Effect of Toll-Like Receptor 9 (TLR9) in Breast Cancer Risk, Along with Hormonal Effects in Patients Receiving Radiotherapy

Asra'a Adnan Abdul-Jalil [1], Marrib N. Rasheed [2], Rana H. Hamoode [3]

Abstract

A Introduction: Antioxidant enzyme polymorphisms and innate immune receptors have been implicated in the development of various cancer forms. This study aimed to assess the potential association between toll-like receptor 9 (TLR9) polymorphisms and female susceptibility to breast cancer. Methods: Forty female breast cancer patients from Iraq and 20 healthy volunteers were enrolled in the study. Gene polymorphisms of TLR9 rs187084 (1237T/C) were analyzed using real-time polymerase chain reaction (RT-PCR). Additionally, a hormonal study was conducted, comparing breast cancer patients exposed to radiation with a control group. The levels of follicle-stimulating hormone (FSH), estradiol (E2), and progesterone were measured. Results: The analysis revealed a non-significant increase in the prevalence of TLR9 wild TT genotypes among breast cancer patients compared to healthy individuals (72.5% vs. 90%, respectively). Conversely, heterozygous CT genotypes were significantly higher in breast cancer patients compared to healthy women (22.5% vs. 10%, P<0.05). In the hormonal study, breast cancer patients exposed to radiation exhibited a significant increase in FSH levels (2.9 vs. 18.8 IU/ml), a significant decrease in E2 levels (0.232 vs. 0.910 pico/ml), and a significant increase in progesterone levels (0.910 vs. 0.732 nanogram/ml). Conclusion: The study concludes that TLR9 rs187084 (1237T/C) polymorphism variants play crucial roles in the susceptibility of Iraqi females to breast cancer. Furthermore, the observed hormonal disruptions in FSH, E2, and progesterone levels highlight potential contributors to breast cancer development, emphasizing the need for further exploration of genetic and hormonal factors in cancer susceptibility.

Keywords: Antioxidant enzyme polymorphisms, Toll-like receptor 9 (TLR9) polymorphisms, Breast cancer susceptibility, Hormonal disruptions, Genetic and hormonal factors

Significance | TLR9 rs187084 polymorphisms crucial in Iraqi women's breast cancer susceptibility.

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Editor Aman Shah Abdul Majid And accepted by the Editorial Board Jan 18, 2024 (received for review Dec 12, 2023)

Introduction

Breast cancer is a prevalent form of cancer in women globally, and its treatment varies based on tumor stage. Metastasis plays a pivotal role in cancer-related fatalities, necessitating a deeper understanding of genetic factors influencing cancer development. Polymorphisms in Toll-like receptors (TLRs) and antioxidant enzymes are associated with cancer. This study focuses on TLR9 polymorphisms and their potential link to breast cancer susceptibility in Iraqi women.

Breast cancer is a prevalent malignancy among women, accounting for approximately 1.7 million new diagnoses and 0.5

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Please cite this article:
Asra'a Adnan Abdul-Jalil, Marrib N. Rasheed, Rana H. Hamoode, (2024). Effect of Toll-Like Receptor 9 (TLR9) in Breast Cancer Risk, Along with Hormonal Effects in Patients Receiving Radiotherapy, Journal of Angiotherapy, 8(1), 1-7, 9424

million deaths globally each year (Ferlay et al., 2015). Standard therapeutic approaches include hormonal therapy, radiotherapy, chemotherapy, and surgery (Howard and Blank, 2012). Survival rates are contingent upon tumor stage at detection (Itano et al., 2015).

Metastasis, contributing to about 90% of cancer-related deaths, is a primary factor in cancer mortality, involving detachment, migration, invasion, and adhesion (Jung and Yoo, 2023). Genetic changes in hosts significantly influence breast cancer risk and response to chemical therapy (Ulrich et al., 2003). Polymorphisms in innate immunity receptors and anti-oxidative enzymes play pivotal roles in cancer development (Klaunig et al., 2010; Pljesa et al., 2018; Eduardo et al., 2016).

Toll-like receptors (TLRs), integral to the innate immune response, exhibit polymorphisms, with TLR9 playing a key role in innate immunity by detecting DNA from endogenous and microbial sources (Zhang et al., 2011; Medvedev, 2013). In breast cancer, TLR9 stimulation has been associated with cell invasion and innate immunity functions (Ilvesaro et al., 2008). However, the full extent of TLR9's role in breast pathophysiology remains unclear.

A hormone, particularly estrogen, exerts its effects by binding to its corresponding receptor, promoting the survival and progression of the cell cycle in hormone-dependent breast cancer. Estrogen also regulates gene expression through non-genomic mechanisms, impacting signal transduction pathways. Imbalances in pro- and anti-apoptotic proteins of the Bcl-2 family contribute to tumor formation, and estrogen's inhibitory effect on apoptosis is implicated in inadequate responses to therapy (Lindsay et al., 2002; Novak et al., 2007). Estrogen receptor presence in tumor cells is a critical determinant of breast cancer outcomes, with estrogen-controlled cells activating mRNA transcription (Ashton et al., 2010; Chrusciel et al., 2019). This study aimed to elucidate the relationship between TLR9 polymorphisms and sexual hormones in the development of breast cancer.

Materials and Methods

Study design

Forty individuals diagnosed with breast cancer at the clinic between May 2021 and April 2022 were included in this study. Prior to treatment, each patient provided essential information, including age, menopause status, number of children, history of breastfeeding, and family history of breast cancer. The control group comprised twenty healthy female volunteers. All participants granted written, informed consent. The study received approval from the Scientific Ethical Committee at the University Of Anbar, adhering to Helsinki Declaration guidelines. Reporting in this article aligns with CONSORT standards.

Specimen collection

Five milliliters of blood were drawn from each participant, with two milliliters in EDTA tubes for DNA extraction to identify TLR9 genetic polymorphisms and three milliliters in activated gel tubes for hormonal tests. Genomic DNA extraction utilized a standard DNA Extraction Kit (Dsbio, China). DNA purity was assessed by the A260/A280 ratio, and concentration and ratios were calculated using a NanoDrop ND-1000.

Analysis of TLR9 polymorphism

TLR9-1237 T/C polymorphism genotypes were identified using RT-PCR on the QIAGEN Real-time PCR System (Rotor-Gene Q, Germany) with qPCR software. Specific primers and probe sequences for the TLR9 rs187084 (1237T/C) were employed. The RT-PCR protocol included a 15-minute incubation at 50°C, followed by a 15-minute incubation at 94°C. The subsequent cycles involved denaturation, annealing, and extension. In the TLR9 gene RT-PCR, the reaction components included forward and reverse primers, probes, probe master mix, DNase-free water, and DNA.

The following primers for LR9-rs187084 (1237T/C) were used: "forward" primer: 5-CACTGTACTGGATCCTGGGG-3 and "reverse primer": 5- TCTTACAAACCTCCCACCCC -3.

Probe sequences for target TLR9 gene. Dye Fam-BHQ: 5-CCCTTAAGAAGCTGACATTCCAG-3 and Dye Vic-BHQ: 5-GCCCTCAAGAAGCTGACATTC -3 (Biosearch Technologies).

RT-PCR

The RT-PCR protocol entails an initial 15-minute incubation at 50°C (Hold 1), followed by a subsequent 15-minute incubation at 94°C (Hold 2). During the first five cycles (Step One), denaturation is conducted at 95°C for 5 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 15 seconds. Subsequently, for the remaining 40 cycles (Step Two), denaturation occurs at 95°C for 5 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 15 seconds. In the genotype RT-PCR for the TLR9 gene, the reaction components consist of 1µl of forward primer, 1µl of reverse primer, 1µl of forward probe, 1µl of reverse probe, 10µl of probe master mix (WizPure™ qPCR Master (PROBE), South Korea), 3µl of DNase-free water, and finally, 3µl of DNA, yielding an overall reaction volume of 20 µl.

Hormonal Analysis

For hormonal analysis, the ELISA method was employed to quantitatively determine Progesterone levels in serum samples. This analysis utilized the Thermo Scientific Varioskan Flash Multimode Reader through an Enzyme-Linked Immunosorbent Assay (ELISA). FSH concentration was assessed using ELISA by KAMIYA BIOMEDICAL COMPANY, while Estradiol concentration was measured using ELISA by Oxis International, Inc.

Statistical Analysis

The impact of different variables on the study parameters was evaluated using SPSS software. The Chi-square test was employed to compare percentages in the study.

Results

Genetic Analysis using RT-PCR

The outcomes of the genetic analysis conducted through RT-PCR utilizing TaqMan with a dual dye (Fam + Vic) revealed noteworthy insights. In breast cancer women, the frequency of wild TT genotypes of TLR9 appeared non-significant in comparison with healthy individuals (TT, 72.5% vs. 90%, respectively). However, heterozygous CT genotypes were significantly higher in breast cancer women compared to healthy women (22.5% vs. 10%, respectively, at $P<0.05$). Conversely, the mutant CC genotype was found in 5% of breast cancer women and was absent in healthy individuals. Importantly, the mutant CC genotype was significantly less frequent ($P<0.01$) compared to wild TT and heterozygous CT genotypes in breast cancer patients. Additionally, the average TLR9 T allele frequency was significantly lower in breast cancer patients compared to controls, with rates of 0.95 and 0.84 percent, respectively (Table 1).

TLR9 rs187084 (1237T/C) Polymorphism and Breast Cancer Risk

Recent research indicates a link between the TLR9 rs187084 (1237T/C) polymorphism and a higher risk of breast cancer in the Iraqi female population. While detailed data on polymorphic rs187084 are not yet available, its location in the gene promoter region suggests a potential impact on the promoter's function. The variant alleles of the TLR9 polymorphism are expected to modify both microbial and endogenous DNA responses, influencing pro-inflammatory mediators. Studies in different populations have associated rs187084 T allele activity with an increased risk of developing endometrial and cervical cancers.

Hormonal Study Results

The hormonal study revealed significant differences between breast cancer patients exposed to radiation and the control group. FSH hormone levels showed a marked increase in patients compared to the control group, with an arithmetic mean of 2.9 IU/ml and 18.8 IU/ml, respectively. This increase was statistically significant at the probability level of 0.05 (Figure 1).

In contrast, E2 hormone levels decreased in patients exposed to radiation compared to the control group, with concentrations of 0.232 pico/ml and 0.910 pico/ml, respectively. This decrease was also significant at the probability level of 0.05 (Figure 1).

Furthermore, there was an observed increase in the level of progesterone hormone in breast cancer patients exposed to radiation, with an average concentration of the hormone at 0.910 nanogram/ml, compared to the control group with 0.732 nanogram/ml. This increase was statistically significant at the probability level of 0.05 (Figure 2, Figure 3).

A detailed analysis of hormonal correlations demonstrated a negative correlation between E2 and FSH and progesterone, while the correlation was positive between FSH and progesterone and negative between FSH and E2, all significant at the probability level of 0.05 (Table 2, Figure 2, Figure 3).

Discussion

Association Between Radiotherapy and Breast Cancer Risk Several published studies have highlighted an inverse correlation between the risk of breast cancer and radiotherapy for both benign and malignant gynecologic diseases in middle-aged individuals. However, these therapeutic interventions have been associated with substantial radiation exposures to the ovaries. Notably, the cells responsible for estrogen production in the ovaries emerge as the likely target cells for radiologic menopause. Despite significant radiation exposures to the ovaries during these treatments, the cells responsible for estrogen production in the ovaries appear to be the pertinent target for radiologic menopause. While postmenopausal ovaries continue to secrete androgens, even when estrogen generation has essentially ceased, high-dose pelvic radiation, as indicated by serum hormone tests in cervical cancer patients, appears to eradicate or notably diminish lingering androgen-producing activity in postmenopausal women's ovaries. These findings suggest a potential sensitivity of estrogen- and androgen-producing cells in the ovary to radiation inactivation, emphasizing the role of androgen production in the development or progression of breast cancer.

Role of Follicle-Stimulating Hormone (FSH) in Breast Cancer FSH, a hormone crucial for promoting follicle growth and maturation in the ovaries, as well as stimulating the production of mature spermatozoa in the testes, has significant implications in various cancer types, including prostate, endometrial, and ovarian cancers Chen et al. (2009) underscore the significant implications of FSH and its corresponding receptor (FSHR) in various cancer types, including prostate, endometrial, and ovarian cancers. The interaction between FSH and FSHR is implicated in cancer cell proliferation, differentiation, and metastasis, achieved through the activation of adenylyl cyclase and subsequent elevation of cAMP levels (Wayne et al., 2007). Notably, high levels of FSH have been associated with a notably poor prognosis in premenopausal breast cancer patients, and FSH has been linked to increased breast cancer risk in women undergoing infertility treatments.

Additionally, FSH plays a pivotal role in regulating gonadal differentiation and function (Hunzicker-Dunn & Maizels, 2006). Despite these associations, a conclusive correlation between circulating FSH levels in women with breast cancer and the

Table 1. Distribution of alleles and genotypes for TLR9 in breast cancer patients and healthy individuals.

Genotypes	**Control (2=20)**		**Patients (n=40)**		**Chi square x^2 value**
	n	%	n	%	
Wild-type TT	18	90	29	72.5	1.77 NS
Hetero CT	2	10	9	22.5	5.12*
Mutant CC	0	0	2	5	---
Chi square x^2 value	---	64**	---	26**	
Allele frequency					
T	0.95		0.84		
C	0.05		0.16		

**Significant at (P<0.01), * Significant at (P<0.05), NS: Non significant

Table 2. Correlation of Hormones level (P>0.05)

		FSH	**Progestrone**	**E2**
FSH	Pearson Correlation	1	0.019	- 0.822**
	Sig. (2-tailed)		0.893	0.000
	N	55	55	55
Progesterone	Pearson Correlation	0.019	1	- 0.084
	Sig. (2-tailed)	0.893		0.543
	N	55	55	55
E2	Pearson Correlation	- 0.822**	- 0.084	1
	Sig. (2-tailed)	0.000	0.543	
	N	55	55	55

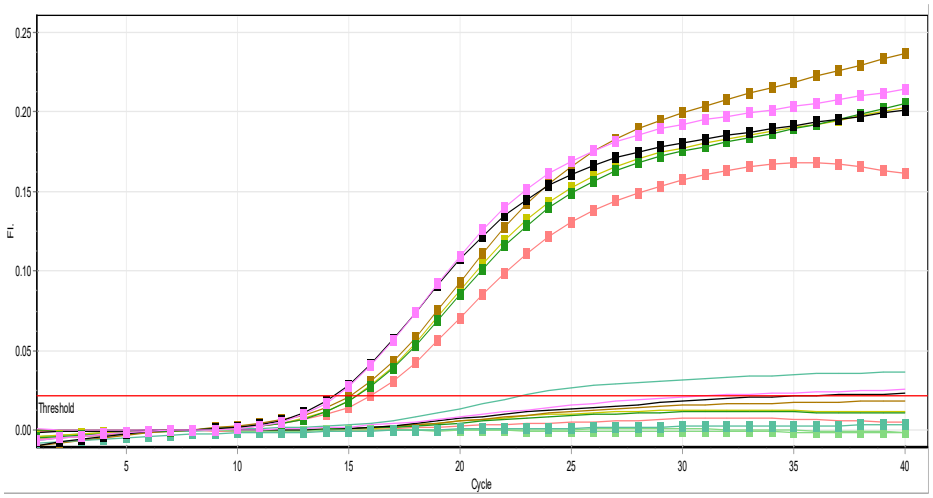

Figure 1. The polymorphism of the TLR9 gene. TaqMan genotyping kit (Fam + Vic) dyes are used in RT-PCR to measure.

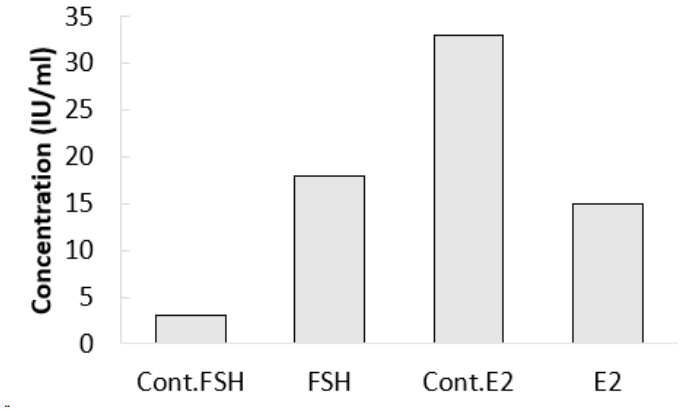

Figure 2. Level of FSH and E2 in patients and control groups. The hormonal study revealed significant differences between breast cancer patients exposed to radiation and the control group.

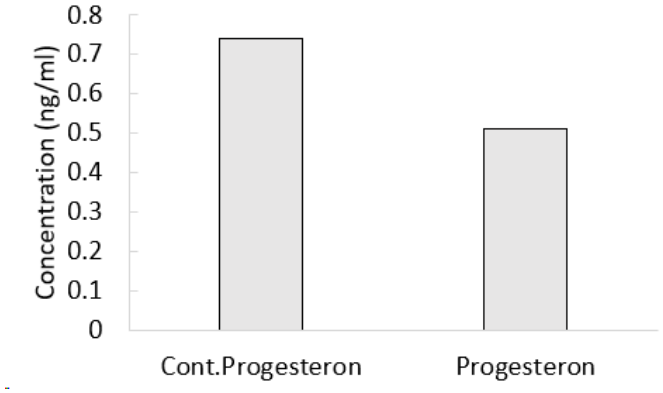

Figure 3. Level of progesterone in patients and control. hormonal correlations demonstrated a negative correlation between E2 and FSH and progesterone, while the correlation was positive between FSH and progesterone and negative between FSH and E2

prognosis of the disease remains elusive. In a study conducted by Wang et al. involving approximately 100 women diagnosed with breast cancer, no statistically significant correlations were observed. However, there was a tendency for patients with serum FSH levels below the median to exhibit a poorer outcome compared to those with higher values (Chimento et al., 2022). Furthermore, FSH prompts the production of Insulin-like Growth Factor I (IGF-I) within mammary stromal fibroblasts, and IGF-I, in turn, is positively correlated with the risk of breast cancer (Eskelinen , et al. 2004).

Progesterone's Role in Breast Development and Cancer Risk Progesterone, an ovarian steroid hormone, plays a crucial role in optimal breast development during adolescence and is indispensable for lactation and breastfeeding. A deeper understanding of progesterone receptor (PR) activity has been gained by utilizing human breast cancer cell line models expressing PR+ or PR-null cells, where wild-type or modified PR is reintroduced (Marquez-Lago and Steinberg, 2022). Additionally, growth factors like EGF or heregulin have been demonstrated to enhance transcriptional synergy with progestins on PR-target genes (Daniel et al., 2007). While numerous genes are under the control of PR expression, not all are directly influenced by progesterone. In response to progesterone/PR-dependent transcriptional repression, many genes undergo downregulation through processes that are not fully understood. The association between the regulation of specific genes in response to progesterone/PR and alterations in cell biology is often weak. Importantly, several genes regulated by PR have been linked to aspects of tumor progression, contributing to a more aggressive phenotype. Additionally, variations in the PR-A:PR-B ratio are commonly observed in breast tumors compared to normal tissue, and these variations are anticipated to exert a significant impact on the genetic program (Huhtaniemi, 2010).

Studies utilizing human breast cancer cell line models suggest that the effects of progesterone vary depending on the tissue, with both progestational and proandrogenic properties. While pharmacologic and physiologic levels of estradiol are widely associated with increased cell growth in the breast epithelium, the potential connection between progesterone and breast cancer risk remains a subject of debate. Elevated serum progesterone levels in postmenopausal women have been linked to a higher risk of breast cancer, particularly when accompanied by higher levels of circulating estradiol. The observed interaction suggests that progesterone's role at normal levels may be influenced by circulating estradiol levels (Choi, et al. 2004).

In vivo studies support the idea that both estradiol and progesterone can stimulate cellular growth in the mammary gland. Progesterone has the ability to impact estrogen signaling by regulating the expression and activity of the estrogen receptor (ER). It can prompt the production of co-regulatory proteins that interact with ER, thereby influencing its transcriptional activity. his modulation consequently changes ER's impact on cellular growth and viability (Satpathi, et al. 2023).

Conclusion

In conclusion, the study indicates that TLR9 rs187084 (1237T/C) polymorphism variants play crucial roles in the susceptibility of the Iraqi female population to breast cancer. The findings highlight the potential relevance of TLR9 polymorphisms in breast cancer growth and emphasize the intricate interplay between genetic factors, hormonal dynamics, and cancer development.

Author contribution

A.A.A.J conceptualized, R.H.H. collected data, M.N.R. analyzed data, and all auhtors wrote and approved the paper.

Acknowledgment

The Authors are grateful to their institutional support.

Competing financial interests

The authors have no conflict of interest.

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