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FASL Gene Polymorphism with Oxidative Stress of Iraqi Females with Breast Cancer

Ferdous A. Jabir¹, WatheqJaber² and Mohammed AbdulwahabAti Al-Askeri^{3*}

¹Biochemistry Dept., College of Medicine, University of Al-Qadisiyah, Iraqz

²Chemistry Dept., College of Science, University of Al-Qadisiyah, Iraq

³Mdical Biotechnology Dep., College of Biotechnology, University of Al-Qadisiyah,Iraq.

Abstract : The aim of current study is to explore the probable role of FASL gene polymorphism and oxidative stress in breast cancer occurring in the Iraqi female patients. Blood samples from patients and healthy volunteers were collected and used for the succeeding experiments. The results were obtained by (T-ARMS PCR) technique for the FASL gene were shown there was a significant difference in 2124 A/G polymorphism of FASL between treated group and control group (P less than 0.05).

Keyword : Brest Cancer, FASL gene, MDA, Oxidative stress, T-ARMS PCR and Polymorphism.

Introduction

Cancer disease begins when normal cells set up to grow out of ordinary body control. Cells in almost any part of the body can become cancer under certain inside and outside factors, and may spread to other parts of the body. Breast cancer is “a malignant tumor that begins in the breast cells”¹.

The key risk factors interrelated to breast cancer susceptibility are related to clinical history, elongated periods of hormonal, age, family history, lifestyle factors and delayed first pregnancy². The other important risk factor for beginning and development of breast cancer is oxidative stress^{3,4}, breast tumors are normally living in an unbelievably pro-oxidative surroundings, while the mammary gland is abundance in close adipose tissue. Therefore, oxidative stress speedily acts on the surrounding area of lipid yielding many of energetic metabolites that can regulate a broad variety of cellular processes. Well-known examples are derived of lipid peroxidation like low-molecular-weight aldehydes that have reported as new supposed indicators of the oxidative status in breast cancer patients are Malondialdehyde, 8-F2-isoprostanes and 4-hydroxynonenal⁵⁻⁷. These prooxidant surroundings appear to be essential through the early stages of disease for probably cancer developing to an advanced stage, besides it may influence adaptability tumor cells against the reactive species (RS) derived from antineoplastic drugs⁸. Oxygen radicals are connected with diverse steps of breast carcinogenesis during formation of adducts, dealing with oncogenes and tumor suppressor genes by DNA damage which affects immunological mechanisms⁹. The indirect creation of “DNA adducts” through launching autocatalytic peroxidation of lipid which cause a great mixture of possible genotoxic products including Aldehydes, Malondialdehyde (MDA), Peroxyl radicals (ROO) and Alkoxy (RO). Thus, DNA is a permanently became damaged and oxidatively amended. Mutations can cause by an oxidative error that is not repaired, increasing the risk of carcinogenesis^[10].

8-hydroxy-2'-deoxyguanosine (8-OHdG) is a very important marker for determining the impact of exogenous or endogenous oxidative affects to DNA. It was studied as a potential factor in initiation and development of carcinogenesis also^[11]. In last few years, 8-OHdG was targeted mostly in several studies as a biomarker for the estimation of endogenous oxidative DNA damage amount and as a risk factor for various diseases involving cancer. The estimation of 8-OHdG based on a simple technique for the estimation of oxidative DNA damage. Many studies showed levels of urinary 8-OHdG were considerably higher in breast cancer patients¹².

Increase expression of a number of genes by reason of certain polymorphisms or mutations increases the breast cancer occurrence risk. In view of the fact that mutations that are familiar to increase breast tumors in families are very rare, recognition of these SNPs is so essential. The most important positions which contain mutations are "STK11, RAD50, BRCA1, BRCA2, CDH1, ATM, PTEN, CHEK2, PPM1D, MLH1, MSH2, MRE11, BARD1, MSH6, MUTYH, NBN, PMS1, PMS2, BRIP1, RAD51C, and TP53" the existence of SNPs in those genes increases the breast cancer risk and correlated diagnostic indicators are between the most credible for estimating prognosis in breast cancer¹³.

FAS or "Apo-1 or CD95" is an active element of the death receptor group, which performs an important role in apoptotic signaling mechanisms¹⁴. FAS protein reacts with its "FAS-ligand (FASL)" to motivate the cell death signal and hence stimulate apoptotic cells death⁴².

Additionally, the sense mutations in "FAS and FASL" genes that weaken apoptotic signal transduction were revealed to be linked with a bigger risk of a range of cancers¹⁵. Consequently the "FAS/FASL system" is possibly important during beginning, growth, and progression of cancer. SNPs, which may have the possibility to change the expression levels of FAS and/or FASL, were suggested to be considerable in the genetic predisposition to cancer¹⁵. This gene locates on site 1q23 and composed of 4 exons across 8 kb and encoding 281 amino acids¹⁶. Many studies scrutinized the role of FAS and FASL gene polymorphisms in the causes of various cancers involving "breast, cervix, bladder, prostate, lung, head and neck and esophagus"¹⁷.

Therefore, FASL is sometimes over-expressed in various human tumors, including "breast cancer"¹⁸. So, we assumed that the polymorphisms in the encoding sequence, "FASL 2124 A/G" may raise the breast cancer probability in an Iraqi population.

Materials and methods

Blood samples have been collected from twenty-one healthy persons and thirty-one patients, the healthy individuals divided into two groups those with no previous family background of cancer (15 persons) considered as a "control group" and those with a family history of breast cancer (sisters of some patients) as related group (6 persons). Blood samples were collected from patients who confirmed by pathohistological examination having breast cancer; those also divided into two groups those were taken therapy (31 persons) as treated group and those without therapy as an untreated group (15 persons). The patients and healthy groups are matched in age as shown in the table (1). Five milliliters of venous blood samples were withdrawn and collected in tubes, two milliliters were mixed in Na-EDTA tubes, and three milliliters were collected in gel tubes, the gel helps in good separating for serum. The serum and whole blood kept frozen at (-20°C) until used for different assays and DNA extraction.

Lipid peroxidation, estimated by MDA formation was determined by the method of Guidet, B. and Shah. (1989)¹⁹. The nitrite/nitrate concentrations were determined by Griess reagent according to the method described by Chaea, S.Y. *et al.* (2004)²⁰. Nitrite/nitrate concentration in serum was determined using the reference standard curve that made.

The levels of 8-OHdG in sera were estimated using ELISA assay by using the 8-OHdG kit, (Elabscience company, USA).

FASL polymorphisms were detected by using "tetra-amplification refractory mutation system-polymerase chain reaction (T-ARMS PCR)" as described previously by Mohammad Hashemi *et al.* (2013)²¹.

PCR product sizes were: “300 bp for the A allele, 197 bp for the G allele and 438 bp for control band” using specific PCR primers as it shown in the table (2).

The statistic analysis between the frequencies of alleles and genotype distributions of the four groups were set by the binary logistic regression at p - value<0.05 using SPSS18 software through calculating the odds ratio (OR) and their confidence intervals values (95% CI) and χ^2 values.

Comparison among groups for biochemical tests conducted by paired sample T-test at (p -value< 0.05) and as a significant variation.

Table(1):The PCR oligonucleotides used in current study

Primer name	Sequence (5' →3')	Tm (°C)
FasLgene polymorphism A/G (rs5030772)	Sense outer primer 5'GGTCTTCTTGGATTAGTCACCCAACCTT 3' Antisense outer primer 5' CACTTTCCTCAGCTCCTTTTTTTCAG 3' Sense inner primer 5' CTGCAGTTCAGACCTACATGATTAGTCTG 3' Antisense inner primer 5' TTA AACCGTAAATGGCAACAGTCTAAAAT 3'	57

Table (2): Samples and groups used in the experiment

No.	Group	Age (mean ±SE)	Range of Age	No. of Cases
G1	Control	42.33 ± 3.54	(23-65)	15
G2	Related	46.67 ± 4.13	(26-53)	6
G3	Treated	48.55 ± 1.27	(33-62)	31
G4	Untreated	46.73 ± 3.06	(23-65)	15

SE= Standard Error

Results:

A-Biochemical tests:

Malondialdehyde (MDA),Nitric Oxide(NO) and 8-Hydroxydeoxyguanosine(8-OHdG) levels in the sera of patients who have breast cancer treated and untreated groups (G3 and G4) were indicated to be significantly higher compared to healthy group (G1) at (p -valueless than 0.001) as illustrated in table (3). Also, when compared the levels in sera of treated group (G3) to the untreated group (G4) were found to be different, less than G4, this difference is statistically significant (P less than 0.05), While when compared the levels in sera of related group (G2) to control group (G1) were found to be different, but this difference is not statistically important (P greater than 0.05), as shown in the table (3).

Table (3): The mean and comparison of concentrations of biochemical tests

variables	G1, n=15 (Control)	G2, n=6 (Related)	G3, n=31 (Treated)	G4, n=15 (Untreated)	Comparisons	P value
NO Cons. (mean ±SE)	33.52 ± 1.04	40.26 ±7.47	54.56 ± 3.27	113.56 ±19.24	G2 with G1 G3 with G1 G4 with G1 G4 with G3	0.506 0.001 * 0.001* 0.006 *
MDA Cons. (mean ±SE)	2.38 ± 0.05	2.78 ± 0. 15	3.02 ± 0.10	3.4 ± 0.16	G2 with G1 G3 with G1 G4 with G1 G4 with G3	0.068 0.001 * 0.000 * 0.001 *
OHdG Cons. (mean ±SE)	121.67±16.30	188.6 ±59.75	243 ± 19.19	377.80±161.24	G2 with G1 G3 with G1 G4 with G1 G4 with G3	0.485 0.000 * 0.000 * 0.036 *

(*) Represent the value is statically significant,(SE) Represent Standard Error

B- Association between various polymorphisms with possible risk of breast cancer

The current study examined the probable association between rs5030772polymorphisms with the risk of breast cancer .The study was conducted by means of “Tetra-Amplification Refractory Mutation System–Polymerase Chain Reaction (T-ARMS-PCR)”^[21].Figure (1) shows the PCR product of FASL gene polymorphism for the four groups (G1,G2,G3and G4), and the PCR product size is, G allele (197 bp),an allele (300 bp) and control band (438 bp) .



Figure (1):Electrophoretic pattern of T-ARMS-PCR for detection of polymorphisms for FASL gene [2124 A/G, rs5030772]; lane No. of (1, 2) refer to G4 (Untreated group);lane(3,12) refer to G1(Control);lane (4,5,6,7,8,9,10) refer to G3 (Treated) ;lane (11) refer to G2Related(sister of the patient No. 10); M: Refer to (100bp DNA Ladder).

1. Related(G2)with Control(G1)

The genotype frequencies and distributions of allele towards 2124 A/G polymorphism of FASL in related (G2) and control (G1)groups are revealed in the table (4). The results indicated that the frequency of “GG” is 0%, “AG” is 100% and “AA” is 0% in G1. The frequency of 2124 A/G polymorphism in G2 was 0% for “GG”, 100 % for “AG” ,and 0% for AA. There was no significant difference in 2124 A/G polymorphism of FASL between G2 and G1 (p=0.233).As shown in the table (4),for G1 the “A” allele frequency was 53.3% and the “G” allele frequency was 46.7%, and in the G2 the frequencies were 50% for “A” allele and 50% for “G” allele. The frequency of allele was not significantly different among G1and G2 (p=1.000).

Table (4): Genotypes and allele distributions for “FASL gene” polymorphism in G2 and G1

Polymorphisms FAS/FASL (-124 A/G)	G1 Control)(N=15(%)	G2 Related)(N=6(%)	χ^{2a}	P-value ^a	OR (95%CI) ^b	P value ^b
AA			0.000	1.000	1.0 ^{ref} (1.0 ^{ref})	
AG	15(100)	6(100)			c	c
GG					c	
A allele	15(50)	6(50)	0.000	1.000	1.0 ^{ref} (1.0 ^{ref})	
G allele	15(50)	6(50)			1.000 (0.262-3.815)	1.000

^a values about genotype distribution, ^b Are calculated relative to subjects with the A/A genotypes and A allele c the variables is constant

2. Treated G3 with Control(G1)

The genotype frequencies and allele distributions of 2124 A/G polymorphism of FASL in treated (G3) and control (G1) groups are shown in the table (5). The results showed that the frequency of “GG” is 0% , “AG” is 100% and “AA” is 0% in G1. The frequency of 2124 A/G polymorphism in G3 was 0% for “GG”, 82.4% for “AG” and 9.7% for “AA”. There was a significant difference in 2124 A/G polymorphism of FASL between G3 and G1.As shown in Table (5) in G1, “A” allele frequency was 50% and the “G” allele frequency was 50%, and in the G3 the frequencies were 58.8 % for “A” allele and 41.2% for G allele. Logistic regression analyses proved that the 2124 A/G“AG” and “GG” genotypes were linked to breast cancer risk compared with the AA genotype (*P* = 0.044). There was a significant difference when comparing the “G” allele with the reference “A” allele (*P* = 0.480).

Table (5) :Genotypes and allele distributions of “FASL polymorphism gene” in G3 and G1

Polymorphisms FAS/FASL (-124 A/G)	G1 Control)(N=15(%)	G3 Treated)(N=17(%)	χ^{2a}	P-value ^a	OR (95%CI) ^b	P value ^b
AA		3(9.7)	4.068 *	0.044 *	1.0 ^{ref} (1.0 ^{ref})	
AG	15(100)	14(82.4)			1.731 (0.000)	0.999
GG						
A allele	15(50)	14(41.2)	0.501	0.479	1.0 ^{ref} (1.0 ^{ref})	
G allele	15(50)	20(58.8)			0.700 (0.260-1.882)	0.480

^a values about genotype distribution, ^b Are calculated relative to subjects with the A/A genotypes and A allele,*the value is statically significant

3. Untreated(G4) with Control(G1)

Genotype frequencies and allelic distributions of 2124 A/G polymorphism of FASL in treated (G4) and control (G1) groups are illustrated in Table (6). The results showed that the frequency of “GG” is 0%, “AG” is 100% and “AA” is 0% in G1. The frequency of 2124 A/G polymorphism in G4 was 0% for “GG”, 100% for “AG”,and 0% for “AA”. There was no significant variation in 2124 A/G polymorphism of FASL in between G4 and G1(*P* = 1.000).As shown in Table (6) in G1, “A” allele frequency was 50% and the “G” allele frequency was 50%, and in the G4 the frequencies were 50 % for “A” allele and 50% for “G” allele. There was a significant difference when comparing the “G” allele with the reference “A” allele (*P* = 1.000).

Table (6):Genotypes and allele distributions of “FASL polymorphism gene” in G4 and G1

Polymorphisms FAS/FASL (-124 A/G)	G1 Control)(N=15(%)	G4 Untreated)(N=11(%)	χ^{2a}	P-value ^a	OR (95%CI) ^b	P value ^b
AA			0.000	1.000	1.0 ^{ref} (1.0 ^{ref})	
AG	15(100)	11(100)			c	c
GG					c	
A allele	15(50)	11(50)	0.000	1.000	1.0 ^{ref} (1.0 ^{ref})	
G allele	15(50)	11(50)			1.000 (0.333-3.005)	1.000

^avalues about genotype distribution, ^bAre calculated relative to subjects with the A/A genotypes and An allele, ^cthe variables is constant

4. Treated(G3) with Untreated(G4)

Genotype frequencies and allelic distributions of 2124 A/G polymorphism of FASL in treated (G3) and control (G4) groups are indicated in the table (7). The results indicated that the frequency of “GG” is 0% , “AG” is 100% and “AA” is 0% in G4. The frequency of 2124 A/G polymorphism in G3 was 0% for “GG”, 82.4% for “AG” ,and 9.7% for “AA”. There was the difference in 2124 A/G polymorphism of FASL in between G3 and G4 but this difference is not significant (p = 0.07).As shown in the table (7) in G4, “A” allele frequency was 50% and the “G” allele frequency was 50%, and in the G3 the frequencies were 58.8 % for “A” allele and 41.2% for “G” allele .There was a “significant difference” when comparing the “G allele” with the reference “A” allele (P = 0.517).

Table (7):Genotypes and allele distributions of “FASL polymorphism gene” in G4 and G3

Polymorphisms FAS/FASL (-124 A/G)	G4 Untreated)(N=11(%)	G3 Treated)(N=17(%)	χ^{2a}	P-value ^a	OR (95%CI) ^b	P value ^b
AA		3(9.7)	3.224	0.070	1.0 ^{ref} (1.0 ^{ref})	
AG	11(100)	14(82.4)			1.269 (0.000)	0.999
GG						
A allele	11(50)	14(41.2)	0.420	0.517	1.0 ^{ref} (1.0 ^{ref})	
G allele	11(50)	20(58.8)			0.700 (0.238-2.060)	0.517

^avalues about genotype distribution, ^bAre calculated relative to subjects with the A/A genotypes and An allele

Discussion

Reactive oxygen species “ROS” have been identified to take part in a significant function in the initiation and development of carcinogenesis and have been involved in carcinogenesis in animal models as well as humans²². Former studies showed that perpetual alteration of genetic material due to oxidative damage causes carcinogenesis^[23]. It has been demonstrated that ROS are correlated with the various steps of carcinogenesis either in the course of structural DNA damage or effecting tumor suppressor genes, oncogenes and immunological mechanisms²².

Our study has proved higher levels of MDA in breast cancer patients, Malondialdehyde (MDA) is the final product of lipid peroxidation, as a result of its high cytotoxicity, it has been proposed to act as a

carcinogenic agent and a tumor promoter. Increase in MDA levels in carcinoma could be used as an important parameter to the risk of this disease fundamentally due to its double roles as a tumor promoter and a mutagen^[22]. Elevated amounts of the final products of lipid peroxidation have shown in tumor tissue itself obviously pointing to the source of elevated “MDA” levels in patients who have with cancer²⁴. Similarly, Mohini Aiyengar Tupurani et al (2013)²² and Aghvani et al, (2006)²⁵ demonstrated average MDA levels were in patients who have breast cancer.

Our study has also revealed higher levels of NO in breast cancer patients. NO is a free “radical gas, water-soluble” which perform an important role in a variety of pathological and physiological mechanisms. It is made by a multifarious family of nitric oxide synthase (NOS) enzymes. The role of “NOS” in tumor biology was specified clearly. It was suggested to have both tumor-promoting impacts in addition to tumoricidal which rely on its real-time reaction, concentration, and site^[26]. These high NO levels may cause by the elevated NOS II activity which was catalyzed by a host protection system versus tumor development. NO-“derived reactive nitrogen species” and NO stimulate nitrosative and oxidative stress which caused DNA damage and the inhibition of DNA repair enzymes during indirect or direct mechanisms^[27]. It was suggested that the biological impacts of NO could be mediated by the productions of diverse NO metabolites. “NO” can cause damage to DNA through the production of N₂O₃ and peroxynitrite (ONOO⁻). Peroxynitrite can cause oxidation and nitration for DNA and may because of single-strand DNA breaks during the attack on the sugar-phosphate backbone. Different Studies have explained that levels of NO can be implicated in stimulating or preventing the cancer etiology²⁸.

Increased NO-generation in a cell may choose mutant p53 cells and participate to tumor angiogenesis by up-regulating VEGF (vascular endothelial growth factor). Also, NO may modulate mechanisms of tumor DNA repair by up-regulating p53, poly (ADP-ribose) polymerase (PARP). A comprehension at the molecular level of the role of NO in cancer will have deep therapeutic implications for the diagnosis and treatment of cancer²⁸. Our study has also authenticated higher levels of NO in breast cancer patients compared to controls, these results are similar to those obtained previously studies^{22,29}.

Our study showed that an association between risk of breast cancer and elevated 8-OHdG levels.

8-OHdG caused by ROS-induced a wide range of oxidative damage of DNA, is widely utilized as an indicator of oxidative damage to DNA, which is essential in carcinogenesis and mutagenesis processes³⁰. Urinary levels of 8-OHdG are therefore considered as an oxidative stress general biomarker. Because elevated levels were noticed among patients with prostate cancer³¹, lung cancer³², urine and serum and 8-OHdG levels in patients with breast cancer^{31,33,34} and colon cancer^{35,36}, when compared to healthy controls. 8-OHdG may be beneficial as an evidence of cancer risk. 8-OHdG can be determined by enzyme-linked immunosorbent assay (ELISA), immunohistochemistry and high-pressure liquid chromatography (HPLC)^{30,46}.

The increase of 8-OHdG levels in patients considers a marker of elevated oxidative stress, lacked antioxidant defense or not insufficient repair of oxidative DNA damage. 8-OHdG is one of the high-oxidized bases was characterized. 8-OHdG in DNA could direct to misincorporation of adenines adverse the 8-OHdG lesion thus inducing “G: C to T: A” mutations in genomic DNA. Elevated 8-OHdG levels in breast cancer patients may be attributable to defects in 8-oxoguanine DNA glycosylase-1 (OGG1) repair gene causing aggregation of mutations, cancer appearance and progression^{37,47}.

Karki et al.(2014) reported that high sensitivity, specificity, and accuracy of bio-indicators of oxidative stress comprising serum 8-OHdG can be utilized as distinguishable markers for reliable diagnosis of breast cancer³⁸. These results are similar to those obtained previously studies^{12,31,37}. The chemotherapy has reduced the oxidative stress and levels of 8-OHdG in patients who have breast cancer. This is consistent with the previous study³⁹.

Our study also investigates the association between -607C/A (rs1946519) and 2124 A/G (rs5030772) polymorphisms with risk of breast cancer. In our study, we found no relationship between FAS expression in the untreated group (G4) or primary stages of breast cancer and the risk of breast cancer, while we found an association between FAS expression in the treated group (G3) or advanced stages and the risk of breast cancer. It was confirmed that the FAS need in the primary tumor was correlated with perilymphatic fat permeation and metastasis process either to the bones or to the surrounding lymph nodes^{40,41,49}.

FAS protein reacts with “FAS-ligand (FASL)” to initiate the death signal molecules and hence stimulate apoptotic cells death^[42]. The interactions between “FAS and FASL” were shown to be included in the creating of an immune response status against tumor by stimulating “FAS-mediated apoptosis in tumor-specific lymphocytes”^[43,50]. Additionally, the sense mutations in “FAS and FASL genes” that weaken programmed cells death signal transduction were proved to be related with an increased risk of a variety of cancers^[44]. Many studies investigated the role of “FAS and FASL gene” polymorphisms in the causes of various cancers involving breast, bladder, prostate, lung, head, cervix, neck and esophagus^{45,48}.

Conclusions

The “FAS/FASL” system possibly important in the beginning, growth, and progression of cancer, and “single nucleotide polymorphisms (SNPs)” which have the possibility to change the expression levels of “FAS and/or FASL” is possible to be considerable in the genetic susceptibility to breast cancer¹⁷.

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