018). IL-6 causes increased proliferation in intestinal epithelium in colorectal cancer, and there is a positive correlation between serum IL-6 levels of patients and tumor burden (Tebbutt et al. 2002). In glioma,

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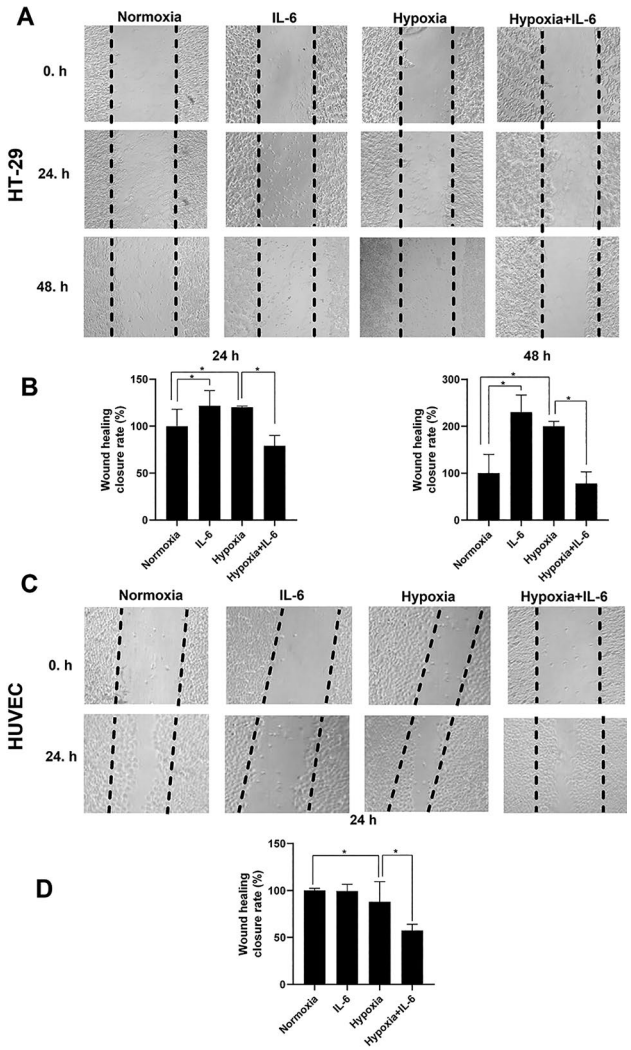

Fig. 3 Effect of a chemical agent, $CoCl_2$, and IL-6 cytokine treatment on wound healing (**A**, **B**) HT-29 cell line (0 h, 24 h and 48 h) and (**C**, **D**) HUVEC cell line (0 h and 24 h). (* $p \leq 0.05$). In experiments with HT-29 cells, untreated groups of HT-29 cells were used as the control group. In experiments with HUVEC cells, untreated groups of HUVEC cells were used as the control group

melanoma, lymphoma, as well as breast, pancreatic, kidney, ovarian, prostate, and colorectal cancers, IL-6 is thought to be a significant tumor-promoting agent. Various studies have shown that IL-6 levels are elevated in the serum of patients and in the tumor tissue itself, IL-6 expression is increased in patients with colorectal cancer, and important oncogenic pathways are activated via IL-6 in cancer cells (Chung et al. 2003). In vitro studies have shown that IL-6 increases the ability of colony formation in colon carcinoma cells in a dose-dependent manner. When IL-6 expression is blocked in-vivo, colon cancer development is suppressed (Schneider et al. 2000).

The expression of the KLK4 gene and protein is strongly linked to the prognosis of cancer (Srinivasan et al. 2022). It has been shown that many of the KLK members show high expression profiles in colon cancer and have prognostic importance for colon cancer. Low KLK10 and KLK7 mRNA expression has been shown to be significantly associated with long-term disease-free survival (Talieri et al. 2009). KLK4 facilitates tumor cell dissemination and enhances the metastatic potential of cancer cells in colorectal adenocarcinoma (Kontos et al. 2013).

In our previous study examining KLK4 expression at mRNA and protein levels in different cancer cell lines, it was observed that KLK4 expression was higher in HT-29 cells compared to SW480 cells (Poyrazlı et al. 2022). It is known that HT-29 cells express androgen receptor and estrogen receptor β. The KLK4 gene is regulated by androgens and is known to be expressed in cancerous tissues, especially in colon cancer. Studies have shown that androgens regulate cellular growth and differentiation in various hormone-dependent tissues, including colorectal tissue (Berta et al. 2004; Ferro et al. (Ferro et al. 2002). The androgen receptor (AR) is a

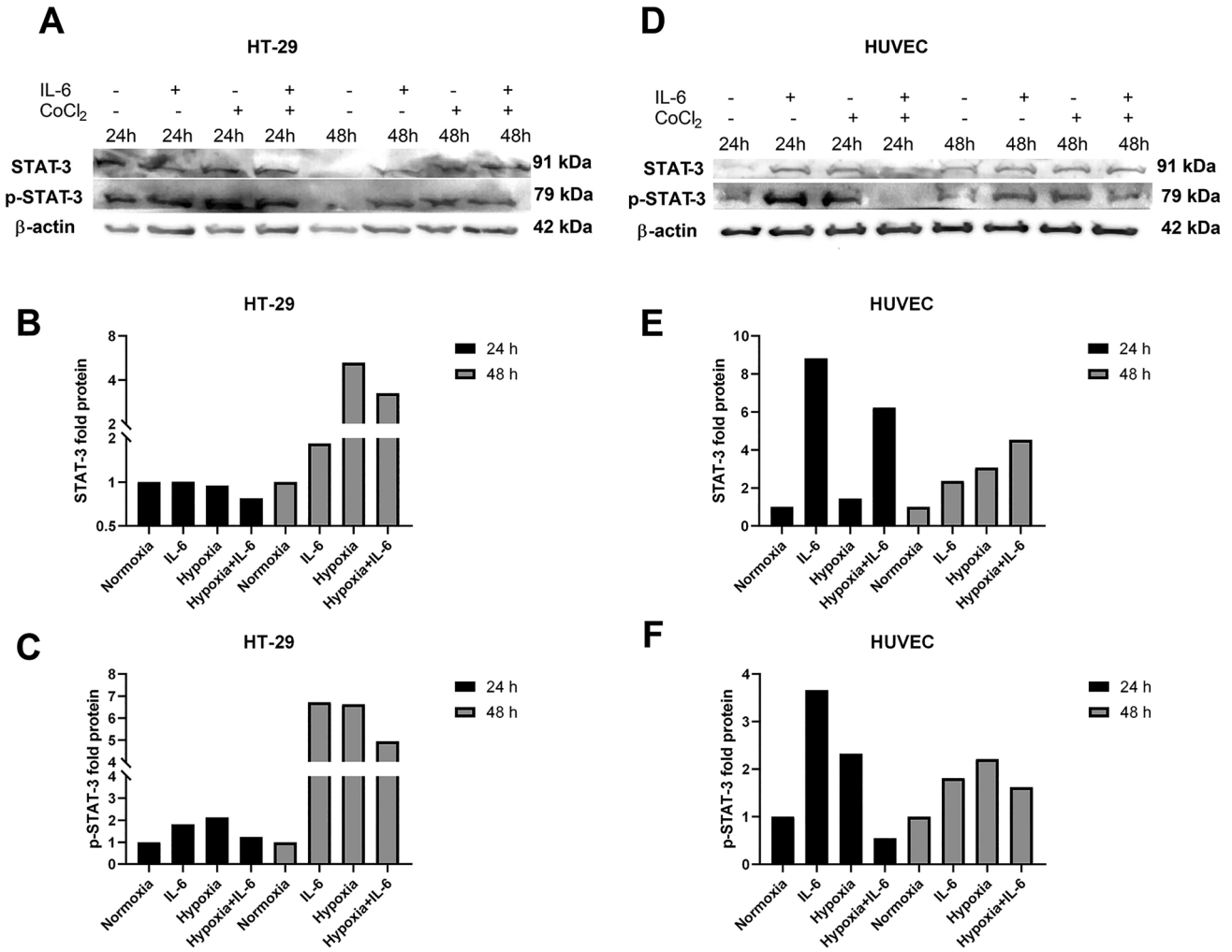

Fig. 4 Effects of IL-6 and $CoCl_2$ on STAT-3 and p-STAT-3 signaling pathways in HT-29 and HUVEC cells. **(A)** Western blot image in HT-29 cell. **(B, C)** Western blot analysis results in HT-29 cell. **(D)** Western blot image in HUVEC cells. **(E, F)** Western blot analysis results in HUVEC cell

ligand-dependent transcription factor that plays a role in controlling cellular proliferation and differentiation (MacLean et al. 1997). Recent studies have suggested that androgens have a protective function in the colon (Huang et al. 2015; Pereira and Khoury 1991; Rao et al. 1991). AR expression has been detected in the colorectal mucosa of experimental animals and humans. In another study, androgen administration was associated with the promotion of colon cancer tumorigenesis in rats (Izbicki et al. 1983). All things considered, steroid hormones appear to have a more complex role in colon cancer (Gu et al. 2009). HT-29 cells serve as a basic research model in colorectal cancer biology because they closely represent the typical genetic and molecular features of colorectal adenocarcinomas. These cells express Duke C stage in colon cancer staging and indicate the progression of the tumor from the intestinal layer to the lymph node invasion (Gala de Pablo et al. 2018; Poyrazlı et al. 2022). HT-29 cells are positive for the expression of c-myc, K-ras, H-ras, N-ras, Myb, sis and fos oncogenes. The p53 protein is mutated (R273H) and there is a G-A mutation resulting in an Arg-His substitution in codon 273 of the p53 gene (Poyrazlı et al. 2022). Increased KLK4 expression detected in the HT-29 cell line derived from colon carcinoma led us to speculate that KLK4 may play an important role in the progression stages. Recently, HT-29 cells have been reported to secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF) α and interleukins (IL) 1β and IL-6, as well as many other factors. In general, the receptors found in this cell line have equivalents in

normal intestinal cells (Verhoeckx et al. 2015). This cell line has some limitations, it is a high glucose consuming cell, so there is an impairment in glucose metabolism. Although they mimic some features of small intestinal enterocytes when differentiated, they are colonic cells. They cannot be compared with normal colonic enterocytes because they express brush border-associated hydrolases. They cannot be compared with absorptive enterocytes because not all hydrolases are present and their ion transport properties are also different (Verhoeckx et al. 2015). Discussing the limitations of this study, it's possible to study earlier time periods than the 24–48 h timeframe. Its effects on cell lines with different colon cancer stages could also be investigated. Further work is needed to conduct in vivo experiments (e.g. inhibition and suppression experiments).

Hypoxia leads to tumor cells becoming regionally and systemically aggressive by decreasing their sensitivity to apoptosis and other cell death signals and increasing signaling that supports angiogenesis, proliferation, and the ability of the tumor cells to spread. Hypoxia has been shown to induce IL-6 release, activation of the IL-6/STAT-3/Bcl2 pathway, and decreased levels of cleaved caspase-3 compared to normoxia (Xu et al. 2019). In another study in hypoxic states in prostate cancer, it was identified that KLK4 expression was up-regulated by hypoxia in PC-3 and LNCaP prostate cancer cells (Poyrazlı et al. 2024). KLK4 has been shown to induce ERK1/2 phosphorylation in HT-29 cells. KLK4 has been shown to be aberrantly statement in colon cancer and has the capability to induce PAR1 signaling in cancer cells. It has been suggested that KLK4 signaling through PAR1 may be an important pathway in colon tumorigenesis (Gratio et al. 2010).

There is no information in the literature about the KLK4 gene and IL-6 regulation, and the current studies are on the relationship between the effects of different plant extracts on HT-29 cells and the IL-6 signaling pathway (Jiang et al. 2015; Lin et al. 2015). In our study, MTT tests revealed that IL-6 and hypoxia had an increasing effect on cell proliferation in HT-29 colon cancer cells, while Scratch tests revealed that IL-6 and hypoxia had an increasing effect on cell migration in HT-29 colon cancer cells in the combined treatment groups. In non-cancer HUVEC cells, a decreasing effect on cell proliferation was also determined. In the literature, it was found that the increase in cell proliferation caused by IL-6 application was eliminated by plant extract applications and that it effectively inhibited the proliferation of human colon carcinoma cells and promoted their apoptosis through modulation of the IL-6/STAT-3 signaling pathway and target genes (Jiang et al. 2015). Another study discovered that HT-29 cells treated with IL-6 had higher levels of colony formation, phosphorylated STAT-3 (p-STAT-3) expression, and cell viability. It was observed that the *Hedyotis diffusa Willd*. extract significantly reduced IL-6-induced phosphorylation of STAT-3 when applied to these cells before IL-6 treatment (Lin et al. 2015). Numerous studies have shown that IL-6 stimulates intracellular pathways through classical and trans signaling pathways. In particular, it has been shown that classical signaling of IL-6 occurs in regenerative or anti-inflammatory processes, while trans-signaling acts mainly in a pro-inflammatory manner, promoting neoplastic transformation (Rose-John 2012). Binding of IL-6 to its receptor (IL-6R or sIL6-R) results in activation of JAK kinases via gp130 dimerization and phosphorylation of the IL-6/IL-6R complex. JAK kinases are receptor-associated kinases (JAK1, JAK2, and Tyk2) that are responsible for cell proliferation and tumor progression (Trikha et al. 2003). JAK phosphorylation results in phosphorylation and activation of transcription activator 3 (STAT-3), a transcription factor critical for cancer transformation and progression, typically through activation of specific target genes involved in neoplastic transformation. Activated STAT-3 binds to the promoters of genes involved in cell survival (Bcl2 and survivin); cell proliferation (c-Myc, cyclin D1, and cyclin B); angiogenesis (HIF-1α and VEGF); extracellular matrix degradation (MMP2 and MMP9); cell adhesion (ICAM-1); and inflammation (IL-6, IL-17, IL-23, and Cox2) and regulates their expression (Rose-John 2012; Turano et al. 2021).

Although there have not been many studies in recent years on the effects of IL-6 and hypoxia on other members of the KLK family, in a study conducted by Wang et al. in 2010, conditioned medium (CM) obtained from WPMY-1 (stromal myofibroblast cell) cells treated with PAR-1 agonists has been reported to induce the expression of prostate-specific kallikrein-related peptidase-3 (KLK3/PSA) and KLK4 in LNCaP cells via IL-6. Consequently, KLK4 triggers transmembrane signaling and has been shown to upregulate IL-6 in WPMY-1 cells via PAR-1 (Wang et al. 2010). In another study, it has been reported that HIF-1α can bind to the hypoxia-responsive element in the KLK3 gene promoter and can alter KLK3 expression (Horii et al. 2007). In another study, it has

been reported that exposure of non-small cell lung carcinoma cells to hypoxia induces the expression of KLK12, which has two HIF-1α binding sites in the 5' flanking region, suggesting that hypoxia and HIF-1α are regulators of KLK12 expression (Kryza et al. 2014).

In our study, hypoxia and inflammation processes, which are effective mechanisms in cancer, were examined together. Hypoxia has been shown to play a role in the development of chemotherapeutic or radiotherapeutic treatment resistance in various tumor forms. Many clinical studies have shown that tumors are hypoxic and that tumor diameter increases with increasing hypoxia. Hypoxic tumor cells can be regionally and systemically aggressive due to decreased are less sensitive to apoptosis and other cell death signals and may increase signaling that supports angiogenesis, proliferation, and the ability of the tumor cells to spread (Vadde et al. 2017). The tumor and its microenvironment contain hypoxic conditions. Many studies have observed that the levels of the pro-inflammatory cytokine IL-6 increase under hypoxic conditions (Ding et al. 2020; Malkov et al. 2021; Shah et al. 2008). There is no information in the literature about the KLK4 gene and IL-6 regulation in colorectal cancer. In this study, IL-6 and KLK4 gene expression, an inflammation-related cytokine, were investigated in colon cancer cell line (HT-29) and healthy endothelial cells (HUVEC) under hypoxic conditions. HT-29 cell line derived from colon carcinoma has some limitations, it is a high glucose consuming cell, so there is an impairment in glucose metabolism. Although they mimic some features of small intestinal enterocytes when differentiated, they are colonic cells. They cannot be compared with normal colonic enterocytes because they express brush border-associated hydrolases. They cannot be compared with absorptive enterocytes because not all hydrolases are present and their ion transport properties are also different (Kontos et al. 2013). These cells possess a number of hormone or peptide receptors, including functional receptors for vasoactive intestinal peptide (VIP), receptors for insulin, alpha-2-adrenergic receptors, EGF, prostaglandins, neurotensin, and catecholamines. All of these hormones or receptors are present in cells grown under normal conditions. Under these conditions, HT-29 cells are undifferentiated: they grow as a multilayered sheet of morphologically unpolarized, undifferentiated cells (Rousset 1986). We show for the first time that KLK4 mRNA and protein levels in colorectal cancer are statistically significantly increased in both the IL-6, hypoxia and combined groups compared to the control group. In colon cancer cells (HT-29), expression of the KLK4 gene increased at both mRNA and protein levels in both separate and combined groups, especially in the 48-h time period, and contributed to the carcinogenesis process. In the HUVEC cell line, IL-6 and hypoxia were found to increase KLK4 mRNA levels at both 24 and 48 h, while in combined applications, it was upregulated only at 24 h. Again, the effect on KLK4 protein level was upregulated at 24 h in the combined groups of IL-6 and hypoxia, while at 48 h a significant increase was observed only in the hypoxia-treated group. In a wound healing experiment conducted on HUVEC cells, it was determined that IL-6 and hypoxia caused a decrease in cell proliferation. This effect was observed in HT-29, while combined applications exhibited a proliferative effect and increased cell migration capacity. To gain insight into the signaling pathways through which this increase in KLK4 in response to IL-6 and hypoxia occurs, protein-level pathway analyses were performed. Pathway analyses in the HT-29 colon cancer cell line revealed increases in both STAT-3 and p-STAT-3, particularly in the 48-hour timeframe, in separate groups treated with IL-6 and hypoxia. Analyses of the same conditions with healthy cells revealed an increase in both 24- and 48-hour timeframes in separate groups treated with IL-6 and hypoxia. These results suggest that the increase in KLK4 expression in colon cancer is likely mediated through STAT-3 activation by IL-6 and hypoxia.

Conclusions

In summary, our study has shown that the KLK4 gene, which has many roles in cancer cells such as cell motility, invasion, cell proliferation and metastasis, has contributed to the IL-6 and hypoxia-induced colon cancer. The findings obtained in our study are that hypoxia and IL-6 up-regulates the KLK4 gene expression via STAT-3 activation. It is a very important finding that the KLK4 gene, which has the potential to be a marker in cancer, responds to hypoxia in colon cancer. Hypoxia is a condition responsible for cancer aggressiveness. The

contribution of the inflammatory mechanism to hypoxia is indisputable. Blocking IL-6, a cytokine associated with inflammation in cancer, will replace the elimination of hypoxia in therapeutic protocols. The contribution of the KLK4 gene in this way should be supported by further studies. Moreover, this study will contribute to the studies to be conducted on the elucidation of the IL-6 signaling process that regulates the inflammation pathway of the KLK4 gene in colon cancer.

Acknowledgements We thank Prof. Dr. Feray Köçkar for her support in pioneering studies on KLK4.

Author contributions FP and SAT designed the research; FP performed the experiments; FP and SAT checked the results; FP and SAT wrote the paper, FP and SAT checked the language of the paper.

Funding No funding was received for conducting this study.

Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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