Scientific Research

Homocysteine as a Biomarker for Predicting Disease-Free Survival in Breast Cancer

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ABSTRACT

Introduction: Breast cancer is the leading cause of cancer mortality among women. Some biomarkers and clinical features are used for the diagnosis and prognosis of this tumor, but no prognostic or predictive marker is routinely available specifically for hormone receptor positive tumors. Homocysteine is well known as a risk factor in atherosclerotic vascular diseases, but its participation in cancer biology is still unclear. The aim of this study was to evaluate serum Homocysteine and Cysteine as biomarkers of disease progression in breast tumor. As a secondary objective, the effect of a short course (one month) of hormonal treatment on Homocysteine, Cysteine and DNA methylation levels was also evaluated. Methods: Blood samples, tumor samples and normal adjacent tissue were collected during the initial biopsy (pre-treatment) and after one month of hormonal therapy (post-treatment). Serum Homocysteine and Cysteine were analyzed by HPLC and tissue global DNA methylation was determined by the Methylation-Sensitive Restriction Enzyme (MSRE) technique. Results: Variations in Homocysteine levels were significantly correlated with Disease-Free Survival. Cox proportional risk model demonstrated that nodal status and Homocysteine levels were independent prognostic factors for disease-free survival (DFS). A significant difference was observed between pre- and post-treatment levels of Homocysteine and Cysteine in advanced tumors, suggesting a prognostic role in patients with poor clinical characteristics. Conclusion: Although more studies are needed to confirm these results, our research suggests that Hcy might be used as a prognostic biomarker for breast cancer.

Keywords: Homocysteine; Cysteine; Breast Cancer; Estrogen Receptor; Prognostic and Predictive Biomarker; Tamoxifen; Anastrozol

1. Introduction

Breast cancer is the leading cause of cancer death among women worldwide [1]. Clinical and pathological characteristics are still the sources of the most important information used to assess prognosis and define treatment (such as tumor staging, hormonal receptors expression and human epidermal growth factor receptor 2 status) [2].

Estrogen receptor positive (ER+) tumors have a better prognosis than most breast tumors, partly due to their response to hormonal therapies [3,4]. These drugs may modulate the estrogen receptor itself or inhibit estrogen production in post-menopausal women. Although it's very effective, hormonal therapies cannot prevent relapse in about 30% of patients with estrogen receptor positive breast tumors, and no prognostic or predictive marker of response is routinely available for this group of patients [5].

Homocysteine (Hcy) is an extensively studied prognostic marker in atherosclerotic vascular diseases [6], but

its contribution to cancer biology is still unclear. Alterations of homocysteine levels may cause an imbalance among the products of the anti-oxidative pathway resulting in increased oxidative metabolism [7]. Homocysteine synthesis is also related indirectly to DNA methylation, a mechanism for gene expression control in normal and tumor cells [8-10].

This work evaluates blood levels of homocysteine and its metabolite cysteine as biomarkers of survival and disease progression in post-menopausal women with non-metastatic breast cancer. We also studied the effect of a short-course of pre-operative (neoadjuvant) endocrine therapy on Homocysteine and Cysteine metabolism in a subgroup of these patients.

2. Patients and Methods

2.1. Patients

Ninety-seven post-menopausal breast cancer patients from two different institutions (Hospital São Paulo and Hospital Pérola Byington in São Paulo-Brazil) were evaluated. Sixty-seven of those patients participated in a randomized study of one month neoadjuvant endocrine therapy (tamoxifen or anastrozol) versus placebo.

The present study was approved by both Universidade Federal de São Paulo and Pérola Byington Hospital Institutional Review Boards, and all patients provided written informed consent. All patients were submitted to a diagnostic biopsy, and only patients with invasive breast carcinomas were included. Tumor samples were also obtained during definitive surgery for the removal of tumor tissue, for those patients participating in the randomized study. Serum and plasma were collected from all patients at the time of the initial biopsy (pre-treatment) and at the day of surgery (post-treatment) for patients in the randomized study.

For experimental purposes, clinical and pathological data were obtained from patients' medical records. Observed clinical parameters were tumor size (T), lymph node positivity (N0, negative lymph node and N1; positive lymph node), clinical Stage (CS, initial stages = I and II; late stages = III), local relapse, distant relapse, date of last event (relapse, death or last visit) and cause of death. Initial tumor size was defined by clinical examination or ultrasound, whichever was greater. Final tumor size was defined by pathological gross examination.

Immunohistochemical characteristics such as estrogen receptor and progesterone receptor status were also recorded.

2.2. Serum Homocysteine and Cysteine Quantification

The total serum Hcy (tHcy) and Cys (tCys) levels were measured according to the method described by Pfeiffer and co-authors [11]. Positive and negative ΔHcy (or ΔCys) were defined as the difference in post-treatment tHcy (or tCys) minus pre-treatment tHcy (or tCys) levels.

2.3. Analysis of Tumor DNA Methylation Levels

One microgram of DNA extracted from tumor sample or adjacent tissue was digested with either MspI or HpaII restriction enzymes (Fermentas, St. Leon-Rot, Germany). Non-digested DNA, and MspI and HpaII digested DNA were visualized in 0.8% agarose gel. Observed bands were quantified with ImageJ and methylation ratio was calculated subtracting HpaII digested DNA quantification from the MspI quantification, divided by non-digested DNA quantification (HpaII-MspI/non-digested DNA).

2.4. Statistical Analysis

Kruskal-Wallis test was used to compare Hcy, Cys and pathological tumor size values, and these variables were correlated using Spearman correlation test when adequate [12].

Disease-free survival (DFS) was calculated from the date of diagnosis until date of first relapse or death, and presented using the Kaplan-Meyer method [13]. Follow-up was measured from date of diagnosis to the date of last visit or date of death. Survival curves were compared using Log-rank test or Breslow test when curve intersection was observed. Univariate and multivariate analysis were performed by Cox regression method.

Multivariate analyses tested all variables found in the univariate analysis with a p-value lower than 0.05, and excluded patients with one or more missing data. All statistical tests were two-sided, and the level of significance was set at 5%. Analyses were done using the Statistical Package for Social Science SPSS (release 15.0, Chicago, Illinois, USA). The analysis adhered to the reporting recommendations for tumor marker prognostic studies.

3. Results

3.1. Clinical Data and Biochemical Quantifications

Patients' characteristics are listed in **Table 1**. Of 67 patients participating in the randomized trial, one was excluded because she received neoadjuvant (pre-operative) chemotherapy.

Homocysteine (Hcy) and cysteine (Cys) levels were measured at the time of the initial biopsy in all patients (entire group). Patients that did not participate in the trial received therapy according to the discretion of their physicians, and had no Hcy or Cys measured after the biopsy.

ABCR

Table 1. Patients' clinical characteristics.

Characteristic	Mean	N (%)
Age (range)	65.90 (36 - 92 years)	88
T (Tumor size)		
1		2 (2.06)
2		67 (69.07)
3		7 (7.22)
4		14 (14.43)
Unknown		7 (7.22)
N (Lymph nodes status)		
Yes		40 (41.24)
No		52 (53.61)
Unknown		5 (5.15)
Clinical Stage		
I ou IIa ou IIb		68 (70.10)
IIIa ou IIIb		20 (20.62)
Unknown		9 (9.28)
Local Relapse		
Yes		6 (6.18)
No		75 (77.32)
Unknown		16 (16.50)
Distant Relapse		
Yes		21 (21.65)
No		59 (60.82)
Unknown		17 (17.53)
Death		
Alive		71 (73.20)
Death for disease		9 (9.28)
Death for another cause		2 (2.06)
Unknown		15 15.46)
Estrogen Receptor Status (ER)		
Yes		58 (59.79)
No		27 (27.84)
Unknown		12 (12.37)
Progesterone Receptor Status (PR)		
Yes		35 (36.08)
No		40 (41.24)
Unknown		22 (22.68)
Initial Tumor Size (cm)	4.26 (1.5 -10.0)	94
Final Tumor Size (cm)	3.19 (0.0 - 7.5)	57
ΔTU (cm) (Initial-Final)	0.58 (−5 - 4)	57

Patients participating in the randomized trial (trial group) had Hcy and Cys measured again at the time of the surgery.

There were no statistical differences in basic characteristics , as well as type of post-operative treatment, between those patients in the randomized trial and those not participating in the trial, and among groups in the randomized trial (data not shown). Because there were no statistical differences between groups, we treated the data as belonging to the same group.

The entire group had biochemical characteristics measured at the time of the initial biopsy, but variations (Δ) were calculate only for trial patients, which had samples collected both at the time of the initial biopsy and at the time of surgery. Results are shown in **Table 2**.

Homocysteine and Cysteine levels, both initial and at surgery, and their variations (Δ) were weak or moderately correlated between themselves (r = 0.579, r = 0.756 and r = 0.621, respectively, with p < 0.001 for all tests). Initial Cysteine levels were weakly and inversely correlated with initial global DNA methylation levels (r = −0.366, p = 0.028). These results are expected in light of the metabolic relationships among Homocysteine, Cysteine and DNA methylation in the methionine cycle [10].

3.2. Variations in Serum Hcy and Cys Levels Correlate with Late Clinical Stages

Figure 1 shows that, for patients with advanced disease (Stage III) or with positive lymph nodes (**Figures 1(c)** and **(d)** respectively), Cysteine levels decreased after use of

Table 2. Biochemical characteristics.

Characteristic	Mean	SD	Minimun	Maximun	N
Hcy (Pre-treatment) (μM)	17.60	10.25	7.10	71.48	83
Hcy (Post-treatment) (μM)	13.65	6.49	4.89	42.94	43
ΔHcy (Pre-post)	−2.89	11.54	−39.43	21.67	40
Cys (Pre-treatment) (μM)	595.40	101.71	389.03	951.65	83
Cys (Post-treatment) (μM)	535.66	170.14	181.06	1055.67	43
ΔCys (Pre-post)	−12.27	189.62	−413.03	463.74	40
MSRE N (Pre-treatment) (%)	44.39	7.03	32.88	56.92	18
MSRE N (Post-treatment) (%)	48.27	11.01	23.62	72.63	26
ΔMet N (Pre-post)	9.29	17.43	−17.21	36.85	7
MSRE T (Pre-treatment) (%)	37.01	11.61	12.97	58.56	40
MSRE T (Post-treatment) (%)	35.01	11.45	10.45	64.99	36
ΔMet T (Pre-post)	−3.39	13.54	−38.51	26.14	23

anastrozol, tamoxifen or placebo (trial group). Homocysteine levels decreased significantly in patients with advanced disease (**Figure 1(a)**), but not with statistical significance in patients with positive lymph nodes (**Figure 1(b)**).

There was no significant statistical correlation regarding Hcy or Cys levels variations and tumor size (data not shown). Interestingly, one patient in the anastrozole group presented a residual tumor with less than 1 mm of size, and other showed no residual tumor after surgery. In the tamoxifen group, one patient as well had no detectable tumor mass after surgery.

Hcy is a crucial component of the methionine cycle, which is linked to DNA methylation [10]. We quantified global DNA methylation levels in patients in the trial group, both at the initial biopsy and at the time of the definitive surgery, in normal and tumor samples. Although global DNA methylation levels were decreased in tumor compared to normal samples as expected [14], no significant correlation was found comparing global DNA methylation levels with initial clinical characteristics (data not shown). Variations in Homocysteine (ΔHcy) showed a statistically non-significant weak to moderate correlation with variations in tumor global DNA methylation levels (ΔMet T) (r = 0.432, p = 0.057).

3.3. Hcy and Cys Levels Did Not Vary Significantly after a Short Term of Neoadjuvant Antiestrogens

Regarding the initial biopsy, we found no significant statistical differences between serum levels of Hcy (r = −0.013; p = 0.938), or Cys (r = −0.069; p = 0.672), and
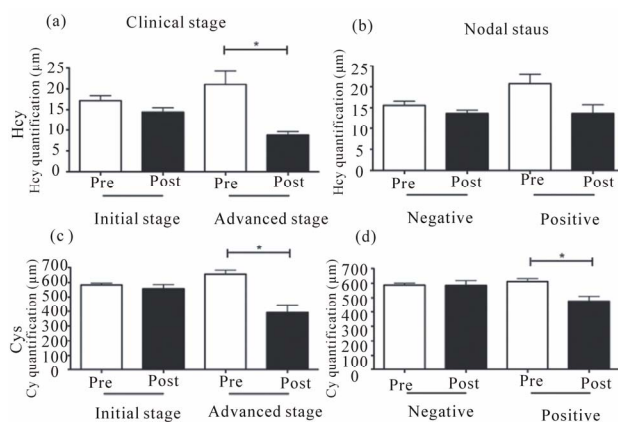

Figure 1. Homocysteine and cysteine levels after hormonal treatment present a larger decrease in patients with advanced clinical stage. Hcy and Cys levels before (pre) and after treatment (post) in initial or advanced clinical stage (a) and (c), respectively, and negative or positive nodal status (b) and (d), respectively. *p < 0.05.

DNA methylation for normal and tumor tissue (r = −0.214; p = 0.645 and r = 0.405; p = 0.62) for the entire group or the trial group.

We also did not find any significant statistical correlation between Hcy and Cys variations (ΔHcy and ΔCys) and the use of antiestrogens or placebo on a multivariate analysis (data not shown).

3.4. High Serum Hcy Variation Predicts Better Disease-Free Survival

Global survival was not significantly correlated with any clinical (data not shown) or isolated biochemical parameters, including Hcy or Cys (**Figure 2**), probably because of a low number of events. However, variations in Hcy levels (ΔHcy), but not in Cys (ΔCys), were significantly correlated with disease-free survival (DFS), and patients with an increase in Hcy levels after treatment (positive ΔHcy) present a higher probability of surviving with no evidence of relapse (**Figure 3**). Cox proportional risk model, including clinical stage, nodal status and Hcy variation (ΔHcy), demonstrated that nodal status (HR = 2.86; 95% CI = 1.16 − 12.79; p = 0.027) and Hcy variation (ΔHcy, HR = 0.96; 95% CI = 0.92 − 0.998; p = 0.042) are independent prognostic factors regarding DFS.
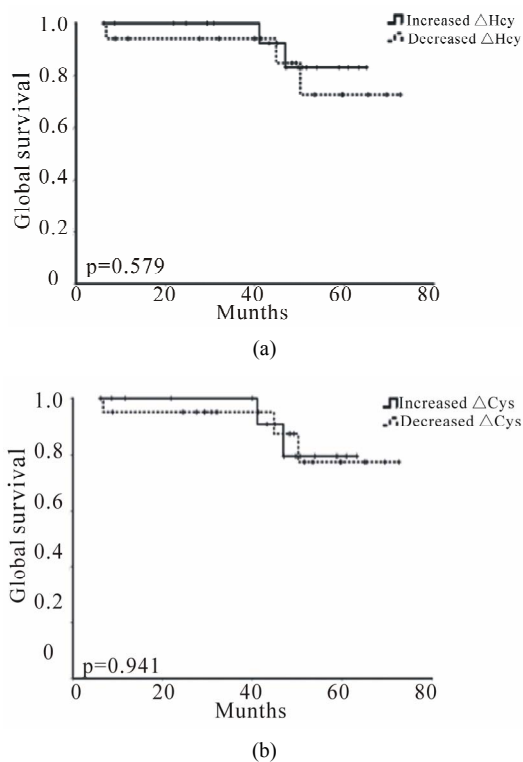

Figure 2. Hcy and Cys variations do not correlate with overall survival (OS) in breast cancer. Kaplan-Meier OS curves for increased and decreased ΔHcy (a) and ΔCys (b) (p = 0.579 and p = 0.941, respectively) + indicates event.

4. Discussion

We found a significant inverse correlation between increased Homocysteine levels, and disease-free survival (**Figure 3(a)**). We also encountered significant positive correlations between variations in Hcy and Cys in trial patients with advanced disease or positive lymph nodes (**Figure 1**), suggesting that Homocysteine levels in these patients may be correlated with response to hormonal treatment. These findings seem contradictory unless we consider that patients with bulky disease, who may have a more noticeable response to antihormonal therapy and therefore a higher variation in Hcy and Cys levels, are also the ones with a much higher chance of relapse. We also postulate that Hcy levels are inversely related to tumor response to antiestrogens, but the low number of patients in this sample did not allow our analyses to support this hypothesis. There are many limitations in this study, including the number of subjects and their heterogeneity, and we approach all findings as hipothesis generating only.

Increased Homocysteine levels have long been studied as a risk factor for cardiovascular disease, and more recently for some neurological problems and pregnancy complications [15]. Plasma Hcy quantification is a relatively simple and fast procedure, and there is some evidence showing Hcy contribution in carcinogenesis [16]
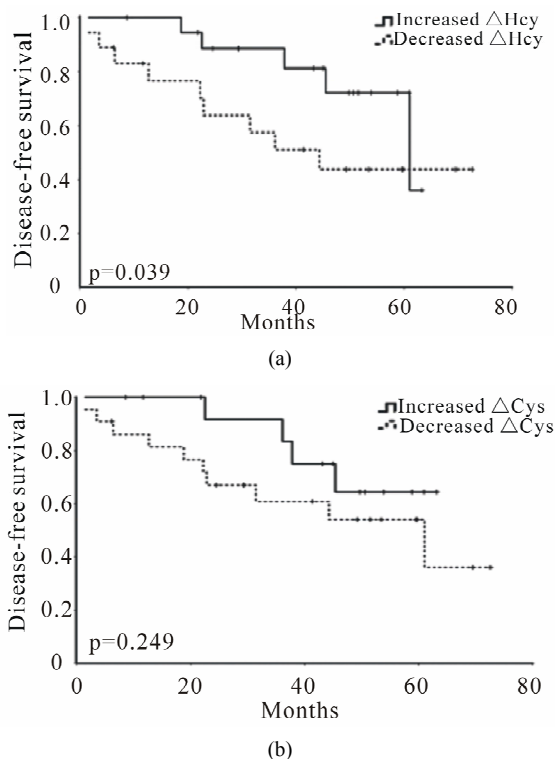

(a)

(b)

Figure 3. Hcy level variations correlate with disease-free survival (DFS) in breast cancer. Kaplan-Meier DFS curves for increased and decreased ΔHcy (a) and ΔCys (b). (*p = 0.039 and p = 0.249, respectively) + indicates event.

and as a risk factor for breast cancer [17]. Few cheap and practical biochemical biomarkers are routinely available to predict prognosis or response to cancer treatment and, in this context, Hcy might be a useful prognostic and/or predictive biomarker.

Breast cancer is a very common type of cancer, and has been studied extensively. Although several biomarkers are described in the literature [18-20], there are many efforts to increase the biomarker list and to personalize cancer diagnosis and treatment. However, most of these efforts look at molecular profiles and depend on molecular genetics facilities or tissue sampling processing, which are expensive and not largely available.

Hcy metabolism can be disrupted by defects in transulfuration or remethylation pathways, which may cause increases in Hcy levels. The accumulation of Hcy levels can result in Cys levels augmentation [17,21]. As expected, our results show a correlation between Hcy and Cys levels, both before and after antiestrogens therapy. A correlation between Hcy and Cys variations (ΔHcy/ΔCys) was also observed.

DNA methylation is a well-studied epigenetic mechanism involved in gene expression control [9]. The imbalance in Hcy levels may result in changes in S-adenosylmethionine (SAM) levels and consequently promote alterations in DNA methylation patterns [8].

Soares and co-authors [22] already demonstrated the importance of hypomethylation as an epigenetic event involved in the breast tumorigenesis. Although we did not observe any correlation among tumor size and global methylation levels in tumor tissues, we observed an inverse correlation between Hcy variation (ΔHcy) and tumor sample methylation variation (ΔMet T), although not statistically significant (data not shown). These parameters may be influenced by several modifying factors such as diet, DNA polymorphisms of the enzymes involved in the methionine metabolism and, even more critical in our study, the interference of two different drugs, tamoxifen and anastrozol [23,24].

We also correlated Hcy and Cys levels variations with clinical-pathological features of breast tumors, as nodal status (N) and clinical stage (CS). Our results show a significant reduction of the Hcy and Cys levels after antiestrogens or placebo in patients with poor clinical characteristics (**Figures 1(a)**, **(c)** and **(d)**). Although we could not find a statistically significant difference for nodal status and Hcy levels (**Figure 1(c)**), we did observe the same biological pattern (reduction of Hcy levels in node positive patients). A study with untreated patients suffering from primary head and neck squamous cell carcinoma (HNSCC) was not able to find any correlation between folate, B12 vitamin and Hcy serum levels and disease progression [25], suggesting that the difference between Hcy and Cys levels and progression may

be specific for different tumor types.

At this moment, few data are available regarding Hcy role in tumor biology. Some efforts have been done to evaluate the relationship between Hcy and Cys levels with the risk of developing breast tumors. Some studies observed that an increased breast cancer risk was associated with high levels of Hcy or Cys in women with low levels of folate [17,21,26]. In addition, high plasma or serum levels of Hcy were also observed in patients with other cancer types [16,27-31].

Although most authors analyzed Hcy levels and other methionine cycle components as factors for developing cancer, only a few studies discussed the possible role of Hcy as a prognostic factor. Bobe and co-workes [28] found that high Hcy concentrations were associated with colorectal adenoma recurrence. No studies could be found correlating Hcy and breast cancer prognosis. We also studied the relationship of Hcy and/or Cys concentration with global methylation levels, in order to search for possible biological mechanisms underlying this association, but we were not able to find any correlation at this time.

To check the possibility that the Hcy and/or Cys present prognostic or predictive value, we evaluated patients with resectable breast tumors, followed for a period of four to seven years. Due to a small number of fatal events, the study lacked power to determine the contribution of the Hcy and Cys as prognostic factors in overall survival (OS). Nevertheless, our results show that an increase in Hcy levels after a short course of neoadjuvant therapy is significantly correlated with a longer disease-free survival (DFS) (**Figure 3**), suggesting that the variation of Hcy is a possible prognostic marker for breast tumor. Interestingly, advanced tumors presented a decrease in Hcy after antiestrogens treatment (**Figures 1(a)** and **(d)**), confirming an association between Hcy and prognosis in breast tumors. A multivariate analysis showed that Hcy levels and nodal status (N), but not tumor size, are independent prognostic factors regarding DFS, implying that the variation in Hcy levels can be useful as an additional marker in the prediction of treatment response in breast tumor patients.

The short-term course of tamoxifen or anastrozol had little influence on overall survival (OS) or disease free survival (DFS) (data not shown). A study from Washington University [32] showed that the use of 4 months of either tamoxifen or letrozol (another aromatase inhibitor, comparable to anastrozol) had no impact on relapse-free survival or specific breast cancer survival. A meta-analysis of 4 studies [33] comparing anastrozol to tamoxifen as neoadjuvant therapy detected a superiority of anastrozol regarding the rate of breast-conserving surgery, which could not be found in our study probably because of the small number of subjects, and the short period of treatment.

High Hcy level is a well-known cardiovascular risk factor and Hcy levels have also been suggested as a potential tumor marker [16]. At least in our knowledge, no report has been published about the value of the Hcy levels variation as a predictive or prognostic marker for breast cancer. We are well aware of the limitations of this study (retrospective analysis of a heterogeneous group of patients), but we have shown for the first time the potential of Hcy levels variation as a disease-free survival (DFS) marker in breast tumor patients. This finding is hypothesis generating, and will be further characterized in the near future by our group.

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