

# Elevated Extracellular Hsp70 Correlates with Increased IL-1 and IL-10 in Iraqi Patients with Colorectal Carcinoma

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## Abstract

Colorectal cancer (CRC) is the most common cancer in the Iraqi population. Cancerous cells are stressed cells that express large amounts of intracellular and extracellular heat shock proteins to correct for distorted proteins. They also directly affect immune cells and produce large amounts of cytokines. This work aimed to study the association between serum levels of heat shock protein 70 (Hsp70), interleukin 1 (IL-1), and interleukin 10 (IL-10) in CRC patients to highlight the importance of this pathway in patients' immune status that may affect the progression of CRC. In the current study, an enzyme-linked immune sorbent assay (ELISA) was performed in 20 Iraqi colorectal cancer patients and 20 healthy volunteers to measure serum levels of Hsp70, IL-1 $\alpha$ , and IL-10. Serum levels of HSP70, IL-1 $\alpha$  and IL-10 were significantly higher in the CRC group than in the healthy control group ( $38.83 \pm 4.7$  and  $14.88 \pm 1.74$  ng mL<sup>-1</sup>), ( $141.4 \pm 23.22$  and  $46.38 \pm 4.4$  ng mL<sup>-1</sup>) and ( $26.95 \pm 10.14$  and  $1.4 \pm 1.07$  ng mL<sup>-1</sup>), respectively, ( $p < 0.0001$ ). According to these findings of this study, Hsp70 can be used as a prognostic biomarker for CRC and may be useful in identifying patients with an increased risk of immune tolerance and CRC development in Iraqi patients.

**Keywords:** colorectal cancer, interleukin-1alpha, interleukin-10, Hsp70.

## Introduction

The most common gastrointestinal cancer is CRC, which is a significant global health concern. According to Globocan 2020, CRC is the second most common cancer in Iraqi women, behind breast cancer, and the third most common cancer in Iraqi men, following lung and prostate cancer [1]. The immune system is made up of special cells and cell products that work collaboratively to protect the body from various pathogens and diseases. It is primarily divided into two systems: innate immunity, characterized by a non-specific and instantaneous response, and adaptive immunity, which

can respond and adapt to specific stress stimulation [2]. Cytokines are signaling molecules (ligands), which are necessary for the coordination of immune responses by serving as ligands and binding to cell receptors, which are quickly synthesized and typically secreted by various immune and non-immune cells, particularly after stimulation, and have an additive, synergistic, or antagonistic effect on many additional neighboring target cells [3]. Cancer develops throughout time, influencing how it interacts with the immune system. Inflammatory bowel diseases cause a chronic inflammatory environment influenced by genetic and environmental factors. It is believed to be a risk factor for cancer progression because tumor supporting molecules such as heat shock protein, cytokines and growth factors, cell survival signals, angiogenic factors, and other carcinogenesis mediators are produced by cells that infiltrate the tumor microenvironment [4]. Intracellular Hsp70 expression increases dramatically dur-

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ing inflammation and plays cytoprotective roles. This is accomplished through traditional chaperone functions such as protein refolding, repair, and direct suppression of apoptosis [5]. Heat shock protein 70 is important in balancing the appropriate response to cell stress because excessive apoptosis can lead to severe human inflammatory diseases. However, in cancer, Hsp70 is known to be overexpressed, disrupting the balance and increasing malignant cell proliferation, invasiveness, and resistance. Heat shock protein 70 has been shown to translocate into the extracellular milieu in response to stress. Furthermore, extracellular Hsp70 (exHsp70) has been shown to have dual pro-inflammatory and anti-inflammatory functions, resulting in increased tissue damage, indicating Hsp70's dual role of Hsp70 [6].

Heat shock protein 70's immunogenicity originates from its capacity to bind antigenic peptides produced by malignancies. Taking into account the clinical findings, it is vital to note that there are two types of Hsp70 circulating in the plasma of cancer sufferers. Exosomal Hsp70 is produced by live tumor cells, while Hsp70 is secreted by dying cancer cells and acts as a damage-associated molecular pattern (DAMP) [7]. Hsp70 and a DAMP produced by necrotic cells have a high immunogenic potential to stimulate an antitumor T cell response. However, prolonged exposure of immune cells to exHsp70 causes immunological tolerance and accelerates cancer progression. A moderate dosage of the Hsp70-peptide complex was observed to be sufficient to boost antitumor immunity, while Hsp70 overabundance leads to tumor development [8]. This research aimed to study whether Hsp70 can be used as a diagnostic biomarker for CRC and the association between serum Hsp70 levels and IL-1 and IL-10 cytokine levels in CRC patients to highlight the importance of this pathway in the patient's immune status that can affect CRC progression.

## Materials and methods

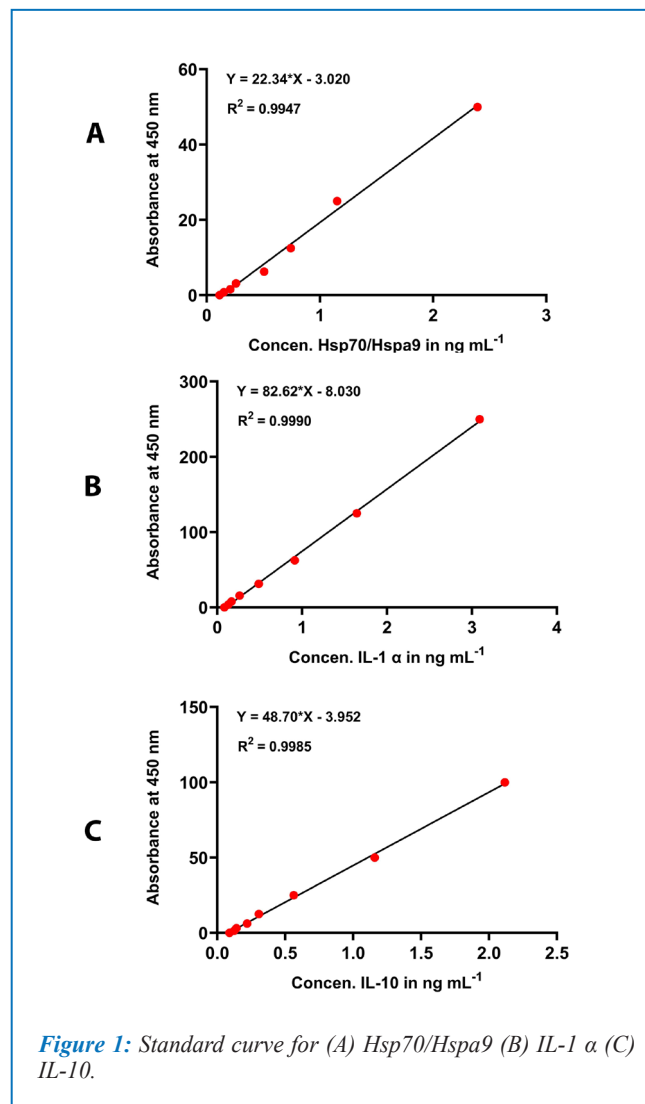
### Subjects and Sample Collection

Blood samples were collected from 20 patients who were diagnosed with colorectal cancer. The ages ranged between (26-84) years (13 of them were male with a median age of 46 and 7 were female with a median age of 65) at Baghdad Teaching Hospital, Baghdad Medical City during the period from January 2022 to March 2022. Twenty apparently healthy volunteers, 15 male (median age 43) and 5 female (median age 51) were considered as a control group. Patients who had received chemotherapy or radiotherapy were excluded from the study. Ethical permission was obtained to carry out the investigation from these hospitals and from all patients included in this study. On December 12, 2021, the Iraqi Ministry of Health's Ethics Committee approved the study under reference number 4707.

### Serum Hsp70 and Interleukins Detections

An aliquot of 3 ml of peripheral blood was drawn in an EDTA-Na<sub>2</sub> tube. The tubes were centrifuged for 15 minutes at 2-8 °C at 1000xg. The sera were separated and stored at 20

C until use. Sandwich ELISA kit obtained from Elabscience, USA (Human Hsp70 Cat. No.: E-EL-H1863, human interleukin 10 Cat. No.: E-EL-H6154 and human interleukin 1 Alpha Cat. No.: E-EL-H0088) were used as per manufacturer recommendations.



### Calculation of the standard curve concentration of Hsp70, IL-1 α and IL-10

To determine the concentrations of Hsp70, IL-1 and IL-10, a standard curve was constructed by plotting the mean values of optical density (OD) for each standard on the vertical y axis against the corresponding concentrations (μg mL<sup>-1</sup>). On the horizontal x-axis, a best-fit curve was drawn through the points represented by the graph (Figure1).

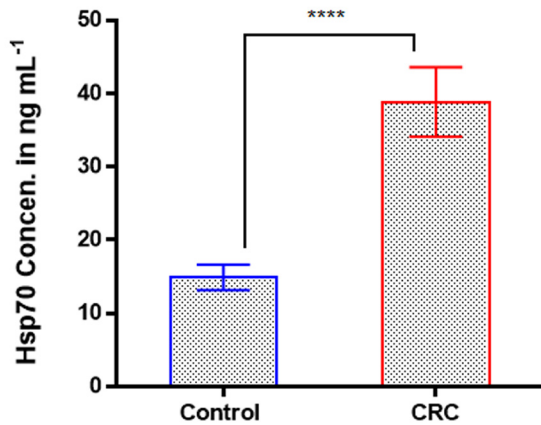
### Statistical analysis

GraphPad Prism version 9 (GraphPad Software Inc., La Jolla, CA) was used to compute statistical significance between different groups. The Student's t-test was used to determine whether the variance in the group was significant or not. The Pearson coefficient r was used to assess the correlation. Data were expressed as mean ± SD and statistical differences were

defined as \*  $p < 0.05$  and \*\*  $p < 0.01$ .

## Results

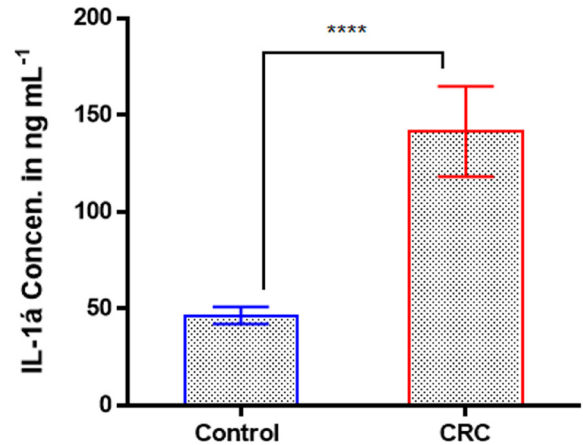
The serum level of Hsp70 in 20 patients accurately diagnosed with colorectal adenocarcinoma and in 20 control individuals is described in Figure 2. Results indicated that the serum level of Hsp70 in CRC patients was significantly increased ( $p < 0.0001$ ) compared with a control group, with a mean concentration of  $38.83 \pm 4.7$  and  $14.88 \pm 1.74$  ng mL<sup>-1</sup>, respectively. This is the outcome of patients who did not receive any therapy, such as a colectomy or chemotherapy.



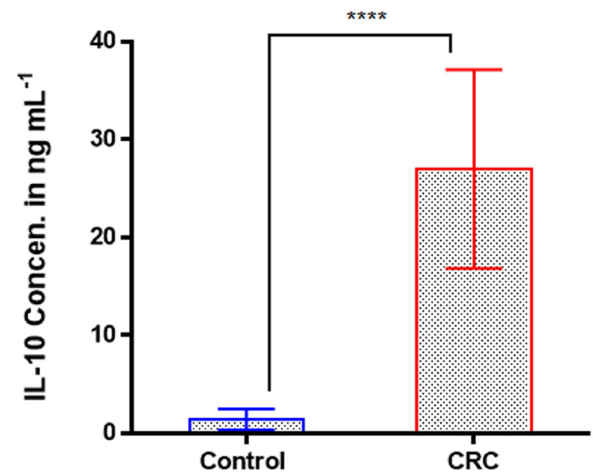
**Figure 2:** Mean serum level ( $\pm$ SD) of Hsp70 in Iraqi patients with CRC compared to the control. \*\*:  $p$ -value  $\leq 0.01$ .

The levels of IL-1 $\alpha$  and IL-10 in serum were measured by ELISA for CRC. The results of the IL-1 $\alpha$  ELISA experiment showed the mean level of IL-1 $\alpha$  in CRC patients was  $141.4 \pm 23.22$  ng mL<sup>-1</sup>, and by comparing with the control group ( $46.38 \pm 4.4$  ng mL<sup>-1</sup>), significant differences ( $p < 0.0001$ , IL-1 $\alpha$  elevation in CRC patients) were observed in figure 3.

The results demonstrate a variation between CRC patients and the control group concerning IL-10 levels. Significant ( $p < 0.0001$ ) increase in IL-10 level was reported in CRC patients ( $26.95 \pm 10.14$  ng mL<sup>-1</sup>), while the reported IL-10 level in the control group was  $1.4 \pm 1.07$  ng mL<sup>-1</sup> (figure 4).



**Figure 3:** Mean serum level ( $\pm$ SD) of IL-1 $\alpha$  in Iraqi CRC patients compared to the control. \*\*:  $p$ -value  $\leq 0.01$ .



**Figure 4:** Mean serum level ( $\pm$ SD) of IL-10 in Iraqi colorectal cancer patients (CRC) compared to the control. \*\*:  $p$ -value  $\leq 0.01$ .

The Pearson coefficient  $r$  was used to assess the correlation between Hsp70, IL-1, and IL-10 and the demographic variables. The findings revealed that there is no correlation between Hsp70, IL-1, and IL-10 and the demographic parameters, as shown in Tables 1 and 2.

**Table1:** correlation between Hsp70, IL-1, and IL-10 expression and control demographic parameters

r (p-value)	Hsp70	IL-10	IL-1
Hsp70	1.00	(0.7144) 0.09	(0.6538) 0.11
IL-10		1.00	(0.6885) 0.1
IL-1			1.00

**Table1:** correlation between Hsp70, IL-1, and IL-10 expression and control demographic parameters

r (p-value)	Hsp70	IL-10	IL-1
Hsp70	1.00	(0.3679) -0.21	(0.5175) 0.15
IL-10		1.00	(0.6436) -0.1
IL-1			1.00

## Discussion

The current project was designed to find a novel biomarker for one of the most difficult tumors, which is colorectal cancer, also studying the effects of excessive exHsp70 on cytokines that promote chronic inflammation in cancer microenvironment. This chronic inflammation disrupts the anti-tumor response of immune cells, which may lead to tumor progression. The dramatic increase in Hsp70 levels in the serum of Iraqi CRC patients is mostly attributed to cellular damage and necrosis of tumor tissue, which cause the release of Hsp70 into the extracellular space. A previous study demonstrated that a release of Hsp70 into the extracellular space made Hsp70 detectable in circulation, and measuring Hsp70 concentration may provide the clinical magnitude of significant tumor information and progression [9].

The results of IL-1  $\alpha$  ELISA showed a high concentration level of IL-1 $\alpha$  when compared to the control group. This result may be because IL-1 $\alpha$  is an important immune and inflammatory mediator. It is also known to be up-regulated in a diverse variety of tumors, and it is believed to encourage tumor adhesion, proliferation, and metastasis by inducing the transcription of genes encoding for proteins responsible for oncogenesis and the creation of growth factors [10]. Non-immune cell types, express IL-1  $\alpha$  constitutively. At a steady state, IL-1  $\alpha$  expression is relatively low, but when exposed to stressful conditions like low pH, hypoxia, and elevated temperature (all these conditions are like cancer microenvironments), IL-1  $\alpha$  expression increases dramatically, triggering inflammatory responses. [11]. The ability of IL-1 to initiate a sterile inflammatory response and secrete chemotactic alarmin or damage-associated molecular pattern (DAMP) from necrotic but not apoptotic cells is one of the major aspects of IL-1's role in the pathogenesis of many inflammatory-related diseases, including colorectal carcinoma, which is still under investigation [12]. The func-

tion of IL-1  $\alpha$  in cancer development has been investigated in various types of cancer as a necessary pro-inflammatory cytokine, and the results suggest that IL-1 is a dual-function cytokine that exerts pro-inflammatory and antitumor functions in various cancers [13]. Overproduction of IL-1 encourages inflammation-related tissue damage and tumor invasiveness. IL-1 can prompt other cell types to make pro-angiogenic and pro-metastatic mediators and thus recreate a function in inflammation-related carcinogenesis [14].

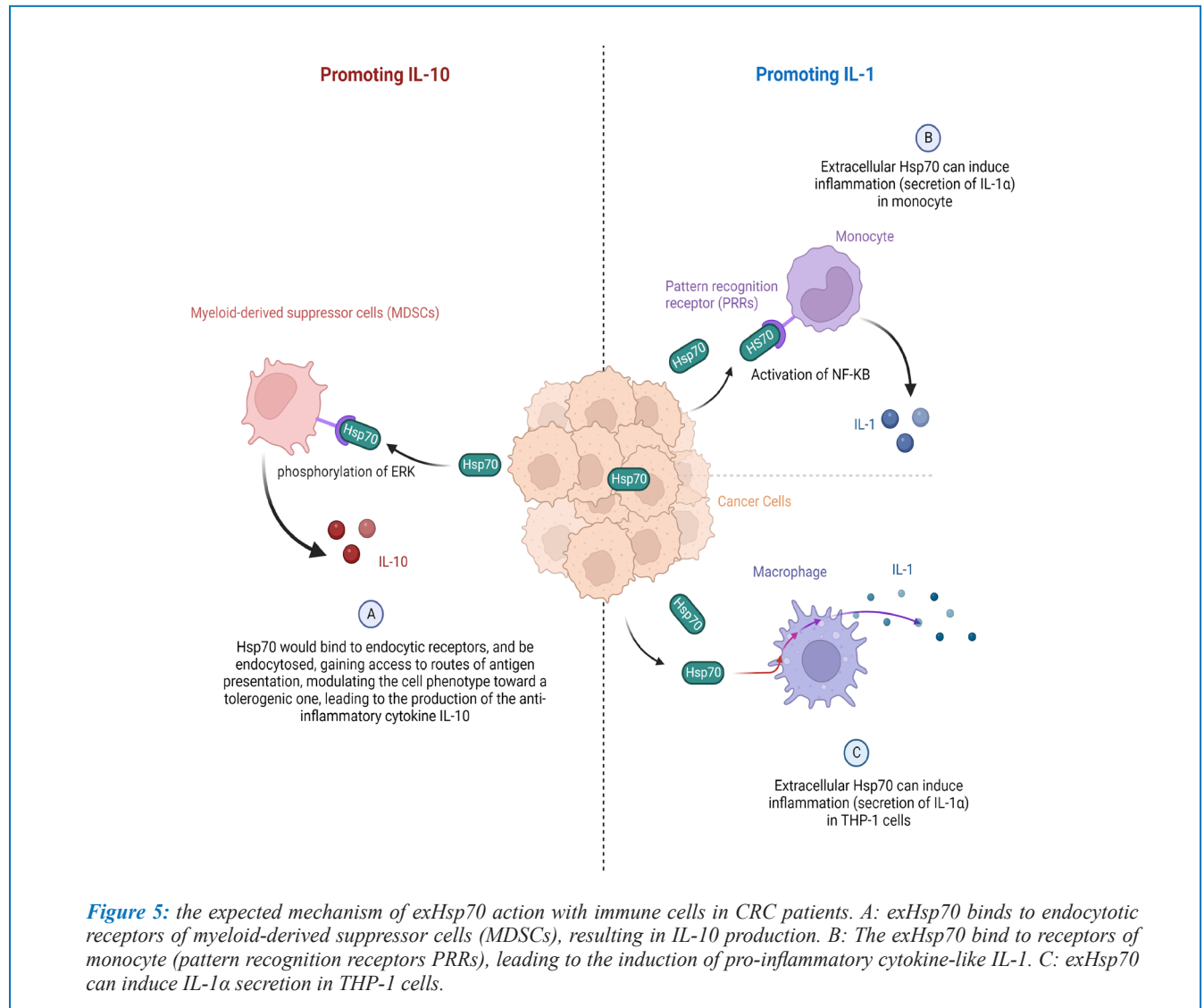
The results of IL-10 levels in the serum of Iraqi CRC patients were very high, perhaps because IL-10 has a dual function and is a potent anti-inflammatory cytokine with immunoregulatory properties. The elevated systemic level of IL-10 agrees with previous research documented in patients with advanced cancer, so IL-10 may serve as a colorectal cancer biomarker [15]. Although IL-10 suppresses the synthesis of pro-inflammatory cytokines, it may also enhance tumor development by boosting cell proliferation and inhibiting cell apoptosis and angiogenesis [16], [17]. Increased IL-10 concentrations were highly related to the progression of colorectal carcinoma. [18]. The highest serum IL-10 concentrations were reported in Stage IV CRC patients, indicating that systemic IL-10 has a pro-tumorigenic effect in CRC progression and may have an important function in tumor-induced immune suppression in CRC patients [19].

IL-10 exhibits two biological functions in the progression of CRC. Through its immunosuppressive action, IL-10 promotes rather than prevents cancer development. The results are consistent with former reports that surgical tumor removal significantly decreased sera IL-10 in patients with gastrointestinal cancer [20]. These findings support the theory that IL-10 is primarily produced by tumor-infiltrating immune cells or cancer patient's tumors. [21]. Nevertheless, this cytokine has both tumor-promoting and tumor-inhibit-

ing properties. At low concentrations, IL-10 exerts an antitumor effect by different mechanisms, such as activating natural killer cells (NK), lymphocytes T, macrophages, and nitric oxide. In contrast, IL-10 manifests immune system deregulation at high concentrations, which allows the tumor to escape from immune recognition [22],[23].

Therefore, the expected mechanism of exHsp70 action with immune cells in CRC patients is explained in Fig. 5 and depends on the results and the references reinforcing it. When exHsp70 binds to the endocytotic receptors of myeloid-derived suppressor cells (MDSCs) and is endocytosed, it sends a signal through TLR2, activating MyD88. Following ERK phosphorylation, a non-specific transcription factor that binds the IL-10 gene promoter is activated, resulting in IL-10 production and, consequently, immunosuppression and modulating the cell phenotype toward tolerogenic (Fig. 5a) [24]. Inducible exHsp70 released from cancer cells has been shown to act by binding to mononuclear cell surface receptors and stimulating the host immune system. The exHsp70 bind to receptors of monocyte (pat-

tern recognition receptors PRRs), leading to the activation of intracellular calcium and induction of pro-inflammatory cytokine-like IL-1 by activation of nuclear factor kappa beta NF- $\kappa$ B (Fig.5b) [5]. The extracellular Hsp70 may bind to specific receptor CD19 of dendritic cells (DCs), then DCs presented antigen by MHC-I to T-cytotoxic cells and induce many pro-inflammatory like IL-1 and TNF $\alpha$ . ExHsp70 may bind non-specifically to dendritic cells, leading to antigen presentation by MHC-II to T-helper cells and the induction of an anti-inflammatory cytokine like IL-10 [25]. Hulina and colleagues in 2018 demonstrated that exHsp70 can induce IL-1 $\alpha$  secretion in THP-1 cells, a human leukemia monocytic cell line widely used to assess macrophage activity modulation (Fig. 5c) [26]. Inducible exHsp70 can act as a critical signal or recruit cells. ExHsp70 responsible for the generation of innate and adaptive immune responses against tumor cells in the early stages of cancer by stimulating monocytes and macrophages to release pro-inflammatory (IL-1) but chronic inflammations lead to immune tolerance in the advanced stages of cancer [27].



## Conclusions

Heat shock protein 70, IL-1 alpha, and IL-10 concentrations in the sera of CRC Iraqi patients were significantly higher than those of healthy volunteers. This exHsp70 and serum cytokine profile may play an important role in tumor development. Furthermore, an increase in Hsp70 has been linked to the progression of CRC, which can be a diagnostic biomarker for Iraqi patients.

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## Conflict of interest

The authors have no conflicts of interest.

## Author contribution

Ahmed Majeed Al-Shammari and Ali Z. Al-Saffar designed and supervised the study, checked, and revised the manuscript draft and final version. Farah E. Ismaeel conducted the lab work and wrote the first draft. Mohamed Farhan Obaid supervised the clinical part of the work and patient samples. All the authors approved the final version.

## Ethical approval

Ethical permission was obtained to carry out the investigation from these hospitals and from all patients included in this study. On December 12, 2021, the Iraqi Ministry of Health's Ethics Committee approved the study under reference number 4707.

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