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ORIGINAL PAPER

RESISTANCE TO BREAST CANCER TREATMENT INDUCED BY TRANSPORT MECHANISMS

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Abstract. Breast cancer represents the most common form of cancer women develop. Although prognosis has significantly improved over the last years, due to increasing trends in early diagnosis and advanced treatment, the medical community still faces a series of severe problems. One of them is resistance to certain chemotherapy agents that constitutes the main cause of death in more than 90% of patients. Despite intense and thorough studies on resistance mechanisms, their relevance for the clinical practice continues to be unclear. The present paper aims to identify a possible resistance mechanism pattern to chemotherapy applied in breast cancers, which includes the attachment of cytostatics to albumin thiols, as a consequence of oxidized protein degradation. Given their chemical structure, these thiols may be responsible for the impossibility of cytostatics to bind to transport albumins, thus inducing therapeutic failures and implicitly resistance. The content of this article focuses on the measurement of albumin thiol levels in patients diagnosed with breast cancer that developed resistance after the first series of antitumor treatment. We also assessed the level of copper-carrying proteins, namely ceruloplasmin involved in oxidation-reduction reactions as well as the overall level of non-enzymatic endogenous antioxidants. The results reveal the occurrence of a significantly damaging oxidative stress that destroys the structure of transport proteins and, indirectly, can create resistance mechanisms.
Key words: Breast cancer, resistance to chemotherapy, transport mechanisms, oxidative stress, albumin thiols, ceruloplasmin, antioxidants

Introduction

According to WHO estimates (International Agency for Research on Cancer- EUCAN) for 2012, breast cancer is a major public health issue, known as the highest incidence rate neoplasia in women from Romania. Of all annually diagnosed forms of neoplasia - 25.22% (figure 1) represent breast cancer which also accounts for the largest mortality rate- 16.74% (figure 2).

The rough incidence for breast cancer in the European Union is 94.2/100.000 women (figura 3)while the mortality rate is 23.1/100.000 women (figura 4).

Despite the fact that prognosis for breast cancer has scaled up considerably during the last years, a high number of female patients still presents an evolution in the disease. The increasing rate of incidence noticed until 2002, as a consequence of mammography introduction and uptake, tends to currently show a decreasing curve [1] also due to a lower use of postmenopausal oestrogen replacements. Even if the tendency in diagnosis increments in women over 50 (375/100.000) in comparison with women under 50 (42.5/100.000), approximately 23% of breast cancers are found in women under this age because this group of population corresponds to 73% of the overall feminine population. [2]

The treatment of the metastatic disease is a challenge for any clinician because many patients are susceptible to therapeutic failure and subsequently to death due to malignancy. In over 90% of patients with metastatic breast cancer resistance to various chemotherapy agents is the main cause of therapeutic failure. [3]

Even if resistance mechanisms have been identified and clarified in cell cultures they are less clear in the clinical practice (Cimoli et. Al 2004,

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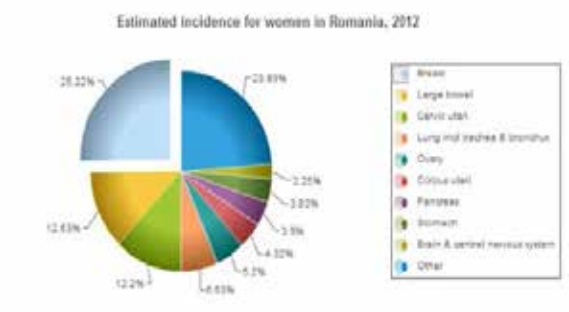

Figure 1. Cancer incidence for women in Romania

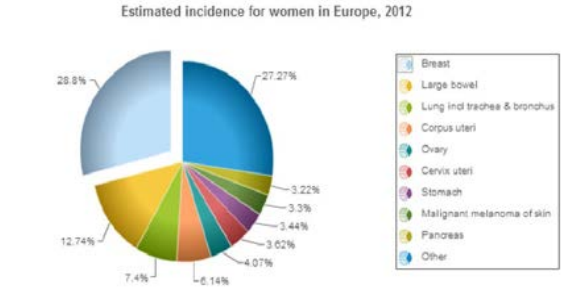

Figure 2. Cancer mortality for women in Romania

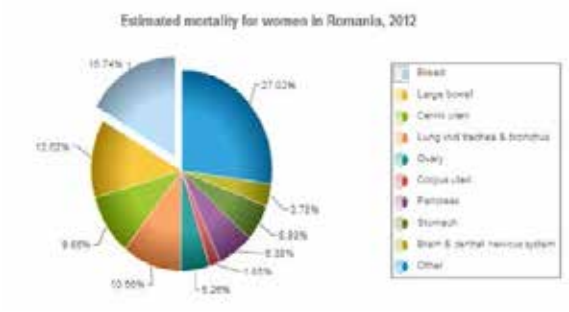

Figure 3. Cancer incidence for women in Europe

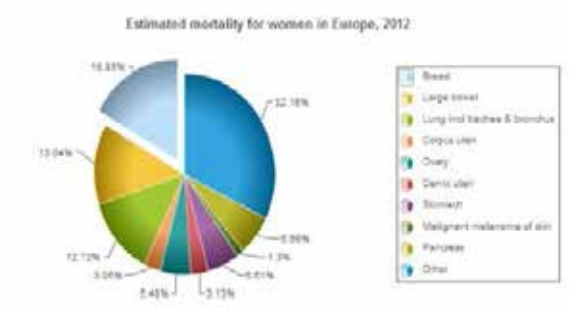

Figure 4. Cancer mortality for women in Europe

Uggla et. Al 2007). Resistance to chemotherapy may appear either prior to treatment administration (primary or innate resistance) or develop in time, after the exposure to a chemotherapeutic agent (acquired resistance). [4] Chemoresistance represents a major limitation for treatment and leaves few effective options [5]. Both innate and acquired resistance to taxane and anthracycline are common, without distinct mechanisms. [6] The most known in vitro mechanism that detects resistance to more than one class of chemically related agents (multidrug resistance) is the overexpression of efflux proteins. The most frequent drug efflux are members of the ATP binding cassette family (ABC): P-glycoprotein Pgp, also known as multidrug resistant protein – MDR or ABCB1, multidrug resistance associated protein 1- MRP-1 also named ABCG, while in breast cancer the resistance protein is BCRP or ABCG2. ABC cassette substrates transporter includes a diverse range of compounds, many of them being structurally independent. These proteins shield the cells and tissue by exporting toxins, including anticancer agents from cells in normal tissue as well as from cancer cells. [7]. Generally, ABCB1 transports large hydrophobic compounds, while ABCC1 and ABCG2 transport both hydrophobic drugs and large non ionic compounds. In relation to breast cancer, ABC proteins were previously involved in resistance to taxane and doxorubicin.

Remission failure and the development of drug-resistant cancer following antiangiogenic therapy were mostly linked to the unintentional induction of a hypoxic tumour microenvironment. [8][9] Estimates reveal that for 40-50% of breast cancer cases, the tumour site is in a hypoxic environment. [10] Hypoxia inducible factor (HIF1a), often detected within solid tumours in response to hypoxia leads to angiogenesis in order to form new blood vessels, necessary to tumour growth. [11] [12] HIF1a may also be activated by nitric oxide, cytokines, growth factors, oncogene expression or by mutations in tumour suppressors. [13]

The present paper aims to identify a possible resistance mechanism pattern to chemotherapy applied in breast cancers, which includes the attachment of cytostatics to albumin thiols as a consequence of oxidized protein degradation. It is well known that tumour tissues produce excess endogenous antioxidants, especially metallothionein and sulphur proteins that once entered in circulation may interact with oxygen reactive species and suffer oxidation-reduction reactions (redox) which eventually can cause excess thiols production. Given their chemical structure, these thiols may be responsible for the impossibility of cytostatics to bind to transport albumins, thus inducing therapeutic failures and implicitly resistance. The content of this article focuses on the measurement of albumin thiols levels in patients diagnosed with

breast cancer that developed treatment resistance. We also assessed the level of copper-carrying proteins, namely ceruloplasmin involved in redox reactions as well as the overall level of non-enzymatic endogeneous antioxidants.

Material and methods

During January 2011 and January 2014 we observed 40 female patients, diagnosed with metastatic breast cancer who received cytostatic drugs for metastatic disease in the Oncology Institute "Prof Al Trestioreanu", Bucharest - Medical Oncology Department I.

Inclusion criteria:

1. Age: >18
2. Histological confirmation of breast cancer
3. Presence of metastatic diseases
4. Immunohistochemical testing
5. Signed informed consent for the introduction of their data in the study
6. The patients received adjuvant cytostatic therapy or treatment for the metastatic disease and presented disease progression in less than six months from the last chemotherapy session or during chemotherapy.

Exclusion criteria:

1. Low IPECOG>3 performance status; serum biochemical markers: bilirubin >1.5X normal values, AST, ALT >2.5X normal values except for hepatic metastases where a value <5X normal values is accepted, CBC when taken into records with ANC (absolute neutrophil count)<1500/mm^3, T <100.000/mm3, Hb <8g/dl
2. Patients with no pathology confirmation of mammary neoplasia
3. Patients with no metastatic disease
4. Patients with different forms of cancer developed during the last 5 years
5. Patients whose disease didn't progress in less than 6 from the last adjuvant chemotherapy or therapy for metastatic disease.

The biological sample used in the study was the serum we collected from our patients subsequent to their signing the informed consent, also approved by the Ethical Committee in Oncology Institute "Prof Al Trestioreanu", Bucharest. Based on the 5th exclusion criterion but following all the inclusion criteria we constituted a control group that had to be as homogeneous as possible, with no resistance mechanisms, from which we collected the same biological samples.

Measurement tests for plasma SH groups rely on the ability to develop a colour complex, measurable spectrophotometrically, with maximum absorbance at 412 nm to acid reaction *5, 5-dithiobis-2-nitrobenzoic acid* (DTNCB), at room temperature.

Normal values range from 370-450 μmol/l. Increased values correlate with an excess production of thiols that represent the result of degradation of oxidized proteins. These thiol groups are highly reactive and through their electronic nature have the ability to attach easily to any circulatory compound whose structural core contains electronic metal groups (e.g. platinum).

Total antioxidants

The used method relies on the serum's ability to reduce iron. At low pH, Felll complex- tripiridil-triazine (Felll –TPTZ) is reduced to the ferrous form with a new complex that develops an intense, measurable blue colour, with a peak of absorption of 539 nm. Any reaction with a potentially positive redox under the mentioned conditions may lead to Felll –TPTZ complex reduction. Using an excess Fe, the limiting factor of Felll –TPTZ complex and of colour formation represents the sample's reducing ability. The reaction measures the reduction of ferric ion complex-2,4,6-tri (2-piridil)-1,3,5- triazine (TPTZ) to a coloured product.

The reaction mixture is prepared on the spot and contains 25 ml of acetate buffer, 2, 5 ml of TPTZ solution and 2, 5 ml of FeCl3.

The reaction is monitored for 4 minutes, focusing on optical density to 593 nm. Results are obtained by multiplying the read absorbance value by a M40 spectrophotometer, with an index correction that takes into account the concentration of used solutions, the optical path, size of cuvettes, etc. Literature data [14] indicate as normal values for this serum type of test: 0,9-1,4 mmol/l. Elevated values may be due to the mobilization of antioxidant protection systems in response to oxidative stress, installed as a disease consequence or due to a diet rich in antioxidants, the patient chooses to adopt after the diagnosis disclosure.

Ceruloplasmin

Ceruloplasmin is a blue colour protein with oxidase activity on polyamines, polyphenols and inorganic ferrous ions (Fe 2+). However, the biologic substratum is Fe2+, ceruloplasmin having the highest affinity for it. The catalytic oxidation of Fe2+ or complexes containing Fe2+ is called ferroxidase activity. There are several ways to determine ferroxidase activity in ceruloplasmin, from which we opted for the Ravin method: p-phenylene diamine reaction in an acetate-acetic acid buffer. Ceruloplasmin carries a complex series of roles, among which: copper ion transport, oxidation of organic amines, Fe2+ oxidation to Fe3+ after its release from transferrin and ferritin, antioxidant activity against lipid peroxidation, endogenous modulation of the inflammatory response, stimulation of cell proliferation and angiogenesis [15]. Ceruloplasmin is an acute phase protein, with an intermediate amplitude response

to other proteins in acute phases, whose level auguments 2-3 times in inflammations, pregnancy, traumas, and surgeries. CP seric concentration is a useful clinical indicator of Wilson's disease. Each CP molecule contains 6 or 7 copper atoms, this being the state for 90-95% of serum copper [16].

The serum sample is incubated for 30 minutes with a known amount of ferric ion in a 0, 2 M environment to 5, 5 ph and a 37 °C temperature. The reaction is stopped with a 0,5% sodium azide solution. The coloured product is quantitatively measured through spectrophotometry, by reading absorbance 540 nm from a control sample. Values are expressed in U.I. The enzyme quantity that converts 1 substrate ***µmol*** per minute is defined as 1 ceruloplasmin unit. Normal values vary between 80-120 U.I. ceruloplasmin.

Results

The first phase was to establish the total values of serum thiols in patients with constant evolution of the disease, during chemotherapy drug treatment compared to patients who developed no treatment resistance.

As Table no. 1 shows, the mean value increased

Control group	Patients who developed resistance
460 µmol/l	547 µmol/l

Table I. Mean levels of thiols in investigated patients

(Normal values: 370-450 µmol/l)

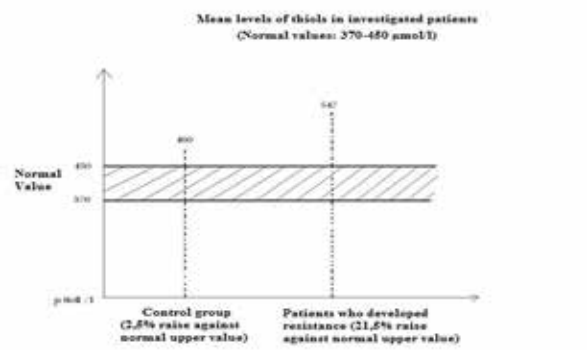

Figure 5. Mean levels of thiols in investigated patients

in both groups. Compared to the accepted maximum limit, the value increase in the control group is 2,2% while in the resistance group 21,5% (figure 5). This enables us to conclude that in the latter group resistance mechanisms can occur due to low transport capacity, where the albumins underwent a supplementary oxidative degradation and no longer have the means to attach and transport chemotherapy agents as effectively as in the non-resistance group.

The obtained values entitle us to determine redox reactions, measurable through the serum's ability to reduce iron. These reactions cause reactive degradation species, responsible for thiol production.

Control group	Patients who developed resistance
1.53 mmol/l	1.60 mmol/l

Table II. Mean value of oxidation-reduction reactions- responsible for thiol production, assessed through FRAS technique
(Normal values: 0,9-4mmol/l)

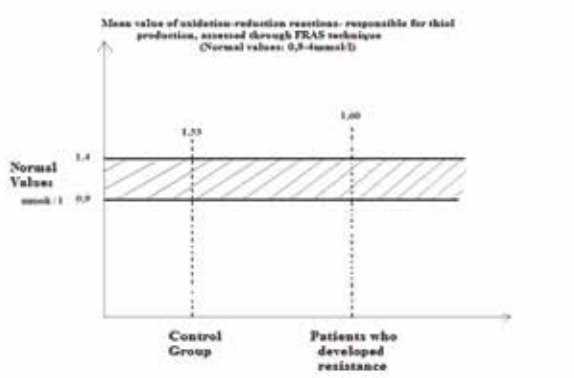

Figure 6. Mean value of oxidation-reduction reactions -responsible for thiol production, assessed through FRAS technique

Control group	Patients who developed resistance
114 U.I.	122 U.I.

Table III. Values of ceruloplasmin activity in patients with breast cancer

(Normal values 80-120 U.I.)

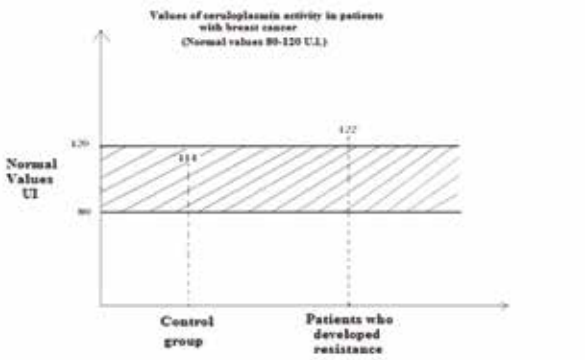

Figure 7. Values of ceruloplasmin activity in patients with breast cancer

In table no. 2 the results reveal a 14% increase in the resistance group compared to the accepted maximum limit, which translates into intensified oxidation processes bred by the tumour mass and its reactivity, taking into account that the tumour tissue induces oxidative stress.

In order to complete the redox panel we also determined the ferroxidase activity in serum ceruloplasmin from the same biological samples.

For the control group we observed values within normal limits while in the group with apparent resistance we noticed a light increase that can be the result of a minor intensification which we believe initiates resistance mechanisms within the transport of chemotherapy agents.

Conclusions

Chemoresistance plays an important role in the weak response and low rate of survival of patients with advanced or metastatic breast cancer. It is both a challenging and devastating phenomenon for oncologists and patients, since it entails various complex mechanisms. Understanding these mechanisms is crucial for the improvement of chemotherapy agents' use, especially of taxanes and anthracyclines in breast cancer. Despite numerous clinical and experimental trials, many questions remain unanswered to date.

A hypothesis is that the excess of one of ABC transporter proteins cannot provide sufficient resistance to chemotherapy, with the possible involvement of other mechanisms that trigger resistance. [17] The clinical relevance of combination chemotherapy to diminish the induction of resistance to chemotherapy agents becomes clear and requires taking into consideration those mechanisms that rely on stress induction as main objective, especially hypoxia. Aggressive cancers may even use the agent in the process of angiogenesis transcription, which ultimately generates the growth of multiple tumour cells, resistant to treatment. Recent studies have demonstrated that the combination between angiogenesis and HIF1 inhibitors can produce better results than using each agent, alone. The occurrence of reactive to oxygen species in low concentration may induce a series of signalling events that create an angiogenic profile of the tumour tissue, ultimately its oxygenation leading to a devastating oxidative stress that destroys the structure of transporter proteins and indirectly, initiates resistance mechanisms.

A thorough understanding of resistance to chemotherapy agents, as well as its inherent processes, engendered either through innate mechanisms or as response to antitumor treatment will open the way to a rational design of targeted therapies, with a maximal effect on tumour cells and the limitation of side effects.

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