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Article

Serum Biomarkers in Breast Cancer: A Comparative Study of Xanthine Oxidase, Thymidine Kinase, and Hormonal Profiles in Iraqi Women

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Abstract: The study was conducted in the laboratories of Marjan Teaching Hospital and several private laboratories in the city of Hilla, Babylon Governorate, from December 1, 2023, to January 15, 2024. It included 35 samples from women with breast cancer, aged between 35 and 70 years, and 35 samples from healthy women of the same age range, serving as control samples. The breast cancer diagnoses were confirmed by specialists at the Cancer Center. Blood samples were collected from both groups (patients and controls) and separated using centrifugation. The levels of xanthine oxidase (XO), thymidine kinase-1 (TK1), and various variables, including CRP, estrogen, progesterone, vitamin D, iron, and calcium, were then measured.

The results of the study demonstrated a significant increase in the levels of XO, TK1, CRP, progesterone, estrogen, iron, and calcium, and a significant decrease in vitamin D levels in the blood serum of the breast cancer group compared to the control group, with a statistical significance of $P \leq 0.001$. This study highlights the significant biochemical differences between breast cancer patients and healthy individuals, suggesting that these biomarkers could potentially be useful for the diagnosis and monitoring of breast cancer. Further research is warranted to explore the underlying mechanisms of these changes and their implications for breast cancer prognosis and therapy.

Keywords: Xanthine oxidase, Thymidine kinase, CRP, Estrogen, Progesterone, Breast cancer

Introduction

Cancer is a broad term encompassing over 100 distinct types of malignant tumors that can develop in various tissues throughout the human body (Iacona, Joseph et al., 2019). It is characterized by the uncontrolled proliferation of a group of cells, leading to the creation of cells that do not adhere to the normal rules of cell division. These aberrant cells continue to grow, forming new, abnormal cells capable of invading adjacent tissues and metastasizing to different parts of the body by penetrating the walls of blood and lymphatic vessels (Matthews et al., 2022). Among different types of cancer, breast cancer is one of the most prevalent non-skin malignant cancers (Yedjou et al., 2019). While it can occur in both women and men, the incidence in women is significantly higher approximately 200 cases in women for every single case in men (Ahmedin, 2010). The majority of breast cancers originate in the breast ducts and can metastasize to other parts of the body, including the enclosed lymph nodes (Horton et al., 2018). Xanthine oxidase (XO) is the final enzyme in the breakdown of purines and plays a crucial role in generating reactive oxygen species (ROS). This enzyme has been identified in various tissues, including the tongue, trachea, esophagus, sweat glands, mammary glands, small intestine, liver, colon, renal tubules, skeletal muscles, lungs, and spleen (Bruder et al., 1984). XO contributes to cancer development by generating ROS, such as superoxide anions and hydrogen peroxide. It has been shown that ROS can initiate and/or promote the carcinogenic condition through inducing specific mutations in proliferating cells (Sakuma et al., 2015; Ali et al., 2024). Additionally, ROS generated by XO stimulate inflammatory pathways, creating a tumor-promoting environment and enhancing processes like cell proliferation, invasion, and angiogenesis (RR). There are some evidences indicating that overexpression of XO is able to facilitate the breast cancer metastasis by promoting epithelial-mesenchymal transition (EMT), enabling cancer cells to spread to other tissues (Gupta et al., 2012). Due to its significant role in promoting oxidative stress and inflammation, XO is considered a potential therapeutic target, with inhibitors like allopurinol being explored to reduce ROS production and slow cancer progression (Verma et al., 2023; Gupta et al., 2012). Thymidine kinase (TK) is a phosphotransferase enzyme found in most living cells (Wintersberger, 1997; Okazaki and Kornberg, 1964). It is a critical enzyme in the pyrimidine salvage pathway, catalyzing the final phosphate transfer (Leija et al., 2016). Thymidine kinase aids in synthesizing phosphate by thymidylate synthase from deoxyuridine monophosphate in the presence of folic acid and vitamin B12, which helps protect the body against cancer development (Andrei and Snoeck, 2011). In addition, the TK is an enzyme critical to the salvage pathway of DNA synthesis. Accordingly, it facilitates the thymidine phosphorylation during DNA replication and repair, particularly in rapidly proliferating carcinogenic cells (Sugitani et al., 2022). Due to its crucial role in supporting rapid cell proliferation, TK activity is significantly elevated in many cancers, making it a valuable biomarker for tumor growth and aggressiveness (Manouchehri Doulabi et al., 2024). High levels of TK, especially the TK1 isoform, are associated with increased tumor burden and poor prognosis in various cancers, such as breast, lung, and prostate cancer (Xie et al., 2022). Recently it has been shown that TK can be considered as a target for anticancer therapies, including inhibitors and innovative gene therapy approaches, like the herpes simplex virus thymidine kinase (HSV-TK) suicide gene therapy, which selectively eliminates the cancer cells (Sułkowski et al., 2018). Estrogen, a steroid hormone derived from cholesterol and secreted by the ovaries, was traditionally believed to be produced exclusively by endocrine glands such as the adrenals, ovaries, and testicles (Abebe et al., 2022). Estrogen promotes breast cancer development by exerting both direct and indirect proliferative effects on cells affected by the disease. These effects may be direct, influencing enzymes and proteins involved in DNA synthesis and activating oncogenes (Lupulescu, 1995). In addition to estrogen, progesterone is another essential female hormone, regulating ovulation and menstruation. It transforms the uterine lining into a secretory one,

inhibits the secretion of gonadotropin from the pituitary gland (which prevents follicle maturation and ovulation), promotes mammary gland growth, relaxes smooth muscles in the uterine lining, maintains pregnancy, and is produced in the ovaries, uterus, and brain (Abdul Rahim Muhammad and Sabah Nasser, 1989). Similar to estrogen, the progesterone is able to promote the hormone receptor-positive breast cancers through binding to progesterone receptors (PR) on breast cancer cells. This signaling pathway can enhance cell proliferation, survival, and invasion (Li et al., 2022; Pedroza et al., 2020). This hormone-receptor interaction leads to the expression of genes that support tumor growth and inhibit apoptosis, allowing cancer cells to thrive and evade normal cell death processes (Lappano et al., 2022). Additionally, progesterone has been found to contribute to the expansion of breast cancer stem cells. The stem cells are believed to play a crucial role in tumor initiation, recurrence, and resistance to therapy (Chen et al., 2020). In addition, this hormone interacts with estrogen signaling pathways, potentially amplifying estrogen's tumorigenic effects (Clusan et al., 2023). Breast cancer tumors have been linked to elevated blood calcium levels (Grattan and & Freake, 2012), as calcium contributes to cancer formation by regulating cell proliferation (Baltaci et al., 2019). As another ion affecting the cancer development, iron is closely associated with tumorigenesis in various human cancers through mechanisms that include promoting mutagenic hydroxyl radical formation, regulating DNA replication, and affecting cell cycle development and repair (Zhang and Zhang, 2015). This has a direct impact on signal transduction in cancer cells and serves as a vital nutrient for cancer cell proliferation (Fu et al., 2022). Given the observed increase in xanthine oxidase (XO) and thymidine kinase (TK) levels in various cancers, particularly breast cancer, the current study aimed to investigate the serum levels of these enzymes in female individuals aged 35-70 years with breast cancer. Additionally, we sought to explore the serum levels of C-reactive protein (CRP), sex hormones (estrogen and progesterone), iron, calcium, and vitamin D in these patients. Finally, we aimed to identify the concurrence of all these factors in patients with breast cancer from the Babylon Governorate.

Materials and Methods

Specimen Collection

This study was conducted in the laboratories of Marjan Teaching Hospital and several private laboratories in Hilla, Babylon Governorate, over the period from December 1, 2023, to January 15, 2024. A total of 70 samples were collected, including 35 samples from women diagnosed with breast cancer and 35 samples from healthy women serving as controls, all aged between 35 and 70 years. The breast cancer diagnoses were confirmed by specialists at the Cancer Center. Blood samples were collected from both groups (patients and controls), followed by serum separation using centrifugation.

XO, TK, and CRP Levels in Blood Serum

The concentrations of XO, TK1, and CRP in serum were determined using the ELISA-Sandwich technique. The assay kits were sourced from Cusabio (Uniprot No: P47989, USA), My BioSource (Cat N: MBS3804422, USA), and CUSABIO (Uniprot No: P04271, China), respectively.

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Estrogen and Progesterone Levels in Blood Serum

To assess estrogen and progesterone levels, the Enzyme-Linked Immunosorbent Assay (ELISA) method was employed. A 100 µl from serum samples from patients were added to

wells along with enzyme-labeled estrogen and progesterone according to manufacturer's recommendation. After incubation, the wells were washed to remove any unbound components. A substrate, provided by kit, were then added to produce a color change. The reaction was stopped with a stop solution, and the optical density was measured at 450 nm using ELISA reader (DANA, 3200). A standard curve was generated using known concentrations of estrogen and progesterone.
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Vitamin D Levels in Blood Serum
The concentration of 25-hydroxyvitamin D (25-OH Vitamin D) in serum samples was determined using an ELISA kit from Euroimmun (Germany).
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Estimation of Iron Concentration in Blood Serum
Serum iron levels were measured using a colorimetric method that converts ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions in a mildly acidic medium. The resulting ferrous ions form a colored complex with Ferrozine, and the concentration was quantified based on the color intensity.

Calcium Concentration in Blood Serum
Calcium concentration was measured using a pre-prepared assay kit, where calcium ions react with 5-nitro-5-methyl-BAPTA under alkaline conditions to form a complex. This complex is then further reacted with EDTA, and the change in absorbance, which is directly proportional to calcium concentration, was measured optically.

Statistical Analysis
The data from the study were statistically analyzed using the XL-STAT software package. The t-test was employed to compare the differences between the two groups, with statistical significance considered at a probability level of $P \leq 0.001$.
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Results
The serum levels of XO, TK1, CRP were increased in breast cancer
The biochemical analyses revealed a significant increase in the serum levels of XO (p=… PLEASE INCLUDE THE EXACT P-VALUE) and TK1 (p=…. PLEASE INCLUDE THE EXACT P-VALUE) in the breast cancer group compared to the intact control subjects (Fig. 1A, 1B). Moreover, the cancer patients represented remarkably (p=…. PLEASE INCLUDE THE EXACT P-VALUE) higher CRP level versus the control subjects (Fig. 1C).
The progesterone and estrogen levels were increased in cancer group
Observations demonstrated a remarkable increase in the serum levels of progesterone (p=…. PLEASE INCLUDE THE EXACT P-VALUE) and estrogen (p=…. PLEASE INCLUDE THE EXACT P-VALUE) in the cancer patients when compared to the control individuals (Fig. 2A, 2B).
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The vitD was decreased and the iron calcium level was increased in breast cancer

The biochemical analyses for vitD and calcium showed a remarkable (p=…. PLEASE INCLUDE THE EXACT P-VALUE) decrease in the serum vitD level in the cancer group versus the control individuals (Fig. 3A). In contrast, the cancer patients showed a significant (p=…. PLEASE INCLUDE THE EXACT P-VALUE) increase in the serum calcium level when compared to the control group (Fig. 3B). similar to calcium level, the cancer group showed a remarkable (p=…. PLEASE INCLUDE THE EXACT P-VALUE) increase in the serum iron level (Fig. 3C).

Discussion

Breast cancer is the second leading cause of death among women in Iraq and worldwide. It impacts various physiological processes, leading to enzymatic and hormonal changes in the body. Notably, XO levels were significantly elevated in Iraqi women with breast cancer compared to healthy controls. This finding aligns with those of a study by Thamer (Thamer, 2018). The observed increase may be attributed to an imbalance between oxidation and reduction within cells, which is often associated with oxidative stress observed in many cancer cells relative to normal cells. Consequently, this oxidative-reductive imbalance may contribute to tumor promotion (Valko et al., 2008). To better understand the condition, one should note that the XO-induced ROS increases under hypoxic conditions, which promotes the inflammatory signaling and tumor progression by enhancing angiogenesis, cell migration, and metastasis (Romagnoli et al., 2010; Balamurugan, 2016). Moreover, the free radicals also play a critical role in cancer development by inducing mutations in DNA, and inhibiting free radicals can potentially reduce or prevent cancer onset (Kalcıoğlu et al., 2004).

The present study also demonstrated a rise in TK1 enzyme levels in the patient group compared to the control group, consistent with findings from Hussein et al. (Hussein et al., 2020). The increase in TK is observed to double preoperatively compared to postoperatively in women with breast cancer (Thwani and Mohsin, 2012). Additionally, research indicates that TK1 levels rise variably in breast cancer patients, depending on the tumor's developmental stage (Ranjan and Sinha, 2014). As such, TK1 may serve as a valuable biomarker for monitoring treatment efficacy in breast cancer patients and could also have therapeutic implications.

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Furthermore, the study revealed a significant increase inCRP levels in both patient group, consistent with findings by Panis et al. (Panis et al., 2012) and Allin et al. (Allin et al., 2011). Elevated CRP levels in women with breast cancer may be due to chronic inflammation, a key factor in the carcinogenesis process. Indeed, inflammatory pathways significantly contribute to breast cancer development (Asegaonkar et al., 2015). CRP is an acute-phase inflammatory protein, and its elevated levels may also result from tumor presence or treatment-related fatigue (Han et al., 2011; Joly et al., 2019).

Progesterone levels were also significantly elevated in the patient group, in agreement with the findings of Ismail et al. (Ismail et al., 2016) and Mousa (Mousa et al., 2013). This increase is attributed to progesterone's role in promoting cell proliferation in breast tissue, where it acts as an initial trigger for growth by increasing growth factor signaling, including epidermal growth factor receptor (EGFR) pathways and associated proto-oncogenes, thus enhancing the proliferation of breast cancer cells (Gottlieb et al., 1997). Progesterone also prepares the reproductive system for pregnancy (Britton et al., 2020). Similarly, estrogen levels showed a significant increase, aligning with the findings of Abdel Fattah (Abdel Fattah et al., 2016) and

Hassan (Hassan et al., 2019). Estrogen is thought to increase breast cancer risk by binding to estrogen receptors (ER) in the nucleus of cancer cells, acting as a transcription factor to regulate cell division and promote cancer cell proliferation (Ho et al., 2008). It also activates pathways responsible for the proliferation and differentiation of mammary cells (Matthews and Gustafsson, 2018).

Conversely, vitamin D levels were significantly reduced in the serum of breast cancer patients, consistent with the findings of González-Fisher et al. (Gonzalez-Fisher et al., 2016) and Shekarriz-Foumani et al. (Shekarriz-Foumani et al., 2016). Vitamin D deficiency negatively impacts breast cancer survival and incidence. Moreover, one study suggests that a diet high in fruits, vegetables, vitamins, and minerals, combined with low saturated fat intake, may reduce breast cancer risk (Deeb et al., 2007;; Vrieling et al., 2014). Vitamin D plays a crucial role in cancer prevention and elimination due to its significant biological functions (Sun et al., 2019).

Iron levels were found to be significantly elevated in the serum of breast cancer patients, consistent with the findings of Rozoqi (Rozoqi et al., 2021) and Salih (Salih et al., 2007). This increase may be due to the heightened metabolic rate and proliferation of cancer cells, which require more iron than normal cells, resulting in increased oxidative stress (Schieber et al., 2014). Elevated iron metabolism is associated with malignant transformation, cancer progression, and drug resistance. Iron overload can lead to programmed cell death, either through the use of iron-chelating agents or by activating self-regulatory mechanisms (Brown et al., 2020).

Calcium levels also showed a significant increase in the serum of breast cancer patients, consistent with the findings of Hassan (Arooj et al., 2012). The elevated calcium levels may be linked to its protective effect against breast cancer, as observed in studies evaluating dietary intake of calcium and other minerals and vitamins (Chen et al., 2010). However, one study noted that calcium and other mineral supplements, such as magnesium and selenium, did not significantly reduce the overall risk of breast cancer in postmenopausal women, suggesting that genetic factors may also play a role in certain types of breast cancer (Cullen et al., 2002).

Conclusion

This study provides valuable insights into the biochemical alterations associated with breast cancer in women from the Babylon Governorate, highlighting the significant changes in serum levels of XO, TK1, CRP, sex hormones (estrogen and progesterone), vitamin D, iron, and calcium. Elevated levels of XO and TK in the breast cancer group suggest their potential roles in oxidative stress and DNA synthesis, respectively, contributing to tumor growth and progression. The increased levels of CRP reflect an enhanced inflammatory response, which may be indicative of chronic inflammation in breast cancer pathology. Similarly, higher concentrations of estrogen and progesterone in patients align with the hormone-driven nature of certain breast cancer types, suggesting a potential for targeted hormonal therapies.

Conversely, the decreased vitamin D levels observed in breast cancer patients reinforce the protective role of this vitamin against cancer development, underscoring the need for adequate vitamin D supplementation or dietary intake as a preventive strategy. Elevated iron levels in the patient group indicate a potential role for iron in promoting oxidative stress and tumor progression, while increased calcium levels may reflect metabolic changes associated with cancer or its treatment.

Overall, the findings of this study underscore the importance of a multi-faceted approach in understanding the complex biochemical environment of breast cancer. They highlight potential biomarkers for early detection and monitoring of breast cancer progression and suggest avenues for therapeutic interventions targeting these altered biochemical pathways. Future research should focus on expanding the sample size, exploring the molecular mechanisms

underlying these changes, and investigating the potential of these biomarkers in guiding personalized treatment strategies for breast cancer patients.

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