Detection of COX-2 in liquid biopsy in patients with breast cancer

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ABSTRACT

Aims To determine the expression of the cyclooxygenase-2 (*COX-2*) gene in patients with breast cancer attended at the Centro Universitário Saúde ABC/Faculdade de Medicina do ABC (CUS-ABC/FMABC) outpatient clinic. Breast cancer is the most common cancer in women worldwide. More than two million new cases are reported annually. An overexpression of *COX-2* has been observed in many cancers. *COX-2* is related to parameters of cancer aggressiveness, including tumour size, positive nodal state and lower survival, and to angiogenesis and resistance to apoptosis.

Methods 15 mL of peripheral blood was obtained from 34 patients and 21 healthy women. The extracellular RNA of QIAamp RNA was submitted to an RNA sequestration kit for RNA reverse transcriptase. Quantitative real-time PCR was performed using *COX-2*-specific oligonucleotides and the endogenous *Glyceraldehyde-3-Phosphate Dehydrogenase* gene.

Results The mean remission time was 53 years. The mean progression time was 33 months. The difference observed between the patient and control groups in median *COX-2* expression ($p<0.001$) was significant.

Conclusions Patients with breast cancer showed a higher mean *COX-2* expression in peripheral blood samples at diagnosis than the control group. Since this information could prove important in the diagnosis and prognosis of breast cancer, further research is required on larger patient samples.

INTRODUCTION

Breast cancer is the second most common cancer among women, second only to non-melanoma skin cancer in both high-income and low-income countries.[1 2] In 2019, US estimates show the emergence of 271 270 new cases of breast cancer.[3] For the 2018–2019 biennium, Brazil estimates 59 700 new cases per year.[2 4] The majority of cases are related to environmental and lifestyle factors, while about 10% are correlated with hereditary factors.[5] Early diagnosis of this neoplasm has a major impact on patient longevity and recovery.[6] Women with invasive cancer have a 5-year survival rate of 90%.[3]

Diagnosing breast cancer in palpable lesions is achieved by mammography and ultrasound, while non-palpable lesions require mammotomy and biopsy.[7–9]

An inflammatory process occurs during the development of cancer, and cyclooxygenase (COX) is among the proteins involved.[10–12] COX is responsible for converting arachidonic acid (AA) into prostaglandin.[13 14] Phospholipids that are present in the membrane release AA through the action of the phospholipase enzyme, which is activated through chemical, traumatic and mitogenic stimuli.[13 14] There are two known isoforms of the enzyme cyclooxygenase, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), which differ in their expression and role in tissue regulation.[10–12] COX-1 is important in several physiological processes, including the regulation of renal homeostasis, gastric mucosal protection and platelet aggregation.[10 12] In contrast, the *COX-2* gene is not transcribed; it is induced; that is, it is silent and is activated in response to inflammatory processes resulting from stimuli, including bacteria, viruses, alcohol, trauma, lipopolysaccharides and tobacco.[15 16]

COX-2 overexpression is observed in numerous types of cancer. It is related to tumour aggressiveness parameters like size, positive nodal status and a shorter survival time.[10 17] The imbalance caused by COX-2 decreases the balance between cell proliferation and apoptosis.[10 13 16] The action of COX-2 in tumourigenesis favours resistance to apoptosis. Studies show that cells can survive in unfavourable conditions.[10 12] Another link between COX-2 and tumourigenesis is angiogenesis, since this is essential to tumour growth.[18 19] Analysis of the literature indicates a scarcity of studies that contribute to achieving early diagnosis and a definitive prognosis for this cancer, together with rising concern regarding the increasing number of deaths. Thus, the purpose of the study was to determine potential correlations between *COX-2* expression and breast cancer.

MATERIALS AND METHODS

Patients

The study included 34 patient samples and 21 control group samples from healthy women. The samples were analysed by the Clinical Analysis and Molecular Biology Laboratories of the Centro Universitário Saúde ABC, Sao Paulo, Brazil.

Patient blood samples were collected on two different occasions, initially for diagnosis and again following the first chemotherapy treatment. A second collection was performed to determine whether important differences in *COX-2* expression continued during the treatment of breast cancer and in relation to healthy women.

RNA extraction

Samples of 20 mL of EDTA blood were collected from each patient and control and were processed

using TRIzol reagent according to the manufacturer's recommendations. Total RNA concentration was estimated by spectrophotometry using GeneQuant DNA/RNA Calculator equipment (Pharmacia, LKB Biotechnology, Sweden).

Complementary DNA (cDNA) synthesis

One microgram of Messenger RNA (mRNA) was used for cDNA synthesis using the enzyme Superscript II RNAse reverse transcriptase (Invitrogen, Life Technologies-Thermo Scientific Researcher, USA) according to the manufacturer's recommendations.

Quantitative rquantitative Reverse Transcriptase PCR (qRT-PCR) was performed using the cDNA obtained, previously diluted at 1:10. The reaction conditions were 1× SYBR Green PCR Master Mix, 0.4 µM of each *COX-2* primer (forward, 5′-CCACCCGCAGTACAGAAAT-3′; reverse, 5′-AAGGAGAATGGTGCTCCAC-3′), 2 µL cDNA, qsp μL diethyl pyrocarbonate (DEPC) water, for a final volume of 15 µL. The Applied Biosystems 7500 Fast Real Time PCR System was used, with the following programme: denaturation, 95°C for 10 min; 45 cycles of 95°C for 15 s; 60°C for 60 s; and melt curve, 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, 60°C for 15 s. To verify *GAPDH* expression, the protocol adopted was 1× SYBR Green PCR Master Mix, 0.30 µM *GAPDH*, 2 µL cDNA, qsp μL DEPC water, for final volume of 15 µL.

Statistical analysis

Absolute and relative frequencies were used to describe the qualitative variables. Quantitative variables (Shapiro-Wilk >0.05) were described by the mean, SD, minimum and maximum values, while for data with non-normal distribution (Shapiro-Wilk <0.05), the median and 95% CI were used. The Mann-Whitney test was used to analyse differences between the COX-2 expressions in the first sample collection from both groups. The Kruskal-Wallis and Mann-Whitney tests were used to determine associations between the variables and COX-2 expression in the second sample collection from both groups. Confidence levels of 95% and 85% were adopted in these analyses. The programme used was STATA V.11.0.

RESULTS

The patient group consisted of 34 (61.8%) samples, while the control group consisted of 21 (38.2%) samples from healthy women. Sample analysis showed that 17 (50%) patients had stage III cancer; 14 (41.2%) had stage II; and 3 (8.8%) had stage I. Analysis of survival showed 21 (61.8%) patients were alive; 5 (14.7%) had died; and 8 were lost due to follow-up (23.5%) cases. Regarding disease progression, 13 (38.2%) of the patients presented tumour enlargement and metastasis, while the remainder (n=21, 61.8%) did not. Hormone receptor expression was observed in 23 (67.6%) patients. The mean patient age was 53.5 years old (±11.8 SD). The mean period for progression was 33 months (±17.1 SD), and 13 (38.2%) patients showed progression (table 1).

Following quantification, mRNA samples from the patient and healthy control groups were converted to cDNA and were used to determine *COX-2* and *GAPDH* expression. *COX-2* expression in the first sample collection was verified and a median value of 2.44 was obtained, while in the second sample collection, a median of 0.274 was obtained (table 2).

The differences observed between the patient and control groups in median *COX-2* expression (p<0.001) and progression (p=0.043) were significant. However, associations between *COX-2* and the remaining variables, stage (p=0.221) and hormone receptor expression (p=0.839), were not significant (table 3).

The differences observed between the patient and control groups in median *COX-2* expression (p=0.008) of the second sample collection were also significant. For the remaining variables, stage (p=0.754), progression (p=0.736) and hormone receptor expression (p=0.544) (table 4), no significance was observed.

Analysis of the first and second *COX-2* expressions in samples from the patient group was performed, and the value obtained was not significant (p=0.174). However, the median *COX-2* expression from the first sample collection was 2.44, while the median from the second was 0.274 (table 5).

Table 1 Sample features

Variables	n	%
Group		
1	34	61.8
2	21	38.2
Stage		
Stage I	3	8.8
Stage II	14	41.2
Stage III	17	50.0
Event		
Alive	21	63.6
Death	5	12.1
Loss follow-up	8	24.2
Progression		
No	21	61.8
Yes	13	38.2
Hormonal receptors		
No	11	32.3
Yes	23	67.6
	Average (SD)	**Minimum–maximum**
Age (years)	53.5 (11.8)	29.0–85.0
Time (progression)	33.0 (17.1)	2.0–50.0

SD, standard deviation.

DISCUSSION

COX-2 is present in all tumour samples, and this expression is higher compared with normal tissue.[11] Regarding *COX-2* expression in the first sample collection, the patients presented a median of 2.44, while the control group presented a median of 0.274. Overexpression was previously detected by immunohistochemistry in a study of 64 patients.[20] In the 71.8% (46) of patients who presented with overexpression, it was related to tumour size, lymph node metastasis, aggressiveness parameter and advanced clinical staging. In the study using mRNA from 30 samples of normal breast and breast cancer tissues was submitted to quantitative reverse transcriptase PCR (qRT-PCR) analysis for *COX-2* detection. *COX-2* expression in normal tissues was rare

Table 2 *COX-2* expression in the first and second collections

COX-2 expression	Median	95% CI
COX-2 (1°)	0.248	0,021 to 1139
COX-2 (2°)	0.274	0,017 to 1204

1° indicates first collection; 2° indicates second collection.
COX-2, cyclooxygenase-2.

Table 3 Association of *COX-2* of the first collection between groups and with variables between patients

Variables	*COX-2* Median	95% CI	P value*
Group			
Patient	2.44	1.05 to 7.36	<0001
Control group (healthy women)	0.01	0.01 to 0.02	
Stage			P**
Stage I	2.01	0.99 to 56.41	0.221
Stage II	6.78	0.94 to 497.81	
Stage III	1.07	0.08 to 6.17	
Progression			P*
No	6.17	1.22 to 38.96	0.043
Yes	1.07	0.01 to 4.86	
Hormonal receptors			
No	2.02	0.02 to 94.99	0.839
Yes	3.25	0.83 to 8.88	

*Mann-Whitney.
†Kruskal-Wallis.
COX-2, cyclooxygenase-2.

(median=0.0), while the median in tissues with breast cancer was 0.56, and overexpression was associated with lymph node metastasis.[21] Another study of 64 breast cancer tissue samples and corresponding normal tissues showed that *COX-2* was overexpressed in 47 (73%) breast cancer samples, and this was related to staging and hormone receptor expression.[22]

COX-2 expression in breast cancer biopsies and its correlation with age, menopausal status, tumour size, lymph node status and other variables was reported in 123 patients, in comparison with a control group of 76 women.[23] This study used biopsies of patients and healthy women, analysed by RT-PCR and immunohistochemistry, to determine that *COX-2* was overexpressed in patients over 50 years old who were postmenopausal, with large metastatic tumours in the lymph nodes.[23] This further corroborates associations between *COX-2* and neoplastic aggressiveness.[23] Inflammatory breast cancer presents a distinctive, aggressive and locally advanced form, with unique characteristics.[24] High levels of *COX-2* mRNA (median 3.68) were detected in biopsies analysed by RT-PCR and immunohistochemistry, coinciding with its protein expression.[24]

Table 4 Association of *COX-2* of the second collection between groups and with variables between patients

Variables	*COX-2* Median	95% CI	P*
Group			
Patient	0,27	(0.1 to 1.20)	0.008
Control group (healthy women)	0.01	(0.01 to 0.02)	
Stage			
Stage I	6,17	0.001 to 99.60	0.754
Stage II	0.08	0.01 to 292.01	
Stage III	0.44	0.01 to 1.94	
Progression			P**
No	0.13	0.01 to 4.88	0.736
Yes	0.44	0.02 to 1.61	
Hormonal receptors			
No	0.01	0.01 to 3.19	0.544
Yes	0.42	0.02 to 1.86	

*Kruskal-Wallis.
†Mann-Whitney.
COX-2, cyclooxygenase-2.

Table 5 Association of *COX-2* moments in patients

Variables	*COX-2* (1°)	*COX-2* (2°)	
Group	Median (95% CI)		P value*
Patient	2.44 (1.05 to 7.36)	0,27 (0.01 to 1.20)	0.174

1° indicates first collection; 2° indicates second collection.
*Wilcoxon.

COX-2 expression has been verified in many biopsy samples from patients with breast cancer. The level of expression presented is always higher than that detected in women free of disease. COX-2 expression is significantly associated with tumour size, metastasis and aggressiveness.[20 21 23] These findings are in agreement with the results obtained by Auwera *et al*,[24] and McCarthy *et al*,[25] both regarding the high expression in patients and in the control group results.

In our study, a decrease in *COX-2* expression was observed between the first and the second sample collections. This decrease is likely associated with the fact that the patient's initial blood sample was collected at diagnosis and in the second was collected after the first cycle of chemotherapy. There are no reports in the literature of this type of analysis of serial collections.

To our knowledge, this work is the first to use peripheral blood samples for *COX-2* detection in patients with breast cancer. This technique allowed us to measure *COX-2* expression at two timepoints, and the ease of obtaining the samples and analysing should be highlighted, given how it contrasts with studies using biopsy tissues, which require greater intervention and preparation time for processing.

CONCLUSION

In this study, higher *COX-2* expression was verified in patients with breast cancer, in two different sample collections, confirming the inflammatory character of cancer and the use of this tool for diagnosis. A decrease in *COX-2* expression was observed between the first and second sample collections from patients, which seems to indicate treatment efficacy and the potential benefits of measuring *COX-2* expression to assess prognosis. *COX-2* detection was correlated with the variable progression, corroborating the importance of this tool in patient prognosis. This is the first time that *COX-2* has been detected in blood samples from patients with breast cancer, and the results highlight how the process makes an important contribution to patient well-being, since collecting this liquid biopsy is far less invasive, less time-consuming and much simpler. These findings show *COX-2* detection could play an important role in the diagnosis and prognosis of patients with breast cancer. Further research is required to confirm these findings and clinical reliability on larger patient samples.

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Contributors CPdS and FSG conceived the study and performed RT-PCR analysis and data analysis, and wrote the first draft. BA, JW and AdOC provided support for statistical analysis. FF and FG contributed to the interpretation of the data. All authors critically reviewed and approved the final version.

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Competing interests None declared.

Patient consent for publication Not required.

Key messages

- Breast cancer is the second most common cancer among women, second only to non-melanoma skin cancer in both high-income and low-income countries.
- Cyclooxygenase is a protein that is involved in inflammatory processes associated with cancer.
- *Cyclooxygenase-2* expression was verified in a sample of patients with breast cancer.

Ethics approval The study was approved by the Research Ethics Committee, under protocol number 1.103.818.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data sharing not applicable as no datasets generated and/or analysed for this study. Deidentified participant data.

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