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ENDOTHELIAL NITRICOXIDE GENE POLYMORPHISMS IN IMPEDED BREAST CANCER TISSUE

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ABSTRACT

The present study aims to investigate endothelial nitric oxide synthase (eNOS) gene polymorphisms in impeded breast cancer tissue, polymorphism detected using amplification VNTR flanking sequence by PCR, the results show significant differences between patients and control in the deletion sequence of intron 4 it was (33.33%) in patients and (12.5%) in control (odd ratio 3.7813 (CI%7.8857 - 1.8131), The present study concluded that deletion in VNTR sequence in eNOS gene associated with breast cancer disease.

KEYWORDS: **Endothelial nitric oxide synthase, VNTR flanking sequence**.

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INTRODUCTION

The breasts form a secondary sexual feature of females and are the source of nutrition for the neonate. They are also present in a rudimentary form in males. The breasts are the site of malignant change in as many as one in ten women, The connective-tissue stroma of the breast forms from the mesoderm, which will form the dermis of the skin and the superficial fascia (telasubcutanea) as well, Fibers forming the suspensory ligaments (of Cooper) will develop from both layers, This development, as well as the appearance of fat in the superficial fascia, does not occur until puberty in the female, Although minor changes occur during each menstrual cycle, pregnancy and lactation bring about the ultimate development of the breasts. [1,2] Nitric oxide (NO) is synthesized is one of three isoform of nitric oxide synthases (NOS) family ;neuronal NOS (nNOS, NOS-I) and endothelial(eNOS, NOS-III), and one inducible NOS (iNOS, NOS-II).The eNOS in the endothelium synthesis little amounts of NO resulted from receptor stimulation or shear stress, NO doesn't cause damage in cell and tissue in because it is cleared by reaction with oxy hemoglobin in the normal state, But in inflammatory and infections, high level of NO was produced which causes damage in host tissues and change the course of diseases.[3-7] The eNOS gene is located on chromosome 7q35–36 and comprises 26 exons spanning 21 kb (6) the variant of this gene may be cases deficiency in some oxidative balance pathways, Many studies have been carried out to determine the relevance between DNA variants in the eNOS gene and some disease, a variety of vascular diseases which include coronary artery disease (CAD) or myocardial infarction (MI), hypertension, stroke, and renal diseases, have been associated with the eNOS gene polymorphisms. [8-13]Cancer is a major health problem and a common cause of death worldwide, it results from uncontrolled cell growth and proliferation caused by mutations in DNA by the carcinogenesis process, or carcinogenic (drugs and chemicals), biological (viruses), or physical (radiation) agents, Mutations in DNA convert proto-oncogenes into oncogenes; then oncogene expression changes, cell proliferation is increased, and ultimately normal cells are transformed into malignant neoplastic cells. The characteristics of cancer cells include loss of contact inhibition, resistance to apoptosis, and insensitivity to cell growth arrest signals. [14-17] The breast cancer is the most common cancer in women and has the highest mortality rate in the world. [18] It is multi factorial, and the major risk factors being age, early menarche, delayed menopause, use of contraceptives or oral medications, hormone therapy, family history, history of benign breast disease, obesity, and having excess weight, lifestyle And oxidative stress. [19-22] Oxidative stress is an imbalance in the ratio between oxidants (free radicals) and antioxidants, which causes increment of damage and destroys in cell function. [23,24] Many cellular processes, including cell proliferation, cell metabolism, signaling pathways, pathways regulating gene expression, and apoptosis are affected by oxidative stress. [25-27] Increasing the free radicals can effect in structure and functions of the main cellular partials like proteins, lipids, and nucleic acids which can lead to tissue destroys, Now it can use as a biomarkers for assessment and diagnosis of all cancers , Especially breast cancer. [28,23,29]

MATERIALS AND METHODS

Sample collection about 30 breast cancer embedded tissue was collect from histopathology unit in Al-Saader medical city, these samples was diagnosis by specialist physician as a breast tumor tissue, also all samples were used to diagnosis tumors in females that don't treated with any anticancer therapy, and 30 blood sample of control were collected from healthy female that have age (35-65 years). DNA was extracted from embedded tissue according to the leaflet of Geneaid manufacture with modification, in briefly (3 REPLICATE): About 40 mg of tissue was put in eppendorf tube contain 1 ml of xylene, then it mixed and incubate at room temperature for 15 min, then Centrifugation at 14000 for3 min, and supernatant was removed, Absolute ethanol was added (1 ml) to mixture then Centrifugation at 14000 for3 min, and supernatant was removed, the mixture was Incubated at 37 C , GT buffer was added (200µl) with homogenize by micro pestle, then Proteinase K (40 µl) was added and incubate for 20min. at 60 C with inverting every 5 min. GBT buffer was added (200 µl) with mixing, then incubate at 60 C for 20 min and Absolute ethanol was added (200 µl) with mixing, then transfer mixture to GD column after that it Centrifuged at 14000 for2 min, the flow-through was discarded and W1 buffer was added to column (400 µl), then Centrifugation at 14000 for 20 sec. the flow-through was discarded also. Wash buffer was added (600 µl) Centrifugation at 14000 for 30 sec. the flow-through was discarded, then it Centrifuged at 14000 for 3 min again, finally DNA was eluted using dH2O (100 µl). Healthy DNA was extracted from whole blood using (Genaid extraction kit), in briefly; A 300 µl of frozen blood was transferred to eppendorf tube, then 40 µl of proteinase k was added and incubated it at 60 C for 20 min, then GB buffer was added (200 µl) and it shaking vigorously, after this absolute ethanol was added (200 µl) and miter was mixed by shaking, then it centrifuged at 15000 rpm for 5 mints. the Supernatant was transferred to GD column, and centrifuged at 15000 rpm for 1 min. the flow -rate was discarded and 400 µl of W1 buffer was added, then centrifuged at 15000 rpm for 1 min, the Fallow rate was discarded also, about 600 µl of wash buffer was added, then centrifuged at 15000 rpm for 1 min. columns were Re-centrifuged after discarded flow ate for 5 min at the same speed to dry column, finally 100 µl of d H2O was added to column and left 2 min at room temperature to absorb it. DNA eluted in new eppendorf tube by centerfield column for 2 min at15000 rpm. After DNA extraction; consternation and purity of DNA were estimated using nanodrpe. PCR conditions were performed as a following; primer that used was to (F-AGGCCCTATGGTAGTGCCTT- and R-TCTCTTAGTGCTGT GGTCAC-), which were based on the sequences flanking VNTR in intron 4 of the eNOS gene, used to amplify the corresponding DNA fragment. PCR cycles were 95Cº for 5 min (one cycle) , 95 Cº for 30 sec, 58Cº for 30 sec, 72Cº for 30sec (30 cycles), then 72 Cº for 10 min (one cycle) [30] .The PCR products

were separated by 1% agarose, 70 V and 20 mA for 45mins. The PCR products were 420 bp wild-type (A allele) contained five 27 bp repeats and the 394 bp mutant type (B allele) contained four 27 bp repeats. [31]

RESULTS

The results of present study show that there was low concentration and high purity of DNA which extract from paraffin embedded tissues it was ranged (7-15 ng\ µl). While concentration of blood DNA were (75-150 ng\µl). High purity was obtained in both blood and embedded tissue DNA it was ranged (1.9-2.2). There were significant variations in intron 4 *eNOS* gene deletion between patients and control, PCR products were 420bp (AA) for wild type of gene and 394bp (BB) for deletion, as show in table (1) and figure (1 , 2), BB allele was more frequent in patient than control it was appeared in 33.33% , 12.5% in patient and control respectively while AA allele was more frequents in control than patient, it was 87.5%, 66.66% respectively.

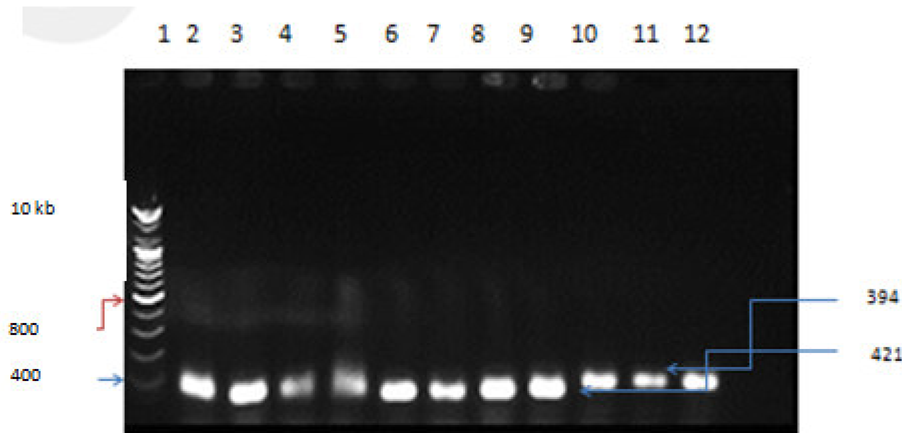

Figure 1
Electrophoresis pattern of intron 4 eNOS gene polymorphisms in study subjects, lane 1 DNA marker (10kb-400 bp), lane 2,3,4,5,12 for control, lane 6,7,8,9,10,11 for patients

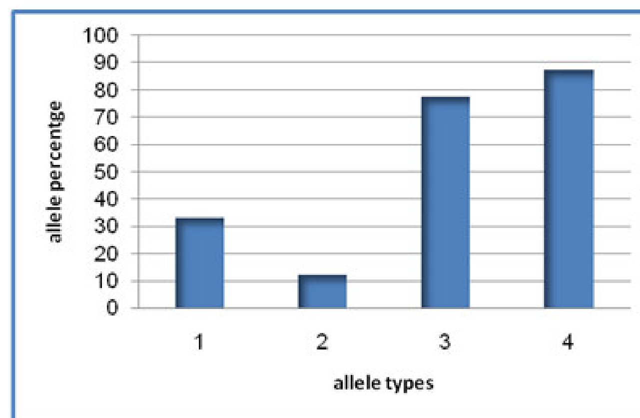

Figure 1
Distribution of alleles percentage in cancer and control group Lane1, 2 AA allele in patients and control respectively. Lane 3,4 BB allele in patients and control respectively.

Table 1
Static distribution of intron 4 eNOS polymorphisms in study subjects

Allele	Patients %	Control %	Odd ratio	CI (95%)	P-value
BB	33.33	12.5	3.7813*	1.8131 - 7.8857	0.0004
AA	66.66	87.5			

**Significant at P-value (0.05)*

DISCUSSION

Oxidative stress has been the important factor which has major role in developments of diseases in present study results show that there is significant association between eNOS gene deletion in study groups. First the all, DNA extraction from impeded tissue have been some problems like low yields and contamination with viral genome if the cancer caused from viral infection also DNA may effected by fixation processing, thus three replicated of each sample must be extracted to avoiding positive false results. In present study high purity with low concentrations of patients sample were obtained, however, many modifications performed to

get better concentrations and purity of DNA like time, proteinase k amounts, heat, and elution DNA , the results show that 200µg of proteinase K was the best and 70C for 30 min the best for proteinase K activity to lyses proteins in cell and cancer tissue, also d H2O was the best for eluted large amount of DNA and for its storage, these optimization performed in study [32] how used different methods for gnomic DAN and forging DNA from blood and tissue of rabbit. Low concentration but high purity of DNA was dependent in PCR reaction in present study for patients, this because that the purity was more important than concentration, PCR consider as amplification system which can amplified smallest amount of DNA with high fidelity DNA polymerase enzyme that have proofreading activity. [33] Many studies consider that imbalance in oxidative stress responsible of DNA mutation which causes accumulation of biomolecules destroyed lead to carcinogenesis. [34] eNOS gene was choose in present study because it have main role in oxidative stress balance, also in Iraqi population there was no studies deal with the common disease that appeared in high percentage in Iraqi women at last decades. Intron 4 NOS gene polymorphisms in present study show significant deletion frequent in patients than control. This variation caused by deletion sequence in the exon 4 of eNOS gene, A allele show that 27 n were deleted from sequence and B allele show normal sequence, in present study deletion allele was higher in patients than control with significant value figure (1,2) and table (1). Previous studies proposed that two polymorphisms of the eNOS gene, might be associated with the change function of this gene, Such functional DNA variants in the eNOS gene may lead to a change in eNOS expression and enzymatic activity.[9] the population studies show association between cancer risk and eNOS gene polymorphisms, meta-analysis and case – control study suggest that eNOS-786T>C and 894G >T polymorphisms are associated with the risk of breast cancer in Chinese Han population.[35] Jang *et al* suggested that the eNOS -786T>C and 894G>T polymorphisms associated with the development of collector cancer in the Korean population. [36] The present study needs more investigation such as DNA sequencing and epigenetic role in regulation of this gene expression. The present study concluded association of eNOS gene deletion with breast cancer.

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