TBARS - a Marker of Lipid Peroxidation Predicts Survival in Patients with Metastatic Urothelial Carcinoma

[1,2]Jan Slopovsky, [3]Jarmila Kucharska, [1,2]Jana Obertova, [1,2]Michal Mego, [1,2]Katarina Kalavska, [2]Silvia Cingelova, [3]Anna Gvozdjakova, [1,2]Patrik Palacka

[1]2nd Department of Oncology, Faculty of Medicine, Comenius University, Bratislava, Slovakia
[2]National Cancer Institute, Bratislava, Slovakia
[3]Pharmaco-Biochemical Laboratory of the 3rd Department of Internal Medicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia

Correspondence: pal_patrick@yahoo.co.uk

Abstract

Background: Oxidative stress plays a significant role in cancer development. TBARS – a marker of oxidative stress is increased in various diseases. Oxidative stress plays a role in bladder cancer development and in cytotoxic effects of chemotherapy. Therefore, in this prospective study we evaluate the prognostic of TBARS in metastatic urothelial carcinoma (MUC) patients before first-line chemotherapy.

Methods: From May 2010 to April 2014, 72 MUC (58 bladder, 14 upper genitourinary tract) patients of whom 57 (79.2%) were men were enrolled into this study. Most of the patients 65 (90.3%) were treated with GC (Gemcitabine, Cisplatin) chemotherapy, carboplatin was used in 6 (8%) patients. Performance status ECOG ≥ 2 had 11(15.2%) patients, visceral metastases were present in 34 (47.2%) patients. Based upon TBARS (Thiobarbituric acid reactive substances) measured in blood plasma samples using the TBARS assay, patients were dichomized according to mean (6.06 μmol.L^{-1}), into low < 6.06 μmol.L^{-1} (N=35) and high > 6.06 μmol.L^{-1} (N=37) groups. Progression-free survival (PFS), overall survival (OS) and their 95% CI were estimated by Kaplan-Meier method and compared by log-rank test.

Results: At median follow-up of 9.6 months, 65 (90%) patients experienced progression and 64 89%) patients died. Patients with low TBARS had significantly better progression-free and overall survival opposed to patients with high TBARS with hazard ratios (HR) of 0.51; 95% and 95 % confidence intervals (CI) 0.31-0.84; *P* = 0.006 for PFS HR 0.44, 95% CI 0.27-0.74; *P* = 0.0009 for OS, respectively). Patients with increased TBARS had significantly higher presence of liver metastases 6.60 vs. 5.88, *P=0.03*. Also, association between high TBARS levels and BMI was found (7.07 vs. 5.0, *P=0.02*). ECOG and TBARS were proven to be independent predictors of PFS and OS.

Conclusion: In this study, high levels of plasma TBARS in MUC patients before first-line chemotherapy were associated with poor survival most likely due to increased disease activity and higher number of liver metastasis. Therefore, this biomarker could be used for identification of patients with worse prognosis and could lead to better patient stratification and treatment decision making.

Key Words: Metastatic Urothelial Carcinoma – Platinum-Based Chemotherapy – Lipid

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 20.4/100 000 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 pTa or pT1 and muscle-invasive bladder cancer (MIBC) stage pT2 or more. Approximately 20-30% of patients are at the time of diagnosis present with 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 oxidative stress development[5].

Oxidative stress is an imbalance between the production and the 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), catalase ect. Various mechanisms affect elevated ROS formation such as abberant metabolism of cancer cells, activation of oncogenes, mitochondrial dysfunction or dysfunctional loss of p53 function[6]. Chronic irritation or inflammation, infections, cytokines and growth factors also increase ROS formation in 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 most importantly 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 process[14]. MDA is the end-product of lipid peroxidation of PUFAs and is used in the TBARS assay as a good indicator of oxidative stress.

The objective of this prospective study was to explore the prognostic value of TBARS in metastatic urothelial carcinoma (MUC) patients, measured before first-line chemotherapy administration. We hypothesized that elevated TBARS levels before the initiation of systemic treatment may affect the activity of the disease as well as alter the effectivity of chemotherapy used in this setting.

Patients and Methods

Study Patients

From May 2010 to April 2014, 72 consecutive MUC patients from National Cancer Institute (NCI) in Bratislava, who signed informed consent, were prospectivelly enrolled into this study. Pathologic, clinic, 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 or adenocarcinoma of the bladder. Aditionally, creatinine clearance of more than 60 mL/min, hepatic function SGOT less than 1.17 U and serum bilirubin less or equal to 25.7 mol/L, adequate bone marrow function described as leucocytes 3,5 x 10^9/L and platelet count 100 x 10^9/L, Hemoglobin above 90 g/dL. Patients with prior systemic therapy, history of prior malignancy (except basal cell or squamous cell carcinoma of the skin), unresolved bacterial infection, or severe cardiovascular disease or pregnant were ineligible. Final data cutoff was October 4, 2019. At the time of final analysis, 65 (90%) of patients progressed and 64 patients (89%) died. Of all patients included in this study, 58 (81%) had bladder cancer and 9 (12%) upper genitourinary tract cancers. Most of the patients were men 57 (79%). Performance status according to Eastern Cooperative Oncology Group (ECOG) ≥ 2 had 11 (15%) patients and visceral metastases were present in 34 (47%) patients. Baseline characteristics are shown in **Table 1.** The most common type of

chemotherapy administered in concordance with good clinical practice were cisplatin (70 mg per m^2 day 1) and gemcitabine (1000 mg per m^2 days 1 and 8, new course on day 22) in 65 patients (90%) and carboplatin AUC 5 (day 1) and gemcitabine (1000 mg per m^2 days 1 and 8, new course on day22) in 6 patients (8%). Dose adjustments were adopted according to toxicities or other relevant medical conditions. This study was done according to Helsinki declaration guidelines for ethical principles for medical research. The study protocol was approved by the Ethical Committee of the National Cancer Institute (NCI).

Plasma isolation

Peripheral blood samples (12 ml) were collected from all participants enrolled into present 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 first line chemotherapy. Patients' blood samples were centrifuged at 1000 g for 10 min at room temperature within 2 h of venipuncture. To avoid cellular contamination, plasma was carefully harvested and centrifuged again at 1000 g for 10 min at room temperature. The cell-free plasma samples were cryopreserved at − 80 °C and further processed in Pharmaco-Biochemical Laboratory of the 3rd Department of Internal Medicine, Faculty of Medicine, Comenius University (FMCU), Bratislava, Slovakia.

TBARS measurement

For oxidative stress assessment the TBARS assay was used. TBARS were determid from plasma. Briefly, 100 µl of plasma was mixed with 1 ml of 0.67% TBA, 1 ml 20% trichloroacetic acid, and 1.5 ml 0.04% 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 min 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 high TBARS and low TBARS groups. Progression-free-survival (PFS) and overall survival (OS)

were calculated from the initiation of first-line chemotherapy administration, to progression or death and subsequently were calculated with their proper 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 used[16].

RESULTS

Association between TBARS and patients/tumor characteristics.

The patient characteristics and their proper associations are shown in **Table 6.** Patients with liver metastasis had significantly higher TBARS levels in plasma compared to patients without liver metastasis (6.60 vs. 5.88, P= 0.03). Patients with TBARS above mean had significantly higher BMI (7.07 vs. 5.0, P=0.02). No other association between TBARS and patient/tumor characteristics were found.

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 9.6months (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 with TBARS levels > 6.06 μmol.L^{-1}, with HR 0.51; 95% CI 0.31-0.84; P=0.006 **(Figure 1A, 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 **(Figure 1B, Table 5)**, 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.

A 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 risk 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, 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 2,4)**.

Discussion

Oxidative stress is induced by an imbalance between pro- and anti-oxidative mechanisms. ROS have the ability to affect membrane bilayers and cause lipid peroxidation of polyunsaturated fatty acids (PUFAs) leading to formation of free radicals such as MDA, hexanal, 4-hydroxynonenal, which have the ability to locally react with macromolecules leading to alteration of their function. If levels of ROS 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 to not only kill cancer cells through formation of DNA adducts, but also through increase in ROS formation and lipid peroxidation, which alteres enzymes and structural proteins and directs a cell-to-cell apoptotic pathway[19]. However, one of the main issues with chemotherapeutic treatments is its toxicity. Cisplatin-a gold standard treatment for MUC, expresses its toxicity through oxidative stress. In presence of hematologic toxicity the dose of chemotherapy is usually lowered or patients skip one cycle, which delays treatment and can affects disease outcomes.

Several prognostic factors have been implicated in this setting such as poor performance status and presence of visceral metastasis[20]. From the molecular perspective, mutation of the p53 gene and high excision repair cross complementation group 1 (ERCC1) status were associated with worse prognosis. Prognostic and predictive biomarkers are necessary for better patient stratification before the initiation of systemic treatments and decreasing the probability of exposing patients to potential chemotherapy related toxicity, without any clinical effect on the disease in all clinical settings.

In this prospective study, patients with high baseline levels of TBARS had significantly shortened PFS and OS in MUC patients (**Figure 1A, 1B)**. This finding can be explained by an

increased disease activity expressed in the increased levels of TBARS. Presence of visceral metastasis was not shown to be independent prognostic factors in this study and did not significantly affect OS and PFS, but as was shown in some studies, visceral metastasis have negative prognostic value either combined (liver, lung) or as single site metastasis [20,21] . In this study, there was a trend toward worse prognosis in patiens with presence of visceral metastasis (lung, liver), however lack of statistical significance is probably due to insufficient number of patients. Also, we found a significant association between TBARS levels and presence of liver metastases (**Table 6**), which is to our best knowledge the first time this association was described and it further supports the prognostic value of increased TBARS levels. Higher levels of MDA measured by the TBARS assay play a role in many diseases however one of the limitations of this method is lack of specificity because factors such as increased BMI, smoking, etc. may confound the results. In this study, increased BMI was associated with increased TBARS levels (**Table 6)**, however it did not affect the prognostic value of TBARS, when adjusting multivariate analysis for BMI (**Table 2, 4)**.

In conclusion, based on the data obtained in this study, patients with increased TBARS levels had shorter OS and PFS, respectively, before the first course of chemotherapy. This finding could be used for early stratification of patients with worse prognosis, which could help physicians to decide whether to use a different therapeutic approach or early inclusion into clinical trials.

Founding Sources

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Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

Slopovsky J:
;Kucharska J:
;Obertova J:
;Mego M:
;Kalavska K:
;Cingelova S:
;Gvozdjakova A:
;Palacka P:

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Figure 1A

Kaplan-Meier survival analysis for progression free survival (PFS) and a marker of lipid peroxidation TBARS

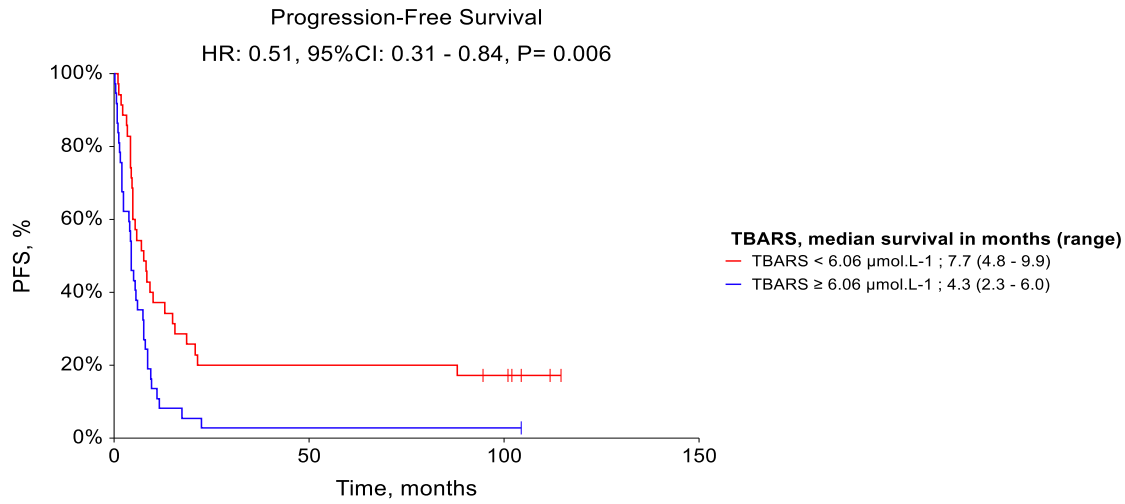

Figure 1B

Kaplan-Meier survival analysis for overall survival (OS) and marker of lipid peroxidation TBARS

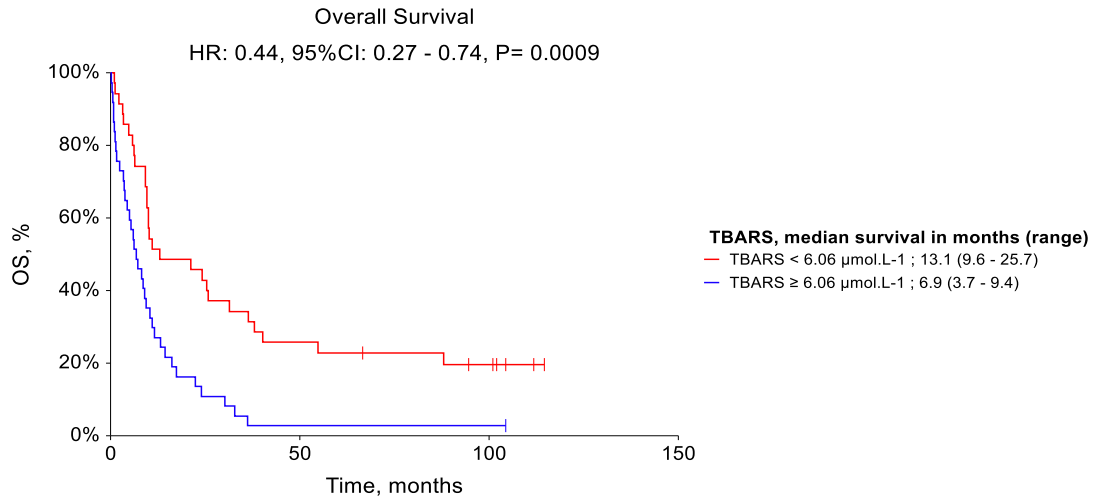

Table 1

Characteristics of Patients at Baseline

N (%)		72 (100%)
Age	Median (range)	66 (39-84)

Men		57 (79)
Progression		65 (90)
Death		64 (89)
Site of Primary Tumor n (%)	Bladder	58 (80)
	Renal Pelvis	9 (12.5)
Histology n (%)	Urothelial carcinoma	72 (100)
Chemotherapy	GC	65 (90)
	Carboplatin	6 (8)
	ddMVAC	1 (1.4)
PS	ECOG ≥ 2	11 (15)
Visceral Metastasis	Lung	25 (35)
	Liver	18 (25
PFS (months)	Median (range)	5.42 (0.26-114.54)
OS (months)	Median (range)	9.6 (0.26-114.54)

Table 2

Multivariate analysis for progression-free survival (PFS) adjusted for BMI above 30

Variable	N	HR	95% CI Low	95% CI High	P-value
TBARS (µmol.L^{-1})	**72**	NA	NA	NA	NA
> 6.06 µmol.L^{-1}	**37**	1.70	1.00	2.90	**0.04***
< 6.06 µmol.L^{-1}	**35**				
ECOG	**72**	NA	NA	NA	**NA**
More than 2	**11**	4.42	2.05	9.52	**0.0001***
Less than 2	**61**				
Visceral metastases (Liver and Lungs)	**72**	NA	NA	NA	**NA**
Present	**9**	1.33	0.60	2.92	**0.47***
Absent	**63**				
Obese	**72**	NA	NA	NA	**NA**
BMI above 30	**10**	0.87	0.42	1.80	**0.71***
BMI below 30	**62**				

Table 3

Univariate analysis for PFS.

Variable	N	Median	95%CI Low	95CI High	HR	95% CI Low	95%CI High	P-Value
TBARS (µmol.L^{-1})	72				0,51	0,31	0,84	0,006
> 6.06 µmol.L^{-1}	37	4,3	2,3	6				
< 6.06 µmol.L^{-1}	35	7,7	4,8	9,9				

Table 4

Multivariate analysis for overall survival (OS) adjusted for BMI above 30

Variable	N	HR	95% CI Low	95% CI High	P-value
TBARS (µmol.L^{-1})	**72**	NA	NA	NA	NA
> 6.06 µmol.L^{-1}	**37**	2.00	1.16	3.46	**0.01***
< 6.06 µmol.L^{-1}	**35**				
ECOG	**72**	NA	NA	NA	**NA**
More than 2	**11**	5.78	2.42	13.84	**0.0001***
Less than 2	**61**				
Visceral metastases (Liver and Lungs)	**72**	NA	NA	NA	**NA**
Present	**9**	1.39	0.59	3.24	**0.45***
Absent	**63**				
Obese	**72**	NA	NA	NA	**NA**
BMI above 30	**10**	1.11	0.52	2.36	**0.70***
BMI below 30	**62**				

Table 5

Univariate analysis for OS.

Variable	N	Median	95%CI Low	95CI High	HR	95% CI Low	95%CI High	P-Value
TBARS (µmol.L^{-1})	72				0.44	0.27	0.74	0,0009
> 6.06 µmol.L^{-1}	37	6.9	3.7	9.4				
< 6.06 µmol.L^{-1}	35	13.1	9.6	25.7				

Table 6

Association between TBARS and patient/tumor characteristics

	N	Mean	Median	SD	SEM	P
TBARS (µmol.L^{-1})	**72**	**6.06**	**6.15**	**1.28**	**0.15**	**NA**
ECOG						
0	22	5.63	5.36	1.03	0.26	0.10
1	39	6.13	6.16	1.29	0.20	
2	9	6.50	6.84	1.42	0.41	
3	2	7.61	7.61	1.78	0.88	
Metastasis (sites)						
Lymph nodes						
N0	4	6.34	6.26	1.11	0.64	0.57
N+	68	6.05	6.15	1.29	0.16	
Skeletal						
absent	48	5.95	5.64	1.35	0.18	0.26
present	24	6.29	6.32	1.10	0.26	
Pulmonary						

absent	47	6.08	6.16	1.16	0.19	0.51
present	25	6.02	5.6	1.49	0.26	
Liver						
Absent	54	5.88	5.64	1.25	0.17	0.03
present	18	6.60	6.47	1.22	0.29	
Pulmonary and Liver (either)						
absent	38	6.03	6.01	1.24	0.21	0.94
present	34	6.10	6.15	1.33	0.22	
Pulmonary + Liver (both)						
absent	62	6.05	6.15	1.30	0.16	0.62
present	9	6.30	6.56	1.13	0.43	
Peritoneum						
absent	60	6.16	6.16	1.32	0.16	0.16
present	12	5.58	5.62	0.94	0.37	
BMI						
TBARS < 6.06 $\mu mol.L^{-1}$	35	5.00	5.23	0.62	0.13	0.000-
TBARS > 6.06 $\mu mol.L^{-1}$	37	7.07	6.77	0.84	0.12	