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Expression of ERCC1, Bcl-2, MT and their Clinical Significance in Advanced Non-small-cell Lung Cancer Treated With Cisplatin-based Chemotherapy

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SUMMARY. The aim of this study was to determine the prognostic value of expression of Excision Repair Cross-complementation Group 1 (ERCC1), B cell lymphoma/lewkmia-2 (Bcl-2) and metallothionein (MT) by immunohistochemically staining tumor specimens from 78 patients with advanced non-small-cell lung cancer (NSCLC) treated with cisplatin-based chemotherapy. The result showed that positive expression of ERCC1 was associated both with short survival (10.45 months vs. 14.38 months, log-rank $p = 0.003$) and poor response to cisplatin-based chemotherapy ($p = 0.003$) when compared with negative group. But expression of Bcl-2 and MT was not associated with response to chemotherapy and survival. Multivariate analysis confirmed that expression of ERCC1 was an independent variable related to overall survival ($p = 0.013$). These data support the role of RECC1 expression as a candidate predictor for the survival of patients with advanced NSCLC treated with cisplatin-based chemotherapy.

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is one of the commonest causes of cancer deaths worldwide [1,2]. Although surgical resection plays a major role in curative treatments, not more than 20% of NSCLC patients were diagnosed in operable stage. Combination chemotherapy with cisplatin is considered a standard treatment for advanced NSCLC patients while only show a limit improvement in survival [3-6]. The major reason for the failure of cisplatin-based chemotherapy is initial or acquired resistance.

Unluckily, it is still not clear about the molecular mechanisms that underlie this chemoresistance. Possible mechanisms of acquired resistance to cisplatin include the following: reduced intracellular accumulation of cisplatin, increased repair activity of DNA damage, altered expression of oncogenes and regulatory proteins, and enhanced drug inactivation by metallothionine and glutathione [7].

Recently, it seems to be helpful to identify some molecular markers, which could predict tumor response to chemotherapy, and then to optimize the treatment for the individual patient. In NSCLC, such prognostic factors would be useful in selecting those patients who are likely to benefit from cisplatin-based chemotherapy, thus preventing about 60% of patients who do not experience response to this treatment from the serious adverse effects.

The aim of the present study was to investigate the predictive value of ERCC1, Bcl-2 and MT for response of cisplatin-based chemotherapy and survival in advanced NSCLC.

KEY WORDS: Bcl-2, Cisplatin, ERCC1, MT, NSCL, Resistance

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METHODS

Case Selection

Microscopic slides from 78 randomly selected cases of advanced NSCLC from 2000 to 2005 were retrieved from Panyu People's Hospital, Guangzhou, China. There were 18 squamous cell carcinomas (SCC), 36 adenocarcinomas (AC), 7 adenosquamous carcinomas and 17 other histological types in the study group. These cases contained 51 men and 27 women. Tumors were staged in accordance with the UICC TNM classification, including p-stage IIIA, IIIB and IV tumors. Clinical follow-up data were obtained from the Medical Record Unit of the Panyu People's Hospital. All patients were treated with cisplatin-based chemotherapy. This research protocol was approved by the Research Ethics Committee of the Hospital (Nº 0056-2).

Data concerning clinicopathologic characteristics, including sex, age, smoking history, histologic type, pathologic stage and survival were obtained by thorough medical record review. Smoker is defined to people having smoked more than 400 cigarettes one year.

Immunohistochemical staining for drug resistance-related proteins

Immunohistochemical staining of formalin-fixed, paraffin-embedded tumor tissue was performed using mouse anti-human monoclonal antibodies against Bcl-2 (clone 124, dilution 1:40, DAKO, Tokyo; Japan), ERCC1 (ERCC1 Ab-2, dilution 1:100, Neomarkers, Fremont, CA, USA), and MT (MT Ab-1, dilution 1:100, Invitrogen, Camarillo, CA, USA).

Sections were deparaffinized in xylene and rehydrated in graded alcohols and water. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 10 min. Sections were treated with protein-blocking solution and then with primary antibodies overnight at 4 °C. After several rinses in phosphate-buffered saline, the sections were incubated in the biotinylated secondary antibody. Bound antibodies were detected by the streptavidin-biotin method. Slides were rinsed in phosphate-buffered saline, exposed to diaminobenzidine, and counterstained with Mayer's hematoxylin. The negative controls for these proteins were made by the omission of the primary antibody during the process of immunohistochemical staining.

The slides were examined independently by two observers (LI J, CAO XL) blinded to both clinical and pathologic data. Expression of the drug resistance-related proteins was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells) (graded on a scale of 0-3; 0 = none, 1 = 1-9%, 2 = 10-49%, 3 = ≥50%) as well as the intensity of staining (graded on a scale of 0-3; 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). The expression of these proteins was considered as positive (high expression) when both scores were 2 or above (Fig. 1).

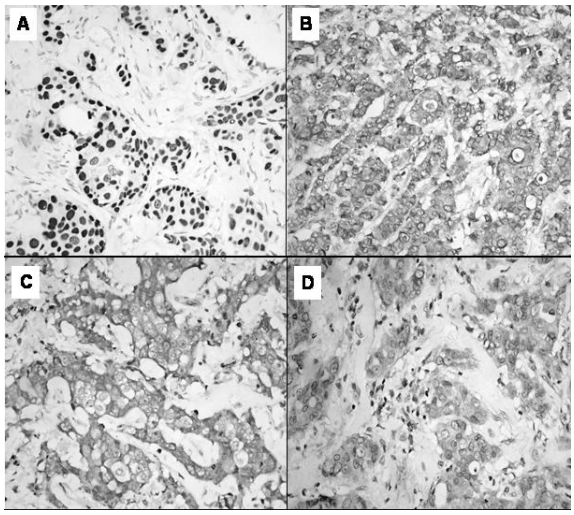

Figure 1. Representative immunohistochemical staining of drug resistance-related proteins in NSCLC (×400). (**A**) High expression of ERCC1: nuclear staining (grade 3). (**B**) High expression of Bcl-2: cytoplasmic staining (grade 3). (**C**) High expression of MT: cytoplasmic staining (grade 3). (**D**) Low expression of ERCC1 (grade 0). Canon PowerShot A720 IS.

Statistical analysis

Overall survival (OS) was calculated using the Kaplan-Meier method. OS was defined as the time from the date of histologic diagnosis to death; data on survivors were censored at the last follow-up. The differences between the survival curves were tested using the log-rank test. The Cox proportional-hazards regression model was used to determine the joint effects of several variables on survival. A comparison of clinicopathologic characteristics was evaluated with the chi-square test. All analyses were performed with SPSS for Windows 16.0 software and a p-value < 0.05 was considered significant.

RESULTS

Patient characteristics

The clinicopathologic characteristics of the 78 patients are listed in Table 1.

Characteristics		Number (%)
Age (years)	Median	57.5
	Range	28-79
Gender	Male	51 (65.4)
	Female	27 (34.6)
Performance status	0	5 (6.4)
	1	52 (66.7)
(ECOG)	2	18 (23.1)
	3	3 (3.8)
Histologic type	Squamous cell carcinomas	18 (23.1)
	Adenocarcinoma	36 (46.2)
	Adenosquamous carcinoma	7 (9)
	Others	17 (21.8)
ERCC-1	-	41 (52.6)
	+	37 (47.4)
Bcl-2	-	40 (51.3)
	+	38 (48.7)
MT	-	42 (53.8)
	+	36 (46.2)
Chemotherapy regimens	Gemcitabine/ cisplatin	33 (42.3)
	Navelbine/cisplatin	7 (9)
	Paclitaxel/cisplatin	39 (48.7)
Stage	3 [a]	29 (37.2)
	3 [b]	30 (38.5)
	4	19 (24.4)

Table 1. Characteristics of patients. ECOG, Eastern Cooperative Oncology Group.

Association of expression of drug resistance-related proteins with clinicopathologic characteristics

Positive expression of ERCC1, Bcl-2, and MT was observed in 37 (47.4%), 38 (48.7%) and 36 (46.2%) patients, respectively. Examples of the immunohistochemical staining patterns are shown in Figure 1. Positivity of ERCC1 showed significant association with smoking (p = 0.001) and response of treatment (p = 0.003) (Table 2). No significant association was found between the positivity of ERCC1 with other clinicopathologic characteristics, including age, gender, ECOG, histologic type, stage, chemotherapy regimens, Bcl-2 and MT expression (Table 2). In addition, neither of positive expression of Bcl-2 and MT represented significant association with clinicopathologic characteristics (data not shown).

Association of expression of drug resistance-related proteins with patient outcome

The follow-up duration of patients was 22 months (range: 3-22 months), and the median follow-up duration was 11 months. 48 patients were dead at the time of analysis. Kaplan-Meier analysis showed the association between positive expression of ERCC1 and poor OS (1-year, 35% *vs.* 65.2%; p = 0.003) (Fig. 2A and Table 3), and in strata analysis, this statistic association was significant in adenocarcinoma (Fig. 2D) and other types of NSCLC (p = 0.009; p = 0.033), not in squamous cell carcinoma (Fig. 2E) or adenosquamous carcinoma (p = 0.919; p = 0.433). Besides, bad ECOG performance status and smoking were also associated with poor OS (1-year; 25.6%/59.0%, p = 0.027 and 36.7%/65.0%, p = 0.029, respectively) (Table 3). However, positive expression of Bcl-2 and MT was not correlated with survival of patients (p = 0.672; p = 0.734) (Fig. 2B-C). Multivariate Cox regression analysis revealed that, among pretreatment characteristics, ECOG performance status (p=0.018) and ERCC1 (p = 0.013) expression were independent predictors of poor OS, while smoking failed to reach statistical significance (p = 0.283), although an association was observed (p = 0.030) in univariate analysis (Table 4).

DISCUSSION

In the present study, we investigated the expression of ERCC1, Bcl-2 and MT in advanced NSCLC and their relationship with response of cisplatin-based chemotherapy. As a result, negative expression of ERCC1 was associated with good response of cisplatin-based chemotherapy (p = 0.003). Moreover, in multivariate analysis, positive expression of ERCC1 (p = 0.013) was an independent poor prognosis factor for patients with advanced NSCLC. In addition, negative expression of ERCC1 showed significant association with longer survival in adenocarcinomas (p = 0.009). However, positive expression of Bcl-2 and MT did not show any correlation with response of chemotherapy and survival in overall NSCLC patients. These findings suggest that immunostaining of ERCC1 may be a useful marker for stratifying NSCLC patients into chemoresponsive and chemoresistant groups to cisplatin-based therapy.

To date, cisplatin-based chemotherapy remains the standard treatment of advanced NSCLC. The nucleotide excision repair pathway

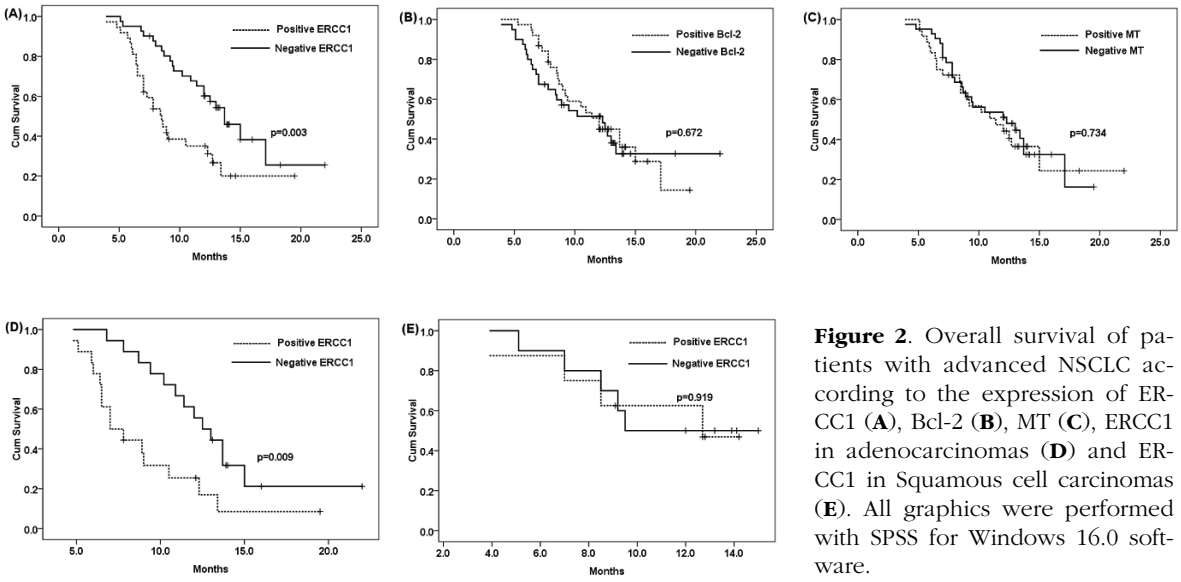

Figure 2. Overall survival of patients with advanced NSCLC according to the expression of ERCC1 (**A**), Bcl-2 (**B**), MT (**C**), ERCC1 in adenocarcinomas (**D**) and ERCC1 in Squamous cell carcinomas (**E**). All graphics were performed with SPSS for Windows 16.0 software.

Characteristics		ERCC-1 expression (%)		*p*
		High	Low	
Age [a]	≤57	15 (40.5)	24 (58.5)	0.112
	>57	22 (59.5)	17 (41.5)	
Gender	Male	24 (64.9)	27 (65.9)	0.927
	Female	13 (35.1)	14 (34.1)	
Performance status (ECOG)	0,1	26 (70.3)	31 (75.6)	0.596
	2,3	11 (29.7)	10 (24.4)	
Histologic type	Squamous cell carcinoma	8 (21.6)	10 (24.4)	0.974
	Adenocarcinoma	18 (48.6)	18 (43.9)	
	Adenosquamous carcinoma	3 (8.1)	4 (9.8)	
	Others	8 (21.6)	9 (22)	
Bcl-2 expression	+	16 (43.2)	22 (53.7)	0.358
	–	21 (56.8)	19 (46.3)	
MT expression	+	16 (43.2)	20 (48.8)	0.624
	–	21 (56.8)	21 (51.2)	
Stage	3 [a]	12 (32.4)	17 (41.5)	0.701
	3 [b]	15 (40.5)	15 (36.6)	
	4	10 (27)	9 (22)	
Chemotherapy regimens	Gemcitabine+cisplatin	19 (51.4)	14 (34.1)	0.189
	Navelbine+cisplatin	4 (10.8)	3 (7.3)	
	Paclitaxel+cisplatin	14 (37.8)	24 (58.5)	
Smoking history	Smoker	11 (29.7)	28 (68.3)	0.001
	Non-smoker	26 (70.3)	13 (31.7)	
Response of treatment	CR, PR	25 (67.6)	14 (34.1)	0.003
	Others [b]	12 (32.4)	27 (65.9)	

Table 2. ERCC1 expression and clinicopathologic characteristics. ECOG, Eastern Cooperative Oncology Group; CR, complete response; PR, partial response; [a] Median age. [b] Stable disease, + progressive disease, – not evaluable.

Prognostic factors		**1-year OS (%)**	***p***
Age [a]	≤57	52.2	0.704
	>57	49.4	
Gender	Male	47.2	0.122
	Female	58.2	
Performance status (ECOG)	0,1	59.0	0.027
	2,3	25.6	
Chemotherapy regimens	Gemcitabine+cisplatin	59.3	0.752
	Navelbine+cisplatin	42.9	
	Paclitaxel+cisplatin	45.1	
Smoking history	Smoker	65.0	0.029
	Non-smoker	36.7	
ERCC-1 expression	+	35.0	0.003
	–	65.2	
Bcl-2 expression	+	50.6	0.672
	–	51.4	
MT expression	+	47.4	0.734
	–	53.7	
Stage	3 [a]	57.6	0.140
	3 [b]	58.2	4
	31.6		
Histologic type	Squamous cell carcinoma	54.5	0.389
	Adenocarcinoma	43.5	
	Adenosquamous carcinoma	57.1	
	Others	61.4	

Table 3. Kaplan-Meier analysis of overall survival. OS, overall survival; ECOG, Eastern Cooperative Oncology Group. [a] Median age. [b] Stable disease, + progressive disease, – not evaluable.

manifests a central contribution in DNA repair and is proved to be involved in resistance to cisplatin-based chemotherapy [8-11]. ERCC1 displays a key role in nucleotide excision repair and removes cisplatin-induced DNA adducts [12-14]. Several clinical studies have shown that ERCC1 expression is associated with resistance to cisplatin-based chemotherapy and poor prognosis in several tumors, including NSCLC [15-24]. By immunohistochemistry, we also found that ERCC1 positive cases were more resistance to the cisplatin-based chemotherapy, and had a shorter overall survival than negative ones. Moreover, a significant correlation between positive expression of ERCC1 and poor outcome of NSCLC was observed only in some histologic types, especially in adenocarcinomas. In fact, to date, there are not many studies concerning about the association between histological type of NSCLC and resistance to cisplatin-based chemotherapy.

Over recent years, it has become recognized that defects in the regulation of apoptosis occupies an outstanding role in the biology and treatment resistance of cancer [25]. The anti-apoptotic proto-oncogene bcl-2 is concerned with apoptosis, by inhibiting programmed cell death (apoptosis) and prolonging cell survival by arresting cells in the G0/G1 phase of the cell cycle. It has been reported that overexpression of Bcl-2 existed in approximate half of all NSCLC cases [26,27]. Data from clinical studies suggest that Bcl-2 not only contributes to a more malignant NSCLC phenotype [28], but also confers resistance to chemotherapy in NSCLC [28,29]. As one of the most frequent chemotherapeuticses in NSCLC treatment [30,31], cisplatin mediated its antitumor effect at least in part by inducing apoptosis [32,33]. Bcl-2 overexpression confers resistance to cisplatin-induced apoptosis in many types of solid tumors and has been reported to correlate with poor response to cisplatin chemotherapy [7]. Nevertheless, no unified conclusion were achieved

Univariate analysis					
Prognostic factors			**Hazard ratio**	**95 % CI**	***p***
All patients	Age [a]	≤57	1		0.706
		>57	1.116	0.632-1.971	
Histologic type	Squamous cell carcinoma	1			0.395
	Adenocarcinoma	1.607	0.756-3.413		
	Adenosquamous carcinoma	0.846	0.228-3.146		
	Others	1.014	0.390-2.633		
Gender	Male	1			0.124
	Female	0.614	0.327-1.150		
Performance status (ECOG)	0,1	1			0.029
	2,3	1.991	1.063-3.729		
Chemotherapy regimens	Gemcitabine+cisplatin	1			0.755
	Navelbine+cisplatin	1.445	0.538-3.878		
	Paclitaxel+cisplatin	1.026	0.561-1.877		
Stage	3 [a]	1			0.144
	3 [b]	1.110	0.560-2.200		
	4	1.925	0.948-3.910		
ERCC-1	+	1			0.003
	–	2.361	1.322-4.216		
Bcl-2	+	1			0.674
	–	0.885	0.502-1.562		
MT	+	1			0.736
	–	1.103	0.625-1.947		
Smoking history	Smoker	1			0.030
	Non-smoker	1.867	1.053-3.310		
Multivariate analysis					
Performance status(ECOG)	0,1	1			0.018
	2,3	2.174	1.142-4.137		
Smoking	+	1			0.283
	–	1.402	0.756-2.600		
ERCC-1 expression	+	1	0.013		
	–	2.232	1.188-4.193		

Table 4. The overall survival univariate and multivariate Cox regression analysis. ECOG, Eastern Cooperative Oncology Group; CI, confidence interval. [a] Median age. [b] Stable disease, + progressive disease, – not evaluable.

with regard to the role of Bcl-2 expression in NSCLC [34]. Several immunohistochemical studies have investigated the prognostic value of Bcl-2 expression in lung cancer. Most studies support the hypothesis of prolonged survival in Bcl-2-positive cases [35-42], but others do not agree [43-45]. In this present study, we did not find the correlation between Bcl-2 expression and survival.

Metallothioneins (MT) are a group of low-molecular weight, cysteine rich intracellular proteins, which are encoded by a family of genes containing at least 10 functional isoforms in human. The expression and induction of these proteins have been associated with protection against DNA damage, oxidative stress and apoptosis. Moreover, MT may potentially activate certain transcriptional factors by donating zinc. Although MT is a cytosolic protein in resting cells, it can be translocated transiently to the cell nucleus during cell proliferation and differentiation [46]. In previous study, it have been demonstrated that overexpression of MT can confer resistance to radiation [47] and antineoplastic drugs [48,49]. It has been supposed that the ability of MT in de-

creasing the efficacy of chemotherapeutic effects may be attributed to sequestrating free radicals, drugs or their metabolites, thus inhibiting the direct interaction of antineoplastic agents with their intracellular targets [46]. In fact, the role of MT in the development of chemoresistance in the clinical setting is still debatable. The protective effect of MT overexpression against antineoplastic agents has been reported in human tumors such as ovarian, testicular and colon tumors [50-54], whereas there are other reports which do not support this perception [46]. In this study, there was no evidence supporting the predictive value of MT expression in advanced NSCLC treated with cisplatin-based chemotherapy.

Although many studies have tried to identify a clinical indicator powerful enough to predict response to chemotherapy and outcome of patients with NSCLC, only TNM stage and performance status are used in clinical practice [55,56]. Likewise, in this present study, we also found that bad performance status was an independent poor prognosis for patients with advanced NSCLC.

In addition, there was also an unexpected finding that ERCC1 expression was associated with smoking, but failed to be an independent poor prognosis of NSCLC in multivariate analysis. The causal relationship between smoking and lung cancer has been accepted since the 1950s, and it has shown that carcinogenic compounds found in tobacco smoke can promote damage to the DNA [34]. Does the DNA damage reversely promote the capacity of DNA repair and then result in the elevated expression of ERCC1 in lung cancer? It seems only feasible in theory but still need warranted.

CONCLUSION

In this study, we showed that ERCC1 could be a helpful biomarker to identify the subgroup of NSCLC patients most likely to benefit from cisplatin-based chemotherapy. However, before putting ERCC1 in daily practice for treatment making-decision, confirmatory retrospective analyses as well as prospective randomised trials are needed.

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