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Original Article

Multidrug resistance protein and topoisomerase 2 alpha expression in non-small cell lung cancer are related with brain metastasis postoperatively

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Abstract: The aim of this study was to investigate association between expressions of multidrug resistance protein (MRP) and topoisomerase 2 alpha expression in non-small cell lung cancer (TOP2A) and brain metastasis operatively. The expression of MRP and TOP2A were performed using immunohistochemistry (IHC) staining, and the results were analyzed in correlation with clinicopathological data. A total of 286 NSCLC patients who underwent curative surgery between 2007 and 2013 were enrolled in this study. Positive expression of MRP and TOP2A were 62.2% and 37.8%. MRP positive expression in NSCLC was significantly correlated with tumor cell differentiation (P=0.028). TOP2A expression was significantly associated with patients' smoking status, tumor histological type (P<0.05). The positive MRP group had significantly inferior survival rates for 2-year BMFS than did the negative MRP group (79.0% vs. 93.4%, P=0.003) by the Kaplan-Meier method and a log-rank test. Similarly, the positive TOP2A expression was inversely correlated with 2-year BMFS (84.2% vs. 93.4%, P=0.030). Multivariate analysis showed that gender, MRP expression and TOP2A expression were independent prognostic factors for BMFS (P<0.05). Positive expressions of MRP and TOP2A in the tumor tissue are associated with increased risk of developing brain metastases in NSCLC.

Keywords: Multidrug resistance protein, topoisomerase 2 alpha, non-small cell lung cancer, brain metastasis

Introduction

Lung cancer is the leading cause of cancer death in both developed and developing countries [1]. Non-small cell lung cancer (NSCLC) represents 85% of patients diagnosed with lung cancer. More than 75% of these patients present with advanced stage disease. Radical surgery is thought to be the main treatment that can provide opportunity of cure and long-time survival. The prognosis for patients with metastatic NSCLC remains poor with a 5-year survival rate at only 10 to 20%, despite aggressive multi-modality therapy [2-4]. Approximately 15-30% of NSCLC patients develop brain metastasis [5, 6]. There has been an increasing incidence of brain metastasis over the last decades due to the better diagnostic methods available and public health awareness. Thus, it is highly desirable to identify novel targets and develop new strategies that inhibit brain metastasis from the primary lung cancer.

Multidrug resistance protein (MRP) and Topoisomerase 2 alpha (TOP2A) are involved in drug resistance in NSCLC [7-9]. MRP family currently has seven members, which transport a wide range of anticancer drugs out of cells and their presence related with drug resistance. Topoisomerases are isomerase enzymes that act on the topology of DNA. In cancers, the topoisomerase II-alpha (TOP2A) is highly expressed in highly proliferating cells. In this present study, immunohistochemical methods were used to investigate expressions of MRP and TOP2A in NSCLC tissues. This study was also to determine whether the expression of MRP and TOP2A can be used as a brain metastasis marker of NSCLC.

Patients and methods

Patients

A retrospective analysis was carried out for 286 NSCLC patients who underwent curative sur-

Table 1. Association between expression of TOP2A, MRP and clinicopathological characteristics of NSCLC patients (n=286)

Variables	Total (n, %)	TOP2A		MRP	
		Positive (n, %)	*P*	Positive (n, %)	*P*
Gender					
Female	65 (22.7)	18 (27.7)	0.057	37 (56.9)	0.315
Male	221 (72.3)	90 (40.7)		141 (63.8)	
Age (year)					
<65	186 (65.0)	72 (38.7)	0.652	122 (65.6)	0.111
≥65	100 (35.0)	36 (36.0)		56 (56.0)	
Smoking status					
No	89 (31.1)	25 (28.1)	0.023	51 (57.3)	0.247
Yes	197 (68.9)	83 (42.1)		127 (64.5)	
Histological type					
Adenocarcinoma	123 (43.0)	30 (24.4)	<0.001	80 (65.0)	0.619
SCC	163 (57.0)	78 (47.9)		91 (60.7)	
Differentiation					
Well	12 (4.2)	5 (41.7)	0.884	6 (50.0)	0.028
Moderate	140 (49.0)	51 (36.4)		98 (70.0)	
Poor	134 (46.8)	52 (38.8)		74 (55.2)	
T stage					
T_{1-2}	220 (76.9)	81 (36.8)	0.548	143 (65.0)	0.079
T_{3-4}	66 (23.1)	27 (40.9)		35 (53.0)	
N stage					
N_0	140 (49.0)	49 (35.0)	0.345	85 (60.7)	0.603
N_{1-2}	146 (51.0)	59 (40.4)		93 (63.7)	
Brain metastasis					
No	258 (90.2)	88 (34.1)	<0.001	154 (59.7)	<0.001
Yes	28 (9.8)	20 (71.4)		24 (85.7)	

gery at Hangzhou first people's hospital between 2007 and 2013. None of the patients had received preoperative chemotherapy and radiotherapy. Patients with distant metastasis prior to surgery were excluded. Patient characteristics, clinicopathologic data, and site of metastasis during follow-up were obtained from medical records and pathology reports. The study protocol was approved by the institutional review board of the hospital. All patients provided informed consent before surgery.

Immunohistochemistry (IHC)

Tissue samples were fixed with formaldehyde solution, embedded in paraffin, followed by regular (4 μm) slicing. IHC was performed by the S-P method according to the manufacture's introduction. The antibodies used in this study were purchased from Beijing Biosynthesis Biotechnology Corporation (Beijing, China). Slides were deparaffinized in xylene and rehydrated through graded ethanol. Briefly, the paraffin embedded sections were dried overnight at 37°C and deparaffiized in xylol, rehydrated and incubated with methanol/0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. Possible background staining was removed by applying normal goat serum, diluted 1:20 for 1 h. The sections were incubated with the primary antibody (50 μL) and kept at 4°C overnight in a humidified chamber. After rinsing in PBS, the biotinylated secondary antibody goat-anti-rat (50 μL) was applied for 60 min at 37°C. Diaminobenzidine (DAB) was employed as a chromogen. The sections were then counterstained with hematoxylin. Each specimen was evaluated independently by two pathologists who were blinded to the clinical status of the patients.

MRP staining was evaluated according to a weighting method, using both the percentage and intensity of tumor cells within the tumor. The percentage of tumor cells was recorded as follows: 0 points, 0-1%; 1 point, 2-25%; 2 points, 26-50%; 3 points, 51-75%; and 4 points, >75%. Tumor intensity was recorded as follows: 1 point, faint yellow; 2 points, yellow; and 3 points, brown-orange. The final weighting score was calculated by multiplying the percentage and intensity scores. A final score of 0-4 was considered to be negative and a score of >4 as positive. TOP2A immunoreactivity was identified in the nuclei of malignant cells. The expression intensity of TOP2A was stratified into two categories scored as follows: negative, <10%; positive, >10%.

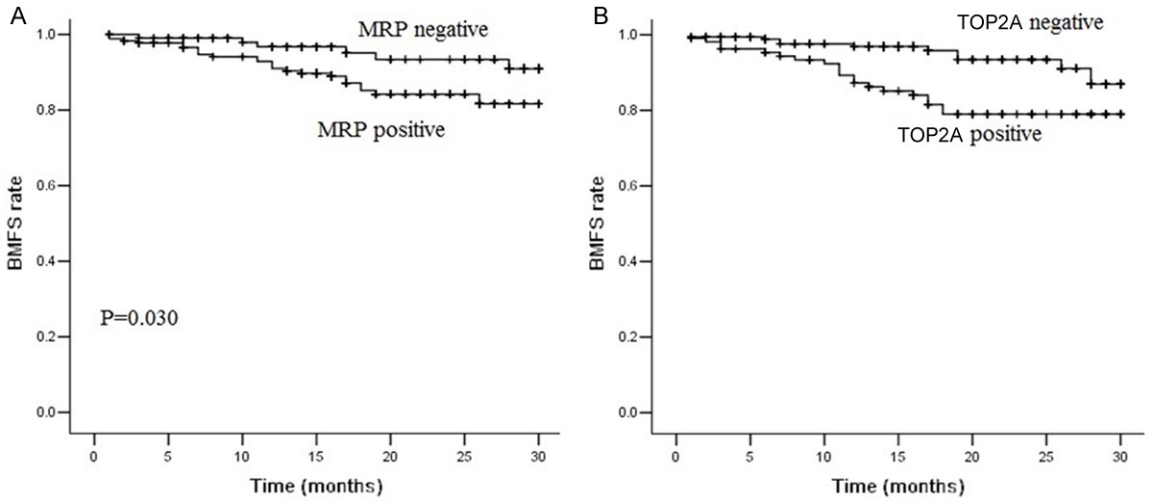

Figure 1. A: BMFS curves are shown in the MRP positive (n=188) and MRP negative (n=98) patients with NSCLC. B: BMFS curves are shown in the TOP2A positive (n=108) and MRP negative (n=178) patients with NSCLC.

Table 2. Univariable analysis on brain metastasis-free survival and overall survival

Variables	2 year BMFS rate (%)	Log-rank	*P*	2 year OS rate (%)	Log-rank	*P*
Gender						
Female	87.6	2.95	0.086	88.6	2.51	0.113
Male	76.6			82.3		
Age (year)						
<65	85.1	0.16	0.688	88.5	12.36	<0.001
≥65	83.8			74.4		
Smoking status						
No	81.0	0.90	0.342	90.1	5.35	0.021
Yes	86.6			80.7		
Histological type						
Adenocarcinoma	81.7	2.89	0.236	88.0	3.83	0.153
SCC	91.0			80.6		
Differentiation						
Well or Moderate	87.1	0.63	0.429	83.6	0.01	0.913
Poor	85.5			82.4		
T stage						
T_{1-2}	87.9	0.22	0.648	85.6	1.16	0.283
T_{3-4}	86.0			81.7		
N stage						
N_0	90.6	3.02	0.082	89.7	5.59	0.018
N_{1-2}	84.1			79.4		
TOP2A						
Negative	93.4	8.66	0.003	87.2	1.60	0.205
Positive	79.0			80.3		
MRP						
Negative	93.4	4.72	0.030	84.5	0.21	0.647
Positive	84.2			84.3		

Statistical analysis

Statistical analysis was carried out using SPSS software version 17.0 (SPSS Inc. Chicago, IL). The Chi-square test was performed to evaluate the association between the clinicopathological variables and MRP, and TOP2A, respectively. Patient survival curves were plotted according to the Kaplan-Meier method and a log-rank test. Multivariate Cox regression analysis was used to identify significant independent prognostic factors. For the brain metastasis-free survival (BMFS) analysis, the duration was defined as the time from diagnosis until the occurrence of brain metastasis. Overall survival was calculated as the time from surgery to death or the last follow-up. A

Table 3. Cox proportional hazards regression on brain metastasis-free survival

Variables	Hazards ratio	95% CI	*P*
Gender			
Male Vs Female	2.51	1.17-5.38	0.018
N stage			
N_{1-2} Vs N_0	1.74	0.83-3.68	0.145
TOP2A			
Positive Vs Negative	3.31	1.52-7.20	0.003
MRP			
Positive Vs Negative	2.76	1.11-6.84	0.029

two-sided *P* value <0.05 was considered statistically significant.

Results

Association of expression of MRP and TOP2A with clinicopathologic variables in 286 NSCLC patients

The clinicopathologic characteristics of the 286 NSCLC patients were shown in **Table 1**. Of these, 123 patients (43.0%) were lung adenocarcinoma and 163 (57.0%) were lung squamous cell carcinoma. Positive expression of MRP and TOP2A were 62.2% and 37.8%. MRP positive expression in NSCLC was significantly correlated with tumor cell differentiation (*P*=0.028). TOP2A expression was significantly associated with patients' smoking status, tumor histological type (*P*<0.05). However, there were no significant differences in terms of age, gender T stage and N stage (*P*>0.05). Furthermore, positive expression of MRP and TOP2A were more frequent in NSCLC tissues with brain metastasis (*P*<0.001).

Association of MRP and TOP2A with brain metastasis-free survival (BMFS) and overall survival (OS)

Further survival analyses of the patient samples indicated that the 2-year OS and BMFS rates were 82.2% and 84.8% for the total study population, respectively. There were 77 (26.9%) patients that developed recurrence or progression of cancer. 28 patients (9.8%) developed brain metastasis. 43 patients (15.0%) died during the study period. Interestingly, the positive MRP group had significantly inferior survival rates for 2-year BMFS than did the negative MRP group (79.0% Vs 93.4%, *P*=0.003, **Figure 1A**) by the Kaplan-Meier method and a log-rank test. Similarly, the positive TOP2A expression was inversely correlated with 2-year BMFS (84.2% Vs 93.4%, *P*=0.030, **Figure 1B**). However, the overall survival rate differences of patients with a positive or negative MRP or TOP2A expression were not statistically significant (*P*>0.05, **Table 2**).

Multivariate analysis was performed for the variables with *P*<0.10 in univariate analyses for brain metastasis. Gender, MRP expression and TOP2A expression were indeed independent prognostic factors for BMFS (*P*<0.05, **Table 3**). These data indicated that MRP and TOP2A may be significant and novel biomarkers for evaluating the outcome in NSCLC patients.

Discussion

The study of biological biomarker for brain metastasis in NSCLC patients is important for improving the survival of patients. In this large scale single institution study, we reported for the first time that MRP and TOP2A were expressed at a higher level in human NSCLC patients and their expression were significantly correlated with brain metastatic features. In the multivariate analysis, MRP and TOP2A expression were indeed independent prognostic factors for BMFS. Based on these findings, we recommend that patients with positive expression of MRP and TOP2A be considered for close monitoring with brain imaging and biochemical surveillance every 3 months for the first 2 years after surgery. Earlier identification of brain metastasis would allow for earlier medical intervention and therefore improve patients' outcomes.

Multidrug resistance (MDR) is a major challenge to the clinical treatment of NSCLC, and detection of MDR proteins may help guide adjuvant chemotherapy in NSCLC and determine the prognosis of patients. MRP is a member of the ATP-binding cassette transport protein superfamily, which acts as an ATP-dependent outward transport pump. The present study found positive expression of MRP in NSCLC tissues were 62.2%, consisted with previous studies [10, 11]. In Xu et al's study [11], the positive rates of MRP expression was 43.4% (66/152), which is markedly correlated with pathological

types and lymph node metastasis. The overall survival rate of patients with positive MRP expression was markedly lower compared with those of patients with a negative MRP expression. However, MRP is not an independent prognostic factor by multivariate analysis. Recently, Chen et al [10] also demonstrated that MRP plays an important role in multidrug resistance in NSCLC and is associated with overall survival. TOP2A serves as the target of several anticancer drugs, such as doxorubicin, VP16 and mitoxantrone [12]. There was a positive correlation between TOP2A expression and resistance to VP-16 and a negative correlation between TOP2A expression and resistance to doxorubicin [7]. In NSCLC, patients with high TOP2A expressing tumors had a significantly worse survival compared with the patients with low or intermediate TOP2A expressing tumors [13]. However, in our current study, MRP and TOP2A expression are not related with overall survival both proven by univariate and multivariate analysis.

Disease stage and tumor histology were the strongest predictor of brain metastasis after curative surgery [14]. The occurrence rate of brain metastasis in NSCLC patients received curative surgery was 9% to 45% [15, 16]. In our study, the incidence of brain metastasis in NSCLC patients who undergone curative surgery is 9.8%, consisted with previous studies [14, 16]. Most patients enrolled in this study were $_{p}$T1-2 and without regional lymph node metastasis, so the rate of brain metastasis were lower than other studies [17, 18]. The median time to onset of brain metastasis was 11.5 months (range: 6-28 months). Immunostaining for MRP and TOP2A may be useful for identifying patients with NSCLC at high risk for developing brain metastasis after curative surgery. MRP, which involved with the blood-brain barrier [19], preventing the influx of agent from the blood into the brain and facilitating the efflux of compounds from the brain into the blood. If tumor cell metastasis inside the brain tissues, chemotherapeutic agents, such as cisplatin and paclitaxel are less likely to be effective.

The major limitation of current study is that the proportion of patients with brain metastasis is much lower than other studies, although the number of patents with early stage disease in this study is larger than in other studies. Second, this study is built retrospectively. A prospective study is required to determine the prognostic of MRP and TOP2A. Furthermore, the follow-up time is relatively short and the number of sample is small. So we cannot do the subgroup analysis. Future studies with larger samples and longer follow-up are warranted to validate our results.

In summary, MRP and TOP2A are expressed in a subgroup of NSCLC and its expression is related to clinicopathological characteristics. Increased expressions of MRP and TOP2A in the tumor tissue obtained at the surgery are related with the risk of developing brain metastasis. Further investigation to access the expressions of MRP and TOP2A in brain metastases is of great interest.

Disclosure of conflict of interest

None.

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