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IMMUNOGENETIC ANALYSIS OF CTLA-4 GENE POLYMORPHISMS AND INTERLEUKIN-10 LEVELS IN IRAQI WOMEN WITH BREAST CANCER: A CASE-CONTROL STUDY

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ABSTRACT

Background: Breast cancer is the most common malignancy in women globally and the second leading cause of cancer death in the developing world. Immune checkpoint molecules, including Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4/CD152), and the anti-inflammatory cytokine interleukin-10 (IL-10) represent key modulators of antitumor immunity that might contribute to immune evasion in breast carcinogenesis.

Objectives: The objective of this study was to investigate the immunological and immunogenetic roles of CTLA-4 and IL-10 in Iraqi women with breast cancer, and to evaluate the association of CTLA-4 single-nucleotide polymorphisms (SNPs) at the +49 (rs231775 A/G) and −318 (rs5742909 C/T) loci with breast cancer risk.

Materials and Methods: A case-control study was conducted on 50 pre-treatment Iraqi women with histologically confirmed breast cancer and 50 age-matched healthy controls (age range: 30–70 years), recruited between September and December 2025. Serum IL-10 and soluble CTLA-4 levels were quantified by enzyme-linked immunosorbent assay (ELISA). CTLA-4 +49 A/G was genotyped by PCR-RFLP; −318 C/T was characterized by Sanger sequencing. Hardy–Weinberg equilibrium was assessed in controls. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated; $p < 0.05$ was considered statistically significant.

Results: Serum IL-10 (55.2 ± 17.9 vs. 22.8 ± 5.4 pg/mL; $p = 0.08$) and soluble CTLA-4 (3.10 ± 0.81 vs. 2.71 ± 0.30 ng/mL; $p = 0.09$) were numerically elevated in patients, though not statistically significant. For SNP +49, the AG genotype was significantly more frequent in patients (50.0%) versus controls (4.0%) (OR = 23.1, 95% CI: 4.9–109; $p < 0.001$). The AA genotype predominated in controls (96.0% vs. 36.0%; OR = 0.02, 95% CI: 0.004–0.09; $p < 0.001$). In invasive lobular carcinoma (ILC) patients, AA genotype was associated with significantly elevated soluble CTLA-4 ($p = 0.01$). SNP −318 distributions did not differ significantly between groups ($p > 0.05$).

Conclusions: In Iraqi women The CTLA-4 +49 AG genotype is significantly associated with breast cancer risk. A subtype-specific immunogenetic signal was detected in ILC patients. These initial results must be confirmed in large, multi center clinically stratified cohorts before translation to the clinic.

Keywords: Breast Neoplasm; CTLA-4; Interleukin-10; Immunogenetics; Single-Nucleotide Polymorphism; Women from Iraq; Immune Checkpoint.

1 INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women, with an estimated 2.3 million new cases within the year as per the GLOBOCAN 2022 projections [1]. In Iraq, it is the most common malignancy among females, and the incidence rates are increasing from 30 to 40 per 100,000 women during 2006–2012, posing a significant burden on the public [2]. Genetic, hormonal, environmental, and immunological factors contribute to the Tens of thousands of people are diagnosed with breast cancer each year in the US. Modern immuno-oncology paradigms model tumor progression as an active process of immune editing, whereby neoplastic cells co-opt regulatory mechanisms to avoid immune surveillance [3]

The immune checkpoint molecules are exploited in particular by tumor to mediate immune escape. Cytotoxic T-lymphocyte antigen-4 (CTLA-4/CD152), a member of the immunoglobulin superfamily, which is mainly expressed on activated T cells and regulatory T cells (Tregs), is an important negative regulator of T-cell activation.CTLA-4 binds with higher avidity than the co-stimulatory molecule CD28 to the ligands CD80 (B7-1) and CD86 (B7-2) that are present on the antigen-presenting cells (APCs), weakening TCR signaling and limiting clonal expansion [4] CTLA-4+ Tregs in the tumor microenvironment (TME) can also remove CD80/CD86 from APCs through trans-endocytosis, impairing effector T-cell priming and promoting immune tolerance [5] Increased density of tumor-infiltrating CTLA-4+ Tregs has been associated with decreased cytotoxic T-lymphocyte activity and worse clinical outcome in breast and other solid malignancies [6]

The 2q33 region contains the gene of the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which itself is approximately 6.2 kb long and encodes for a protein containing a signal peptide, a single extracellular immunoglobulin-like ligand-binding domain, a transmembrane domain, and an intracellular signaling domain [7] Out of the known polymorphisms, the +49 A/G substitution in exon 1 (rs231775) and the -318 C/T promoter polymorphism (rs5742909) have been the most studied. The +49 A/G polymorphism leads to a threonine-to-alanine substitution at position 17 of the signal peptide and is associated with altered protein glycosylation, efficiency of intracellular trafficking, and density of surface expression [8] The −318 C/T polymorphism is located in a regulatory sequence of the CTLA-4 promoter and affects CTLA-4 transcriptional activity. Population studies have shown that the T allele has a higher promoter efficiency and in some populations, higher mRNA levels have been observed [9]. Meta-analyses have shown that the CTLA-4 +49 A/G polymorphism is linked to an increased risk of several types of malignancy, such as breast cancer, although the odds ratios reported by the investigated studies diversified to a great extent according to ethnicity and geography which indicated the need for specific population -based investigations[10,11]

Interleukin-10 (IL-10) is a multipotent immunoregulatory cytokine secreted by macrophages, dendritic cells, Tregs, and several other leukocyte subsets.IL-10 inhibits APC co-stimulation and pro-inflammatory cytokine secretion, enhances Treg differentiation and suppresses CTL activity within the TME, resulting in the generation of immunosuppressive tumor protecting microenvironment [12]High serum IL-10 levels have been linked to late-stage disease and poor prognosis in breast cancer, indicating that it may have value as a prognostic biomarker[13]

Breast cancer immunogenetics has attracted worldwide attention among researchers; however, information on the Iraqi and Middle Eastern populations is still insufficient.This study was designed to: (1) measure the levels of serum IL-10 and soluble CTLA-4 in breast cancer patient and healthy control groups in Iraq; (2) analyze the genotype and allele frequencies of CTLA-4 +49 A/G and −318 C/T, with an estimation of the odds ratio; and (3) assess the possible subtype-specific associations of the +49 genotype with soluble CTLA-4 protein concentration in people with invasive ductal (IDC) and invasive lobular carcinoma (ILC).

2 MATERIALS AND METHODS

2.1 Study Design and Participants

This was a hospital-based case-control study carried out in [Hospital Name], Baghdad, Iraq, during the period of September 2025 to December 2025. Fifty Iraqi women with newly diagnosed breast cancer (histologically confirmed) were included as cases and 50 age-matched (±5 years) healthy women free of malignancy and immune-mediated disease were recruited as controls. Eligibility criteria required histopathological confirmation of malignancy, absence

of prior systemic therapy, and no concurrent autoimmune disorder, active infection, or pregnancy. Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Ethics Committee of Ibn Sina University of Medical and Pharmaceutical Sciences and conducted in accordance with the Declaration of Helsinki.

2.2 Serum Cytokine Quantification

Peripheral venous blood (10 mL) was collected under sterile conditions prior to any anti-cancer therapy. Serum was separated by centrifugation (3000 rpm, 10 min, 4°C) and stored at −80°C until analysis. Serum concentrations of IL-10 and soluble CTLA-4 were measured in duplicate by sandwich enzyme-linked immunosorbent assay (ELISA; manufacturer details to be specified) according to standard protocols. Intra- and inter-assay coefficients of variation were verified prior to sample processing. Results are expressed as mean ± standard deviation (SD).

2.3 DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a standardized salting-out method. DNA integrity and purity (A260/A280 ratio: 1.8–2.0) were verified spectrophotometrically. CTLA-4 +49 A/G (rs231775) was genotyped by PCR-RFLP: a 163-bp amplicon was digested with BbsI restriction endonuclease, generating diagnostic fragments of 163 bp (AA homozygote), 119 + 44 bp (GG homozygote), or all three bands (AG heterozygote), resolved on 2% ethidium bromide-stained agarose gel. Representative samples from each genotype category were confirmed by Sanger sequencing. For CTLA-4 −318 C/T (rs5742909), the promoter region was directly sequenced on an ABI 3500 Genetic Analyzer and aligned to the reference sequence (GenBank: L15006) using Sequencher 5.4.

2.4 Statistical Analysis

Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY). Normality was assessed by the Shapiro–Wilk test; non-normally distributed continuous variables were compared using the Mann–Whitney U test. Categorical variables were analyzed by chi-squared test or Fisher's exact test as appropriate. Hardy–Weinberg equilibrium (HWE) was tested in controls using a chi-squared goodness-of-fit test. Binary logistic regression was used to calculate ORs with 95% CIs for genotype-disease associations. Subgroup comparisons of soluble CTLA-4 by histological subtype and genotype were evaluated with the Kruskal–Wallis test and Dunn's post hoc correction. A two-tailed $p < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Participant Characteristics

The study comprised 50 breast cancer patients (mean age: 48.3 ± 10.2 years) and 50 age-matched controls (mean age: 46.7 ± 9.8 years; $p = 0.42$). Histological subtypes included invasive ductal carcinoma (IDC; n = 36, 72%) and invasive lobular carcinoma (ILC; n = 14, 28%). All patients were treatment-naïve at the time of recruitment. Control genotype distributions satisfied Hardy–Weinberg equilibrium for both SNP +49 ($\chi^2 = 2.14$, $p = 0.14$) and SNP −318 ($\chi^2 = 0.87$, $p = 0.35$), confirming population representativeness.

3.2 Serum IL-10 and Soluble CTLA-4 Levels

Serum IL-10 was numerically higher in patients (55.2 ± 17.9 pg/mL) compared with controls (22.8 ± 5.4 pg/mL; $p = 0.08$, Mann–Whitney U test). Soluble CTLA-4 was similarly elevated in patients (3.10 ± 0.81 ng/mL) versus controls (2.71 ± 0.30 ng/mL; $p = 0.09$). While neither difference attained statistical significance, the approximately 2.4-fold elevation of IL-10 in patients is biologically noteworthy and warrants evaluation in a larger adequately powered sample (Table 1; Figure 1).

Table 1: Serum IL-10 and soluble CTLA-4 levels in breast cancer patients and healthy controls.

Parameter	Patients (n=50) Mean ± SD	Controls (n=50) Mean ± SD	p-value	Sig.
IL-10 (pg/mL)	55.2 ± 17.9	22.8 ± 5.4	0.08	NS
Soluble CTLA-4 (ng/mL)	3.10 ± 0.81	2.71 ± 0.30	0.09	NS

NS = not significant. Values presented as mean ± SD. p-values derived from Mann–Whitney U test.

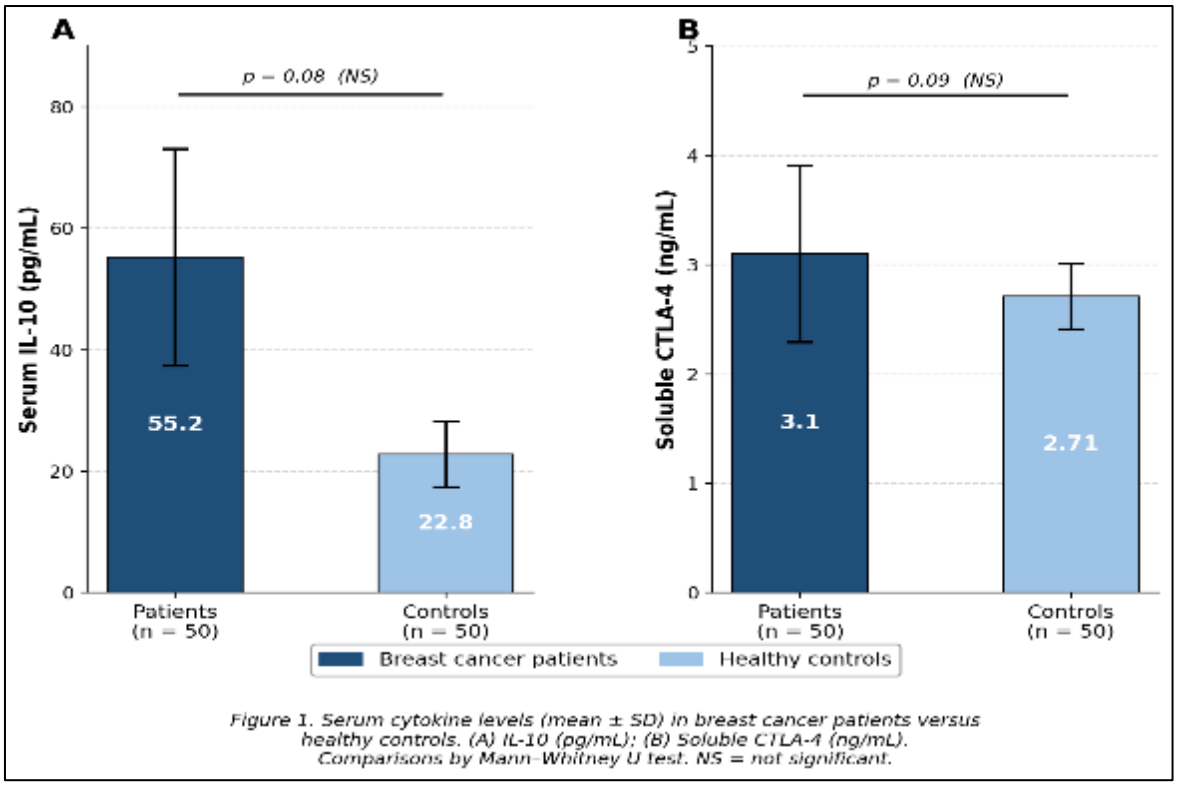


Figure 1: Serum cytokine levels (mean ± SD) in breast cancer patients versus healthy controls. (A) IL-10 (pg/mL); (B) Soluble CTLA-4 (ng/mL). Comparisons by Mann–Whitney U test. NS = not significant.

3.3 CTLA-4 +49 A/G Genotype and Allele Distribution

The genotype distribution of CTLA-4 +49 A/G was strikingly different and significantly divergent between patients and controls (Table 2; Figure 2). The AG genotype was found in 50.0% of patients but only 4.0% of controls (OR = 23.1, 95% CI: 4.9–109; $p < 0.001$), while the GG genotype was only found in patients (14.0% vs. 0%; Fisher's exact, $p = 0.006$). In contrast, the AA genotype was most common among controls (96.0% vs. 36.0% in patients; OR = 0.02, 95% CI: 0.004–0.09; $p < 0.001$). The G allele frequency was 39.0% in patients and 2.0% in controls ($p < 0.001$), indicating a significant over-representation of G-allele carriers among breast cancer cases.

3.4 CTLA-4 −318 C/T Genotype Distribution

There were no significant differences in the distribution of the −318 C/T genotypes between the two groups (Table 2). The CC genotype was observed in 60.0% of patients and 56.0% of controls ($p = 0.68$), the CT in 22.0% and 30.0% ($p = 0.37$), and the TT in 18.0% and 14.0% ($p = 0.59$) of patients and controls, respectively. The frequency of the C allele was similar in patients (71.0%) and controls (71.0%).These findings indicate the lack of an independent effect of the −318 C/T polymorphism on breast cancer risk in this population, but the limited power of the study does not allow drawing of a definitive conclusion.

Table 2: CTLA-4 +49 A/G and −318 C/T genotype distributions with odds ratios

SNP Locus	Genotype	Patients n (%)	Controls n (%)	p-value	OR (95% CI)
+49 A/G	AA	18 (36.0%)	48 (96.0%)	<0.001*	OR=0.02 (95% CI: 0.004–0.09)
+49 A/G	AG	25 (50.0%)	2 (4.0%)	<0.001*	OR=23.1 (95% CI: 4.9–109)
+49 A/G	GG	7 (14.0%)	0 (0.0%)	0.006*	Fisher's exact
−318 C/T	CC	30 (60.0%)	28 (56.0%)	0.68	NS
−318 C/T	CT	11 (22.0%)	15 (30.0%)	0.37	NS
−318 C/T	TT	9 (18.0%)	7 (14.0%)	0.59	NS

* $p < 0.05$, statistically significant. OR = odds ratio; CI = confidence interval; NS = not significant. Fisher's exact test applied where expected cell count <5.

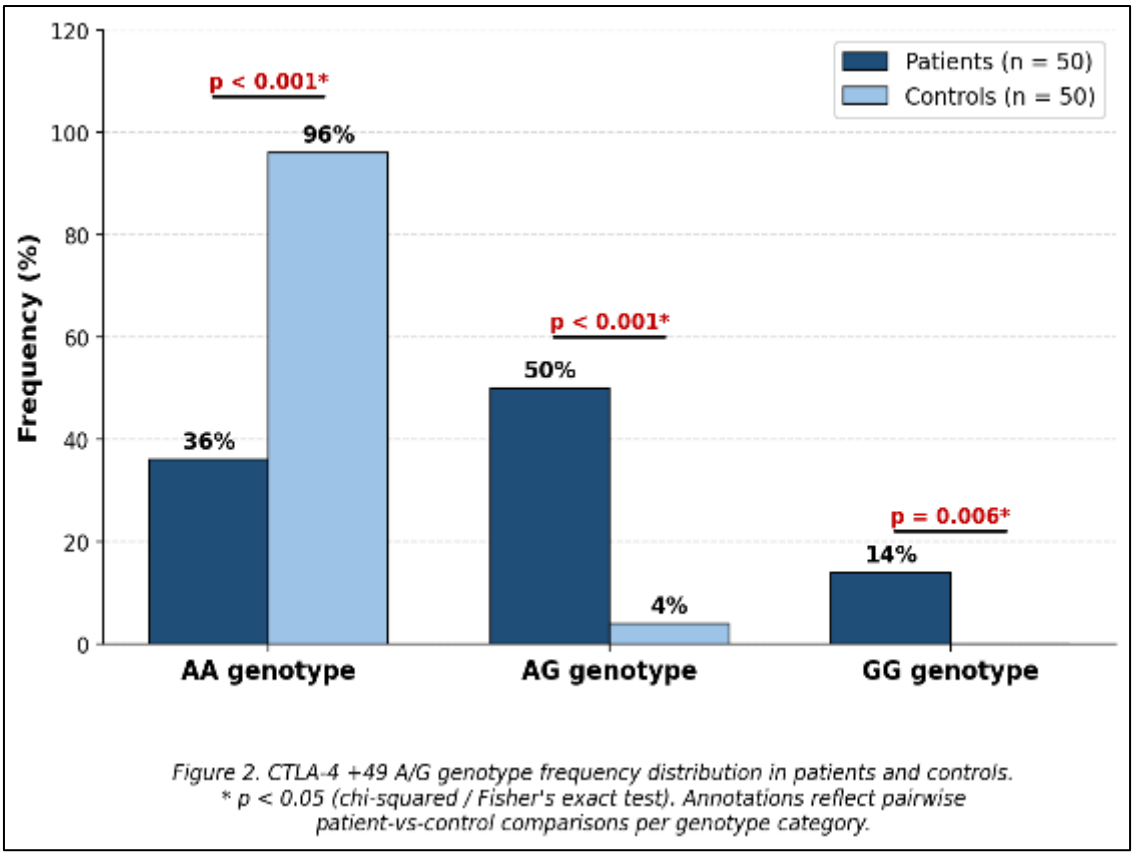


Figure 2: CTLA-4 +49 A/G genotype frequency distribution in patients and controls. * p < 0.05 (chi-squared / Fisher's exact test). Annotations reflect pairwise patient-vs-control comparisons per genotype category.

3.5 Genotype-Specific Soluble CTLA-4 Levels by Histological Subtype

When stratified, there were unique genotype–protein associations by histological type(Table 3). In the IDC group, there was no statistically significant difference in soluble CTLA-4 levels between AA and AG/GG carrying patients (3.22 ± 0.94 vs. 3.01 ± 0.72 ng/mL; p = 0.42). Contrarywise, ILC carrying AA genotype showed significantly elevated soluble CTLA-4 with respect to AG/GG carriers in the ILC subgroup and versus control (4.11 ± 0.63 vs. 2.85 ± 0.58 ng/mL; p = 0.01) pointing to a possible subtype- specific immunogenetic regulation of CTLA-4 expression and secretion.

Table 3: Soluble CTLA-4 levels stratified by CTLA-4 +49 A/G genotype and histological subtype.

Histologic Subtype	Genotype (+49)	n (%)	Soluble CTLA-4 (ng/mL)	p-value	Sig.
IDC (n=36)	AA	14 (38.9%)	3.22 ± 0.94	0.42	NS
IDC (n=36)	AG/GG	22 (61.1%)	3.01 ± 0.72	—	—
ILC (n=14)	AA	4 (28.6%)	4.11 ± 0.63	0.01*	Sig.
ILC (n=14)	AG/GG	10 (71.4%)	2.85 ± 0.58	—	—

* p < 0.05. IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; Sig. = significant; NS = not significant. p-values from Kruskal–Wallis test with Dunn's post hoc correction.

4 DISCUSSION

This case-control study highlights the first immunologic and immunogenetic profiling of CTLA-4 and IL-10 in an Iraqi breast cancer cohort, an ethnic group that has not been addressed in the breast cancer immunogenetic literature. The strongest and most consistent result was a very significant association with G-allele carriage at CTLA-4 +49 (rs231775) and breast cancer, and the OR for the AG genotype was 2.31 (95% CI: 4.9–109; p < 0.001). This is much larger than the effect sizes reported previously in pooled meta-analyses, which may be explained by the small sample or perhaps by the specific allele frequency architecture of the Iraqi population. A subtype-specific association was observed as well: among ILC patients with the AA genotype, soluble CTLA-4 was significantly higher (p = 0.01), a finding bearing possible mechanistic implications.

4.1 CTLA-4 +49 A/G Polymorphism: Susceptibility and Mechanistic Considerations

The significantly high frequency of the AG genotype in patients (50.0% vs. 4.0%) and the near absence of G-allele homozygotes in controls is a striking finding. The +49 A/G variant changes the amino acid sequence (threonine to alanine) of the CTLA-4 signal peptide at codon 17, which alters N-linked glycosylation at a nearby asparagine residue and affects the rate of intracellular trafficking as well as the density of surface expression on the membrane [8] Recent findings imply that CTLA-4 variants associated with the G-allele might have different transport kinetics, possibly affecting inhibitory signaling in a manner that has yet to be fully elucidated. Immunologically, reduced surface expression of CTLA-4 may paradoxically suppress inhibitory signaling and thus limit its effectiveness in Tregs against effector T cells; on the other hand, some reports show that G-allele carriers have higher expression of the soluble isoform which could theoretically affect CD80/CD86 concentration in the TME in a competitive manner [10] The consistent overrepresentation of G-allele carriage in cancer cases across Middle Eastern and Asian populations, including in this study and that of El-Ghonaimy et al. in Egyptian women [14] is in stark contrast to the more diverse observations seen in European populations indicating population-specific LD and background genetic effects [11]

4.2 Subtype-Specific Immunogenetic Effects in ILC versus IDC

The strong correlation of the AA genotype with high levels of soluble CTLA-4 in ILC but not in IDC represents a novel finding and should be explored further in mechanistic studies. Although the immune microenvironment of ILC and IDC are remarkably different with ILC being associated with lower tumor-infiltrating lymphocyte (TIL) density, lower immunogenic neoantigen expression, and differential immunotherapy responses [15] The immune recognition of ILC perhaps may be impacted by the CDH1 inactivation characteristic to this disease and immune related pathways such as antigen presentation through E-cadherin mediated signaling loss, potentially modulating, in a genotypically dependent manner, expression of the immune checkpoints. It is also conceivable that in the context of the immunologically different ILC microenvironment, the AA-associated CTLA-4 signal peptide variant has an enhanced capacity to produce soluble isoform from Treg populations and thus contribute to local immune suppression. Given the ILC subgroup consisted of only 14 patients, these observations are clearly of preliminary nature and necessitate confirmation in prospective studies including larger ILC populations.

4.3 Serum IL-10 and Soluble CTLA-4: Biological Plausibility of Non-Significant Trends

About 2.4-fold increase in serum IL-10 in the patients (55.2 vs. 22.8 pg/mL; $p = 0.08$) is biologically consistent with well-recognized mechanisms of TME immunosuppresssion. Tumor-associated macrophages and Tregs represent the major source of IL10 within the tumor, and systemic leakage can be detected in the peripheral serum as the disease progress [12] Published studies show, that IL-10 upregulation is more strongly associated with advanced tumor stage and lymph node metastasis [16] indicating that a treatment naive group of mixed stages of disease may blur the detectable serum differences. Statistical significance could not be reached in the current sample size with these effect sizes (estimated $n \approx 120–150$ per group to achieve 80% power at $\alpha = 0.05$, based on the observed SD). While this number-based elevation recapitulates the direction of the effect estimated in several previous investigations [13,16,17], thus providing biological plausibility of these initial signals. Prospective analysis in a powered, stage stratified cohort using paired tissue-level IL-10 quantification would greatly bolster the evidentiary.

4.4 CTLA-4 −318 C/T Polymorphism: Null Findings in Context

No significant association was observed between the distribution of CTLA-4 −318 C/T genotypes and breast cancer status, which is in agreement with a number of previous studies that reported null results for this promoter polymorphism [18] The functional consequences of the −318 T allele on CTLA-4 transcription are strongly cell type-specific and are influenced by co-regulatory elements, epigenetic modifications, cell type-specific availability of transcription factors, and interaction with other loci such as the +49 variant [9] The relatively similar frequencies of the C allele in patients and controls (71.0% each) also indicate that this polymorphism is not an independent risk factor in the studied Iraqi population. Power considerations: the current study, with only 50 cases and 50 controls, had sufficient power to detect large effects (ORs ≥ 3.0) but had low power to detect moderate effects (ORs 1.5–2.0) with an acceptable type II error rate. More research with substantially larger sample sizes is needed to definitively determine the role of the −318 variant in breast cancer risk among the Iraqi population.

4.5 Clinical Implications

These observations have a number of translational clinical consequences. First, the strong association between the CTLA-4 +49 G allele and breast cancer risk in the Iraqi population suggests that this SNP might be considered as a putative risk prediction marker in Middle Eastern populations, provided that validation at the multi-center level as well as complete clinicopathological characterization is conducted. Second, the immunogenetic environment at CTLA-4 loci may also associate with responses to immune checkpoint inhibitor therapy. Pharmacogenomic analyses suggest that CTLA-4 genotype may alter ipilimumab activity and immune-related adverse events in patients with solid tumors [19] As immunotherapy is increasingly investigated in breast cancer, particularly triple-negative disease, including CTLA-4

genotyping in clinical trial designs focused on the Iraqi and regional population may lead to actionable biomarker data. Fourth, the observed IL-10 trends in this work encourage the pursuit of combined CTLA-4 blockade and IL-10 pathway modulation as immunotherapeutic options in breast cancer.

4.6 Study Limitations

This study has a number of key limitations that limit the generalizability of its findings. The sample size (n = 100 total) is too small to have adequate statistical power for genetic association analyses, especially for rare genotypes and subgroup comparisons. There was no clinical stratification of our cohort according to tumor stage, molecular receptor status (ER, PR, HER2), grade and lymph node involvement, all well-known drivers of immune microenvironment composition and potential confounders of genotype-phenotype associations. Serum cytokine measurements are a systemic proxy for TME immune dynamics; tissue-based quantification would provide greater mechanistic resolution. We did not functionally validate genotype-dependent CTLA-4 expression at the mRNA or protein level in patient-derived samples. The single-center design and modest recruitment window also reduce the external validity. Future studies should use a multi-center design, formal power calculations, full clinicopathological characterization, functional immunological assays, and potentially extended haplotype analysis of the CTLA-4 gene region.

5 CONCLUSIONS

This case-control study demonstrates a significant and substantial association between the CTLA-4 +49 AG genotype and breast cancer susceptibility in Iraqi women (OR = 23.1, 95% CI: 4.9–109; $p < 0.001$), alongside a putative protective role of the AA genotype in healthy controls. A subtype-specific immunogenetic signal was identified wherein the AA genotype was associated with significantly elevated soluble CTLA-4 in ILC patients ($p = 0.01$) but not IDC patients, suggesting histological subtype may modulate the functional consequences of CTLA-4 genetic variation. Serum IL-10 and soluble CTLA-4 levels were numerically elevated in patients, consistent with an immunosuppressive tumor milieu, though these differences were not statistically significant in this pilot cohort. The −318 C/T polymorphism was not independently associated with disease status. These preliminary findings offer a suggestive immunogenetic basis for CTLA-4-mediated immune regulation in Iraqi breast cancer, warranting prospective multi-center validation in larger, clinically stratified cohorts before clinical translation can be considered.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

The author declares no conflicts of interest.

Statement of ethical approval

Approved by the Institutional Ethics Committee of Ibn Sina University of Medical and Pharmaceutical Sciences. All procedures followed the Declaration of Helsinki.

Author Contributions

Z.A.H.: conceptualization, data collection, laboratory analysis, statistical analysis, manuscript drafting and revision, and final approval of the submitted version.

Data Availability

Data are available from the corresponding author upon reasonable request.

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