Original research

Plasma thiobarbituric acid reactive substances predicts survival in chemotherapy naïve patients with metastatic urothelial carcinoma

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ABSTRACT

Article info

Oxidative stress plays a significant role in development and progression of cancer, including urothelial carcinomas. TBARS (Thiobarbituric acid reactive substances) represents a marker of oxidative stress increased in various diseases. In this prospective study, we tested the hypothesis of plasma TBARS concentration and correlation with survival in chemotherapy naïve MUC (metastatic urothelial carcinoma) patients. Most of subjects (N = 65) were treated with gemcitabine and cisplatin (GC) chemotherapy. Performance status ECOG ≥ 2 had 11 patients, visceral metastases were present in 43. Based upon the mean of plasma TBARS, subjects were dichotomized into low and high groups. Progression-free survival (PFS), overall survival (OS) and their 95% CI were estimated by Kaplan-Meier method and compared by log-rank test. At median follow-up of 9.6 months, 65 patients experienced progression and 64 died. Subjects with low TBARS had significantly better PFS (HR 0.51) and OS (HR 0.44) opposed to high TBARS. Patients with low TBARS had significantly higher rate of neutropenia G4 and less liver involvement. High TBARS correlated with BMI above 30 kg/m². Performance status and plasma TBARS were proven to be independent predictors of PFS and OS. In this study, high TBARS in MUC patients were associated with poor survival, likely due to more aggressive disease activity as reflected in increased liver involvement. Therefore, this biomarker could be used in clinical practice for early identification of patients with worse prognosis, better patient stratification, and treatment decision making.

Introduction

Bladder cancer is the most common malignancy of the urinary tract and due to its poor prognosis and highest recurrence rates compared to any cancer, it imposes a great public hazard and substantial health-care burden [1]. Worldwide, it is the 9th most common cancer with crude incidence of 20.4/100000 in men [2]. Transitional cell subtype is seen in 90% of all cases, the rest 10% is comprised of squamous-cell carcinoma and adenocarcinoma. In general, transitional cell carcinoma is classified as non-muscle invasive bladder cancer (NMIBC) stage pT1 or pT2 and muscle-infiltrating bladder cancer (MIBC) stage pT2 or more. Approximately 20–30% of patients at the time of diagnosis present with MIBC and with or later develop metastasis, currently treated mostly by a standard gemcitabine-cisplatin chemotherapeutic regimen [3].

Urothelial cancer development is a multifactorial process ranging from genetic to environmental stimuli like tobacco smoke, heavy metals and other xenobiotics [4]. Some of these factors, e.g. smoking, are able to activate immune system cells and therefore induce chronic inflammation, which promotes the development of oxidative stress [5].

Oxidative stress is an imbalance between the production and elimination of reactive oxygen species (ROS). ROS are responsible for alteration of macromolecules. To prevent oxidative damage, cells possess various antioxidant defense mechanisms such as superoxide dismutase (SOD) and catalase.

Various mechanisms have the ability to affect ROS formation, such as aberrant metabolism of cancer cells, activation of oncopogenes, mitochondrial dysfunction or loss of p53 function [6]. Chronic irritation or inflammation, infections or cytokines and growth factors also increase ROS formation in

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cancerous tissues [7]. Low levels of ROS can have beneficial effects on cell processes such as pathogen killing or tissue repair [8]. However, increased levels of ROS can lead to damage of DNA, lipids, and proteins, which can result in tissue oxidative damage, death or subsequent cancer development [9]. Oxidative stress effects on cancer initiation, promotion, and progression are well-known [10]. High levels of ROS are cytotoxic, but during the course of carcinogenesis, cancer cell are able to develop mechanisms to evade cell death caused by ROS and subsequently, these mechanisms help cancer cells to develop resistance to treatment and to increase their survival in hypoxic environments [11].

Lipids are major components of cell membranes and play an important role in cell membrane stabilization and signal transduction [12]. Lipid peroxidation defined as oxidative deterioration of polyunsaturated fatty acids (PUFAs), is a process, which can damage DNA and help in cancer development [13]. Reactive aldehydes such as 4-hydroxy-2-nonenal, acrolein, and malondialdehyde (MDA), can affect cell proliferation through formation of DNA-DNA or DNA-protein crosslinks, which can result in replication errors, mutations and genomic instability, if not repaired before DNA replication [14]. MDA is the end-product of lipid peroxidation of PUFAs and is used in the TBARS (Thiobarbituric acid reactive substances) assay as a good indicator of oxidative stress. In healthy population the physiologic values of TBARS are equal to or less than 4.5 μmol.L⁻¹.

The objective of this prospective study was to explore the prognostic value of TBARS measured before first-line chemotherapy in MUC patients. We hypothesized that elevated TBARS levels before the initiation of systemic treatment may affect the activity of the disease, as well as alter the efficacy of chemotherapy used in this setting. Finding new potential biomarkers is helpful for better prognostic assessment before initiation of treatment. Biomarkers should be easily measurable and overall easily assessed. Such new biomarkers would help clinicians to better stratify patients before treatment and/or offer them early clinical trial inclusion. There is a lack of prognostic biomarkers in MUC patients.

Patients and methods

This study was approved by the Ethical Committee of the National Cancer Institute (NCI) in Bratislava, Slovakia and carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Study patient population

From May 2010 to April 2014, 72 consecutive MUC patients from National Cancer Institute (NCI) in Bratislava were prospectively enrolled into this study after informed consent signing. Pathologic, clinical, and radiologic data were collected by physicians into electronic data files and their accuracy was validated for each patient by an independent investigator.

Patients were eligible if they had histologically proven urothelial carcinoma of bladder, renal pelvis, ureter or urethra, and additionally, creatinine clearance of more than 30 mL/min, hepatic function SGOT less than 1.17 U and serum bilirubin less or equal to 25.7 μmol.L⁻¹. Adequate bone marrow function described as leucocytes 3,5 × 10⁹/L and platelet count 100 × 10⁹/L, hemoglobin above 90 g/dL. Patients with prior systemic therapy assessed. Such new biomarkers would help clinicians to better stratify patients before treatment and/or offer them early clinical trial inclusion. There is a lack of prognostic biomarkers in MUC patients.

The concentrations of TBARS were calculated using MDA as a reference value of TBARS measured before first-line chemotherapy in MUC patients.

The common type of chemotherapy administered in concordance with good clinical practice were cisplatin (70 mg per m² day 1) and gemcitabine (1000 mg per m² days 1 and 8, new course on day 22) in 65 patients (90%) and carboplatin AUC 5 (day 1) and gemcitabine (1000 mg per m² days 1 and 8, new course on day 22) in 6 patients (8%). Dose adjustments were adopted according to toxicities or other relevant medical conditions.

**Plasma isolation**

Peripheral blood samples (12 mL) were collected from all participants enrolled into this study. Samples were collected into Vacutainer® EDTA Blood Collection Tubes (BD Biosciences, Franklin Lakes, NJ, USA) at baseline in the morning on day 0 or day 1 before the first dose of the first line chemotherapy. All plasma samples were frozen down to − 80 °C and stored for maximum 28 days. Median retention time of frozen samples was 21 days (range 14–28). Median samples analyzed per one TBARS assay experiment was 5 (range 2–7). Patient blood samples were centrifuged at 1000g for 10 min at room temperature within 2 h of venipuncture. To avoid cellular contamination, plasma was carefully harvested and centrifuged again at 1000g for 10 min at room temperature. The cell-free plasma samples were cryopreserved at − 80 °C and further processed in Pharmacobiocchemical Laboratory of the 3rd Department of Internal Medicine, Faculty of Medicine, Comenius University (FMCU) in Bratislava, Slovakia.

**Measurement of TBARS**

For oxidative stress assessment the TBARS assay was used (OxiSelect TBARS Assay Kit). TBARS were determined from plasma. Briefly, 100 μL of plasma was mixed with 1 mL of 0.67% thiobarbituric acid (TBA), 1 mL 20% trichloroacetic acid, and 1.5 mL 0.04% butylated hydroxytoluene (BHT) in test tubes. The mixtures were incubated in a boiling water bath for 20 min. After cooling to room temperature, the reaction mixture was centrifuged at 4000g for 10 minutes and the absorbance of the supernatant was measured at 532 nm using the same UV–visible Spectrophotometer. The concentrations of TBARS were calculated using MDA as a reference standard [15].

**Statistical analysis**

Based on the mean of TBARS of 6.06 μmol.L⁻¹, patients were dichotomized into low < 6.06 μmol.L⁻¹ (N = 35) and high ≥ 6.06 μmol.L⁻¹ (N = 37) groups. Progression-free-survival (PFS) and overall survival (OS) were calculated from the initiation of the first-line chemotherapy administration to progression or death, and subsequently were calculated with their survival in hypoxic environments [11].
proportional 95% CI by the Kaplan-Meier method and compared with log-rank test. A multivariate Cox proportional hazards analysis was used to evaluate prognostic parameters with respect to the risk of death or progression. For statistical assessment NCSS 2019 software was employed [16].

Results

Association between TBARS and patient/tumor characteristics

The patient characteristics and their proper associations are shown in Table 2. Subjects with neutropenia grade 4 (G4) after chemotherapy, had significantly decreased levels of TBARS measured in blood plasma (4.70 vs. 6.16 μmol.L⁻¹, P = 0.02). Patients with liver metastasis had significantly higher TBARS levels in plasma compared to patients without liver metastasis (6.60 vs. 5.88 μmol.L⁻¹, P = 0.03). Patients with TBARS above mean had significantly higher BMI (7.07 vs. 5.0 μmol.L⁻¹, P = 0.02). No other associations between TBARS and characteristics of patients or tumor were found.

Table 2

| TBARS (μmol.L⁻¹) | N  | Mean  | Median  | SD  | SEM  | P value |
|------------------|----|-------|---------|-----|------|---------|
| ECOG             | 2  | 6.06  | 6.15    | 1.28| 0.15 | NA      |
| 0                | 22 | 5.63  | 5.36    | 1.03| 0.26 | NA      |
| 1                | 39 | 6.13  | 6.16    | 1.29| 0.20 | 0.10    |
| 2                | 9  | 6.50  | 6.84    | 1.42| 0.41 | 0.41    |
| 3                | 2  | 7.61  | 7.61    | 1.78| 0.88 | 0.88    |
| Toxicity         |    |       |         |     |      |         |
| Anemia G3        | 59 | 6.07  | 6.16    | 1.28| 0.17 | 0.66    |
| No               | 13 | 6.01  | 5.38    | 1.29| 0.36 | NA      |
| Yes              | NA | NA    | NA      | NA  | NA   | NA      |
| Anemia G4        | 69 | 6.09  | 6.17    | 1.27| 0.15 | 0.21    |
| No               | 3  | 7.07  | 6.43    | 1.17| 0.73 | 0.16    |
| Yes              | 65 | 6.01  | 6.26    | 1.56| 0.21 | 0.16    |
| Neutropenia G3   | 50 | 6.11  | 6.50    | 1.33| 0.18 | 0.64    |
| No               | 22 | 5.96  | 5.73    | 1.17| 0.27 | 0.02    |
| Yes              | 67 | 6.16  | 6.16    | 1.22| 0.15 | 0.02*   |
| Neutropenia G4   | 5  | 4.70  | 4.26    | 1.31| 0.55 | 0.02    |
| Toxicity         |    |       |         |     |      |         |
| Alopecia G3      | 65 | 6.10  | 6.16    | 1.28| 0.16 | 0.38    |
| No               | 7  | 5.68  | 5.12    | 1.25| 0.48 | NA      |
| Metastasis sites |    |       |         |     |      |         |
| Lymph nodes N    | 4  | 6.34  | 6.26    | 1.11| 0.64 | 0.57    |
| N+               | 68 | 6.05  | 6.15    | 1.29| 0.16 | 0.57    |
| Skeletal         | 48 | 5.95  | 5.64    | 1.35| 0.18 | 0.26    |
| Present          | 24 | 6.29  | 6.32    | 1.10| 0.26 | 0.03    |
| Pulmonary        | 47 | 6.08  | 6.16    | 1.16| 0.19 | 0.51    |
| Absent           | 25 | 6.02  | 5.6     | 1.49| 0.26 | 0.03    |
| Present          | 54 | 5.88  | 5.64    | 1.25| 0.17 | 0.03*   |
| Present          | 18 | 6.40  | 6.77    | 1.22| 0.29 | 0.037   |
| Lungs and liver (either) | 38 | 6.03  | 6.01    | 1.24| 0.21 | 0.043   |
| Absent           | 34 | 6.10  | 6.15    | 1.33| 0.22 | 0.03    |
| Present          | 62 | 6.05  | 6.15    | 1.30| 0.16 | 0.62    |
| Present          | 9  | 6.30  | 6.56    | 1.13| 0.43 | 0.06    |
| Peritoneum       | 60 | 6.16  | 6.16    | 1.32| 0.16 | 0.16    |
| Absent           | 12 | 5.58  | 5.62    | 0.94| 0.37 | 0.043   |
| Present          | 35 | 5.00  | 5.23    | 0.62| 0.13 | 0.0001* |
| BMI              | 37 | 7.07  | 6.77    | 0.84| 0.12 | 0.001   |

TBARS: Thiobarbituric acid reactive substances; ECOG: Eastern Cooperative Oncology Group; SD: standard deviation; SEM: standard error of mean; NA: not applicable.

*a means statistically significant.

Prognostic values of TBARS on progression-free survival and overall survival

The median follow-up was 9.6 months (range: 0.26–114.54), the median PFS was 5.42 months (range: 0.26–114.54), and the median OS was 9.6 months (range: 0.26–114.54). Patients with TBARS levels <6.06 μmol.L⁻¹ had a median progression-free survival of 7.7 vs. 4.3 months compared to patients with TBARS levels ≥6.06 μmol.L⁻¹. HR 0.51; 95% CI 0.31–0.84; P = 0.006 (Fig. 1A and Table 3). Statistically significant decrease in overall survival was observed in population of patients with increased levels of lipid peroxidation measured by the TBARS assay, HR 0.44, 95% CI 0.27–0.74; P = 0.0009 (Fig. 1B and Table 4), median survival time for patients with TBARS levels <6.06 μmol.L⁻¹ was 13.1 months, whereas median survival of the group of patients with TBARS levels ≥6.06 μmol.L⁻¹ was 6.9 months.

Multivariate Cox proportional hazards analysis for independent prognostic factors assessment

TBARS and ECOG were proven to be independent prognostic factors for OS and PFS, respectively, with hazard ratios for TBARS of HR 1.70, CI 1.00–2.90; 2.00, P = 0.04 for PFS and HR 2.00, CI 1.16–3.46; P = 0.01 for OS. For ECOG HR: 4.42, CI 2.05–9.52; P = 0.0001 for PFS and HR: 5.78, CI 2.42–13.84; P = 0.0001 for OS (Table 5 and Table 6).

Discussion

Oxidative stress is induced by an imbalance between pro- and antioxidative mechanisms. ROS have the ability to affect membrane bilayers and cause lipid peroxidation of polyunsaturated fatty acids (PUFAs) leading to formation of molecules such as MDA, hexanal, 4-hydroxynonenal, which have the ability to locally react with macromolecules leading to alteration of their functions. If ROS levels are increased enough and antioxidant mechanisms become overwhelmed, ROS irreversibly damage DNA, lipids, proteins, which in turn leads to genetic and epigenetic alterations that drive tumorigenesis. Signaling pathways such as the epidermal growth factor receptor signaling pathway, nuclear factor erythroid 2-related factor 2, the mitogen-activated protein kinases MAPK/ERK, phosphatidylinositol-3-kinase, phospholipase C, and protein kinase C, p53 are altered by oxidative stress [17].

As was shown in previous studies conducted on this topic, oxidative stress plays a major role in cancer development and subsequently in cancer progression and dissemination [9,18]. Moderate levels of oxidative stress stimulate progression and dissemination of tumors however, intrinsically increased levels of ROS in cancer cells, which are necessary for maintaining their proliferation, makes them also more susceptible to ROS induced death, when further increasing the levels of ROS above their threshold. This finding can be exploited in cancer treatment because some cytotoxic drugs, such as cisplatin was recently shown not only to kill cancer cells through formation of DNA adducts, but also through increase in ROS formation and lipid peroxidation, which alters enzymes and structural proteins, and directs a cell-to-cell apoptotic pathway [19].

One of the main issues with chemotherapeutic treatments is its toxicity. Cisplatin, a gold standard in MUC patient treatment, expresses its toxicity through generation of DNA adducts but partly also through oxidative stress. In this study, subjects with neutropenia grade 4 after platinum-derived combined chemotherapy had lower levels of TBARS measured in plasma compared to patients without neutropenia grade 4 toxicity. This finding might be explained by reduced neutrophil numbers, which are significant oxidative stress producers in a human body [20].

In this prospective study, MUC patients with high baseline levels of TBARS had significantly shortened PFS and OS (Fig. 1A and B). This finding can be explained by an increased activity of this malignant disease
expressed in the increased levels of TBARS. As it was shown in some trials, presence of visceral metastases is associated with negative prognosis, either combined (liver and lung) or as single site metastases [21,22]. In this prospective study, there was a trend toward worse prognosis in patients with presence of lung and liver involvement, however presence of visceral metastases was not shown to be an independent prognostic factor and did not significantly affect OS and PFS. The lack of statistical significance is probably due to insufficient number of subjects enrolled into this trial.

However, in this study population, a significant association between TBARS levels and presence of liver involvement was found (Table 2). To

**Table 3**

Univariate analysis for progression-free survival (PFS).

| Variable        | N  | Median | 95% CI Low | 95% CI High | HR  | 95% CI Low | 95% CI High | P value |
|-----------------|----|--------|------------|-------------|-----|------------|-------------|---------|
| TBARS (μmol.L⁻¹) |    |        |            |             |     |            |             |         |
| ≥ 6.06 μmol.L⁻¹ | 37 | 4.3    | 2.3        | 6.0         | 0.51| 0.31       | 0.84        | 0.006*  |
| < 6.06 μmol.L⁻¹ | 35 | 7.7    | 4.8        | 9.9         |     |            |             |         |

TBARS: Thiobarbituric acid reactive substances.

* means statistically significant.
our best knowledge, this is the first study which associated TBARS with liver metastasis and this finding further supports the prognostic value of increased TBARS levels. Plasma TBARS determination method applied in this study has some limitations, mostly in the term of the lack of specificity. Factors, as increased body mass index (BMI) may confound the results. In this trial, association between increased BMI and increased TBARS levels (Table 2) did not affect the prognostic value of TBARS when adjusting multivariate analysis for BMI (Table 5 and Table 6). TBARS concentrations in plasma were reported to modestly be increased with age and certain vitamin supplementations [23]. However, age of patients enrolled in this study was approximately homogeneous and most of the patients were men.

In conclusion, based on data obtained in this study, chemotherapy naïve MUC patients with increased TBARS had worse prognosis in terms of shorter PFS and OS, respectively. Measurement of plasma TBARS at baseline might be useful for early identification of patients who are candidates for different treatment options within or out of clinical trials. The authors speculate that therapeutic targeting of signaling pathways involved in regulations of oxidative stress could contribute to increase in effectivity and/or reduce the toxicity of current platinum-based treatments in metastatic urothelial carcinomas.

CRediT authorship contribution statement

Slopoposky, J: Data accuracy validation, statistical analysis, manuscript writing; approved the final version of the manuscript to be published.
Kucharska, J: Performed analysis of TBARS and calculated obtained data; approved the final version of the manuscript to be published.
Obertova, J: Enrollment of patients, data collection; approved the final version of the manuscript to be published.
Mego, M: Enrollment of patients, manuscript reviewing; approved the final version of the manuscript to be published.
Kalavka, K: Performed sample collection and processing; approved the final version of the manuscript to be published.
Cingelova, S: Statistical analysis; approved the final version of the manuscript to be published.
Svetlovska, D: Data accuracy processing and validation; approved the final version of the manuscript to be published.
Gvozdkajakova, A: Rewieved manuscript; approved the final version of the manuscript to be published.
Furka, S: Data accuracy processing and validation; approved the final version of the manuscript to be published.
Palacka, P: Study design, enrollment of patients, data collection and processing, statistical analysis, manuscript writing and reviewing; approved the final version of the manuscript to be published.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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