RESEARCH ARTICLE

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Is Cyclooxygenase 2 Effective in Breast Cancer Diagnosis?

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Abstract

Background: Breast cancer is considered the leading cause of death in women worldwide. When cancer is detected early, the survival rate increases significantly. Molecular identification of genes alterations and expression of specific proteins has become a key indicator of breast cancer diagnosis and treatment. *COX2* is considered as one of biomarkers for different types of cancer. **Objective:** This study aimed to validate the correlation between *COX2* mutation and breast cancer. **Methods:** Using conventional PCR, *COX2* was isolated from 57 patients after lumpectomy and from blood samples. The samples were collected from patients admitted to Al-Basrah Teaching Hospital. Forty-two samples were malignant and fifteen were benign which were used as controls for mutation and for expression. In addition, tissue from inside of tumors and far from tumor were tested to compare *COX2* expression by performing of RT-PCR. **Results:** The results showed that 5 out of 12 (30.3%) samples sent to DNA sequence were mutated, three of them had deletions, while the others had substitution. All mutations were in exon2 and no mutation in exon1. Expression of *COX2* in malignant was detected by RT-PCR and showed very significant difference between malignant and benign in all areas of samples, inside tumor, far from tumor tissue and blood. Significant differences in expression of *COX2* in malignant lesions and blood samples of malignant, and benign tissue can be considered as a good biomarker for detection of breast cancer in suspected patients. Relatively high expression level of *COX2* were recorded in patient with aged 57-66 with 39% inside malignant tumor and 13.53% far from tumor and 23.1% in blood compared with only 7.25%, 8.4% and 5.3% in benign samples in the same age group, respectively. **Conclusion:** These results provide insight into the ability to use *COX2* as a biomarker for suspected breast cancer patients in Iraq.

Keywords: Breast cancer- *COX2*- Diagnosis

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Introduction

Breast cancer is one of the most common cancers in women worldwide. Over 1.5 million women are diagnosed with breast cancer every year throughout the world; it is considered as the first leading cause of cancer deaths among women. Development of breast cancer is a multi-step process involving multiple cell types, and its prevention remains challenging in the world [1, 2].

Early diagnosis of the disease can lead to a good prognosis and a high survival rate. Breast cancers can be classified into biologically and clinically meaningful subgroups according to histological grade and histological type [3]. It seems logical to consider factors known to play an important role in predicting 5-year survival of breast cancer patients and to question their importance in survival 10 years after diagnosis and even longer. These factors may be categorized as patient factors including age, comorbidity, time of diagnosis and race, tumor related factors such as size, histological type, grade, lymph node involvement, vascular invasion, recurrence and metastasis, tumor markers including hormonal receptors,HER-2 expression, gene expression profile , mitotic activity index, and and molecular factors BRCA 1,2 [4]. Apart from the traditional immunomarkers, (*COX2*) is being studied extensively in breast cancer tissue [5].

Cyclooxygenase (COX) group of enzymes are important for the conversion of arachidonic acid to prostaglandins. Cyclooxyrgenase-1 (COX-1) is constitutively expressed at a constant level throughout the cell cycle in most of the tissues. The inducible isoform, Cyclooxyrgenase-2 (*COX-2*), is often over expressed in breast cancer, Various research articles suggest that prostaglandin metabolites may contribute to maintenance of tumor viability, premalignant hyper proliferation, tumor growth, transformation, invasion and metastatic

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spread. *COX-2* is the key involved enzyme, as it triggers prostaglandin synthesis, which has been shown to be over expressed in many human malignant tumors [6].

Studies showed that expressed significant high levels of *COX-2* in malignant breast cell, while benign breast tissue at least 1 cm from a malignant lesion did not express *COX-2*. Elevated *COX-2* expression was associated with a large tumor size, a high histological grade, a negative hormone receptor status, a high proliferation rate along with axillary node metastases. In addition to the well-established pathophysiological role of *COX2* in inflammation, evidence implies that this enzyme may be involved in multiple biologic events throughout the tumorigenic process. *COX2* gene has been characterized as an immediate early gene associated with cellular growth, differentiation and angiogenesis. Emerging data suggest that *COX2* is involved at various steps of the process of malignant transformation and tumor progression [7].

Cyclooxygenase-2 promotes carcinogenesis, raises the recurrence rate of cancer, and reduces survival in cancer patients. It also increases the resistance of malignant cell to radiotherapy and chemotherapy [8].

Over-expression of *COX2* in epithelial carcinomas has been detected in several types of tissue, such as colon, gastric, esophageal, lung, liver, pancreas, prostate, ovary, and breast. Tumor cells with elevated *COX2* levels are highly resistant to apoptosis, have increased proliferation, invasion and migration [9].

In this study we will discuss the effect of *COX2* in the diagnosis and prognosis of breast cancer.

Materials and Methods

This study was conducted in Basrah teaching hospital, from April 2021 to February 2023. Samples of tissue and blood were collected from 57 female patients in surgical ward, aged 17-66 years who were admitted with breast mass, whether it's benign or malignant after informed consent was obtained from them except those who were admitted for mastectomy following excisional biopsy and those who were kept on chemotherapy. In view of the result of Fine needle biopsy (FNA), excisional biopsy or mastectomy was done to the patients with free margin of at least 1 cm from the lesions.

Tissue samples were immersed in formalin solution or normal saline, and blood was collected in EDTA tube, and then transported to the molecular laboratory in AL-Zahraa College of Medicine for extraction of *COX2* for molecular identification.

Tissue extraction

A biopsy of about 1gram of the breast specimen was cut from suspected area of each sample and other samples were taken far from suspected cancer tissue by about 1cm away. The tissues were washed by phosphate buffer saline twice, then cut in small pieces and washed again, tissues were mashed up in sterile mortar with 200 ml Nuclease-Free Water to get homogenized liquid.

We added 0.75 ml of TRIzolTM LS Reagent per 0.25 ml of sample volume then homogenize the sample by pipetting up and down several times to lyse samples.

Isolation of COX2 DNA by PCR

Two referenced forward and reverse primers compatible for *COX2* were performed to detect *COX2* by using conventional polymerase chain reaction (Gradient thermal Cycler, DLAB, USA, TC1000-G). Amplification of *COX2* was done by using Master mix GoTag® (Promega Cat No. # M7122) in final volume 50 µl with 1 µl of extracted tissue as a DNA templet and 0.5 µl from each *COX2* forward and reverse primers. The thermocycler machine was programmed to meet the PCR protocol as follows: Initial denaturation step for 1 minute at 94˚C, then 30 cycle of second denaturation at 94˚C for 30 sec followed by annealing step at 58˚C, according to the advice of primer supplier company (Promega, Korea, Cat No.#162770), then extended step at 72ºC for twenty second followed by further 10min extension time.

Gel electrophoresis

A concentration of 3% gel electrophoresis was performed to separate *COX2* DNA and compare by their base pair size (Table 1). SYPR® Safe (Promega, Korea, Cat. #CS411) was performed to visualize DNA bands under UV trans illuminator cabinet (Wealtec, USA, MD-25/HD-25). Density of gel bands was estimated by using IMAGEJ software which was download from website (https://imagej.nih.gov/ij/download.html) [10].

DNA sequencing

Twelve samples of PCR products, visualized in right size in gel electrophoresis, were sent to DNA sequencer company (Base-Gene company, Netherland). Sequence analysis information was performed by the company through their specific website and confirmed by using online tool(https://www.baseclear.com/genomics/fragment-analysis/).

Detection of COX2 expression by RT-PCR

Relative quantification RT-PCR was used to measure gene expression of *COX2*.

Blood extraction

1 ml of each blood sample was performed to lysis cells and extract RNA according to (Dongsheng iotech, N1121) RNA blood mini kit protocol. Concentration and purification of RNA was confirmed by nanodrop spectrophotometer (Avans, NAS_99) under absorbance at 260 nm and 280 nm. A ratio of ~2 was used and considered RNA pure sample.

cDNA synthesis

1 µg of the extraction cellular RNA was performed to synthesis cDNA by Oligonucleotide primers that were performed to detect *COX2* by RT-PCR product 232 bp length were shown in Table 1.

The procedure of RT-PCR was adopted by 1X SYBR Green PCR Master Mix, and started by step one in 60 min of reverse transcription at 55 °C then inactivate of the transcriptase at 95°C for 8 min, followed by amplification for 40 cycles of denaturation at 95 °C for 20 sec, then annealing step at 55 °C for 20 sec, and extension step at 72 °C for 50 sec.

Table 1. Oligonucleotide Primers with Their Site of Amplicons and Their Sizes by Base Pairs with Their References

Amplicon	Purpose	Primer sequence (5'-3')	ize bp	Reference
COX2 EXON1	PCR	F-TAAGGGGAGAGGAGGGAAAA	372	[11]
		R-AGGAGGTCAGAGCGGAAACT		
COX2 EXON2	PCR	F- AAAAATTGTATTTCCATGACTACCTAT	855	[11]
		R- TCTTGCTGATCCAAATCCAA		
COX2 conserved regions	PCR	F-GATCACTTCAAAATGAATTCAGGAT	135	[12]
		R-GCTACGAAGATAGATTACAGTTATG		
COX2	RT-PCR	sense- TGA AAC CCA CTC CAA ACA CAG	232	[13]
		Antisense-TCA TCA GGC ACA GGA GGA AG		
β-actin	RT-PCR	sense-GTT TGA GAC CTT CAA CAC CCC	320	[13]
		Antisense- GTG GCC ATC TCT CTT GCT CGA AGT C		

Table 2. Type of Samples According to Age group

Age group (Yrs)	Type				Total	
	M	%	B	%	No.	%
17-26	4	7.01	1	1.75	5	8.76
27-36	6	10.52	2	3.51	8	14
37-46	10	17.54	7	12.3	17	29.84
47-56	11	19.3	2	3.51	13	22.8
57-66	11	19.3	3	5.26	14	24.6
Total	42	73.67%	15	26.33%	100	

M, malignant; B, benign

The total RNA solution was subjected to RT-PCR analysis using the Qiagen one-step RT-PCR kit (#Cat. 210210). The total RNA specimens 1μg were performed for reverse transcription and amplified in 25 μl of reaction mixture, and the reaction conditions for *COX2* and human gene and protein abbreviation ACTB (β-actin).

Statistics

The data were analyzed by SPSS version 26, the data were presented as frequencies and percentage, and the association was measured by Chi square and Fisher's exact test (when the expectation frequencies less than 5). The data value less than 0.05 was considered as significant.

Results

This study covered 57 patients diagnosed with breast mass sent for fine needle aspiration (FNA). Patients age ranged from 17 to 66 years old (mean 48 years old). The patients were grouped into five groups according to the age. Samples were classified as 42 (73.6%) malignant and 15 (26.3%) benign tumors as shown in Table 2. The biopsies of all patients were taken from the suspected tumor area as a cancer tissue and far from that, from tissue looking normal, as a control tissue. 57 samples of tumor and 57 as control samples were used.

Detection of COX2 DNA.

PCR products were subjected to gel electrophoresis and showed bands for *COX2*-exon1; *COX2*-Exon 2 and *COX2* conserved region in their related base pair size as shown in Figure 1. Samples were compared with 1000 bp DNA ladder.

DNA sequences

Twelve PCR products, 5 of each *COX2* of EXON1, *COX2* EXON2 and 2 from *COX2* conserved regions were cut from gel and purified by Qiaquick®-gel-extraction-kit and sent to DNA sequencing company. The results showed that *COX2*- EXON1 was compatible with *COX2* sequence from Homo sapiens (GenBank access code: NG_028206) (GenBank access code: AGCE_01110177) and showed no mutation as well as bands that got from conserved region showed compatible to *COX2* reference (Table 3). On the contrary, 5 samples out of 5 (100%) of *COX2* in Exon2 showed mutation, 3 of them were malignant and showed deletion mutation in 9 bp (316-324). This mutation caused removal of three amino acid Leu-Lyc-Leu in area 106, 107 and 108; segment sequence was (5'CTG AAG CTA3'). Another 2 mutations showed one substitution in benign sample in base pair number 217 (CCT>CTT) and this leads to change the amino acid proline to Leucin (ΔP72L), and the other substitution was in malignant sample and mutated a base pair number 153 (CAG>CTG) which change amnio acid Glutamine to Leucin (ΔG51L), (Figure 2). All these mutations were

Table 3. Number of Samples that Showed Deletion, Substitution and Non-Mutated.

Number	type of mutation
3	Deletion
2	Substitution
7	None

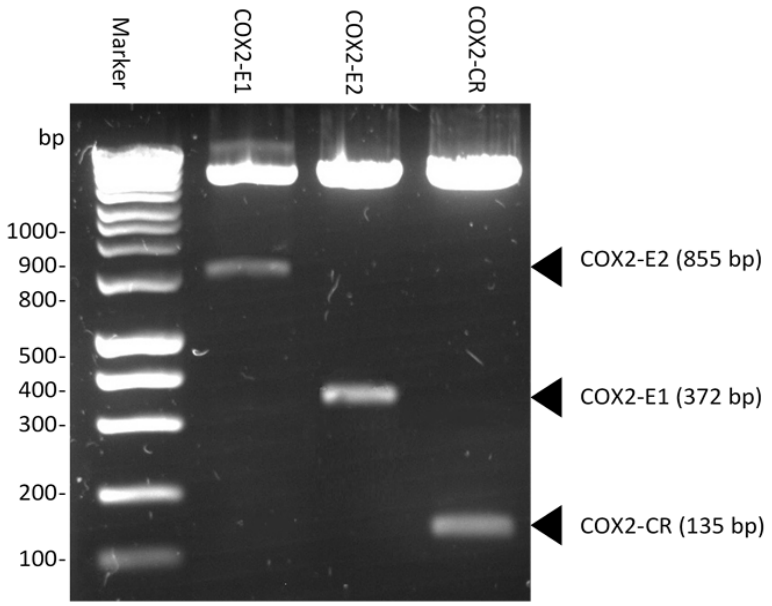

Figure 1. Gel Electrophoresis 3% Visualized PCR Products, *COX2* of EXON2 (855bp), *COX2* EXON1 (372bp) and *COX2* Conserved Regions (CR) 135bp.

Table 4. DNA Sequence Results Showed Mutation and Non-Mutation Samples with Their Age

Year	Type	Results	Site	Mutation
17	M	Deletion	Exon 2	Δ316-324
26	M	None	Exon 1	--
26	B	None	CR	--
34	M	None	CR	--
35	B	None	Exon 1	--
40	B	Substitution	Exon 2	ΔP72 L
42	B	None	Exon 1	--
43	M	Deletion	Exon 2	Δ316-324
52	B	None	Exon 1	--
57	M	Substitution	Exon 2	ΔG51L
59	B	None	Exon 1	--
60	M	Deletion	Exon 2	Δ316-324

shown in the C-terminal of *COX2*. Sequence results also showed no mutation in conserved region of *COX2* in all the 2 samples that were sent. Benign samples showed non-mutated in 3 out of 4 (75%) and just one of them was mutated, while 4 malignant out of 6 (66.6%) carried mutation either deletion or substitution (Table 4).

Estimating expression of COX2

Expression of *COX2* in samples were identified by performing of RT-PCR according to [14] in order to detect mRNA of samples and recorded signal of Cycle Threshold (CT) value of samples and then converted to relative expression by the equation below. The average CT value of the endogenous protein β-actin was 32, which was used as a control to compare its expression with *COX2* in each sample.

$$\text{Relative expression} = 2^{(\text{CT } \beta\text{-actin}) - (\text{CT COX2})}$$

Three samples group were used (Tumor site; far from tumor and blood) from each malignant and benign sample. The results explained that the expression of *COX2* was increased as shown in Table 5) and Figure 3.

Expression of *COX2* in tumor site of malignant tissue increased in an average of 3.25 times from that fare from the tumor site. Also, it increased in an average of 0.6 times from expression in blood of patient. In addition, *COX2* was expressed 1.58 more times in blood compared with tissue far from cancer site. The statistical analysis showed highly significant differences of the expression of *COX2* between cancer tissue and far from tissue and from cancer tissue and blood (p-value <0.05) (Table 5). On the other hand, expression of *COX2* in benign showed no significant differences between site of tumor, far from tumor as well as blood samples (Table 5).

Expression of cDNA of COX2

Expression of *COX2* confirmed by loading of same amount of cDNA samples in gel electrophoresis (Figure 4A). The expression was found to be increased significantly in tumor of malignant tissue compared with its expression in tumor of benign tissue (p-value<0.05). Whereas the expression of *COX2* in blood was higher in malignant patients compared with blood taken from patients with benign tumor. The expression in blood showed significantly different (p-value<0.05) (Figure 4B). Bands of *COX2* were shown in their estimated size (372bp) compared with marker 1000bp. Endogenous β-actin cDNA were used to estimate equality of PCR product and their bands were showed in about 323bp.

Discussion

Breast cancer is considered as one of the common

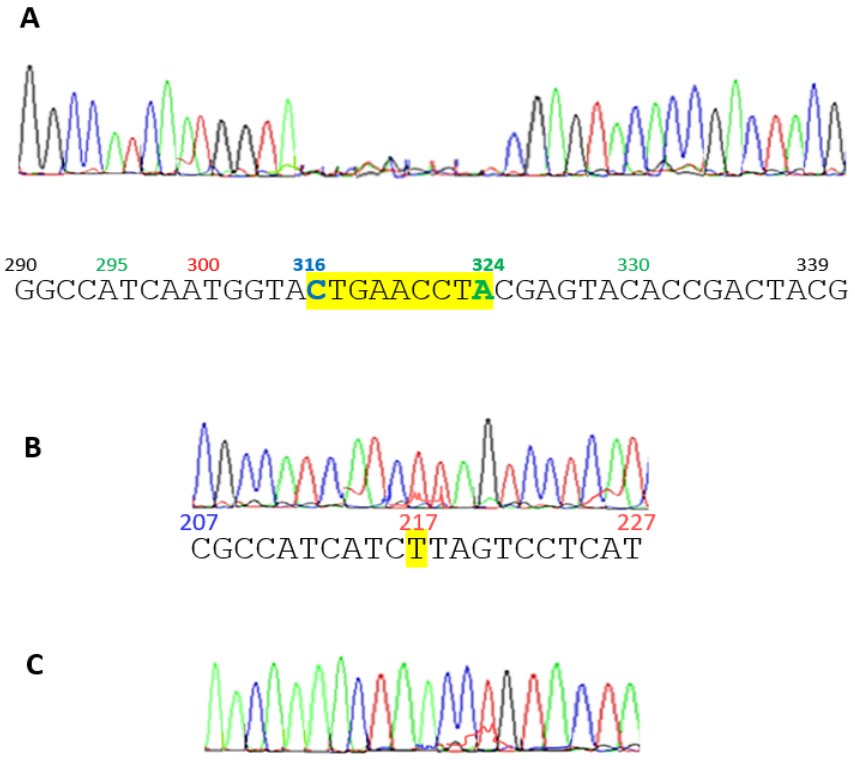

Figure 2. DNA Sequence Curve Showed Mutation of *COX2*, (A) mutation deletion of 9 bp (316-324), (B) Mutation of substitution in bp217(CCT>CTT), (C) mutation of substitution in bp153 (CAG>CTG). Black = Guanine; Blue = Cytosine; Green = Adenine and Red is Thymine.

cancers in women worldwide. It is leading incident cancer and cause of death among Iraqi women [14, 15].

In a study revealed a link between breast cancer and the cellular protein *COX2* which plays a role in various physiological processes, including inflammation, development, and progression of cancer, including breast cancer [16].

This study adopted 57 breast cancer biopsies which were collected after lumpectomy. Samples showed 42 malignant and 15 benign in percentage of 73.6% and 26.4% respectively. The study showed that the highest malignant patients were 19.3% in two age groups (47-56 and 57-66) years. A new study conducted in India was recorded that malignant concentrated in age 51-61 years in a percentage about 50% [17].

Conventional PCR was used to detect *COX2*, and the result identified bands of *COX2* which isolated in size 855bp which lies in Exon2 and 372 in Exon 1 and 135bp in conserved region. The study conducted that genes in exon1 were conserved, and no mutation has been shown in this area. In contrast, Exon2 appeared carry two types of mutations; deletion of the 9-base pair from 315 to 323 which leads to remove amino acid sequence Leu-lyc-Leu. Correlation of *COX2* mutation in different types of cancer was confirmed, four amino acid deletions in EXON 6 and 7 were isolated from Iranian patient with pancreatic cancer in about 21.9% of all cases under study [5]. Point mutation in Thymine number 8473 to Cytosine in sequencing of *COX2* was identified in nasopharyngeal, bladder, esophageal and skin cancers [18], The same research group found several mutations in *COX2* related to malignant breast cancer; G765C, G1195A and T8473C were detected in breast cancer [19]. A novel mutation was detected in breast cancer and linked with cancer progression was in exon 2 when single mutation occurred (C169G) and repeated in about 15% of samples in study conducted Fawzy MS et al. [20].. These results confirmed that *COX2* mutation associated with breast cancer and exon2 is one of

Table 5. Relative Expression of *COX2* in Malignant and Benign from Tumor Site, Far from Tumor and Blood of Different Age of Patients

Age group	Expression of COX2 Malignant				Expression of *COX2* Benign			
	COX2 in Tumor	*COX2* Far site	*COX2* in blood	p-value	*COX2* in Tumor	*COX2* Far site	*COX2* in blood	p-value
17-26	33.5	6.6	19.8		9.7	7.2	5.2	
27-36	32.9	6.5	21.7	<0.05	7.3	7.2	5.6	
37-46	34.2	6.2	22.6		10.5	9	5.21	<0.05
47-56	38.9	13.56	21.6		6.4	6.8	5.4	
57-66	39	13.55	23.1		7.25	8.4	5.3	

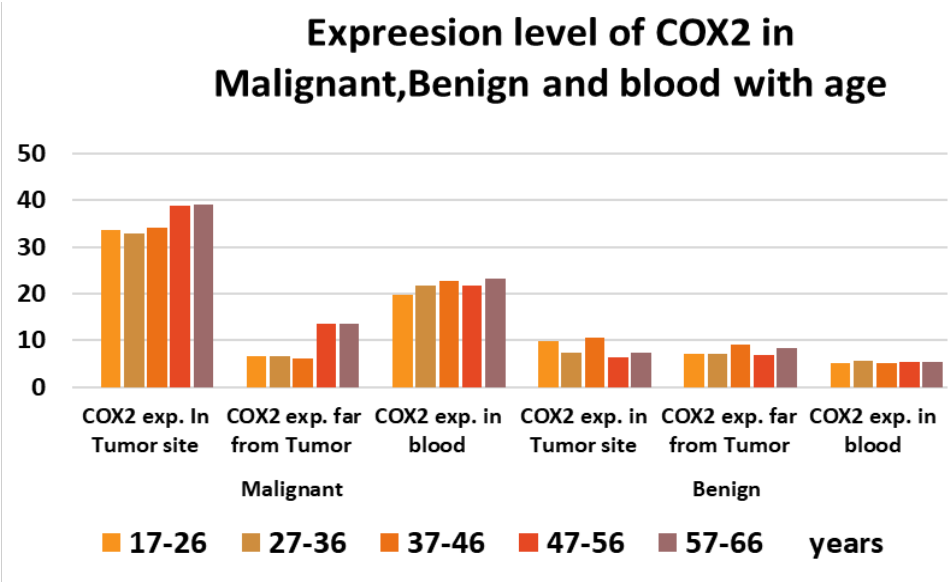

Figure 3. Bar Chat Elucidates the Relative Expression of *COX2* in Malignant and Benign Samples According to Age Groups

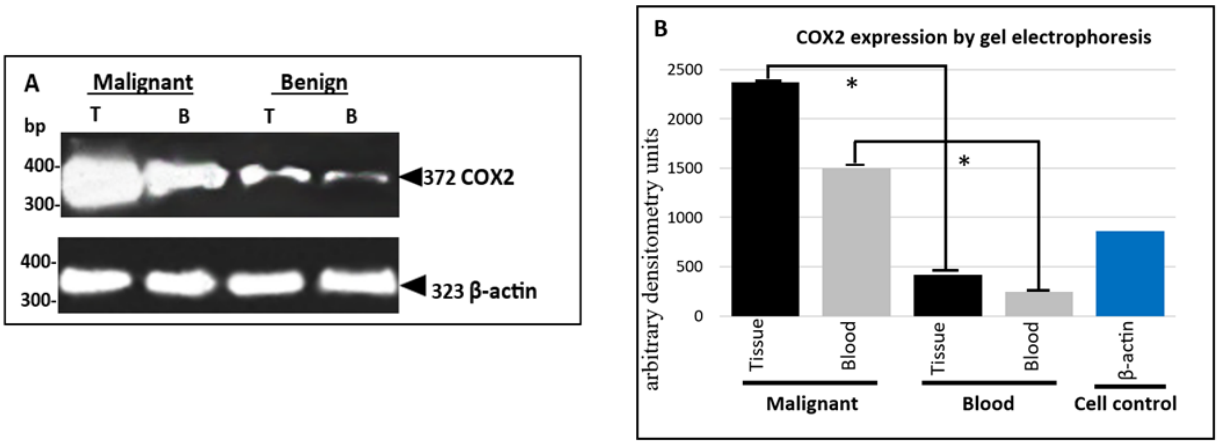

Figure 4. Expression of *COX2* in Gel Electrophoresis in Tumor and Blood in Malignant and Benign Samples. (A) Gel electrophoresis showed bands of *COX2* and β-actin. (B) Bar chart explain the arbitrary densitometer of gel electrophoresis bands by using IMAGJ software. * Significant differences p-value <0.05.

the areas affected by mutation and related to progression. These results compatible with what this research found, the exon 2 carry point mutations in two areas G51L and P72L, and deletion of 9 bp (314-324) which contradicts Leu-Lyc-Leu deletion as shown in table [4].

The mutation P27L was detected in benign samples and this result is subject to many speculations, it may be because of false negative fine aspiration cytology (FNA) diagnosis, which its sensitivity around between 86% to 93% [21] in addition to the false negative was found in several mammography results. Another expectation of this mutation (P72L) probably not be associated with any pathological changes, especially since it was not mentioned in literature before.

Expression of *COX2* was one of the aims of this study and the results showed that the expression evaluated in malignant compare with benign related to site of tumor, far from tumor tissue and blood as shown in table [5] This result was confirmed by RT-PCR and gel electrophoresis as mentioned in Figures 3, 4. Breast cancer stage, progression and metastasis may be classified according to *COX2* expression, [22] used RT-PCR to record expression of *COX2* in breast cancer and conclude that high expression level was detected remarkably in metastatic and invasive breast carcinoma. This result was supported by several studies by using different technique, by immunohistochemistry [23]. found 36% of breast cancer samples were recorded as a high expression level of *COX2*. Similarly another study detected 25% of breast cancer samples were associated with high *COX2* expression in immunohistochemistry [24, 25]. A study conducted by RT-PCR confirmed that 84.6% of malignant carcinoma was shown increased in *COX2* gene expression [24]. Related to the age, overexpression of *COX2* was clearly identified in female over 50 years by 91.2% of breast cancer [9].

Detection of *COX2* expression in blood was also aimed in this study in order to study the ability to use it

as a biomarker. The result showed significant differences between expression of *COX2* in blood of malignant and benign sample in all age groups as explained in Table 5 and Figure 3. The *COX2* expression in blood was used and similar results were conducted by several studies> [26-28].

Detection of *COX2* expression in blood samples of patient with breast cancer was confirmed and found to vary depending on age, stage and progression [29, 30] and these results compatible with the results of this study (Table 5).

In conclusion, the study identified that expression of *COX2* was vary inside breast cancer tissue and far from the area of cancer as well as in blood in each benign and malignant tissues. The results are promising to use expression of *COX2* by RT-PCR as a specific biomarker for cancer and for grade of cancer or progression. *COX2* expression in blood of malignant samples also showed significant differences with benign and can be used as a primary marker.

RecommendationThese results need to be confirmed by increasing the number of the samples and classified samples with their criteria like; age of patients and stages of cancer.

Author Contribution Statement

All authors contributed equally in this study.

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None.

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