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Investigating the role of CD47 gene regulation in Iraqi cancer patients: a promising prognostic factor

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Abstract

Background: Innate immune checkpoints have been one of the most studied and promising approaches in cancer immune therapy. Therefore, it is considered valuable when investigating these checkpoints at the gene expression and protein levels. However, this approach still has variations from study to study due to various types of cancer. Therefore, it is hypothesized that CD47 could be one of those checkpoints that affect the survival of cancer cells. CD47 is an integrin like marker that has been identified for its role as "do not eat me signal" which enhances the ability of cancer cells to escape from being phagocytized by macrophages.

Aim of the study: This study aims to investigate CD47 expression in ten solid tumors and five blood cancers at the gene level and at the protein level.

Methods: Clinical specimens were collected from patients with different cancers attending Kirkuk oncology center. The patients were categorized into two main groups: solid tumor group and blood-derived cancer group together with their control counterparts. CD47 gene expression was investigated using q RT-PCR techniques while its protein levels were investigated using ELISA techniques.

Results: The results showed a significant up-regulation of CD47 gene expression in gastric cancer, cholangiocarcinoma; CCA (bile duct), chronic myeloid leukemia (CML), and breast cancer, respectively. These results were also confirmed at the protein level, suggesting that those types of malignancy are more susceptible to an increase in CD47 expression than other types of cancer that were involved in this study.

Conclusion: CD47 tends to be over-expressed in solid tumors and blood cancers, and could be used as diagnostic and prognostic markers, especially for solid tumors.

Keywords: *CD47, CML, gene expression, ovarian cancer, solid tumor*

Introduction

Cancer is considered one of the most health threatening issues in the world; therefore, understanding its pathology and the mechanistic pathways involved in its spread is an essential way to tackle this problem. Cancer cases are growing more and more in Iraqi population recently. According to the annual report of the Iraqi cancer registry during 2021, the highest cancer incidence was reported in the city of Al-Sulaymaniyah at a rate of 118.87 per 100000 people. Gender-based records also Al-Sulaymaniyah registered highest records in males 113.55 per 100 kp, while Kerbala city recorded 62.93/100kp. Kirkuk city showed the lowest cancer incidence among all other Iraqi cities in both males and females respectively (51.20/100kp, 71.62/100kp)(1).

CD47 is a protein present on the surface of numerous cells throughout the body. It instructs circulating immune cells known as macrophages not to engulf these cells. The CD47 protein is used by the body to protect cells that should be protected and to help dispose of old or unhealthy cells (2)

Since CD47 is an ubiquitous glycoprotein, it is widely expressed in all human tissues, initially from bone marrow cells and fatal precursors such as megakaryocytes. Furthermore, this gene could be found in different human tissues, such as

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the kidney, liver, spleen, pancreas, bile duct, gallbladder, and microglial cells (3).

CD47, alternatively referred to as an integrin-associated protein (IAP), is a transmembrane protein (C terminus is cytoplasmic and the N-terminus is extracellular) CD47 is expressed by all cells in the body, including red blood cells that do not normally express integrin. Therefore, it is now mentioned as CD47 rather than IAP (4).

It binds as a ligand for the signal regulatory protein-alpha (SIRPα) which is expressed in myeloid cells (5), including dendritic cells (DCs) and macrophages (6), as well as in cytotoxic T lymphocytes (7).

These two proteins combine to produce a "don't-eat-me" signal that prevents macrophages and dendritic cells (DCs) from phagocytosing target cells that express CD47 acting as a crucial regulatory switch for these cells' phagocytic activity (8). Due in part to their phagocytic activity, macrophages are a varied group of immune cells that are necessary for tissue homeostasis and protection against pathogens (9).

Interest in CD47 is increasing and it is thought to be a promising target for cancer therapy. Research on CD47 in cancer has revealed that many malignancies express high quantities of CD47 on their surface, which shields the cancer cells from phagocytosis by macrophages and helps them avoid immunity surveillance (10).According to Elade et al (2020) cancer cells frequently exhibit and overexpress CD47 in an effort to fight against phagocytosis (11),. According to studies specifically on MDS, suppressing the CD47 signal causes MDS cells to be selectively phagocytosed because it reduces their "don't eat me" signal(12).

Its distinguished that the interaction between CD47 ligand and SIRPα orchestrate the phagocytic activity via immune cells, such as macrophages, which lead either to apoptosis or to be phagocytized(13). The CD47 "don't eat me" signal acts as a molecular marker on the surface of cancer cells. By binding to SIRPα on macrophages, CD47 transmits an inhibitory signal that inhibits phagocytosis. This interaction essentially signals the macrophage not to engage the CD47-presenting cell. Many cancer cells exploit the CD47-SIRPα pathway by regulating CD47 expression. By doing so, they hijack the normal regulatory machinery of immune cells and effectively avoid phagocytosis(14). This property allows tumor cells to survive and continue to multiply, which contributes to tumor growth and progression. This phagocytic phagocytosis depends on the transcriptional expression of pro- and anti-autophagy signals on its target. Starting in the early 1970s, it was found that tumor growth could be promoted by tumor-associated macrophages, activating macrophages to enhance the immune response against cancer cells. This activation can lead to the release of pro-inflammatory cytokines and the recruitment of other immune cells(15). Therefore, it is considered one of the promising approaches in cancer research to follow the signaling pathway that underlies cancer cell survival. This study aims to investigate the role of CD47 in the gene expression step and in the protein synthesis step.

Materials and Methods

Patients and samples:

Blood samples were collected from patients attending the oncology center of Kirkuk, Iraq during the period from December 2022 to June 2023. All information and consent forms were issued under the supervision of the ministry of health regulations. The study included 152 subjects who were divided into two main groups: the control group (50 healthy people) and the patient group (62 solid tumors, 40 blood cancers). A total of 4 ml of blood samples were obtained from each subject under aseptic conditions, 2 ml of each blood samples were stored in EDTA tubes in -20 freezer for the gene expression experiments and 2 ml were allowed for clotting and centrifuged then serum samples were aliquoted and stored at -20 freezer for ELISA experiments. Inclusion criteria: this study involved newly diagnosed cancer patients who did not take any medication at the time of sampling.

RNA extraction

RNA was extracted from each blood sample following the manufactures instructions (GENEzol™ TriRNA extraction Kit, Genoid, cat# GZX050, Korea). Three volumes of GENEzol reagent were first added to 200µl of blood sample in the ratio of 1:3 in order to eliminate erythrocytes and obtain leukocytes only then samples were vortexed and incubated 5 min RT, centrifuged at 12000xg for 1 min then samples were taken to new micro centrifuge tubes and one volume of absolute ethanol was added and mixed by vortex and then RB columns were prepared and fixed to collection tubes. This was followed by transferring samples to the RB columns, centrifuged for 1 min at 14000 xg, the flow through was eliminated and the filtered column placed back on the collection tube, then three steps of RNA washing were added by adding 400µl,600µl, 600µl wash buffer and centrifuged for 30 sec. at 14000 xg between each wash, then the flow through was discarded and the RB column was placed in new collection tubes where 25 µl of RNAase free water was added to each column and left for 3 min at RT then centrifuged at 14000 xg and the eluted samples were kept at -20 until use.

Gene expression

RNA samples were then used for the gene expression study. GoTaq® 1-Step RT-qPCR Kit, Promega, cat# A6020 Kit was used for q RT-PCR and specific primers were designed for CD47 gene from NCBI PRIMER BLAST tools were forward primer 5`-CCTCGCTGTGGTTGGACTGA -3` and reverse primer 3`-TCCTCTACAGCTTTCCTAG-GAGGT -5`. B globin gene was used as a reference gene (Forward 5`-ACACAACTGTGTTCACTAGC-3`, Reverse 5`-CAACTTCATCCACGTTCACC-3`). The fold change of the relative mRNA was calculated using the comparative threshold method (ΔCt control= Ct target gene (CD47 gene) - ct reference gene(β-globin), Δct patients= Ct target gene (CD47 gene) - ct reference gene (β-globin), ΔΔct = Δct patients- ΔCt control and the fold change = 2-ΔΔct (16) . PCR conditions were initial denaturation 95 ºC for 2 min,

(denaturation 95 ºC for 30 sec, annealing 55 ºC for 30 sec and extension 72 ºC for 40 sec) 40 cycles using q Tower3 G, Germany.

Measurement of CD47 levels in patient's serum

ELISA was performed using Human CD47 ELISA Kit from Mybiosource, cat# MBS8248341, USA. The protein levels were obtained using the manufacturer's instructions and protocol. In each well of the 96-well plate 200µl from each sample was added and standers were added and the plate was covered and incubated for 90 min RT. After the incubation time, the wells were washed three times with wash buffer and the plate was drained carefully on tissue paper. 100µl of biotin-labeled detection antibody was added to each well and incubated for one hour at 37 ° C followed by three washing steps and 100µl of streptavidin-HRP was added and incubated for 30 min at 37 °C. After this, five final washes were performed and an amount of 100µl TMB substrate was added to each well and incubated for 30 minutes in the dark before adding 100 µl of stop solution to each well. CD 47 concentration was measured at 450nm on the ELISA plate reader.

Statistical analysis

All the data presented in this article was performed using GraphPad prism software. Data represented M±SEM and the significance of differences was tested between all groups using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test.

Results:

Relative expression of CD47 mRNA in cancer patients

Tracking the expression of the CD47 gene, the current study showed a remarkable increase in CD47 mRNA from cancer types. Higher elevation recorded in solid tumors including colon, ovarian, gall bladder, skin, brain, lung, and cervical cancers. All increased significantly ($p < 0.001$) when compared to their controls. The highest fold change in solid tumors was recorded in ovarian cancer (1755.77 ±2.89), followed by colon cancer (1464 ± 17.59), brain cancer (1210.32 ± 5.49), lung cancer (1118.51 ± 10.15), cervical cancer (814.45 ± 17.95), skin cancer (309.77±11.35), whilst breast cancer showed the lowest expression (0.08 ± 0.005) as shown in Figure (1).

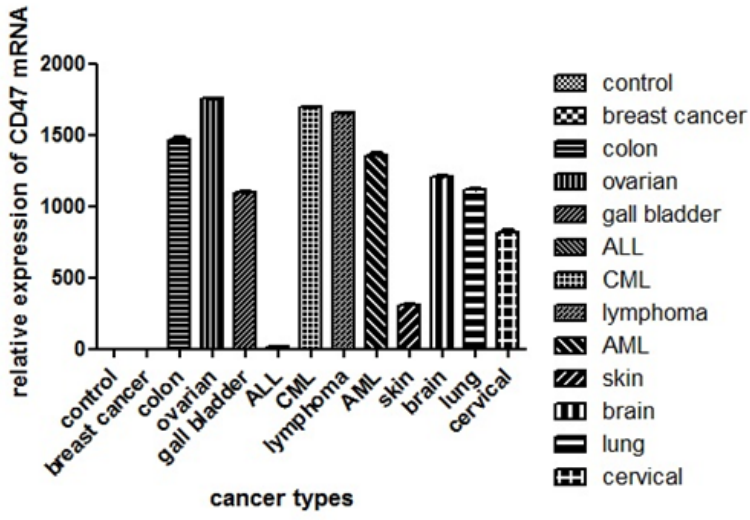

Figure 1: Up-regulation of CD47 mRNA in cancer.

On the other hand, blood-derived cancers show a significant increase in CD47 expression levels which peaked at 1696 ± 3.10 in chronic myeloid leukemia followed by lymphoma (1658 ± 1.64), AML (1353 ± 26.59). The least expression shown in acute lymphatic leukemia (12.33 ± 0.25).

Investigation of the CD47 protein

In order to further confirm our results, ELISA techniques were used to investigate and study CD47 protein concentrations in the patient's serum samples. The results in Figure 2 show a significant increase ($p < 0.001$) in CD47 protein concentrations in cancer patients compared to its levels in controls. Their levels reached the peak in stomach cancer were Mean ± SE (4127.040 ±72.372) compared to their values in the control group (209.303 ±75.814). Bile duct cancer patients were the second cancer that showed a marked increase in CD47 protein levels (2899.290±105.537) followed by CML cancer (1763.967 ± 35.144) and breast cancer (816.737±64.530). On the other hand, CD47 levels were not statistically affected in patients with Lymphoma and NHL patients (328.340±92.533, 187±12.222) respectively compared to their relative controls.

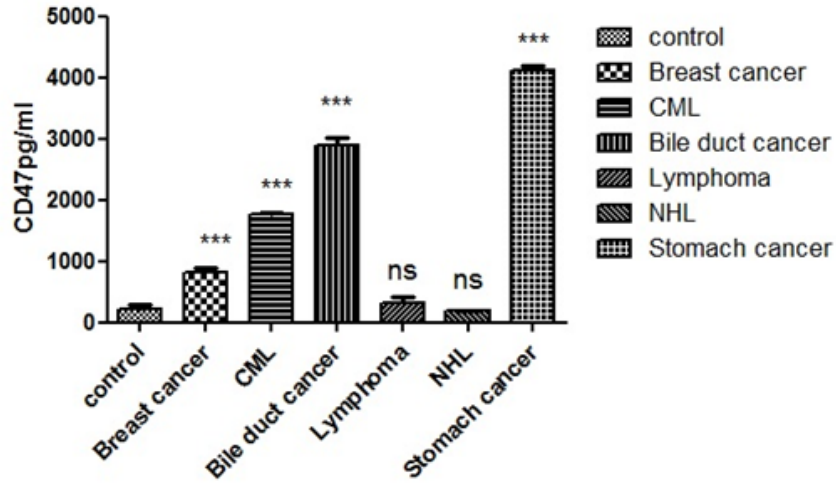

Figure 2: CD47 protein concentration is altered in cancer

Serum samples were collected from patients and controls and CD47 protein concentration was performed using a specific precoated antibody ELISA kit.

Patients with lymphoma and NHL did not show an increase in CD47 protein concentration in this study, which may be due to their taking of the chemotherapy drug.

Discussion:

The main objective of this study was to develop research-based data to investigate the innate immune checkpoint CD47 in newly diagnosed cancer patients to confirm the current knowledge available about this marker and determine its expression in Iraqi cases, especially in the local community of Kirkuk city. The advantages of this type of research are considered crucial and the first step in evaluating the importance of targeting the CD47 signaling pathway, which was mentioned earlier in this article, that this marker interacts with a receptor called SIRPα on immune cells such as macrophages. This interaction is a bridge through which cancer cells escape from being phagocytosed. Therefore, it is beneficial to start tracking CD47 expression in solid and blood cancers. From this point on, the current study is the first to shed light on this important strategy in the area. Regarding the downstream signaling, this research first investigated the gene expression using q RT-PCR.

Current study results for CD47 gene upregulation in ovarian cancer are in agreement with another recent study by Yu et al (2021) were they found a close relation between CD47 increase and immune cell infiltration in ovarian cancer tissues (17) suggesting its role in ovarian cancer heterogeneity which could be used as a prognostic biomarker which in turn could add new prospective in ovarian immunotherapy.

The second type of cancer that recorded high levels of CD47 gene expression was colon cancer, and these results could be supported by other researcher's results in colorectal cancer (18, 19), possible reason could be the effect of CD47 overexpression on immune cell infiltration and apoptosis rate (20). This explanation is confirmed via blocking PDL-1 and CD47 which enhances the therapeutic activity of chemotherapeutical drugs (21).Our results of CD47 upregulation in brain was in line with other researcher who demonstrated that the increase of CD47 expression in glioblastoma could be talked by a combination of using an inhibitor for CD47 alongside with the treatment with temozolomide which enhanced the phagocytic activity of macrophages (22).

Many other studies are in line with the results of the current study on CD47 upregulation in cancer cells. A study conducted by Yoshida et al. (2015) showed that CD47 positive malignant cells recorded higher proliferation activity than cells with lower concentrations of CD47 in gastric cancer indicative of an adverse prognostic agent in gastric cancer immunotherapy (23). Another research in row with our results stated that CD47 is a novel target for bile duct cancer (which is also called cholangiocarcinoma; CCA) treatment via blocking CD47 which in turn enhances macrophage ability to phagocytose cancer cells (24). The same scenario was approved as well in acute myeloid leukaemia; AML(25).another recent project developed a local inhibitory checkpoint antibody to block the interaction between CD47 and SIRPα in AML, which could also be used for the elimination of side effects of healthy cells during immunotherapy (26) Overall, the current study is the first of its kind to record CD47 overexpression in cancer types in patients in Kirkuk city and Iraq. However, limitations are unmissable in any research. The current study faced many limitations such as matching both q RT-PCR and

ELISA experiments for all types of cancer, as the ELISA kit was expensive. In addition to the limited time available for the study. This type of research requires more time, effort, and expenses. The results of this study require further studies such as investigating the proliferation of cancer cell lines and evaluating CD47 and its receptors in macrophages during phagocytosis. The phagocytic activity of macrophages and its correlation with up-regulation of CD47 are also suggested.

Conclusions

CD47 is highly upregulated in ovarian, colon, brain, lung, and gallbladder cancers while it did not show any effect in breast cancer with a slight change in skin cancer. Blood-derived cancers, on the other hand, did not show an effect of CD47 in acute lymphatic leukaemia; ALL. However, the CD47 gene and protein showed a dramatic increase in chronic myeloid leukemia and a lower content in acute myeloid leukemia. Lymphoma patients showed a significant up-regulation of CD47 in the patients under investigation at the gene expression levels and protein levels, indicative of an important role of CD47 in cancer immunotherapy due to its blockage. More detailed and continuous studies are needed to further investigate the signaling pathways involved in the CD47 link with SIRP-α. In summary, this study establishes CD47 as a promising target for researchers in order to develop new CD47 inhibitors that allow better recognition of cancer cells by innate immune cells.

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Conflict of interest: None.

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