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DISTURBANCE OF SYSTEMIC ANTIOXIDANT PROFILE IN NON-SMALL CELL LUNG CARCINOMA

Running title: Antioxidants in Lung Cancer

James C. Ho MRCP, Moira Chan-Yeung MD FRCP, S.P. Ho BSc,

Judith C. Mak PhD, Mary S. Ip MD FRCP, *Gaik C. Ooi MRCP FRCR,

[+]Maria P. Wong FRCPath, Kenneth W. Tsang MD FRCP, W.K. Lam MD FRCP

University Departments of Medicine, *Diagnostic Radiology, and [+]Pathology

The University of Hong Kong, Queen Mary Hospital, Hong Kong SAR, China

Address for correspondence:

Dr. James C. Ho MRCP FCCP

Clinical Assistant Professor

Division of Respiratory and Critical Care Medicine

University Department of Medicine

The University of Hong Kong

Queen Mary Hospital

Pokfulam, Hong Kong SAR, China

Tel: (852) 2855 5414 **Fax:** (852) 2855 5411 **Email:** jcmho@netvigator.com

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ABSTRACT

This study aimed to determine the alterations of antioxidant activities in erythrocytes from patients with non-small cell lung carcinoma (NSCLC).

We conducted a comparative study of the systemic antioxidant activities in red blood cell lysate from subjects with NSCLC and healthy control subjects. Antioxidants (catalase, superoxide dismutase [SOD], and glutathione peroxidase [GPx]) were measured using chemical kinetic reactions under spectrophotometry.

There were 189 cases of mostly advanced stage IIIB or IV NSCLC and 202 healthy controls being studied. In subjects with lung cancer, there were similar catalase activity, lower SOD activity (median [interquartile range]: 13.4 [9.0, 27.2] vs 48.7 [27.0, 64.3] U/gHb, $p<0.001$), and higher GPx activity (median [interquartile range]: 175.2 [126.6, 288.3] vs 49.2 [39.5, 59.2] mU/gHb, $p<0.001$) compared with controls. The antioxidant activities in lung cancer subjects were not associated with age, gender, smoking status, or tumour cell types. However, more advanced disease (stage IV compared to IIIB) was associated with lower SOD activity. Using multivariable analysis, the presence of lung cancer independently predicts SOD and GPx activities.

In conclusion, NSCLC in Chinese is associated with alterations in systemic antioxidant activities, which may play an important role in carcinogenesis.

KEYWORDS

Catalase, glutathione peroxidase, lung cancer, superoxide dismutase

INTRODUCTION

Free radicals and reactive oxygen species (ROS), especially superoxide anion and hydroxyl radical, have been implicated in the pathogenesis of various diseases including cancer development [1]. The interaction between superoxide and nitric oxide can produce potentially harmful and powerful oxidants, including peroxynitrite [2]. Human lungs are particularly vulnerable to the potential damage by ROS because of their constant exposure to environmental oxygen and exogenous free radicals, for example in cigarette smoke or air pollutants. In order to protect against the deleterious effects of ROS, a well-developed antioxidant system exists in the lung, which includes superoxide dismutases (SOD), catalase, and glutathione-dependent enzymes like glutathione peroxidase (GPx) [3]. SOD enzymes include the intracellular manganese SOD and copper-zinc SOD, and an extracellular SOD that exists in epithelial lining fluid and blood vessels [4]. SOD converts superoxide anion to hydrogen peroxide (H_2O_2) and catalase converts H_2O_2 to water and oxygen [5]. GPx also helps in the detoxification of H_2O_2 and lipid peroxides, requiring glutathione and other cofactors. A previous study has shown that ingestion of food rich in antioxidants was associated with a decreased incidence of lung cancer [6]. However, the landmark primary prevention trials using vitamins A and E have not been able to demonstrate beneficial effect on lung cancer prevention [7,8] . Therefore, a better understanding of the exact role and alteration of antioxidant profiles in lung cancer is essential to enable future therapeutic use of antioxidants in management of lung cancer.

A recent study has identified a decreased catalase and increased manganese SOD expressions in resected non-small cell lung carcinoma [9]. However, there has been

limited data on the systemic antioxidant profiles in lung cancer, which are represented mostly by the antioxidants in erythrocytes and plasma. In a previous study of 36 non-pulmonary malignant solid tumours, catalase activity in erythrocytes was significantly elevated suggesting an adaptive response to oxidative stress [10]. Since lung carcinomas, like other types of solid tumours, are characterized by a rich supply of circulating blood, we postulated that the antioxidant activities in the systemic compartment as reflected by those in erythrocytes were altered in the presence of lung tumours. Therefore, we conducted a comparative study on the systemic antioxidant activities in patients with confirmed non-small cell lung carcinoma (NSCLC).

MATERIALS AND METHODS

Subject recruitment

Consecutive subjects with histologically or cytologically confirmed diagnosis of NSCLC were recruited before initiation of any anti-cancer therapy in the Queen Mary Hospital, a tertiary University-based referral centre for lung cancer in Hong Kong, from July 1999 to December 2001. The revised lung cancer staging system in 1997 was adopted [11]. The healthy controls were recruited as part of a population-based lung function study of nonsmokers and smokers conducted by 8 hospitals in Hong Kong. In brief, individuals over the age of 18 years were recruited by telephone using random digit dialing. Only those subjects without a history of lung cancer or other chest diseases were recruited as controls. There were no matching of age or gender between lung cancer patients and healthy subjects. Trained research assistants interviewed patients and controls using a questionnaire to obtain information on demographic data, smoking habits, presence or

absence of chest symptoms and past history of chest diseases and surgery. Ever-smoker was defined as one who had smoked at least one cigarette a day, pipe, water pipes, cigars, and hand rolled cigarettes, for one year or more. Informed consent would be obtained from all patients and controls taking part in the study. All patients and controls were Chinese. The study was approved by the Institutional Review Board of the University of Hong Kong.

Blood collection

Ten millilitres of venous blood in lithium heparin was taken from each lung cancer patient and control. Red blood cells were separated from plasma and buffy coat by immediate centrifugation at 2500 rpm for 10 minutes and stored at -70°C until subsequent assays of antioxidant activities. Red blood cell lysate was prepared by washing packed red blood cells 3 times with cold normal saline under centrifugation at 3000g and then lysed with 4 volumes of cold deionized water. Haemoglobin (Hb) concentrations were assayed by a commercially available kit (Sigma, St. Louis, USA).

Antioxidant activities

Catalase

The quantitation of catalase activity in the red cell lysate was based on the reaction with hydrogen peroxide as previously described [12]. In brief, the initial rate of disappearance of hydrogen peroxide (0 to 60s) was recorded spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was defined as the rate constant of the first-order reaction. The catalase activity was expressed as milliunits (mU) per g haemoglobin (Hb).

Superoxide dismutase (SOD)

SOD activity in the red cell lysate was determined from the rate of reduction of cytochrome c [4], with one unit (U) of SOD activity defined as the amount of SOD required to inhibit the rate of cytochrome c reduction by 50%. The final reaction volume was 3 ml, and included 50 mM potassium phosphate buffer, 2 mM cytochrome c, 0.05 mM xanthine, and a 0.1 mM ethylenediamine tetra-acetic acid (EDTA) solution. Xanthine oxidase (Sigma, St. Louis, MO) was added at a concentration sufficient to induce a 0.020 change in absorbance per minute at 550 nm. The SOD activity was expressed as U per g Hb.

Glutathione peroxidase (GPx)

Total GPx activity was determined spectrophotometrically in the red cell lysate through an indirect coupled assay [13]. The reactions were carried out using the Bioxytech GPx-340 assay kit (Oxis, Portland, OR). The red cell lysate was put in the proprietary assay buffer and reagent consisting of glutathione, glutathione reductase, and reduced β–nicotinamide-adenine dinucleotide phosphate (NADPH). The reaction was initiated by the addition of 350 μL of 0.007% tert-butyl hydroperoxide. The decrease in absorbance at 340 nm over 3 min, as NADPH is converted to nicotinamide adenine dinucleotide phosphate (NADP), was proportional to the GPx activity. One unit (U) of activity was defined as the activity that catalyzes the oxidation of 1 nmol NADPH/min, with a molar coefficient of extinction of 6.22×10^6 M^{-1} cm^{-1} used for NADPH. The GPx activity was expressed as mU per g Hb.

Statistics

The basic demographic data were expressed as mean ± standard deviation (S.D.), and the specific antioxidant activities in median and interquartile range (IQR). Independent sample t-test and Chi squared test, where appropriate, were used to compare the demographic data between cases and controls. The comparison of specific antioxidant activities between cases and controls, taking into account of confounding variables, was achieved using multivariable analysis of General Linear Model. Pearson correlation analysis was used for testing relationships between different antioxidants among cases. Non-parametric test (Mann-Witney U test) was used to compare the specific antioxidant activities between groups with different clinical characteristics among cases. The analysis was performed using the SPSS® 10.0 Version package (SPSS Inc., Chicago, IL, USA). A p value of less than 0.05 was taken as of statistical significance.

RESULTS

Clinical characteristics

There were 189 patients with NSCLC and 202 healthy controls, all were Chinese, recruited in this study (Table 1). The lung cancer cases were significantly older and had more ever-smokers than controls, but with similar gender distribution. The predominant type of NSCLC was adenocarcinoma (55.6%) and the majority (90%) in stage IIIB or IV diseases.

Antioxidant activities in NSCLC cases

In NSCLC cases, there were no correlations between erythrocyte catalase, SOD and GPx activities. There were significantly lower median [IQR] SOD (13.4 [9.0, 27.2] versus 48.7 [27.0, 64.3] U/gHb), and higher median [IQR] GPx (175.2 [126.6, 288.3] versus 49.2 [39.5, 59.2] mU/gHb) activities in erythrocytes of NSCLC cases compared with controls (Table 2). However, the erythrocyte catalase activities were not significantly different between NSCLC cases and controls. By using multivarible analysis of General Linear Model, presence of lung cancer was an independent predictor of erythrocyte SOD and GPx activities but not age, gender and smoking status.

In ever-smokers, there were significantly lower median [IQR] SOD (16.5 [8.8, 31.6] versus 48.8 [21.9, 70.7] U/gHb) and higher median [IQR] GPx (166.7 [103.6, 275.1] versus 46.5 [38.7, 58.6] mU/gHb), but similar median [IQR] catalase (7.6 [4.5, 16.2] versus 26.4 [17.2, 34.3] mU/gHb) activities in erythrocytes of NSCLC cases compared to controls (Table 3). Similarly, in nonsmokers, the median [IQR] erythrocyte catalase (8.0 [4.2, 14.2] versus 19.0 [14.0, 27.7] mU/gHb) activity was similar, while median [IQR] SOD (12.6 [9.2, 23.5] versus 48.7 [30.1, 62.3] U/gHb) activity was significantly lower, but median [IQR] GPx (189.9 [142.7, 335.0] versus 50.9 [39.7, 60.0] mU/gHb) activity was significantly higher in NSCLC cases compared to controls (Table 3).

Clinical predictors of antioxidant activities in NSCLC cases

There were no differences in antioxidant activities (catalase, SOD, and GPx) in NSCLC cases with respect to age, gender, smoking status, and tumour cell types (Table 4). However, the more advanced tumour staging was associated with lower median [IQR]

SOD activities in erythrocytes (12.2 [8.2, 25.0] versus 17.0 [9.4, 44.0] U/gHb in stage IV versus stage IIIB). There was a trend for GPx activity to be higher in those with stage IV disease.

DISCUSSION

In this study we have demonstrated increased GPx, but decreased SOD activities in erythrocytes in patients with NSCLC compared with healthy controls. This alteration in systemic antioxidant profile was independent of age, gender, and smoking status. The presence of clinically evident metastatic (stage IV) disease was associated with significantly lower erythrocyte SOD activity and a trend for higher GPx activity compared with locally advanced (stage IIIB) disease.

Previous studies suggested a general trend of decrease in various antioxidants (selenium [14], vitamins A and E [15], SOD [16], GPx [14], and glutathione-S-transferase [14]) in blood obtained from lung cancer patients compared with controls. In a recent case-control study, erythrocyte Cu-Zn SOD and catalase activities were significantly increased in subjects with non-small cell lung cancer (NSCLC) compared to healthy controls, while GPx activity was only numerically higher in NSCLC subjects [17]. However, these apparently discrepant results from our study could be related to lack of measurement of Mn SOD and ethnic differences in functional polymorphisms of antioxidant genes [18,19]. Surgical removal of lung cancer resulted in augmentation in plasma total radical-trapping antioxidant parameters (TRAP) due to unidentified antioxidant components and protein sulfhydryl groups, reflecting the relief of oxidative stress caused by the malignant

tumours [20]. Chronic inflammation has been suggested to contribute to lung carcinogenesis [21] and inflammatory cell infiltration appeared abundantly in the direct vicinity of resected lung tumours [22]. The extent of inflammation was also found to significantly affect the antioxidant status in lung cancer, with negative correlations between C-reactive protein and retinol, alpha-tocopherol, and lutein [23]. Recently, a study of the antioxidant status in resected non-small cell lung carcinomas and adjacent tumour-free lung tissues suggested altered expressions of major antioxidants in tumour tissues [9]. Specifically, there were upregulated manganese (Mn) SOD and downregulated catalase activities, protein and RNA expressions in lung tumour tissues. The *in vivo* results were also reproduced by cytokine stimulation in A549 cells. Therefore more efficient conversion of superoxide into hydrogen peroxide, together with decreased removal of the latter, would result in accumulation of hydrogen peroxide within the tumour cells. Hydrogen peroxide has been implicated in causing genetic damage [24], while superoxide mediates apoptosis [25,26]. The high levels of MnSOD with decreased catalase may create an anti-apoptotic intracellular environment which is especially susceptible to increased frequency of mutations, a situation likely to lead to cell transformation and cancer.

The finding of increased erythrocyte GPx activity in patients with NSCLC as in the current study suggests an enhanced removal of hydrogen peroxide in the peripheral blood compartment, which may help in counteracting for the increased accumulation of hydrogen peroxide within the lung tumours. It has been suggested that hydrogen peroxide has a stimulatory effect on SOD activity [27]. Therefore, with the enhanced removal of

hydrogen peroxide in erythrocytes, there will be less accumulation of hydrogen peroxide leading to the diminished erythrocyte SOD activity. With this postulate of the systemic antioxidant profile serving as a compensatory mechanism in the presence of lung tumours, the more advanced disease with larger tumour bulk may be associated with greater alteration in the systemic antioxidant profile, accounting for the significantly lower erythrocyte SOD activity in stage IV compared to stage IIIB disease.

Cigarette smoking, the most important cause of lung cancer, has been shown to be associated with depletion of some plasma antioxidants including vitamin C, α-tocopherol, carotenoids, glutathione-S-transferase, and glutathione peroxidase [28,29]. This negative effect of cigarette smoking on plasma micronutrients was not limited to active smokers, in which serum carotenoids were also found to be reduced in passive smokers [30]. Smoking cessation was also shown to restore the initially reduced plasma vitamins A, C, E, uric acid, total thiols, and carotenoids [31]. However, some of these effects might be related to the differences in diet between smokers and nonsmokers. Having adjusted for dietary antioxidant intake, recent studies have only consistently demonstrated depletion of ascorbic acid in plasma from smokers, but not for other antioxidants [32,33]. Interestingly, smoking a single cigarette has been demonstrated to acutely lower significantly the concentrations of major serum antioxidants (ascorbic acid, cysteine, methionine, and uric acid) [34]. In addition, an age-dependent adaptive response to antioxidants has been suggested leading to significant reduction of plasma GPx activity only in older smokers, who could no longer sustain a counteracting effect to oxidative stress [35]. In our cohort of NSCLC cases, smoking status was not found to be a

predictor of all 3 antioxidant activities, which may result from the interplay of the factors above and possibly the effect of the presence of lung tumours.

However, the findings in our study are limited by the clinical characteristics of our patient cohort with predominantly adenocarcinoma and advanced disease. That adenocarcinoma is becoming the predominant cell type in lung cancer has been recognized in Hong Kong and elsewhere in the past two decades [36,37]. Larger study with inclusion of more non-adenocarcinoma cell types and early stage diseases may help better delineate the role of pathologic cell types and disease stages in systemic antioxidant profiles. Future study is warranted to investigate the clinical significance of systemic antioxidant activities as markers of disease status.

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Table 1 Clinical characteristics and demographics of 189 non-small cell lung cancer (NSCLC) patients and 202 control subjects

	Controls	NSCLC cases	p value
N (Number)	202	189	
Age (years)	49.5 ± 15.6	57.0 ± 11.7	**<0.001**
Gender (male (%))	119 (59)	126 (67)	>0.05
Smoking status (N (%))			
Ever-smoker	78 (39)	105 (56)	
Non-smoker	124 (61)	84 (44)	**0.026**
Tumour cell types (N (%))			
Adenocarcinoma		105 (55.6)	
Squamous cell		32 (16.9)	
Large cell		16 (8.5)	
Unclassified NSCLC		36 (19.0)	
Overall TNM stage (N (%))			
I-II		5 (2.6)	
IIIA		8 (4.2)	
IIIB		61 (32.3)	
IV		109 (57.7)	
Unknown		6 (3.2)	

Table 2 Antioxidant activities in red blood cell lysate in non-small cell lung cancer (NSCLC) patients and control subjects

	Controls	NSCLC cases	*p value
Catalase (mU/gHb)	20.9 (15.4, 31.1)	7.6 (4.5, 15.5)	0.68
N	202	189	
SOD (U/gHb)	48.7 (27.0, 64.3)	13.4 (9.0, 27.2)	**<0.001**
N	202	186	
GPx (mU/gHb)	49.2 (39.5, 59.2)	175.2 (126.6, 288.3)	**<0.001**
N	181	186	

SOD, superoxide dismutase; GPx, glutathione peroxidase; Hb, haemoglobin

*Adjusted for age, gender and smoking status

Data shown in median (interquartile range)

Table 3 Antioxidant activities in red blood cell lysate in non-small cell lung cancer (NSCLC) patients and control subjects according to smoking status

	Smoking status	Controls	NSCLC cases	*p value
Catalase (mU/gHb)	ES	26.4 (17.2, 34.3)	7.6 (4.5, 16.2)	0.45
N		78	105	
SOD (U/gHb)	ES	48.8 (21.9, 70.7)	16.5 (8.8, 31.6)	**<0.001**
N		78	104	
GPx (mU/gHb)	ES	46.5 (38.7, 58.6)	166.7 (103.6, 275.1)	**<0.001**
N		74	105	
Catalase (mU/gHb)	NS	19.0 (14.0, 27.7)	8.0 (4.2, 14.2)	0.90
N		124	84	
SOD (U/gHb)	NS	48.7 (30.1, 62.3)	12.6 (9.2, 23.5)	**<0.001**
N		124	82	
GPx (mU/gHb)	NS	50.9 (39.7, 60.0)	189.9 (142.7, 335.0)	**<0.001**
N		107	81	

SOD, superoxide dismutase; GPx, glutathione peroxidase; Hb, haemoglobin; ES, ever-smokers; NS, nonsmokers

*Adjusted for age and gender

Data shown in median (interquartile range)

Table 4 Effects of clinical characteristics on antioxidant activities in red blood cell lysate in non-small cell lung cancer patients

Characteristics	N	Catalase (mU/gHb)	p value	SOD (U/gHb)	p value	GPx (mU/gHb)	p value
Age (years)							
≥ 57	98	7.7 (4.4, 16.4)		19.0 (8.4, 32.6)		172.5 (104.8, 268.4)	
< 57	91	7.4 (4.7, 14.9)	0.85	12.0 (9.2, 20.4)	0.10	186.6 (136.8, 330.5)	0.14
Gender							
Male	126	7.8 (4.5, 22.6)		14.8 (9.0, 26.8)		167.2 (109.0, 276.4)	
Female	63	7.3 (3.9, 13.4)	0.32	12.7 (8.7, 30.2)	0.59	178.2 (138.4, 330.2)	0.21
Smoking status							
Ever-smokers	105	7.6 (4.5, 16.2)		16.5 (8.8, 31.6)		166.7 (103.6, 275.1)	
Non-smokers	84	8.0 (4.2, 14.2)	0.66	12.6 (9.2, 23.5)	0.16	189.9 (142.7, 335.0)	0.06
Tumour cell types							
AD	105	7.3 (4.3, 13.4)		12.7 (8.7, 32.7)		175.2 (130.0, 320.7)	
Non-AD	84	8.2 (4.5, 17.6)	0.25	15.4 (9.3, 24.7)	0.59	179.6 (116.0, 268.5)	0.50
Overall TNM stage							
IIIB	61	7.7 (4.7, 14.5)		17.0 (9.4, 44.0)		180.2 (129.9, 280.7)	
IV	109	6.9 (4.2, 15.1)	0.46	12.2 (8.2, 25.0)	**0.03**	189.9 (121.3, 313.8)	0.63

AD, adenocarcinoma; Hb, haemoglobin; SOD, superoxide dismutase; GPx, glutathione peroxidase

Data shown in median (interquartile range)