Androgen Receptor Marker among Iraqi Patients with Triple-Negative Breast Cancer Exposed to Depleted Uranium

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Two recent major wars and a 12-year economic embargo as well as several years of war on terrorism have had a damaging effect on Iraq’s land, air, water, food, and health infrastructure. The presence of depleted uranium (DU) in Iraqi soil, water, and the overall food chain is documented by measuring the DU in animal organs and fish as well as the water in the most populated cities in the middle and south of Iraq. Breast cancer is the most common tumor type among Iraqi women living in war zones, and triple-negative breast cancer (TNBC) constitute the most aggressive molecular subtype among breast tumors. The objective of this pilot study is to determine the prevalence and prognostic target of androgen receptor (AR) positivity in TNBC patients living in war regions polluted with a high level of DU. Methods: This observational, retrospective pilot study included 50 cases of TNBC patients living in the war region. The expression of AR, CK5/6, and CK8/18 biomarkers was evaluated using an immunohistochemistry study on formalin-fixed paraffin-embedded tumor samples from TNBC patients. The serum level of CA-153 and vitamin D was measured. Results: AR was positive (IHC>12%) in 12% of TNBC patients. K5/6 expression was considered if the score was >2. This expression of K5/6 was positive in 80% of cases, and CK8/18 was negative in 80% of cases. Serum vitamin D level was significantly lower in TNBC patients compared to controls. Since the two Gulf wars, there has been a steady increase in the incidence of breast cancer in Iraq. Conclusion: The middle and south of Iraq contain a heavily war-related, DU-polluted environment. Based upon the findings of this study, in regions exposed to high levels of DU, AR overexpression in TNBC patients is similar to studies that have been conducted on populations not exposed to DU.

Keywords: Breast Cancer; Depleted Uranium; Gulf War.

Cancer in the developing world is projected to increase by 70% over the next 20–25 years. Breast cancer (BC) represents 20–30% of cancer among women and is likely to account for a major part of that increase1. Cancer is largely considered a disease of older people, and the change in lifestyle from a traditional to a Westernized lifestyle exposes women to higher individual risk2. In general, the categorization of breast cancer is based on receptor expression, including progesterone receptor (PR), estrogen receptor (ER), and Her2/neu receptor (Her2) expression. TNBC is characterized as tumors that do not express ER, PR, and HER-2 genes, which account for 10-24% of invasive high-grade breast cancers with different histological types. Patients with TNBC tend to have a short, disease-free interval, a higher recurrence rate after diagnosis,
a relatively higher rate of relapse, and a worse overall survival rate than other forms of breast cancer. Reduced overall survival, especially for the lack of targeted therapies, tends to be more common in younger women. The relatively high rate of relapse of TNBC has intensified interest in understanding its pathogenesis and the research of new molecular signatures tailored to this specific BC subtype. Furthermore, studies attempted to further classify TNBC through a detailed analysis of gene expression profiling, receptor expression, and histopathological features to identify potential new therapeutics for TNBC patients. This approach has led to the adoption of seven subtypes of TNBC in addition to the androgen receptor as a prognostic marker and therapeutic target for TNBC patients. The AR is expressed by approximately 10-15% of TNBC.

Garay et al have demonstrated, both in vitro and in vivo, that AR-positive (AR+) TNBC is dependent on androgens for growth, and inhibition of AR signaling results in growth inhibition of the target cells. Androgen deprivation therapy (ADT) is a standard-of-care for prostate cancer and Enzalutamide is an ADT agent and has shown a growth inhibitory effect on AR+ TNBC.

Immunohistochemical and molecular analyses demonstrated that a subset of TNBC expresses the AR, while the low or lack of AR expression prognostic marker correlated with aggressive tumor behavior and poorer overall survival.

In the first years of diagnosis, TNBC patients with the residual disease tend to have a poor prognosis with a high probability of relapse and lower overall survival. Recent evidence suggests a significant heterogeneity within the TNBC subtype. Meanwhile, more studies have focused on genetic targets and demonstrated high rates of altered expression and genotoxicity due to DU exposure. This is expected to worsen the campaign to combat cancer in Iraq, including TNBC. Currently, in clinical practice and clinical trials, targeted treatment approaches based on the expression of molecular markers including androgen receptors are being implemented.

Briefly, it is critically important that readers of this pilot study understand this overview of the seriousness of DU contamination in Iraq and the damaging effects on Iraqi civilians’ health. Iraq suffered from two major and tragic wars where munitions containing DU were used. The Royal Society of London estimated that more than 350 tons of DU were used in the 1991 war on Iraq by the US and Coalition forces. Other reports indicated that DU levels were estimated to be around 320–800 tons in the aftermath of the first Gulf War in 1991 with further comparable levels occurring in 2003. During the US invasion of Iraq in 2003, both the USA and UK governments acknowledged that at least 150 tons of DU munitions had been used in Iraq.

Studies indicated that DU was found in the Iraqi soil and food chain. This was documented by measuring the uranium in sheep organs, fish, and also in seasonal dust storms affecting the most populated Iraqi cities in middle and southern Iraq. Additionally, it was reported that the concentration of DU in the blood of leukemia patients and the teeth of healthy children was significantly higher than in clinically healthy control samples. It was also reported that the uranium concentration in clinically healthy human blood samples of people living in the southern city of Basra (heavily bombarded by the US and allied forces in 1991 and 2003) was higher than in people living in Babylon city in the middle of Iraq, which did not see the same level of bombardment.

Meanwhile, DU concentration was found high in nursing mothers’ milk and in the hair of Iraqi parents of children with congenital anomalies. Additionally, DU was also found in the semen and body fluids of US veterans who served in the wars in Iraq. Bombardments were consistently in heavily populated areas in the middle and southern Iraq, extensively exposing individuals to DU, although this was challenging to document accurately. At the time of publishing, breast cancer is the most common tumor type among Iraqi women living in these previous war zones.

Since the 1990s, reports from southern Iraq reported a steep rise in the incidence of cancers, especially in children. According to Iraq’s High Commission for Human Rights, the city of Basra is registering 800 new cases of cancer each month, attributing the cause to “multiple reasons, including environmental pollutants in the water and soil, that resulted from the effects of wars.”

Cellular DNA and specifically proto-oncogenes and tumor suppressor genes are
sensitive to damage from environmental factors such as DU. This may lead to uncontrolled cell division that may form tumors. DU is known to induce genomic instability such as DNA double-strand breaks, chromosome aberrations, and micronuclei formation.

This pilot study is the first to look into the prevalence of the biomarker, AR positivity, and to evaluate its prognostic target in TNBC cancer in women living in the Middle Euphrates region of Iraq exposed to high levels of DU. The results of this work are compared to findings on the co-expression of these biomarkers in studies on breast cancer from other parts of the world, in a population not exposed to DU. Reports have demonstrated an association between AR expression and vitamin D serum levels. Vitamin D may act directly in tumorigenesis in breast tissue, expressing the CYP27B1 enzyme, which is responsible for converting the inactive precursor of vitamin D to the active form 1,25-dihydroxycholecalciferol [1,25(OH)₂D₃]. This vitamin can have autocrine or paracrine activity, protecting the breast tissues from malignant transformation.

The normal serum value of 1,25(OH)₂D₃ is 20 ng/ml (50 nmol/L). Values under 20 ng/ml are classified as deficient. Low sun exposure, reduced dietary vitamin D intake, and older age are the most common factors associated with vitamin D deficiency. The serum level of vitamin D may be linked with the recurrence and survival of cancer, which makes it a possible target for treatment and a prognostic tool for TNBC. Meanwhile, other reports suggested using AR agonists in breast cancer treatment will depend on ER and HER-2 status, and a combination of AR and vitamin D receptor (VDR) agonists can be additive with chemotherapy.

**SUBJECTS AND METHODS**

**Patients and Specimens**

This prospective study included 50 consecutive cases of TNBC. From 2016 to 2017, patients who met the criteria of ER-, PR- and HER-2 status and were confirmed by two experienced pathologists based on immunohistochemical staining were enrolled in this study as TNBC. In our institution, the percentage of tumors classified as TNBC is approximately 15-20% of the total number of breast cancer cases treated. All cases of TNBC and non-TNBC breast samples were reviewed according to WHO classification criteria using standard tissue sections and appropriate immunohistochemical slides. Medical records for all cases of TNBC samples were reviewed for clinical information, including histologic parameters that were determined from the H&E slides. The following clinical and pathological parameters were evaluated for each tumor included in the study: patient age at initial diagnosis, tumor size, histologic subtype, histologic grade, nuclear grade, nodal status, number of positive lymph nodes, tumor stage, and tumor recurrence. In addition, all specimens were characterized for all routine diagnostic immunophenotypic parameters.

**Measurement of Vitamin D**

Vitamin D levels in the form of 1,25(OH)₂D₃ were measured in TNBC patients and healthy control samples using a combination of enzyme immunoassay competition methods with final fluorescent detection. Quantitative determination of 1,25(OH)₂D₃ concentrations was performed using the enzyme-linked fluorescent assay (ELFA) based on the VIDAS System (bio Mérieux-Lyon, France), following the manufacturer’s protocol. Briefly, the blood samples were mixed with a pre-treatment reagent to separate vitamin D from its binding protein. The pretreated sample is then collected and transformed into the well that contains the alkaline phosphates labeled anti-vitamin D antibody (conjugate). The vitamin D antigen present in the sample and the vitamin D coating the interior of the solid phase receptacle compete for binding sites on the anti-vitamin D antibody (conjugate). The vitamin D antigen present in the sample and the vitamin D coating the interior of the solid phase receptacle compete for binding sites on the anti-vitamin D antibody alkaline phosphatase conjugate. In the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the solid phase receptacle. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliflorone). Fluorescence was measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of vitamin D antigen present in the sample.

**Immunohistochemistry Analysis**

Immunohistochemical staining was done on slides from formalin-fixed, paraffin-embedded tissues to evaluate the expression of ER, PR, HER-2, K5/6, K8/18, and AR markers according to the
method described by Collina et al., 2016 with slight modification. Paraffin slides were de-paraffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in 0.01M citrate buffer (pH 6.0) in a 97°C bath for 20 minutes. After antigen retrieval, the slides were allowed to cool. The slides were rinsed with Tris-Buffered Saline (TBS) and the endogenous peroxidase was inactivated with 3% hydrogen peroxide. After protein block (Bovine Serum Albumin 5% in Phosphate-Buffered Saline, 1x), the slides were incubated with primary antibody to human ER (Monoclonal Mouse Anti-Human ER, Clone ID5, dilution 1:35, Dako North America, Inc., Carpinteria, CA, USA), PR (Monoclonal Mouse Anti-Human PR, Clone 636, dilution 1:50, Dako North America, Inc., Carpinteria, CA, USA), and K5/6 (Monoclonal Mouse Anti-Human K5/6 Ag Clone MIB-1, dilution 1:75, Dako North America, Inc., Carpinteria, CA, USA) for 30 minutes. For CK 8/18, mouse monoclonal antibody keratin 8/18 Ab-2 ready-to-use (clone K8.8+ DC10; like 5D3, Lab Vision, Neo Marker) and AR (monoclonal mouse anti-human AR antibody clone AR441, dilution 1:75, # M3562; Dako North America, Inc., Carpinteria, CA, USA) were used. The sections were rinsed in TBS and incubated for 20 minutes with a biotinylated secondary antibody (Novocastra RE7103), a biotin-conjugated secondary antibody formulation that recognizes mouse and rabbit immunoglobulins. Afterward, the sections were rinsed in TBS and incubated for 20 minutes with Streptavidin-HRP (Novocastra RE7104) and peroxidase reactivity was visualized using a 3,3′-diaminobenzidine (DAB). Finally, the sections were counterstained with hematoxylin and mounted. The results were interpreted using a light microscope.

Evaluation of Immunohistochemistry

Antigen expression was evaluated independently by a pathologist using light microscopy. The observer was unaware of the clinical outcome. For each sample, at least five fields (inside the tumor and in the area exhibiting tumor invasion) and >500 cells were analyzed. Using a semi-quantitative scoring system microscopically and referring to each antigen scoring method in other studies, an observer evaluated the intensity, extent, and subcellular distribution.

Statistical Analysis

Data analysis was done by using SPSS (Statistical Package for Social Sciences) version 20 (SPSS, Inc., Chicago, USA), where we used mean plus standard deviation and percentages as descriptive statistics. For analysis, we used an independent sample t-test for continuous variables and Yates corrected the chi-square test for categorical data. Fisher’s exact test was used to analyze the correlation between the triple-negative phenotype and AR expression. P-values <= 0.05 were regarded as significant.

RESULTS

The incidence of TNBC in the Middle Euphrates region was 15-20%, with 12% of this incidence being AR-positive. TNBC was strongly associated with younger age. The number of patients with various types of cancer and those with breast cancer is demonstrated in Figure 1. The majority of patients were middle-aged between 41 and 65 years old and had a mean age of 49.2 ± 9.65 (30-65) years. The median age was 48 years. Most of the cases were invasive ductal carcinoma (IDC), accounting for 78.5% of all cases. Useful immunohistochemical markers for characterizing basal-like carcinomas are CK5/6 (Figure 2), androgen receptor (Figure 3), and CK8/CK18 (Figure 4).

The demographic characteristics of the study participants are presented in Table 1. Five years of survival with various variables are shown in Table 2. No significant difference was observed with various variables when comparing the presence or absence of androgen receptors as indicated in Table 3. An evaluation of the association between 5-year survival and androgen receptor, a trend toward increased survival of AR+ vs AR- is shown in Table 4. Serum vitamin D level was tested in both the TNBC patients and controls and the results indicated a significantly low level of serum vitamin D in TNBC patients compared to controls, while no significant difference in the BMI of patients versus healthy controls was found. Figure 4 demonstrates immunostaining of the CK8/18 in negative and positive TNBC patients included in this study.
Fig. 1. Number of cancer patients diagnosed and treated at the Middle Euphrates Cancer Therapy Center in Najaf, Iraq. Total; total number of cancer patients in a year and BC; the number of breast cancer patients in the same year.

![Graph showing number of cancer patients per year](image)

Fig. 2. Immunohistochemical staining of TNBC patient with CK5/6. A. CK 5/6 control (10X), B. CK 5/6 positive (20X), and C. CK 5/6 positive (40X)

![Immunohistochemical staining images](image)

Fig. 3. Androgen Receptor TNBC immunophenotype: A. High expression of AR (10X); B and C. Details of high expression of AR (20X and 40X respectively)

![Immunohistochemical staining images](image)
DISCUSSION

For the last six years, the number of cancer patients increased steadily, with most patients coming from battleground areas of the 1991 and 2003 US-led military invasion of Iraq where DU was used in armor-piercing rounds to disable enemy tanks during these wars\(^3\). Inhalation, ingestion, dermal contact, and open wounds are the most likely routes of DU intake into the human body.

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Table 1. Demographic characteristics of studied patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Values Mean ± SD (range)</th>
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<tr>
<td>Age/years</td>
<td>49.2±9.65 (22-72)</td>
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<tr>
<td>BMI Kg/m²</td>
<td>25.8±2.6 (18.7-32.9)</td>
</tr>
<tr>
<td>Type</td>
<td>Ductal 41 (82%)</td>
</tr>
<tr>
<td></td>
<td>Lobular 9 (18%)</td>
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<td>5-year survival</td>
<td>Yes 46 (92%)</td>
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<tr>
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<td>No 4 (8%)</td>
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Table 2. Five-years survival among different variables

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<th>Mean</th>
<th>Std. Deviation</th>
<th>P value</th>
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<td>11.9</td>
<td>4.7</td>
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<td>No</td>
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<td>14.2</td>
<td>4.7</td>
<td></td>
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<td>CA 15-3</td>
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<td>46</td>
<td>78.6</td>
<td>104.6</td>
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<td>.4</td>
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Fig. 4. CK8/18 immunostaining in TNBC. A. CK8/18 negative internal control (10X); B. CK8/18 positive internal control with preserved membranous pattern in invasive duct carcinoma.
Table 3. Androgen receptor and different variables

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AR</th>
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<th>Mean</th>
<th>Std. Deviation</th>
<th>P value</th>
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<tr>
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<td>30.9</td>
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<td>44</td>
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<td>53.9</td>
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Table 4. Association between 5-year survival and androgen receptor

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<th>P value</th>
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<td>91.3%</td>
<td>50.0%</td>
</tr>
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<tr>
<td>Total</td>
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<td>4</td>
<td>100.0%</td>
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Yates chi square = 2.677

Table 5. Comparison between cases and controls regarding vitamin D and BMI

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<td>Vitamin D ng/ml</td>
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<tr>
<td></td>
<td>Controls</td>
<td>100</td>
<td>46.9</td>
<td>8.1</td>
</tr>
<tr>
<td>BMI Kg/m²</td>
<td>Cases</td>
<td>50</td>
<td>25.8</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>100</td>
<td>26.2</td>
<td>1.7</td>
</tr>
</tbody>
</table>
in AR-negative tumors. Gonzalez-Angulo et al have evaluated the impact of the AR expression on treatment outcomes of 347 patients with breast cancer and demonstrated that high levels of AR were associated with higher age at diagnosis, the presence of low nuclear grade, and the expression of ER and PR. Among different subtypes of breast cancer, a significant difference in AR expression was observed. Additionally, it was discovered that high expression levels of AR were observed in ER and/or PR positive subtypes and low expression levels in the TNBC, which is similar to the results of our study. Meanwhile, our results indicated no significant difference in overall survival between AR- and AR+ of TNBC patients, while both the disease-free survival and overall survival were similar in AR+ and AR- patients and were similar to previously reported studies.

Our TNBC patients’ results with a low level of serum vitamin D are similar to other reported studies. Other reports indicated that the ratio of patients with TNBC was higher in the region with more sunlight exposure. This conclusion is different from our results; while our region in Iraq is exposed to long hours of sunlight, the ratio of TNBC patients is similar to the ratio reported in other parts of the world. Since the majority of women in Iraq wear clothing that covers their entire body, this may interfere with skin exposure to direct sunlight and, in turn, vitamin D deficiency. Further study is recommended to evaluate the effect of sunlight and vitamin D on the development of breast cancer in Iraq. Other studies also suggested that using a combination of AR and Vitamin D receptor (VDR) agonists can be additive with chemotherapy in the treatment of TNBC. In our study, even though the serum level of vitamin D is significantly lower than controls, giving vitamin D supplementation had no significant effect on the status of TNBC patients. With the limited resources of our study, we were unable to confirm a direct link between the increase in the incidence of breast cancer and the exposure of patients to depleted uranium.

CONCLUSION

Current research strategies are aimed at better understanding both the risk factors and the biology underlying triple-negative breast cancer to develop preventive measures and improve treatment strategies for this challenging subtype of breast cancer in women living in DU-polluted areas. Limitations of this study are the small sample size, the lack of resources to affirm the direct link between DU and breast cancer, and the political sensitivity of the topic. Our recommendation is to initiate a comprehensive program to investigate the Iraqi environment – especially war zones where DU ammunition was used – to identify DU-contaminated areas and assess the health risks. This investigation would inform a remediation plan with the help of UNEP and WHO, similar to the plan executed in Serbia and Kosovo. A long-term plan should be available to assess the long-term health risks to Iraqi citizens living in DU-polluted areas similar to that used by the Depleted Uranium Follow-up Program at the US Department of Veteran Affairs.

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Conflict Interest

The authors have no conflicts of interest to declare.

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Authors’ contributions

Authors contributed equally to this work.

Statement of Ethics

Informed consent from each patient and an approval letter from the Institutional Review Board at Kufa University were obtained.

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