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Assessment of hypoxemia status by measuring serum level of hypoxia inducible factor 1 alpha in relation to tumor suppression protein p53, estradiol and tumor proliferation markers of breast cancer in Thi-Qar province/Iraq

Zainab Ali Khadem[a,*] |Shatha Abdul Wadood AL-Shammaree[b] |Mohanad Abdulretha[c]

[a]*Department of Clinical Biochemistry, College of Medicine, University of Thi-Qar, Thi-Qar, Iraq*

[b]*Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq*

[c]*Department of Surgery, College of Medicine, University of Thi-Qar,Thi-Qar, Iraq*

Hypoxemia means low levels of oxygen in the blood and it alters cancer cell metabolism and causes multiple intracellular signaling pathways. Investigate hypoxia status by measuring the concentration of HIF-1α as a prognostic factor of hypoxia and its relation to tumor suppressor protein p53, estradiol, tumor grade, tumor size, and lymph node metastases of adult female with breast cancer. This study is a case control study which includes sixty- five adult female patients with breast mass. Out of 65 patients, a 44 (68%) were with fibroadenoma in the age range of 18-42 (32.55±6.40) and the other 21 (32%) cases with breast cancer type invasive ductal carcinoma (IDC) aged 32-80 (56± 14.4). Most IDC cases were in grade III and sizes T2 and T3. The other 50 healthy females, as a control group were in mean age of 44.56±16.85. Preoperative blood samples were collected for biochemical analysis by the ELISA method. A significant elevation in serum HIF-1α, P53 and E2 ($p<0.001$) in IDC cases as compared to fibroadenoma and the control group, there was a positive relation between HIF-1α, P53, and E2. HIF-1 α, P53, and E2 were significantly elevated in the patients with grade III and tumor stage T3 than grade II and stages T2 and T1. A significant elevation was found in the subgroup of positive LNM for all preoperative serum levels of parameters compared with the negative LNM patients group. HIF-1α, p53, and E2 were useful markers of invasion depth of tumor or LNM in breast cancer staging and the interactions between HIF-1α, p53, and E2 signaling pathways may be of major clinical significance in cancer therapies through targeting the lowering of severity of hypoxia and angiogenesis.

***Corresponding Author:**
Zainab Ali Khadem
Email: zainabalsalmy111@gmail.com
Tel.: +9647721014811

KEYWORDS

Hypoxia; breast cancer; invasive ductal carcinoma; HIF-1α, tumor suppression protein p53; estradiol and lymph node metastases.

Introduction

About 25% of women were exaggerated with breast diseases and the majority occurred as a new breast mass which was related to a wide range of causes, this type of malignant disease has been the most common and highly mortality rate among the other cancer types [1,2].

Oxygen is an essential element for cellular metabolism and energy yield. The reduction of the normal oxygen level or supply to tissue is

called hypoxia [3,4]. Breast tumors are the most solid tumor types and have been shown to exhibit regions of hypoxia which is a characteristic feature of cancer and inflammation [5]. Hypoxia causes a locally failure complicated with metastasis. It is important to assess the hypoxia statuses in this cancer type which are associated with molecular genetic defects [6]. Malignant cells rapidly grow so it needs the high energy demand and causes multiple newly formed blood vessels to take nutrients inside the solid tumor mass causing angiogenesis [7]. These cell types grow in hypoxia condition, in an event known as "Warburg Effect" in which cells have energy as ATP, through the use of a fermentation pathway instead of the citric acid cycle, this is a universal phenotype of cancer cells [8].

HIF-1α is only expressed during hypoxic conditions as well as a measurement as oxygen-dependent components of the HIF transcriptional complex [9], the stability and activity of the alpha subunit of this factor are regulated by its post-translational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation [10,11].

Tumor suppressor protein p53 is a prominent transcription factor which regulates many cellular metabolism and functions, it has been classified as a "guardian of the genome" because of its vital role in response to DNA damage and can be interacted with multiple signaling transduction and amplification pathways associated of the following this response to abnormal propagation signals, DNA damage, hypoxia, and osmotic stress [12]. The p53 function as transcription factor which activates a variety of genes responsible of DNA damage repair, apoptosis, cell cycle arrest, and many other gene clusters related with diverse processes such as membrane functions, transcription, cell adhesion, cell mobility, and the other different metabolism which affected by p53 activity. The mutation in p53 is commonly proved in many cancer types and is an additional cause and risk factor of tumor progression, resistance to chemotherapy, and poor prognosis [13]. There are different results and observations about the fact of serum P53 level in benign and malignant breast tumors [1]. While breast tumor growing up, hormones such as 17β-Estradiol or estrogen (refer to both hereafter as E2), a steroid hormone play an important roles which have a correlation and are prominent in mammary gland development, it's the main risk factors in breast cancer, because of its actions which mainly mediated by two receptors as estrogen receptor-α (ERα) and -β (ERβ) [15]. On other hand, the conversation of estrogens to quinone metabolites, which directly bind to DNA cause mutations at the depurinated sites, then results in an error in DNA repair. In addition, the accumulation of these mutations aggressively would contribute to the development of breast cancer [16-17]. There were a few studies on the relation between HIF-1α and estradiol which can prove either negatively or positively affects the hypoxia signals and path ways in different cellular contexts.

This study was designed to investigate hypoxia status by measuring HIF-1α as a prognostic factor of hypoxia in serum of adult female with benign and malignant breast tumors in relation to p53, estradiol, and several factors which are a predictive criteria for the prognosis such as grade, tumor size and the status of lymph node.

Materials and methods

This study is a case control study which is carried out in Nasiriyah city in Thi-Qar province at Al-Hussein Teaching Hospital and Al-Habboby Teaching Hospital, during the period from January to August 2021, a sixty-five adult female patients who were suffering from breast mass, admitted to the operating treatment. Histopathological examination

data such as tumor type, grade, stage, and LNM of breast tumor samples were recorded from medical records in histopathology unit. Control group included fifty healthy females who didn't have a history of breast mass, and their age range was between (18-80) years, and also their mean age was (44.56±16.85).

Biochemical analysis: About 3 mL preoperative blood samples were obtained and put in a sterile glass gel tubes, and then it was allowed to be clotted at 37 °C for about 30 minutes, then it was centrifuged at 3000 rpm for 10 minutes and the serum was separated and kept at −120 °C until which can be used for biochemical analysis [18]. Serum markers HIF-1α, P53, and E2 were deducted by the ELISA method according to the manufacturer's instructions by using kits from Elabscience, the United States, code no. E-EL-h6066 and E-EL-H0910, respectively for HIF-1α and P53. The E2 kit was from Monobind, USA, code no. 4925-300. The results were expressed as picograms per milliliter (pg/mL).

Statistical analysis

SPSS version 19 was used for statistical analysis. At first, the normality of the data was assessed by the Shapiro–wilk normality test, then Chi-square and percentage were used for clinical data. ANOVA and t-test used for analyzing the comparison between parameter concentrations, and finally the Pearson's correlation coefficient was employed to analyze the correlation between variables. A probability (p) value≤0.01 was considered statistically significant and p>0.01 meant no significant differences.

Results

Out of 65 patients, a 44 (68%) were associated with benign breast tumor diagnosed by histopathology as fibroadenoma aged between 18-42 (mean age 32.55±6.40) and the other 21 (32%) were associated with breast cancer treated by mastectomy, diagnosed by histopathologists as invasive ductal carcinoma not to be otherwise specified (IDC:NOS) type, at age range between 32-80 (57±14.4), as displayed in Figure 1.

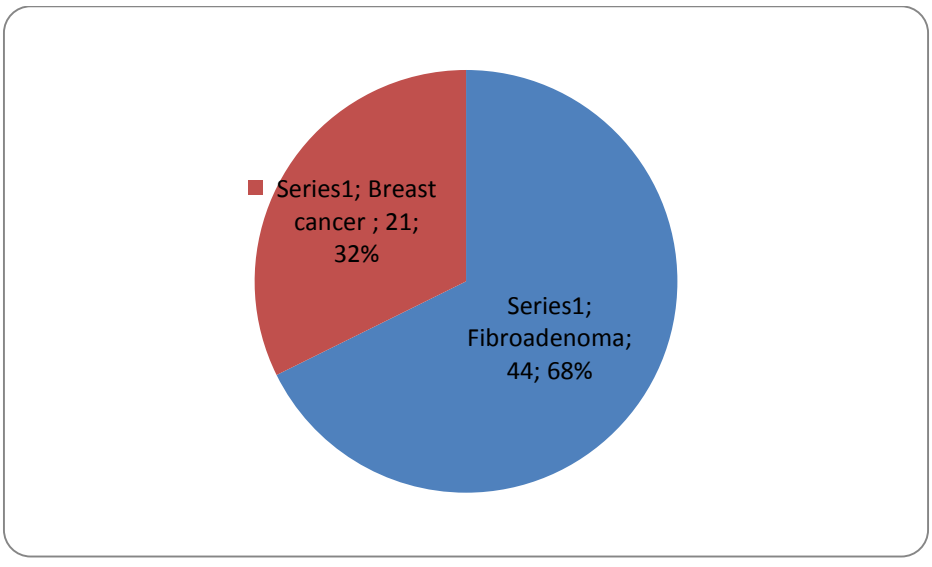

FIGURE 1 A pie chart depicting the distribution of cases according to the breast tumor type

There were 12 (57%) of the 21 breast cancer patients and their tumor tissue was associated with high grade and also it was poorly differentiated (grade III), and 9(43%) were moderately differentiated or intermediate grade (grade II). Noteworthy, only 5 patients (24%) were with T1 and the high percentage 8 (38.1%) with T2 and also 8 (38.1%) were with T3. LNM examinations

indicate that 8 (38%) patients with negative LNM and 13 (62%) positive cases for LNM.

The mean concentration of HIF-1α, p53, and E2 preoperative serum levels (pg/mL) for breast tumor patients and the control group were listed in Table 1 and Figure 2. In the control group, HIF-1α was undetectable 0.00 pg/mL, so the standard division was 0.00. Hypoxia markers were elevated in both breast tumor patients, and there were significant ($p<0.01$) differences between fibroadenoma and IDC groups. Both P53 and E2 were increased to a significant degree ($p<0.01$) in the IDC malignant group compared with the benign and control groups.

There were a highly positive correlations between HIF-1α and P53 (r=0.933), and HIF-1α with E2 (r=0.95), of 21 cases of breast cancer type IDC, its significance at the level 0.01, as displayed in Figures 3 and 4.

TABLE 1 Serum concentration (pg/mL) of HIF-1α, p53, and E2 of control and the 65 female patients with breast tumor. Concentration expressed as mean±S.D. value of probability<0.001 was referred to significant degree

Preoperative serum levels	Study groups Control (n=50)	Fibroadenoma (n=44)	Malignant IDC* (n=21)	P-value
HIF1α (pg/mL) means ±S.D	0.00±0.00	61.67±0.34	730.77±22.60	0.00
P53 (pg/mL) means±S.D	56.41±0.14	933.51±14.86	1742.20±23.6	0.00
E2 (pg/mL) means±S.D	103.16±10.43	110.71±5.46	331.54±15.87	0.00

* IDC: Invasive Ductual Carcinoma.

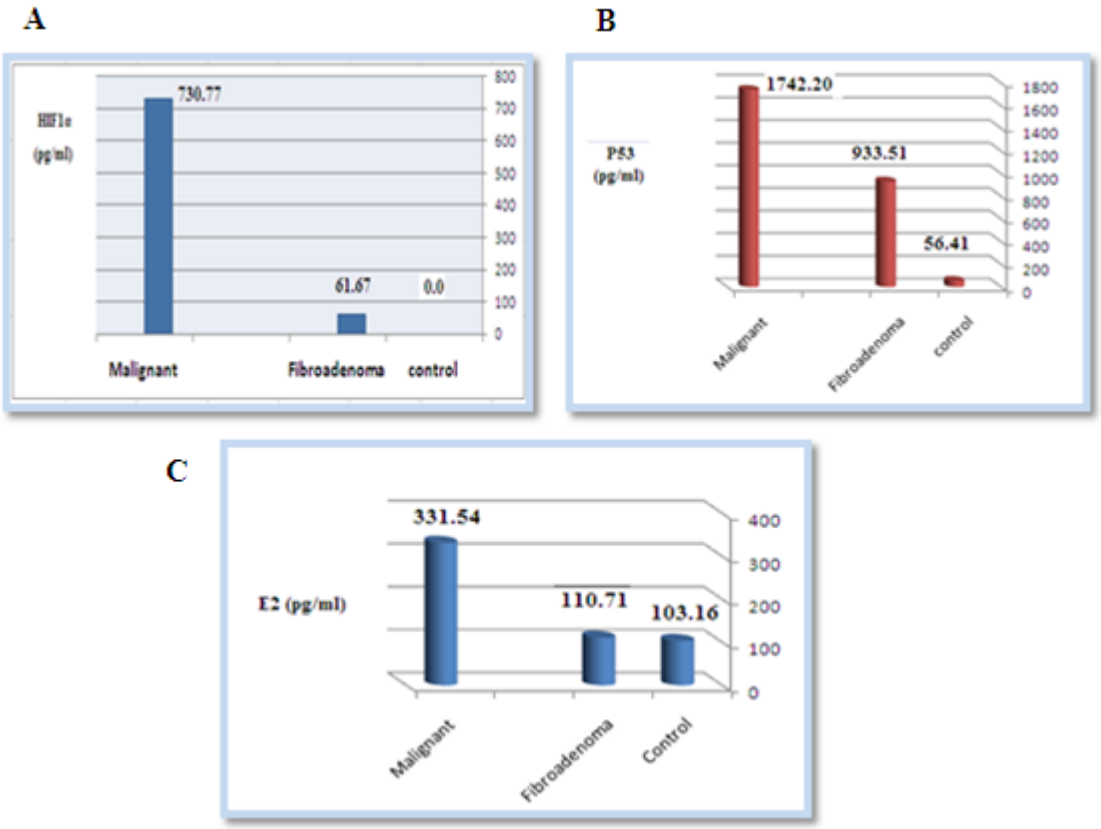

FIGURE 2 Preoperative serum levels (pg/mL) of biomarkers in control and breast tumor patients; (A) levels of HIF-1α, (B) p53 and (C) E2

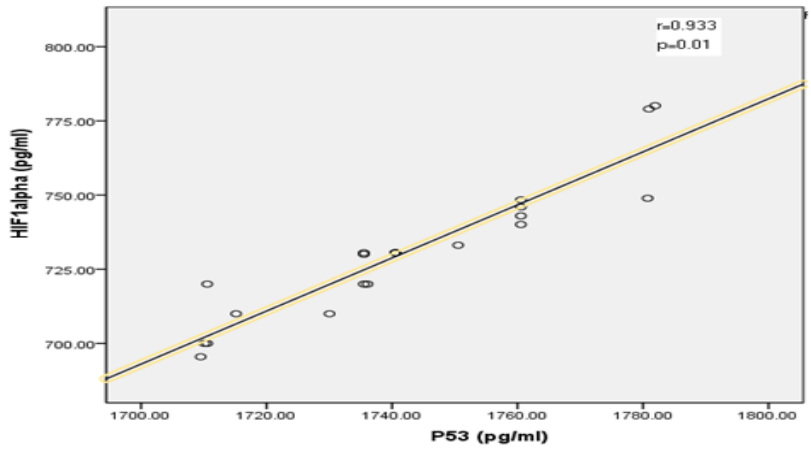

FIGURE 3 The correlation between serum HIF-1α (pg/mL) and serum level of p53 pg/mL in 21 cases of adult female patients with breast cancer type IDC

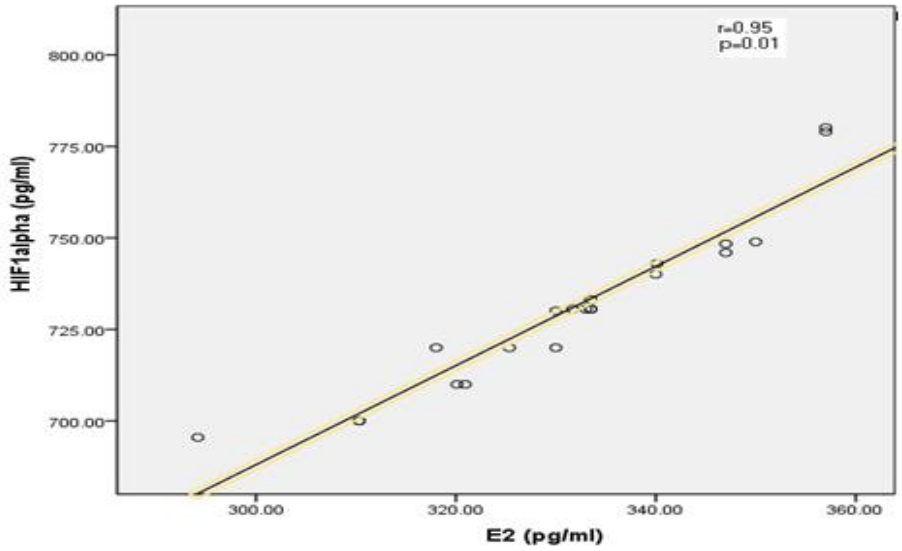

FIGURE 4 The correlation between serum HIF α 1(pg/mL) and serum level of E2 pg/mL in 21 cases of IDC adult female patients

In Figure 5A, B, and C, it is evident that preoperative concentration (pg/mL) of HIF-1α (745.06±17.63), p53 (1757.78±16.84), and E2 (341.94±9.41) were higher (P-value =0.00) in the patients with the poorly differentiated (grade III (n=12)) group than the moderately differentiated (grade II, (n=9)) group. Of HIF-1α (711.72±11.64), p53 (1721.43±12.41), and E2 (317.69±11.39).

In Table 3, the preoperative serum HIF-1α (Figure 6), p53 (Figure 7), and E2 (Figure 8), compared in terms of tumor size, parameters were significantly higher in the patients with T3 than T1 and T2.

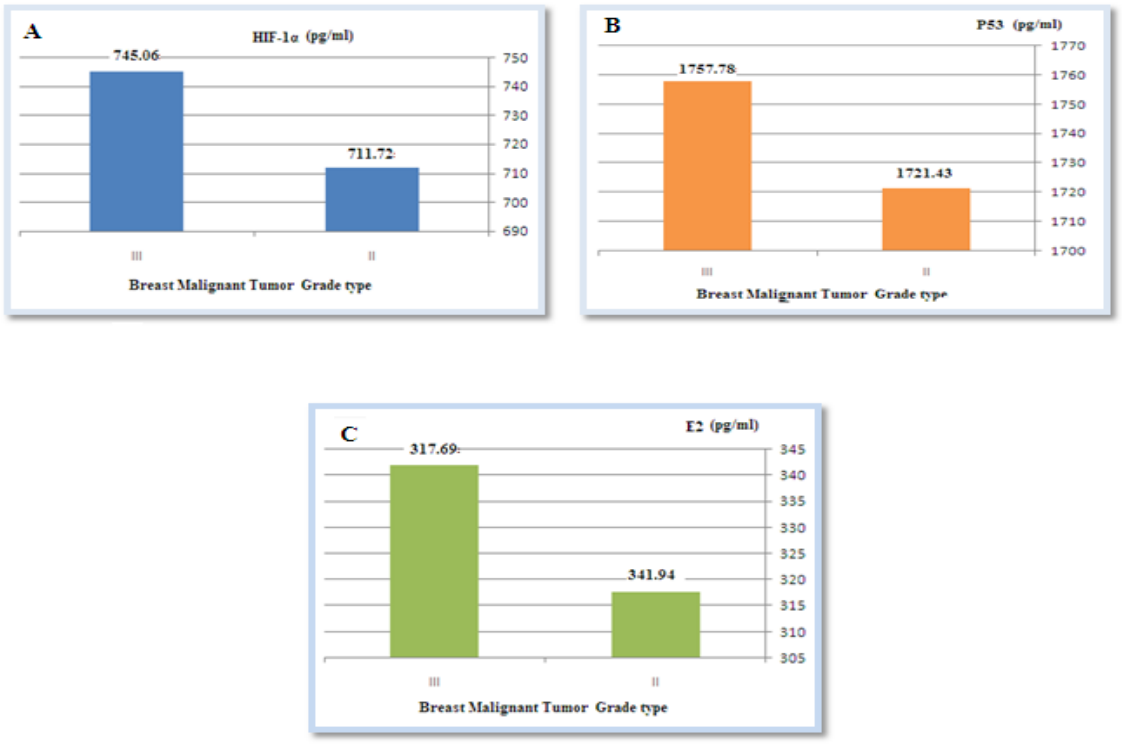

FIGURE 5 Preoperative Serum HIF-1α (A), p53 (B), and E2 (C) levels compared in terms of clinical pathological parameters tumor grade of breast cancer. Breast Malignant tumor Grade type II: Moderate differentiated, III: Poorly differentiated

TABLE 3 Preoperative serum levels of HIF-1α, P53 and E2 compared in terms in relation to tumor size

Parameters (pg/mL) means±S.D	Breast Malignant tumor size type*			P-value
	T1 (n=5)	T2 (n=8)	T3(n=8)	
HIF-1α	708.00±8.36	723.46±12.24	752.32±17.55	0.003
P53	1715.27±8.50	1734.21±10.27	1767.04±12.21	0.000
E2	315.94±5.23	326.40±13.29	346.45±8.32	0.010

*Breast Malignant tumor size type: T1: ≤20 mm, T2: >20 mm ≤ 50 mm, and T3: >50 mm.

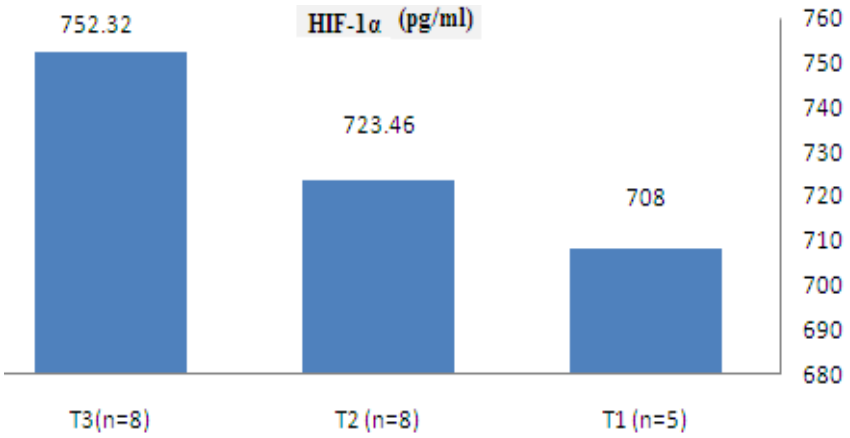

FIGURE 6 Preoperative serum levels of HIF-1α according to tumor size (T1: ≤20 mm, T2: >20 mm ≤ 50 mm, T3:>50 mm).

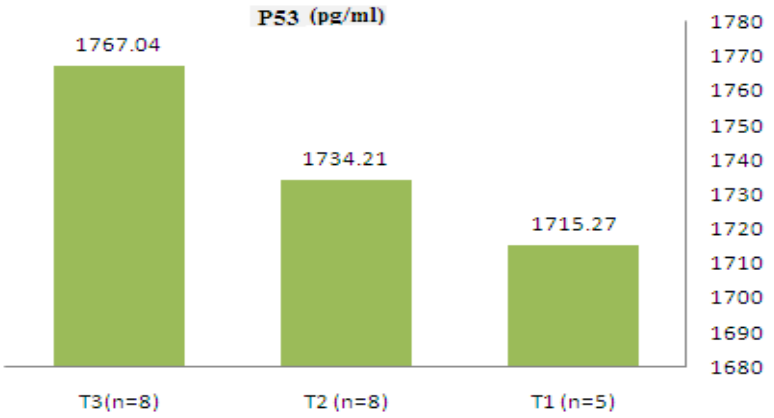

FIGURE 7 Preoperative serum levels of P53 in according to tumor size (T1: ≤20 mm, T2: >20 mm ≤ 50 mm, T3:>50 mm).

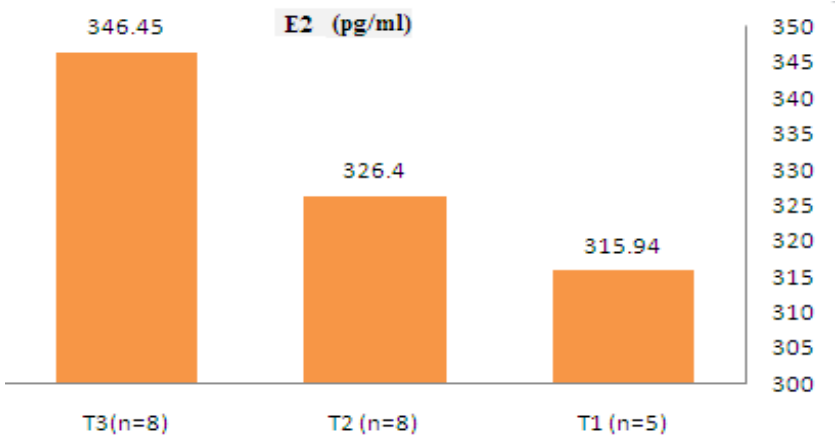

FIGURE 8 Preoperative serum levels of E2 according to tumor size (T1: ≤20 mm, T2: >20 mm ≤ 50 mm, T3: >50 mm)

In Table 4 and Figure 9, it is obvious that prognostic value for HIF-1α, p53, and E2 was related to the lymph node metastasis (LNM) of breast cancers group and a significant elevation was found in the subgroup of positive LNM for all HIF-1α, p53, and E2 levels compared with negative LNM.

TABLE 4 Preoperative serum levels of HIF-1α, P53, and E2 compared with Lymph node status of breast cancer **(IDC)** patients

Parameters (pg/mL) means±S.D	**lymph node metastasis (LNM)***		**P value**
	Negative (n=8)	**Positive (n=13)**	
HIF-1α	709.44±10.05	743.91±17.39	0.00
P53	1719.68±12.01	1756.07±17.26	0.00
E2	316.15±11.14	341.02±9.60	0.00

* Lymph node metastasis (LNM): negative: less progressive stage; there is no metastases to lymph node, positive: a progressive stage metastases level I, II axillary lymph node, and more extensive

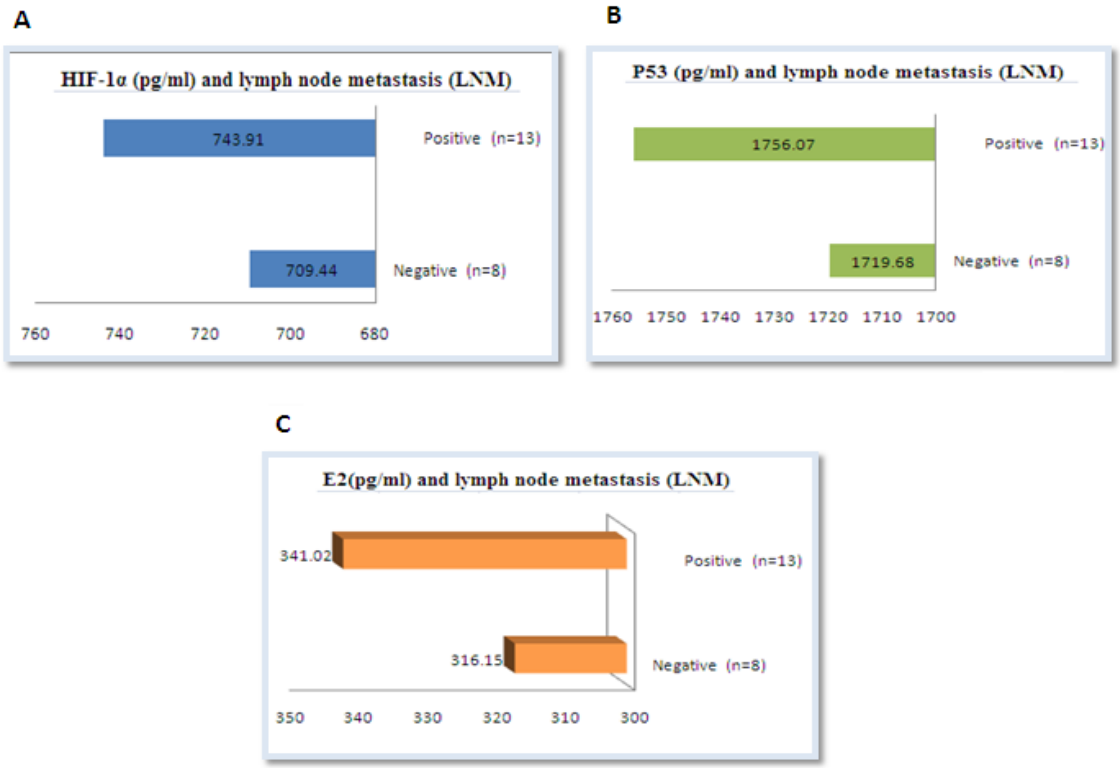

FIGURE 9 Preoperative Serum HIF-1α (A), p53 (B), and E2 (C) in relation to Lymph node status of breast cancer **(IDC**) patients

Discussion

Our results indicate that the level of HIF-1α in the serum of the control group was undetectable because in normal condition, oxygen is found in the best level and HIF-1α protein is incessantly degraded via the ubiquitin pathway, so it has a short half-life [20]. Elevated HIF-1α in fibroadenoma and IDC groups means that breast tumor was associated with hypoxia conditions. In fact, most solid tumor have a salient feature, and in this saturation, HIF-1α hydroxylation is impaired, the ubiquitination of HIF-1α is blocked, and complicated with "inflammatory hypoxia" [6,21]. The increase in oxygen requirement, poor supply, and increased metabolic demand lead to the increase in hypoxia at sites of inflammation which was associated with increased inflammation, the synthesis rate of inflammatory mediators, enzymes, and cytokines [22]. Pro-inflammatory cytokines like tumor necrosis factor regulates HIF-1α expression and synthesis [23].

In healthy subjects, we found that serum P53 was barely detectable because normally, the "standby" mode activation of p53 operates occurs and responds to a variety of cellular stresses such as DNA damage and expression of activated oncogenes to prevent the outgrowth of aberrant cells, by inducing cell cycle arrest, DNA repair or programmed death [24]. In contrast, most tumor cells associated with the elevated levels of p53 in benign and malignant patients prove mutations in the p53 gene in more than 50% of patients with malignant tumors, including amino acid substitutions [25]. An accumulation of p53 protein means p53 dysfunction and too much P53, as in cancer cases, can be severe. In malignant tissue, tumorigenesis enhances and affects the mutant p53 proteins [26]. Our adverse results contributed to another study reveals no different results in serum p53

levels in normal and malignant breast tumor [14].

An elevated plasma estradiol level is strongly related to the risk of developing the disease and regulates the growth of many breast tumors such as fibroadenoma which is believed to be stimulated by estrogen [27]. In women with estradiol 20–25% above the mean values, the incidence of breast cancer is 2 to 3 times higher [28]. Estrogens have been exhibited to increase angiogenesis and genotoxic effects in experimental breast cancer and normal human breast tissue [15]. Major mechanisms which are postulated to be involved in their carcinogenic effects are stimulation of cellular proliferation through their receptor-mediated hormonal activity and direct genotoxic effect (increasing mutation rates through cytochrome P450-mediated metabolic activation, and induction of aneuploidy) [29].

Malignant cases (IDC) were associated with significant elevation ($p<0.001$) in HIF-1α, p53, and E2 as compared with fibroadenoma cases. These abnormalities and changes were related to the nature and magnitude of the stress [26].

Both P53 and HIF-1α were linked absolutely. The interaction between p53 and HIF-1α plays a key role in the hypoxic response [30]. There are five response elements in the p53 promoter in which HIF-1α transcriptional up regulates p53 by binding to it [31]. It was reported that tumors bearing p53 mutations are generally characterized by higher HIF-1α levels and mutant p53 appears to stimulate HIF-1 α stabilization by blocking its interaction with mouse double minute 2 homolog (MDM2) under the hypoxic condition [32]. Cancer progression through mutant p53 can lead to a relationship with the hypoxia and HIF signaling pathway [33].

There was a positive interaction between HIF-1α and E2 signaling pathways. The secretion of estrogen responsive proteins via HIF-1α regulation by breast cancer cells leads to an increase in endothelial cell migration and tubulogenesis in vitro [34].

Many factors facilitate hypoxia status in blood. The three major tumor localization, tumor size, and blood flow, all affect the oxygen accessibility, and also the rapid growth of solid tumors often result in the development of hypoxic regions [35]. The proliferation and potentially associated with more aggressive tumors and increased micro vessel density occurs as incessant growth and vascular abnormalities lead to an insufficient perfusion of the tumor mass [35,36]. All that explains the increase in HIF-1α, P53, and E2 levels in cases associated with higher tumor grade III and stage T3 and thus, these results are accepted with the others [33,37], while the other studies indicates that there was no statistically significant differences in p53 detection in serum among the poor, semi-, or well-differentiated breast carcinomas [36].

Determent of the lymph node metastasis (LNM), distant metastasis, and swollen lymph nodes (lymphadenopathy) are the useful prognostic factors for assessing the relapse-free or overall survival [40]. Hypoxia plays a critical role in the progression degree of the tumor including invasion and metastasis, a significant elevation in HIF-1α, P53, and E2 as associated with the positive LNM subgroup in which HIF-1α is a central regulator of lymph angiogenesis and swollen lymph nodes [41].

Conclusion

Based on the results of the proposed study, it is clear that HIF-1α, p53, and E2 may be useful markers for invasion depth of tumor or LNM involvement in breast cancer staging and the interactions between HIF-1α, p53, and E2 signaling pathways as a major clinical significance in cancer therapies through targeting the lowering of severity of hypoxia and angiogenesis.

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Orcid:
Zainab Ali Khadem:
https://www.orcid.org/0000-0002-9003-3249

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