Oxidative stress status in metastatic breast cancer patients receiving palliative chemotherapy and its impact on survival rates

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Abstract

Antineoplastic agents are known to induce the production of free radicals leading to cell damage. These adverse effects may fuel up the acquisition of new mutations and the development of treatment resistances. We selected 30 metastatic breast cancer patients receiving palliative chemotherapy and paired blood samples, before and after chemotherapy, were extracted. We analyzed DNA, lipid and protein oxidative damage markers and determined the extent of antioxidant and repair defenses activation at the systemic level. We found that DNA repair activity of the KU86 enzyme was significantly lower after chemotherapy and antioxidant capacity of the plasma was significantly higher after treatment. Cox regression analysis revealed a significant effect of KU86 activity on the survival rates of those patients who received antrhacyclines as part of their treatment. The high clinical heterogeneity of metastatic breast cancer patients warrants further studies to clarify the role of DNA repair and systemic antioxidant capacities during chemotherapy.

Introduction

Breast cancer is the most common neoplasm in women and a leading cause of cancer-related deaths worldwide [1]. The improvement of diagnostic and therapeutic tools have made possible to detect breast cancer even in pre-invasive stages and lead to a significant decrease in breast cancer mortality over the past decades [2]. A variety of cytotoxic agents are used in the neoadjuvant, adjuvant and metastatic settings, providing a significant palliation of the illness [3]. Apart from the beneficial actions of chemotherapy, the adverse consequences of its action on normal tissue are constant as antitumor drugs are indiscriminate, leading to severe toxicities that limit the chemotherapy dose and consequently, the chemotherapy efficiency in some cases as well [4, 5]. Among these adverse consequences, it is well known that the majority of the antioneoplastic drugs induce the generation of free radicals [5]. The cumulative production of free radicals leads to oxidative stress, which is the term used to describe a physiologic situation characterized by a cellular redox imbalance, which has been found to be present in cancer cells compared to normal cells. Oxidative stress is related to oncogenic stimulation because it causes damage in different cellular components such as lipids, protein and DNA, which promote DNA mutation and alters crucial cellular processes such as enzymatic catalysis or signal transduction [6, 7]. Moreover, the activation of enzymatic and non-enzymatic antioxidants contributes to generate a particular microenvironment that notably influences tumor behavior, tumor response to chemotherapy and thereby cancer patient's clinical outcome [6, 7, 8]. We have recently published a work [9] whose results suggest that DNA repair activity and systemic total antioxidant capacity may influence the clinical outcome of breast cancer patients undergoing neoadjuvant or adjuvant chemotherapy.

Based on the above mentioned premises and our previous observations in other groups of breast cancer patients, the main objectives of the present work were to test whether chemotherapy influences oxidative damage and antioxidant markers levels in the plasma of metastatic breast cancer patients and if these perturbations have any effect on their survival rates.

Methods

Patients and samples.

Thirty patients diagnosed of metastatic breast cancer, were enrolled in this study after signing informed consent. Metastatic breast cancer patients are a very heterogeneous group composed of a significant percentage of total breast cancer patients. This group of patients is characterized by a persistent chemo-resistance and the highest morbidity and mortality rates. No standard care therapy exists for this population and it is not selected to be studied as frequent as non-metastatic breast cancer patients, despite it would greatly benefits from any improvement in toxicity reduction and therapy effectiveness [10]. Together with the fact that we have previously investigated the effect of chemotherapy on the redox status of non-metastatic breast cancer [9] and wanted to confront our previous results with those obtained in the metastatic setting, these are the main reasons that brought us to select the metastatic population for this study.

The pathologic and clinical information was extracted from the medical reports achieved in the Oncology Department Registry. Matched blood samples were collected from each patient before and after four cycles of chemotherapy. This study was conducted following the guidelines of the local Ethical Review Board and in accordance with Good Clinical Practices and the tenets of the Declaration of Helsinki.

Blood collection and processing.

Approximately 5 ml of blood was taken from the patients by venous puncture. The blood was drawn into an EDTA-containing tube (Vacutainer®EDTA Tubes. BD, New Jersey, USA) and centrifuged at 1000 × *g* for 15 min. Plasma was kept in a separate tube and frozen at -80ºC. White blood cells were removed, washed in 3 ml of RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA), carefully overlaid on 5 ml of Histopaque 1077 (Sigma-Aldrich) and centrifuged at 700 × *g* for 30 min. The cells were kept until analyzed at –80ºC in a cryoprotectant solution containing 90% fetal calf serum (Sigma-Aldrich) and 10% dimethylsulfoxide (Sigma-Aldrich).

Alkaline single-cell gel electrophoresis (comet assay).

DNA strand breaks were detected using the alkaline comet assay, or single-cell gel electrophoresis [11, 12]. Isolated lymphocytes were resuspended in 85 µl of 1% low–melting-point agarose (Invitrogen, Scotland, United Kingdom; w/v, in phosphate buffered saline, 37°C, pH 7.4) and pipetted onto an agarose-precoated microscope slide embedded with 85 µl of 1% high–melting-point agarose (Invitrogen; w/v, in phosphate buffered saline, pH 7.4). Agarose was allowed to set for 5–6 min at 4°C and the slide was incubated for 1 h in a lysis solution (2.5 mol/l of NaCl, 10 mmol/l of Tris, 100 mmol/l of Na_2EDTA, NaOH to pH 10, and 1% [v/v] Triton X-100; Sigma Diagnostics). The slides were then placed in a double row in a 260-mm–wide horizontal electrophoresis tank (Consort, Parklaan, Belgium) containing 0.3 mol/l of NaOH and 1 mmol/l of Na_2EDTA for 40 min before electrophoresis at 25 V for 30 min at an ambient temperature of 4°C (the temperature of the running buffer not exceeding 15°C). The slides were then washed three times for 5 min each with 0.4 mol/l of Tris-HCl (Sigma

Diagnostics), pH 7.5, at 4°C before staining with 20 µl of 4’6-diamidine-2-phenylindol dihydrochloride (Sigma Diagnostics; 5 µg/ml).

Quantification of the comet assay.

The nucleoids stained with 4’6-diamidine-2-phenylindol dihydrochloride were scored using a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany) [12]. One hundred comets from each gel (scored at random) were scored using computerized image analysis (Komet 3.0; Kinetic Imaging Ltd., Liverpool, United Kingdom) and the percentages of fluorescence in the comet tail (representing the fraction of DNA in the tail) and head (representing the fraction of DNA in the head) were measured.

Thiobarbituric acid–reactive substances measurement.

The extent of lipid peroxidation was evaluated on blood plasma by measuring the concentration of thiobarbituric acid–reactive substances (TBARS) as previously described [13]. Results were expressed as nmol of TBARS per ml of blood plasma.

Plasma protein carbonyl assay.

The levels of plasma protein carbonyl groups were assessed using Protein Carbonyl Kit (Cayman Chemical Company, MI, USA). Briefly, 100 µl of blood plasma were transferred to two tubes. One tube was the sample tube and the other was the control tube. After adding 400µl of 2,4-Dinitrophenylhydrazine (DNPH) to the sample tube and 400µl of 2.5 M HCl to the control tube, both of them were incubated in the dark at room temperature for 1 h. Afterwards, 0.5 ml of 20% trichloroacetic acid was added to each tube and incubated in ice for 5 min. This mixture was centrifuged at 10.000 × *g* for 10 min at +4ºC, obtaining a pellet that was resuspended in 0.5 ml of 10% trichloroacetic acid and incubated in ice for 5 min and again centrifuged at 10.000 × *g* for 10 min at +4ºC. The pellet obtained was resuspended in 0.5 ml of (1:1) ethanol/ethyl

acetate mixture and centrifuged at 10.000 × *g* for 10 min at +4ºC twice. Finally, the pellet obtained was resuspended in 250 µl of guanidine hydrochloride and centrifuged at 10.000 × g for 10 min at +4ºC, obtaining a supernatant of which 220 µl were transferred to a 96-well plate and absorbance read (SYNERGY HT, Multi-Detection Microplate Reader. BioTek Instruments, Inc., Vermont, USA) at 370 nm. Total protein concentration in the plasma samples was measured using Biuret Colorimetric Assay Kit (Spinreact S.A. Barcelona, Spain). The results were expressed as nmol of carbonyl proteins per mg of total proteins in the plasma.

Determination of the total antioxidant capacity of the plasma.

Total antioxidant capacity was assessed using the method described by Re et al. [14]. Essentially, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) was produced by incubating an 7 mM aqueous solution of ABTS (Fluka, St. Louis, USA) in 140 mM potassium persulfate buffer (Merck, Darmstadt, Germany) in the dark and at room temperature, overnight. The mixture was diluted in phosphate buffered saline 1× (Invitrogen) until the absorption of the solution reached 0.70±0.02 at 734 nm. After that, the samples were diluted in phosphate saline buffer (Merck) (1:10) and transferred to a 96-well plate. Diluted ABTS solution was added, mixed and incubated in the dark and at room temperature during 10 min. The determination was carried out using a BIO-TEK analyzer (SYNERGY HT, Multi-Dection Microplate Reader. BioTek Instruments, Inc.). The resulting values were compared with a calibration curve constructed diluting the synthetic antioxidant Trolox (Fluka) (concentration range 0-20 µM Trolox in the well). The results were expressed as µM Trolox.

DNA repair activity of enzymes RPA and KU86.

The activity of the DNA repair enzymes Replication Protein A (RPA) (single strand breaks) and KU86 (double strand breaks), was assessed by an inmunoenzymatic assay using the Active Motif RPA and KU70/86 DNA Repair Kits (Active Motif, Tokyo, Japan). Each kit contains a 96-well plate to which a single stranded DNA oligonucleotide or a double stranded linear DNA molecule containing a blunt end, has been immobilized. RPA and KU86 contained in the nuclear extract bind specifically to this DNA molecule, which is detected through the use of a primary antibody. Addition of a secondary antibody conjugated to horseradish peroxidase provides a sensitive colorimetric readout quantified by spectrophotometry. Previously, cell concentration in the samples was measured by using the hematologic analyzer Sysmex KX21 (Sysmex Corporation Tokyo, Japan). Afterwards we extracted and quantified the nuclear proteins of blood lymphocytes using the Nuclear Extract Kit (Active Motif) and the Bicinchoninic Acid Kit (Pierce Biotechnology, Illinois, USA), respectively.

Statistical Analysis.

Data were expressed as the mean ± standard error of the mean of 30 patients per group. Student's t test for related samples was used to determine significant differences before and after chemotherapy. When normal and homogeny criteria were not followed, Wilcoxon test was applied. Statistical significance was established at $P \leq 0.05$ and $P \leq 0.01$. With respect to survival analysis, DFS was the primary end point of this study. DFS was defined as the time, measured in months, elapsed from the end of first chemotherapy cycle to date of first event or to the date of censoring if eventless. Disease relapse or death as a result of any cause was considered as an event. OS was a secondary end point and was calculated from the date of the first chemotherapy cycle to date of death or date of censoring if alive. Univariate and multivariate analyses were performed for the Cox regression model for survival using DFS and OS as end points.

Given that all oxidative stress and antioxidant markers were analyzed twice for each patient, before and after chemotherapy, paired data were available for each of these markers. An extended Cox regression model with time-dependent covariates was employed for univariate and multivariate analyses [15]. Categorical variables such as previous hormonotherapy (yes vs no), previous chemotherapy (yes vs no), monoclonal antibodies (yes vs no) and type of drugs received -Antrhacyclines (yes vs no), Taxanes (yes vs no) and other chemotherapeutics which included alkylating agents, alkaloids and antimetabolites (yes vs no)- were added as non-time-dependent covariates to the Cox regression model. Potential prognostic factors were included in the multivariate model following both statistic and clinico-biological criteria. Factors with $P \leq 0.1$ in the univariate analysis were included in the multivariate analysis together with significant interactions, as that between hormonotherapy and total antioxidant capacity of the plasma and the interaction between antrhacyclines and KU86 activity. The latest selection was performed according to the results of previous studies performed by us [9, 16] and others [17, 18], which indicate in one hand the possible influence of KU86 activity as a protector factor during chemotherapy [9], especially with DNA damage agents as antrhacyclines [16], and on the other hand that circulating sex hormone levels may have a significant impact on the total antioxidant capacity of the plasma and/or particular antioxidants [17, 18]. Stepwise backward elimination method with model removal set at $P \leq 0.05$ was used in the multivariate analysis to obtain the final model. All statistical analyses were carried out using SPSS 15.0 software.

Results

Characteristics of the study population are shown in Table 1. Essentially, most of the patients were aged between 40 and 70 years old at diagnosis and there was a high

proportion of women with ductal carcinoma, later-stage, high histological grade, estrogen receptor-positive, progesterone receptor-negative and Her2 receptor-negative. Table 1 shows that metastatic patients received a heterogeneous chemotherapeutic treatment depending on the clinic-pathological characteristics of their disease and the systemic treatment received previously, as determined by the medical oncology team.

Under our experimental conditions, DNA oxidative damage in lymphocytes of peripheral blood and lipid peroxidation, determined as TBARS production in blood plasma, were not significantly different before and after chemotherapy (Figures 1A and 1C, respectively). The concentration of carbonyl proteins in plasma is presented in Figure 1B and showed similar results profile as the presented for DNA oxidative damage and TBARS production. On the other hand, the analysis of the activity of the DNA repair enzymes RPA and KU86 (Figure 2) yielded the following results. KU86 activity (Fig. 2A) was lower after chemotherapy ($P<0.05$). RPA activity (Fig. 2B) did not significantly change after treatment. Trolox-equivalent antioxidant capacity of the plasma (TEAC) measured by the ABTS assay (Figure 3) showed increased values after chemotherapy ($P<0.01$).

With respect to the effect of oxidative status during chemotherapy and clinical outcome, we observed the following results. Disease free survival (DFS) and Overall survival (OS) were evaluated with a mean a follow-up time at the end of observation of 21.86 months (range, 7 to 45 months). Univariate analysis selected several drugs as anthracyclins, monoclonal antibodies and those included in the group “Other drugs” (Table 2). With respect to OS, only those treatments which included drugs different from anthracyclines, taxanes and monoclonal antibodies were seleceted. On multivariate analysis, increasing KU86 activity was associated with better DFS (HR= 0.918; 95%

CI, 0.867 to 0.972; *P*= 0.003) in those women that underwent anthracycline-based chemotherapy (Table 3).

Discussion

A number of studies have shown that breast cancer patients are characterized by significantly higher level of oxidative stress markers than healthy controls and that chemotherapy treatment further reinforce this effect [5]. Increased breast [19, 20], bladder [19], and multiple [19] cancer risks has been associated with higher DNA damage measured by the comet assay. It has also been reported that breast cancer patients have increased DNA damage, by itself or because it is more likely that breast cancer patients have impaired DNA repair mechanisms, and that the administration of a single cycle of chemotherapy already results in a significant increase in DNA damage, as measured by the alkaline comet assay [21, 22, 23]. Results found for DNA damage matched with those found for protein oxidation and lipid peroxidation. Oxidative stress is closely related to the carbonyl stress, which is characterized by the increase of the reactive carbonyl compounds due to their increased formation and/or due to their decreased degradation and elimination [24]. Oxidative and carbonyl stress may contribute to the process of carcinogenesis [25]. High plasma protein carbonyl levels has been positively correlated with high breast cancer risk [26] and protein oxidation correlated with morphologic hyperplasic changes in a model of estrogen-induced carcinogenesis [27]. Moreover, Tesarová et al [28] in 2007 demonstrated that patients with breast cancer had since the early stage I (with no respect to the grade and the expression of both estrogen and Her2/neu receptors) increased serum concentrations of carbonyls. In this study, serum levels of carbonyls were further increased in patients with clinical stages III-IV compared to the patients with clinical stages I-II.

Our results suggest that DNA damage and protein carbonyl levels are not further increased by chemotherapy. Nevertheless, apart from ours [9], it has not been published any study evaluating protein oxidation in breast cancer patients after chemotherapy to our knowledge. Comparing the levels of oxidative DNA and protein damage in blood plasma from metastatic breast cancer patients with those previously obtained from neoadjuvant and adjuvant breast cancer patients [9], it is noticeable that the oxidative profile of metastatic patients resembles that of adjuvant patients but it is markedly different from the oxidative profile of neoadjuvant patients, in such a way that only neoadjuvant patients experienced a significant raise in DNA and protein damage levels after chemotherapy. Moreover, it is also noticeable that the mean pre-chemotherapy level of DNA damage registered by neoadjuvant patients (27.57 ± 1.92 % DNA in tail) is around half of those registered by adjuvant and metastatic patients (41.14 ± 2.6 and 43.75 ± 2.49 % DNA in tail, respectively). In parallel, neoadjuvant patients registered a comparable reduction in protein damage (0.086 ± 0.007 nM/mg total protein) with respect to adjuvant and metastatic patients (0.144 ± 0.012 and 0.175 ± 0.009 nM/mg total protein, respectively). These data suggest that those patients not previously subjected to chemotherapy or surgery, are the most affected at the level of DNA damage and protein oxidation after the first chemotherapy treatment cycles. However, those previously subjected to surgery, as adjuvant patients or those subjected to several cycles of chemotherapy and/or surgery, as metastatic patients, presented high starting levels of protein oxidation that are not further raised after a new treatment batch.

With respect to lipid peroxidation, data obtained in previous studies using the same method [29, 30] show a significant increase after chemotherapy. Nevertheless, most of these studies do not consider if patients received surgical treatment or not, and none of them evaluated the changes in this parameter in the metastatic group. One

possible explanation for the absence of differences in TBARS levels of metastatic groups is the low specificity of the method, which may not has detected the probable differences in TBARS concentration after chemotherapy in these groups. On the other hand, it can be possible that previous clinical interventions, such as surgical treatment, would have induced the activation of antioxidant defenses able to repair the lipid oxidative damage and, as a consequence, TBARS concentration in plasma was reduced at a point from which new damage induced by chemotherapy was detectable by this method.

Regarding DNA repair activity and systemic antioxidant capacity, metastatic patients showed a significant decrease in KU86 activity and a significant increase in TEAC after chemotherapy. Again, if we compare these results from those previously obtained by our group in the neoadjuvant and adjuvant setting [9], it worths to be highlighted that TEAC after chemotherapy becomes increasingly higher from neoadjuvant to metastatic group in such a way that those patient groups who have undergone more clinical interventions, the metastatic setting in this case, exert the highest levels of TEAC. In contrast, KU86 activity showed significant decreased levels after chemotherapy in the metastatic group. It is possible to find in the bibliography some works about DNA repair capacity in cancer patients following chemotherapy treatment that point out the inefficiency of breast cancer patients to repair oxidative damage in DNA [23, 31, 32]. Nevertheless, these works analyze the repair activity shortly after exposition to chemotherapeutic agents, whereas our study analyzes a more prolonged effect in time. With respect our previous data from neoadjuvant and adjuvant patients [9], KU86 activity significantly decreases only in neoadjuvant and metastatic settings ($P<0.05$) but mean post-chemotherapy levels of KU86 activity are comparable among all patients, despite the starting levels of KU86 activity are maximal in the

metastatic patients. Then, according to our results, the data derived from previous works [23, 31, 32] may reflect early effects of chemotherapy on DNA oxidative damage rather than the inefficiency of DNA repair enzymes in breast cancer patients.

In general, our results suggest that previous clinical interventions induce a level of oxidative damage to an extent that it is not increased by the chemotherapeutic treatment. This hypothesis is sustained by the fact that the level of oxidative damage did not increase in the metastatic setting after chemotherapy and was comparable to that obtained in the adjuvant setting despite, in general, metastatic patients received more chemotherapy cycles and were subjected to more aggressive clinical interventions. These data suggest that breast cancer patients are subjected to high levels of oxidative stress as they become treated surgically and systemically. The oxidative damage causes the activation of antioxidant defenses that counteract in some extent the raise of oxidative damage, but successive clinical interventions result in additional oxidative damage that would overcome the antioxidant defenses, maintaining a high level of damage during all the phases of breast cancer treatment.

Finally, based on previous results recently published by our research group [9], we performed a survival analysis in order to make formal inferences about the influence of oxidative stress status along chemotherapy on metastatic breast cancer patient's survival. Univariate Cox regression with time-dependent covariates showed that an increase in KU86 activity during systemic treatment has a protective effect against disease recurrence in those women subjected to anthracycline-based chemotherapy. This latter interaction is in accordance with the severe genotoxicity often attributed to antrhacyclines and highlights the protective role of DNA repair at the systemic level. A recent work has reported that increasing DNA repair activity in tumor cells is significantly related with chemo-resistance arguing that transformed cells potentiate the

activity of these enzymes to overcome drug-induced toxicity [33]. Nevertheless, we have analyzed the activity of DNA repair enzymes in blood plasma of patients undergoing chemotherapy, what may principally correlate with systemic toxicity. Secondarily, high levels of microenvironmental oxidative stress have been correlated with tumour cell migration, angiogenesis and metastasis [7]. A highly oxidative environment may contribute to survival and progression of eventually disseminated tumor cells, known as circulating tumor cells (CTCs), which are thought to be responsible of distant metastasis [34]. This data are in accordance with our results which suggest that those metastatic patients with a decreased systemic DNA repair capacity are at greater risk to experience disease recurrence than those with improved systemic DNA repair capacity.

In regard to systemic antioxidant capacity and opposite to the results obtained for neoadjuvant and adjuvant breast cancer patients [9], the survival rates of metastatic breast cancer patients seem to be unaffected by the antioxidant activity of TEAC. A plausible explanation for these differences may account for the large clinical and therapeutic heterogeneity of the metastatic group in comparison with the neoadjuvant and adjuvant groups. Some metastatic breast cancer patients have undergone previous surgery and/or systemic treatment, either in neoadjuvant or adjuvant sequence, some others have been diagnosed as metastatic patients and have not undergone so many clinical interventions and many of them have received different chemotherapeutic agents. It is noticeable that despite the same clinical heterogeneity also affected the analysis of KU86 activity with respect patient’s outcome and survival, these design and experimental inconvenience has not masked the correlation between these two variables.

Conclusions

This study accounts for the evaluation of the impact of chemotherapy on the oxidative stress status of metastatic breast cancer patients and its influence on their clinical outcome. It was found that chemotherapy significantly decreases the activity of the DNA repair enzyme KU86 and increases the total antioxidant capacity of the plasma. These observations and the potential deleterious effect of a higher level of oxidative stress on the evolution of this disease are in accordance with the significant influence of increased KU86 activity on the improvement of the DFS rates achieved by metastatic breast cancer patients receiving anthracycles-based chemotherapy. Deeper analysis of these results and their comparison with those previously obtained by our group in other treatment settings, pointed out the probable importance of the cumulative oxidative damage achieved trough successive clinical interventions undergone by patients. From the results of this analysis it is clear that further studies focused on this topic are needed in order to further elucidate the antioxidant and repair molecular mechanisms which improve patient's survival rates and reduce toxicity. New targeted and personalized therapies according specific characteristics of cancer patients may arise from future studies based on these results.

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Figure captions:

Figure 1.- Oxidative damage markers in metastatic patients, before and after chemotherapy. (A) Percentage of DNA in the tail of lymphocytes, as measured by the comet assay. (B) Plasma protein carbonyl levels. (C) TBARS production in the blood plasma fraction.

Figure 2.- DNA repair capacity in neoadjuvant, adjuvant and metastatic patients, before and after chemotherapy. (A) DNA repair activity of the KU86 enzyme. (B) DNA repair activity of the RPA enzyme. Intragroup statistical differences owed to chemotherapy are indicated as * (P<0.05).

Figure 3.- Trolox-equivalent antioxidant capacity of the plasma in metastatic patients before and after chemotherapy. Intragroup statistical differences owed to chemotherapy are indicated † (P<0.01). *TEAC*, trolox equivalent antioxidant capacity.