Diagnostic potential of hypoxia-induced genes in liquid biopsies of breast cancer patients

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In tumor cells, higher expression of glucose transporter proteins (GLUT) and carbonic anhydrases (CAIX) genes is influenced by hypoxia-induced factors (HIF). Thus, we aimed to study the expression profile of these markers in sequential peripheral blood collections performed in breast cancer patients in order to verify their predictive potential in liquid biopsies. Gene expressions were analyzed by qPCR in tumor and blood samples from 125 patients and 25 healthy women. Differential expression was determined by the 2(−ΔCq) method. Expression of HIF-1α and GLUT1 in the blood of breast cancer patients is significantly higher (90–91 and 160–161 fold increased expression, respectively; p < 0.0001) than that found in healthy women. Their diagnostic power was confirmed by ROC curve. CAIX is also more expressed in breast cancer women blood, but its expression was detected only in a few samples. But none of these genes could be considered predictive markers. Therefore, evaluation of the expression of HIF-1α and GLUT1 in blood may be a useful laboratory tool to complement the diagnosis of breast cancer, in addition to being useful for follow-up of patients and of women with a family history of breast cancer.

Breast neoplasms, the leading cause of cancer mortality among women, is a clinically heterogeneous disease and 10–15% of patients present an aggressive disease with distant metastasis in the first 3 years after detection of the primary tumor. Tumor progression is characterized by rapid cell growth accompanied by alterations in the tumor cell microenvironment that requires high bioenergetic expenditure. The most efficient cellular energy generation process is glucose metabolism. Therefore, adequate supply of oxygen and glucose is essential for cellular functioning. The main regulator of glucose transport in tumor cells is GLUT. This family is composed of 14 members, which exhibit tissue specificity to different hexoses, and at least one GLUT protein is expressed in each of the different human cell types. GLUT1 is ubiquitously expressed and is responsible for the basic cellular supply of glucose.

Notwithstanding the adequate uptake of glucose, not all cells have the adequate supply of oxygen at their disposal in order to function. Hypoxia is the term used to describe oxygen deficiency in some tissues. To survive hypoxia, cells either develop mechanisms to restore adequate tissue oxygenation, or adapt to new physiological conditions by switching to anaerobic metabolism. The Warburg Effect is known as aerobic glycolysis, in which even in the presence of oxygen, glucose is converted to lactic acid. Thus, while some tumor cells exhibit oxidative phosphorylation, most of the glucose uptake by these cells (approximately 66%) is metabolized by fermentation, a process ten times faster than the complete oxidation of this molecule. Therefore, hypoxia-enhanced glycolysis is seen as an essential component of tumor malignancy and is considered a hallmark of invasive tumors, in addition to protecting the tumor against conventional treatment methods, indicating a worse prognosis.

Hypoxia-Inducible Factors (HIF) are a family of hypoxia-activated transcription factors that regulate the effects of cellular oxygen sensors and a series of genes encoding glycolytic pathways. The change in the expression of glycolytic proteins mediated by HIF causes the metabolic reprogramming of the tumor cell allowing its survival under hypoxic conditions and resistance to chemo and radiotherapeutic treatments. Clinically, hypoxia and hypoxia-induced factors (HIF) are associated with increased distant metastases and worse prognosis in several tumors.
responsible for the reversible conversion of CO2 into HCO3 in the presence of water19,20. So far, 15 CAs isoforms are expressed in normal tissues21. CAIX is essential for the elimination of acidic products of glycolysis, which promote acidification of hypoxic or perinecrotic regions, suggests a role in the process of adaptation of the tumor to low O2 tensions, and is associated with worse prognosis and with chemoresistance22.

In breast cancer patients, detection of tumor cells spread from the primary tumor (circulating tumor cells; CTC) in peripheral blood has been associated with reduced general and progression-free survival. CTC detection and characterization techniques have the potential to play the role of “liquid biopsy” that will allow clinicians to track tumor development/progression and determine the best treatment for that patient23. Furthermore, it has previously been shown that circulating tumor cells have a tumor-independent response to hypoxic conditions24.

On account of the above, the aim of this study was to evaluate the expression of HIF-1α, GLUT1 and CAIX in peripheral blood samples taken from breast cancer patients as well as in samples of tumor tissue and peripheral blood from healthy donors.

**Material and methods**

**Subjects.** To evaluate the expression of HIF-1α, GLUT1 and CAIX and their association with pathological variables such as clinical stage, hormone receptors, HER2 and recurrence, breast cancer patients from the oncology department of FMABC were included in this study. Women older than 18 years with breast cancer confirmed by pathological examination, without previous chemotherapy or radiotherapy treatment and without a history of diabetes and renal and/or cardiovascular diseases were included. Patients who did not meet the criteria for inclusion or who chose not to participate in the study were excluded. From each patient, peripheral blood samples were analyzed at diagnosis and at 3 and 6 months after initiating the chemotherapy treatment, as well as in samples of tumor tissue and peripheral blood from healthy donors.

**Total RNA isolation.** From tumor samples, two 10 µm sections per block were used to obtain RNA, using RNeasy FFPE kit (Qiagen, DE) according to manufacturer’s directions. From peripheral blood, 5 mL were collected by venipuncture in an EDTA tube and RNA was obtained using TRIzol reagent (TRIzol LS Reagent, Thermo Fisher, USA) according to manufacturer’s directions. The extracted material was quantified by a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific Inc.).

**cDNA synthesis.** RNA samples (initial 1 μg) were converted into cDNA using the Quantitect Reverse Transcription kit (Qiagen, DE), according to the manufacturer’s protocol.

**qPCR.** HIF-1α, GLUT1 and CAIX gene expressions were evaluated by real-time PCR (qPCR). Sequences of the designed primers and their amplicon characteristics are described in Table 1. To normalize the relative expression of the target genes, mean values of RPL13a (a ribosomal protein) gene expression were used.

The initial standardization of real-time PCR amplifications was performed in an Applied Biosystems 7500 Real Time PCR Systems thermocycler (Applied Biosystems, USA) in a final volume of 15 μL containing: 1 ×

### Table 1. Characteristics of specific primers.

| Gene | Nucleotide sequence (5'–3') | Amplicon (bp) |
|------|-----------------------------|--------------|
| HIF-1α | F- tgtacccaatagccgagg<br>R- gctagcgcagggagaa | 227 |
| GLUT1 | F- cccagaggggtgaggag<br>R- cctgccattcatcagc | 201 |
| CAIX | F- ctttggccaggttgacpgg<br>R- tgggagtctggctgaat | 205 |
| RPL13a | F- tggaggaccctggttagtgc<br>R- cctggaggagagggagaag | 126 |
SYBR Green mix (Quantitec SYBR Green PCR kit, Qiagen, DE), 10 pmol of each specific primer and 2 μL cDNA (initially diluted 10 times). The cyclic parameters consisted of an initial hotstart step at 95 °C for 10 min, followed by 40 repetitions of 95 °C for 15 s and 60 °C for 25 s. The calibration curve for each gene under study was made using serial dilutions of cDNA synthesized from 1 μg of mRNA from the MCF7 (tumorigenic), MCF-10A (non-tumorigenic) and MDA-MB-231 (metastatic) cell lines.

Expression of the genes studied in the different biological matrices was determined using the formula $2^{(-\Delta Cq)}$.

**Statistical analysis.** Qualitative variables were presented as absolute and relative frequency. For the quantitative variables with no normal distribution of the data, medians and 25 and 75% percentiles, respectively were used; as for variables with normal distribution (Shapiro–Wilk, p > 0.05) was used to describe mean, standard deviation, minimum and maximum.

To evaluate the behavior (expression) of the genes proposed in breast cancer patients and donors (healthy women = control group), association of the diagnostic variables with the markers from the first collection was studied using the Mann–Whitney test. Finally, the Friedman test was performed to analyze the evolution of HIF and GLUT1. For all analyzes, a confidence level of 95% was used. The program utilized was Stata version 11.0 and GraphPad Prism version 6.0.

All methods were carried out in accordance with relevant guidelines and regulations.

**Ethics approval.** This work was approved by Centro Universitário Saúde ABC/faculdade de Medicina do ABC Ethical Committee (Approval number 2.433.922). All methods were carried out in accordance with relevant guidelines and regulations.

**Results**

In order to study the expression of HIF-1α, GLUT1 and CAIX genes in liquid biopsy of patients with breast cancer, samples from 125 patients and 25 healthy donors were evaluated. As state before, healthy donors were women without a history of neoplasias, diabetes and renal and/or cardiovascular diseases. The mean age of donors was 50 y.o. (50 ± 8 y.o.), pairing with patients. The clinical characteristics of breast cancer patients are described in Table 2.

| Clinical Characteristics | n | % |
|--------------------------|---|---|
| **Clinical stage**       |   |   |
| I                        | 36| 28.8|
| II                       | 59| 47.2|
| III                      | 30| 24.0|
| **Recurrence**           |   |   |
| Negative                 | 113| 91.9|
| Positive                 | 10 | 8.1 |
| **Estrogen receptor**    |   |   |
| Negative                 | 31 | 25.2|
| Positive                 | 92 | 74.8|
| **Progesterone receptor**|   |   |
| Negative                 | 51 | 41.5|
| Positive                 | 72 | 58.5|
| **Her_ihc**              |   |   |
| Negative                 | 28 | 22.9|
| +/+3+                    | 39 | 32.0|
| ++/+3+                   | 24 | 19.7|
| +++/+3+                  | 31 | 25.4|
| **Median (p.25; p.75)**  | Min.; Max |
| Age                      | 52.5 (47; 61) | 27; 78 |
| Follow-up time (months)  | 28 (24.5; 32) | 4; 49 |

Table 2. Clinical characteristics of the patients. +/3+, ++/3+ or +++/3+ are standard positivity index. p.25; p.75: 25–75; Min. minimum, Max. maximum.
women) and in breast cancer patients at the time of diagnosis (Fig. 1). In breast cancer patients, expression of HIF1-α showed a 90–91-fold increased expression, whereas GLUT1, a 160–161-fold increased expression, approximately ($p < 0.0001$). Although statistically significant, the difference in expression of CAIX between the two groups was smaller and, as its expression in the samples is not consistent, this gene was left out of further analysis.

A ROC Curve for HIF-1α and GLUT1 was generated (Fig. 2). The HIF-1α marker presented the largest area under the curve [0.9987 (95% CI 0.9683–1007; $p < 0.0001$)], with the cut-off value of $2^{-\Delta Cq} > 0.004938$, with a sensitivity of 98.15% (95% CI 93.47–99.77%) and a specificity of 96% (95% CI 79.65–99.90%). Gene expression was accessed by $2^{-\Delta Ct}$ formulae before the beginning of the treatment.

Discussion

To date, increased expression of HIF-1α, GLUT1 and CAIX is associated with invasion, metastasis and worse prognosis. However, to date, few studies have described the detection of the expression of HIF-1α and GLUT1 genes in peripheral blood samples from patients with solid tumors. Thus, our result confirms that peripheral blood is a suitable biological matrix for detecting the expression of these genes in patients with breast tumor. Moreover, expression of HIF-1α and GLUT1 is significantly higher in peripheral blood samples from breast cancer patients than that found in healthy women, a result that points to their expression detection in this biological matrix as a potential diagnostic tool of this disease. As stated earlier, increased expression of...
Expression of GLUT1 may influence the sensitivity of tumor cells to chemotherapy. In fact, in tumor pieces, increased expression of GLUT1 is significantly higher in patients with positive disease progression and is therefore a marker of chemoresistance. The same is seen for HIF-1α. Treatment of breast cancer cells with chemotherapy results in increased germ tumor cells among the surviving cells, a fact that depends on the activity of hypoxia-induced factors. Thus, to assess whether there is a relationship between progression and expression of HIF-1α and GLUT1, patients were then divided according to disease progression into two groups (positive progression and negative progression). However, there was no difference in expression between these two groups. Patients were then divided according to the degree of clinical staging to assess whether the increase in gene expression is related to staging of the disease (determined at the diagnosis), however no difference in expression was found between these groups as well.

Analyzing breast tumor samples, Bos et al. demonstrated that HIF-1α expression is greater in samples with a more advanced stage, a result that positively associates this factor with tumor aggressiveness. In patients with acute myeloid leukemia, GLUT1 expression has also been shown to be greater in patients without remission than in patients in complete or partial remission, which results in the predictive value of GLUT1. Still corroborating the role of GLUT1 as a predictive marker in a meta-analysis, Zhao et al. analyzed 41 studies with a total of 4797 patients, in whom increased GLUT1 expression was significantly associated with worse prognosis in different
types of cancer. However, our results indicate that in the peripheral blood, the expression of HIF-1α and GLUT1 cannot be considered as a tool for predictive evaluation or prediction of tumor aggressiveness.

Thus, our results suggest that in liquid biopsies, the differential expression of HIF-1α and GLUT1 has diagnostic but not predictive potential. CAIX, by its turn, is also more expressed in breast cancer women than in healthy individuals. However, its expression was detected only in a few samples, so it was not included in various analyzes of this work. Therefore, evaluation of the expression of HIF-1α and GLUT1 in the blood may be a useful laboratory tool to complement the diagnosis of breast cancer.

Conclusions

The HIF-1α and GLUT1 genes can be considered good markers for breast cancer diagnostic evaluations in liquid biopsies, since their expression is significantly increased in patients with excellent sensitivity and specificity values. The CAIX gene, however, was expressed in few samples, with no association with clinical data. The expression profile of the markers under study is compatible with the characteristics presented by the included cell lines, which technically and functionally validates the study of these genes.

Data availability

Data will be made available under reasonable request.

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Author contributions
C.H.F.P., M.M.P. and G.S.A.A. performed all molecular reactions; J.F.A.E., L.V.A.S. and G.L.V. performed statistical analyses and figures/graphics; A.G. and F.L.A.F. selected and included patients; B.C.A.A. wrote the main manuscript text; all authors reviewed the manuscript text.

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Competing interests
The authors declare no competing interests.

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