



Oxidative DNA damage of lymphocytes in peripheral blood and ascites in cancer patients

J. Wang MMed,^{*a} *S.S. Xing* PhD,^{†a}
S.B. Guo MMed,[‡] *W. Jin* MMed,[§]
 and *W. Zhang* PhD^{||}

ABSTRACT

Background

Patients with malignant ascites (MA) usually experience poor quality of life, and treatment of this symptom remains a challenge. Oxidative stress, which can cause oxidative damage to DNA, plays a pivotal role in carcinogenesis; however, the relationship between oxidative stress and DNA damage to tumour-associated lymphocytes (TALS) in MA is unclear.

Methods

We measured the total antioxidant capacity (TAC) of plasma and MA supernatant in 31 cancer patients with MA, and we used a comet assay to assess DNA damage to both peripheral blood mononuclear cells (PBMCS) and TALS. Measurements in age- and sex-matched healthy volunteers were used as controls.

Results

The TAC of plasma was remarkably lower in cancer patients (9.73 ± 1.96 U/mL) than in healthy control subjects (11.31 ± 1.50 U/mL, $p < 0.001$). The TAC of MA supernatant (6.34 ± 1.57 U/mL) was significantly lower than that of plasma in cancer patients (7.42 ± 1.36 U/mL, $p < 0.001$). The comet percentage of PBMCS was higher in cancer patients ($17.26\% \pm 6.04\%$) than in healthy control subjects ($9.44\% \pm 4.47\%$, $p < 0.01$). In cancer patients, the comet percentage of TALS ($36.14\% \pm 17.85\%$) was significantly higher than that of PBMCS ($17.26\% \pm 6.04\%$, $p < 0.001$). In cancer patients with MA, negative correlations were observed between plasma TAC and DNA damage to PBMCS ($r = -0.505$, $p = 0.004$) and between the TAC of MA supernatant and the comet percentage of TALS ($r = -0.588$, $p = 0.001$).

Conclusions

Results indicate the presence of significant oxidative damage to the DNA of lymphocytes in peripheral blood and ascites from patients with MA, being especially higher in the cells from ascites. The lower TAC of MA supernatant may be related to a higher degree of DNA damage to TALS. The present study suggests that an oxidant–antioxidant imbalance may be one of the mechanisms leading to the DNA damage detected in peripheral blood and local TALS in patients with MA, which may provide a novel approach to the treatment of MA.

KEY WORDS

Malignant ascites, tumour-associated lymphocytes, total antioxidant capacity, DNA damage

1. INTRODUCTION

Malignant ascites (MA), a condition in which fluid containing cancer cells accumulates in the abdomen, is frequently observed in patients with progressive carcinomas such as ovarian (37%), pancreatobiliary (21%), gastric (18%), esophageal (4%), colorectal (4%), and breast (3%) cancers¹. The appearance of rapid and persistent effusion in MA is caused by peritoneal involvement or metastases. Patients with MA usually show decreased appetite, bloating, nausea, vomiting, abdominal distension, fatigue, weight loss, and even cachexia, all of which seriously affect quality of life and survival. However, the treatment of MA remains a challenge².

Oxidative stress, defined as loss of the balance between production of reactive oxygen species (ROS) and antioxidant defenses, is considered to cause oxidative damage to lipids, proteins, and DNA. The interaction of ROS with DNA is now recognized to result in an impairment of the genetic material in the cell nucleus. In the absence of an adequate antioxidant system, the accumulated DNA damage may result

^a These authors contributed equally to the present work.

in DNA mutation. Mutations in DNA and decreased efficacy of DNA repair cause further dysfunction and apoptosis in many kinds of cells, including lymphocytes, possibly playing a pivotal role in cell carcinogenesis and dissemination of cancer cells³.

Tumour-associated lymphocytes (TALS), the lymphocytes accumulating in the abdomen, are a special type of tumour-infiltrated lymphocytes. They can serve as a suitable model for the study of tumour-infiltrated lymphocytes⁴. Many studies have shown that TALS are functionally impaired (for example, by having a poor immune response to autologous tumour cells^{5–8}), and ROS seem to be related to TAL immunosuppression⁹. However, the relationship between oxidative stress and DNA damage to TALS in MA is still unclear.

Because DNA damage plays a major role in carcinogenesis, and because measurement of total antioxidant capacity (TAC) has been proved to be a useful tool for estimating the antioxidant capacity¹⁰, we set out to assess DNA damage to TALS in MA and the TAC of plasma and ascites from patients with carcinoma.

2. METHODS

2.1 Study Population

The present study conforms to the principles of the Declaration of Helsinki¹¹ and was approved by the Ethics Committee of the Yidu Central Hospital of Weifang City. From 2008 to 2009, the study enrolled 31 cancer patients with MA before cancer therapy (“research group”). The study also enrolled 31 age- and sex-matched healthy volunteers as a control group. To eliminate factors that might affect free-radical antioxidant activity, we excluded smokers and alcoholics from both the research group and the control group. Also excluded were individuals with chronic or acute diseases such as hypertension; diabetes mellitus; hyperlipidemia; diseases of the liver, kidney, and endocrine glands; and immunologic disorders. Informed consent was obtained from all participants.

The disease status of all patients in the research group was confirmed by positive carcinoma cytology in the effusions. Each 500 mL ascites was collected according to standard operating procedures. From each sample, 10 mL removed by centrifugation was used for TAC and protein determination; the remainder was used for isolation of TALS. Blood samples were also drawn after an overnight fast for measurement of plasma TAC and preparation of peripheral blood mononuclear cells (PBMCs).

2.2 Preparation of PBMCs

The PBMCs were isolated by centrifugation (20 minutes, 1000g) in a density gradient of Ficoll–Hypaque cushion. The cells from the interface were washed twice with phosphate-buffered saline (PBS) and suspended

in PBS at a final concentration of 5×10^5 cells/mL. The viability of PBMCs was tested using the trypan blue exclusion method and was found to be not less than 95%. Plasma was frozen at -20°C for the detection of TAC.

2.3 Preparation of TALS

The TALS from ascites were isolated by discontinuous density using a previously described method¹². Briefly, after centrifugation (10 minutes, 150g), the cell pellet was washed twice with PBS, layered on 100% Ficoll–Hypaque (specific gravity: 1.077), and further centrifuged (20 minutes, 1000g). Tumour cells and mononuclear cells were collected from the interface. The TALS were separated on a discontinuous density gradient (Ficoll–Hypaque: 100% and 75%) by centrifugation at room temperature (30 minutes, 1400g). The TALS were collected from the top layer of 100% Ficoll–Hypaque and washed twice with PBS. Their viability was tested using the trypan blue dye exclusion method and was found to be not less than 95%.

2.4 Measurement of TAC in Plasma and Ascites

In plasma and ascites, TAC was measured on the basis of the ability of antioxidants in the samples to reduce Fe^{3+} -TPTZ to Fe^{2+} -TPTZ, a stable blue product proportional to the TAC, which was measured at 593 nm¹³. One unit of TAC is represented by an increase in the absorbance of the reaction mixture of 0.01 per millilitre of sample per minute. The measurement was taken using a commercial test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing City, PR China) according to the manufacturer’s instructions. At least 3 independent experiments were performed in all samples, and results are expressed as units per millilitre of plasma or ascites.

2.5 Single-Cell Microgel Electrophoresis

To determine the DNA damage to TALS and PBMCs, a single-cell microgel electrophoresis assay (“comet assay”), was performed under alkaline conditions essentially according to the procedure of Singh *et al.*¹⁴. Isolated TALS and PBMCs were suspended in agarose and spread onto glass microscope slides pre-coated with agarose. The agarose was allowed to set at 4°C for 5 minutes. Slides were incubated in ice-cold lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, pH 10.0, and 1% Triton X-100 with 10% DMSO) to remove cell proteins, leaving DNA as “nucleoids.” After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with a fresh solution (300 mmol/L NaOH and 1 mmol/L EDTA, pH > 13) for 20 minutes at 48°C to allow for DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted in an

electrophoretic solution containing 30 mmol/L NaOH and 1 mmol/L EDTA, pH greater than 13 at an ambient temperature of 4°C (temperature of the running buffer did not exceed 12°C) for 20 minutes at an electrical field strength of 0.73 V/cm (28 mA). The slides were then washed in water, drained, stained with ethidium bromide, and covered with cover slips. To prevent additional DNA damage, all the steps were conducted under dimmed light or in the dark. Within 24 hours, the comets were viewed at 200× magnification using a fluorescence microscope (Olympus BH-2; Beckman Coulter, Fullerton, CA, U.S.A.) with excitation by green light (546 nm) and a barrier filter of 590 nm; photomicrographs were taken. For each sample, 3 slides were scored, with at least 100 cells were counted randomly on each slide. No DNA-damaged cells retained a circular appearance. During electrophoresis, DNA with strand breaks migrated toward the anode, giving the cell a “comet” appearance. Using the method of Everett *et al.*¹⁵, the slides were analyzed under blind conditions by at least 2 independent scorers. Median scores are presented. Readings of the mean scores were in agreement.

2.6 Statistical Analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences (version 12.0; SPSS, Chicago, IL, U.S.A.). Quantitative variables are presented as mean ± standard deviation. The paired *t*-test was used to analyze continuous normally distributed variables. The correlation analysis used linear correlation. A 2-tailed $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Patient Characteristics

The ages of the 31 cancer patients enrolled in the study (15 men, 16 women) ranged from 48 to 72 years (mean: 53.2 ± 14.5 years). Histologically, 11 had ovarian cancer, 9 had gastric cancer, 7 had hepatic cancer, and 4 had pancreatobiliary cancer.

3.2 TAC in Plasma and DNA Damage to PBMCs

As shown in Table I, plasma TAC was 14.0% lower and DNA damage to PBMCs (“comet percentage”) was 82.8% higher in cancer patients with MA than in healthy control subjects. A negative correlation was observed between plasma TAC and DNA damage to PBMCs in cancer patients with MA ($r = -0.505$, $p = 0.004$, Figure 1).

3.3 TAC in Ascites and DNA Damage to TALS

As shown in Table II, the TAC in MA supernatant was significantly lower than that in plasma from cancer

patients. In addition, the comet percentage for TALS was significantly higher than that of PBMCs in the patients. A negative correlation was observed between the TAC in MA supernatant and the comet percentage for TALS ($r = -0.588$, $p = 0.001$, Figure 2).

4. DISCUSSION

Compared with healthy subjects, cancer patients with MA had lower TAC and higher relative DNA damage to lymphocytes in both peripheral blood and ascites, indicating that oxidative stress is present not only systemically but also at the tumour site. In addition, TAC in plasma and MA supernatant were negatively correlated with the degree of DNA damage to PBMCs and TALS alike.

Reactive oxygen species are a class of chemicals with the properties of active oxygen atoms or group of atoms; they include all the active forms of oxygen. Under physiologic conditions, scavenging of ROS is performed by a large number of antioxidant systems, including antioxidant enzymes and nonenzymatic antioxidants. An imbalance between oxidant and antioxidant status, resulting either from increased production of ROS or inactivation and excessive consumption of antioxidant systems, causes oxidative stress. Under conditions of oxidative stress, cellular biomolecules such as lipids, proteins, and DNA become damaged and participate in many pathologic

TABLE I Plasma total antioxidant capacity (TAC) and comet percentage from a single-cell microgel electrophoresis assay (“comet assay”) of peripheral blood mononuclear cells in 31 healthy subjects and 31 cancer patients with malignant ascites

	Healthy subjects	Cancer patients	t Value	p Value
TAC (U/mL)	11.31±1.50	9.73±1.96	3.585	0.001
Comet score (%)	9.44±4.47	17.26±6.04	25.359	0.000

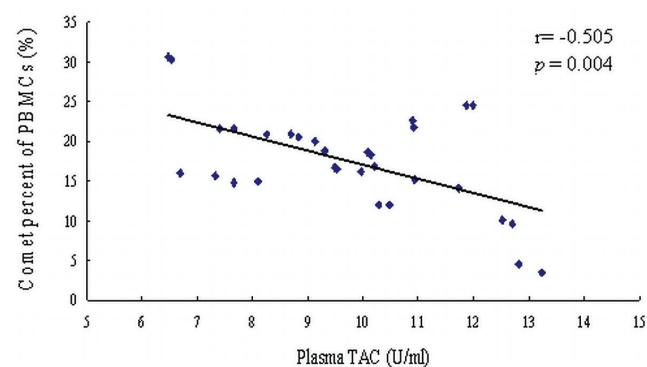


FIGURE 1 Negative correlation between plasma total antioxidant capacity (TAC) and comet percentage from a single-cell microgel electrophoresis assay (“comet assay”) of peripheral blood mononuclear cells (PBMCs) in cancer patients with malignant ascites.

TABLE II Total antioxidant capacity (TAC) and DNA damage in plasma and ascites in 31 cancer patients with malignant ascites

	Plasma	Ascites	t Value	p Value
TAC (U/mL)	9.73±1.96	7.42±1.36	4.957	0.000
Comet score (%)	17.26±6.04	36.14±17.85	8.73	0.000

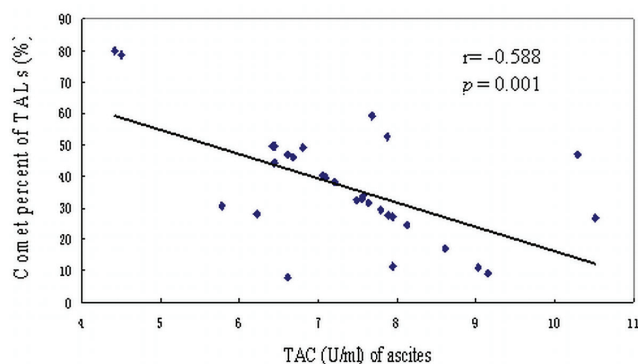


FIGURE 2 Negative correlation between total antioxidant capacity (TAC) of ascites supernatant and comet percentage from a single-cell microgel electrophoresis assay (“comet assay”) of tumour-associated lymphocytes (TALS) in cancer patients with malignant ascites.

processes¹⁶. Accumulation of DNA damage and inefficacy of DNA repair are now recognized to play a major role in neoplastic transformation and metastasis, serving as therapeutic targets in the treatment of cancer¹⁷.

Many experiments have shown that cancer patients suffer from oxidative stress. For example, just recently, Wang *et al.*¹⁸ evaluated the total oxidant:antioxidant status in patients with thyroid cancers. Those authors showed that serum total antioxidants were significantly lower in patients with thyroid cancer than in control subjects and that serum total oxidant levels and indices of oxidative stress were significantly higher in the patients. Further analysis demonstrated that oxidative stress was the best indicator for distinguishing cancer patients from patients with benign thyroid disease or from healthy subjects, and total oxidant status and the oxidative stress index were good indices for discriminating patients with thyroid cancer from controls¹⁸. Those findings suggested that oxidants are increased and antioxidants are decreased in patients with thyroid cancer, indicating that oxidative stress may be a new risk factor and useful for monitoring clinical status in thyroid cancer patients¹⁹.

In the present study, our data show a decline in TAC in both plasma and MA supernatant—but especially in MA supernatant—from cancer patients (Table II). As an index of the capacity of a given tissue to modulate the damage associated with increased

production of free radicals, TAC is widely used to assess the degree of oxidative stress in many pathologic conditions²⁰. Thus, our results provide evidence that oxidative stress is present not only systemically but also at the tumour site in patients with MA, with its presence in ascites being the most serious, which is consistent with a previous report in patients with malignant pleural effusion²¹.

Comet assay—a rapid, simple, and sensitive technique for measuring and analyzing DNA breakage in single mammalian cells—is widely used to investigate the effects of ROS on DNA. We therefore further evaluated oxidative DNA damage in PBMCs and TALS from patients with MA. We verified significantly greater levels of DNA migration, reflecting DNA damage, in PBMCs from cancer patients with MA than in PBMCs from healthy subjects. Interestingly, when assessing the extent of DNA damage in the patients alone, the comet percentage for TALS was significantly higher than that for PBMCs, which indicates more serious oxidative stress in the ascites. In addition, we observed a negative relationship between TAC in the ascites supernatant and DNA damage to TALS (Figure 2). It is reasonable to assume that the lower TAC of ascites supernatant may be related to the higher degree of DNA damage to TALS caused by oxidative stress.

Several limitations should be noted. This is a single-centre study with a relatively small number of patients, and thus there is a risk of patient selection bias. In addition, the comet assay, like almost every method used to measure DNA damage and repair, has its own limitations because it can directly detect only strand breaks or other DNA modifications that result in strand breaks at alkaline pH. It cannot detect several other categories of DNA damage, including mismatches and alkali-insensitive base modifications. The method can directly detect DNA–DNA and DNA–protein crosslinks provided that they are the sole damage to a cell, but if they coexist with strand breaks, some additional experimental steps such as cleavage with a protease should be performed to determine the kind of DNA damage being measured. However, the data drawn from the present study indicate high oxidative stress not only systemically, but also at the tumour site in patients with MA, with stress in the ascites being the most serious, which is consistent with a previous report in patients with malignant pleural effusion²¹.

5. CONCLUSIONS

Taken together, our results indicate the presence of significant oxidative DNA damage to lymphocytes in both peripheral blood and ascites from cancer patients, with the damage being especially high in ascites. The lower TAC in ascites supernatant may be related to the higher degree of DNA damage to TALS. The present study suggests that an oxidant:antioxidant imbalance may be one of the mechanisms leading to

the DNA damage detected in whole blood and local TALS in patients with MA. Because DNA damage plays a major role in carcinogenesis, our results may provide a novel approach to the treatment of MA.

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7. CONFLICT OF INTEREST DISCLOSURES

The authors declare that there is no financial conflict of interest that could be perceived to prejudice the impartiality of the research reported.

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Correspondence to: Wei Zhang, Department of Endocrinology, Shandong Provincial Qianfoshan Hospital, No. 66 Jingshi Road, Jinan 250014, and Shi-Biao Guo, Department of Endocrinology, Yidu Central Hospital of Weifang City, Weifang 262500 PR China.

E-mail: zhw08aoyun@yahoo.com.cn, zhwwlby@163.com

* Department of Experimental Center, Yidu Central Hospital of Weifang City, Weifang, PR China.

† Shandong University of Traditional Chinese Medicine, Jinan, PR China.

‡ Department of Endocrinology, Yidu Central Hospital of Weifang City, Weifang, PR China.

§ Department of Gastroenterology, Yidu Central Hospital of Weifang City, Weifang, PR China.

|| Department of Endocrinology, Shandong Provincial Qianfoshan Hospital, Jinan, PR China.