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Original Article

Expression of ATM, CHK2 and BRCA1 predicts the clinical outcome of non-small cell lung cancer in patients receiving platinum-based chemotherapy

Yinyan Li, Lingjuan He, Yang Wang, Ying Li, Si Yang, Jian Chen, Linrun Wang, Yan Lou

The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, People's Republic of China

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Abstract: Background: The aim of this study was to assess the predictive value of tumor expression of ataxia telangiectasia-mutated (ATM), checkpoints kinase 2 (CHK2) and breast cancer susceptibility gene1 (BRCA1) on clinical outcome in patients with advanced non-small cell lung cancer (NSCLC) receiving platinum-based chemotherapy. Methods: Immunohistochemistry was used to analyze the expression of ATM, CHK2 and BRCA1 isolated from paraffin-embedded tumor biopsies of 101 NSCLC patients. All NSCLC patients received first-line platinum chemotherapy. Results: There was a significant relationship observed between ATM and CHK2. However, a correction was observed between the expression of CHK2 and patients' age. Patients below 60 years had a lower CHK2 expression than those above 60 years. Patients with low CHK2 or BRCA1 expression had a longer OS as compared to patients with high CHK2 or BRCA1 expression. Median OS was 34 months in the squamous NSCLC and median OS was 14 months in the mixed NSCLC. Conclusions: Our results suggest that CHK2 and BRCA1 expressions are associated with the prognosis in NSCLC patients treated with platinum-based chemotherapy. Clinical outcomes could be improved by additional targeting CHK2 to refine BRCA1 based predictive modeling from benefit of platinum-based chemotherapy.

Keywords: Non-small cell lung cancer, ATM, CHK2, BRCA1, DNA repair, prognostic biomarkers

Introduction

Lung cancer is a major cause of mortality from malignant disease because of its high incidence, malignant behavior, and lack of major advancements in treatment strategies [1]. Platinum-based chemotherapy, such as cisplatin or carboplatin, is considered as the most frequently chemotherapy for advanced NSCLC [2]. However, patients diagnosed with the same stage of NSCLC and histological type of cancer show different resistances to chemotherapy and different prognoses. Therefore, detection of molecular markers may facilitate the selection of drugs for postoperative adjuvant chemotherapy to increase the benefit to patients. Both cisplatin and carboplatin act by damaging DNA through various mechanisms [3], and several candidate genes that are involved in cellular DNA-repair are potential predictors for platinum-based chemotherapy [4].

Breast cancer susceptibility gene1 (BRCA1) is a key factor involved in nuclear excision repair [2]. Previous reports show that BRCA1 is involved in resistance to platinum in tumor cells and low BRCA1 mRNA level is associated with higher response and longer progression-free survival (PFS) in NSCLC patients [3]. Its predictive role has been confirmed in several solid tumors, including NSCLC [4]. Ataxia telangiectasia-mutated (ATM), a serine/threonine proteins kinases, is member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family. It is needed for the initiation of the double-strand break (DSB) repair by homologous recombination (HR) [5]. Upon induction of DSBs, ATM is activated and phosphorylates several DSB response proteins and initiates a series of downstream reactions [6]. The reduced apoptotic response, altered DNA repair signaling and cell cycle perturbations in NSCLC are possible factors contributing to their thera-

py resistance [7]. The authors report on the probability of inadequate activation of ATM, leading to compromised cell cycle checkpoint. ATM mutant is the high risk of cancer, as to the role of ATM in lung cancer, several epidemiological studies have implicated the relationship between ATM variants and risk of cancer [8]. The checkpoints kinase 2 (CHK2) is a tumor suppressor that plays a crucial role in regulating cell-cycle checkpoint and apoptosis following DNA damage. ATM can phosphorylate CHK2, and this activated CHK2 will subsequently phosphorylate BRCA1 through its SQ cluster domain [9]. CHK2 mutations occur in a range of human tumors, indicating that CHK2 is a major rate-limiting factor for cancer suppression in many tissues. Interestingly, down-regulation of CHK2 expression via promoter methylation has occurred in a proportion of sporadic cancers such as lung, breast, colon as well as ovarian [10]. Mutations of the CHK2 are rare in lung tumor [11].

Previous studies have reported the association between BRCA1 and NSCLC. However, there is still insufficient knowledge about the individual response to standard platinum-based chemotherapy in patients with lung cancer. To further assess the association between expression of ATM, CHK2, BRCA1 and clinical outcome of NSCLC, we conducted a perspective study with a series of NSCLC cases treated with platinum-based chemotherapy.

Materials and methods

Study participants

The study included 101 patients with non-small cell lung cancer receiving first-line platinum chemotherapy. Briefly, patients were recruited between January 2008 and December 2012 at the First Affiliated Hospital of Zhejiang University. We retrospectively collected all available tumor samples and the corresponding clinical data. The inclusion criteria of tumors as follow: histologically confirmed NSCLC, first-line treatment with cisplatin at a dose of 75 mg/m^2 or carboplatin AUC=5 on day 1 and gemcitabine at a dose of 1200 mg/m^2 on days 1 and 8 or paclitaxel 175 mg/m^2 on day 1, availability of formalin-fixed, paraffin-embedded primary lung cancer tissue blocks, availability of OS data. The Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University approved the study. The participants signed an informed consent form prior to enrollment to the study.

We calculated progression-free survival (PFS) from the date of the chemotherapy to progressive disease. It was assessed by chest X-ray and computed tomography imaging. Overall survival (OS) was calculated from the start date of chemotherapy to the date of death or last clinical follow-up. Median PFS and OS were estimated using the Kaplan-Meier curves. A univariate Cox regression analysis, with hazard ratios and 95% confidences intervals, was used to assess the association between each potential predictive factor and survival.

IHC-staining

The selected sections were deparaffinized via a gradual alcohol and xylene series followed by rehydration in distilled water. Antigen retrieval was performed by adding citrate buffer (Ph6.0) and heating in a microwave oven for 20 min at 100°C. The sections were subsequently incubated in a 3% hydrogen peroxide solution to block endogenous peroxidase activity and washed with a phosphate-buffered saline solution. Following incubation with blocking solution for 20 min, the sections were incubated again with primary and then secondary antibodies at appropriate dilutions. The present study used three kinds of antibody (anti-ATM; cat no. ab47575; dilution, 1:100; anti-CHK2; cat no. ab47433; dilution, 1:100; anti-BRCA1; cat no. ab16780; dilution, 1:100). The specimens were counterstained with hematoxylin, mounted and examined by light microscopy (Olympus BX50, Japan).

Protein expression was divided into two groups according to the differential stained grades in the tumor cell nuclei or cytoplasm. It was defined as negative if immunoreactivity was 10% less than tumor cells. Otherwise, it was defined as positive.

Statistical analysis

All statistical analyses were carried out using SPSS software (Version 13.0; SPSS Inc, Chicago, IL, US). Proteins expression levels were regarded as continuous variable. Continuous variables were expressed as mean and SD, and categorical variables were expressed by percentage. Chi-square tests were calculated to

Table 1. Clinical characteristics of patients in the study

Characteristic	Number	Percent (%)
Median age		
<60	50	49.5
≥60	51	50.5
Gender		
Female	27	26.7
Male	74	73.3
Smoking status		
Yes	61	60.4
No	40	39.6
Performance stage		
0	76	75.2
1 and 2	25	24.8
Histology		
Squamous	28	27.7
Adenocarcinoma	55	54.5
Mixed NSCLC	18	17.8
Stage		
I	7	6.9
II	47	46.5
III	23	22.8
IV	24	23.8
Metastasis sites		
Lymph Node	78	77.2
Lung	3	3.0
Bone	3	3.0
Brain	10	9.9
Liver	5	5.0
Others	5	5.0

assess the correlation between ATM, CHK2 and BRCA1 proteins expression. Statistical comparisons were performed with clinical characteristics of NSCLC. Cox proportional hazards regression was conducted to assess the effects of those protein expression on PFS and OS of NSCLC, with results expressed as hazard ratio (HR) and its 95% confidence interval (CI). Survival distributions were analyzed by the log-rank test and plotted by Kaplan Meier survival curve. A value of $P<0.05$ was considered statistically significant.

Results

Patient characteristics

The clinical characteristics of the study population were summarized in **Table 1**. A number of 101 NSCLC patients who received surgical treatment at the First Affiliated Hospital of Zhejiang University, China, between January 2008 and December 2012 were randomly selected for this study. A total of 101 NSCLC patients were included in cases who were histopathologically diagnosed at diverse tumor stages and had been through platinum-based adjuvant chemotherapy. Patients were predominately males (73.3%) and the median age was 60 years (range: 33-76 years). 60.4% patients were smokers, and 39.6% patients were non-smokers. The physical status (PS) of each patient was graded. 75.2% NSCLC patients obtained a score 0, whereas others gained score within the range of 1-2. Of the 101 NSCLC cases, 27.7% were presented with squamous cell carcinoma, 54.5% with adenocarcinoma, and 17.8% cases mixed NSCLC. The histopathological examination confirmed 6.9% cases of stage I, 46.5% cases of stage II, 22.8% cases of stage III, and 23.8% cases of stage IV. 6.9% cases received postoperative adjuvant chemotherapy and radiotherapy. Median follow-up duration of the 101 NSCLC patients was 20 months (range: 6-34 months), and the last follow-up visit was on October 30, 2014. 86 patients showed disease progression after surgery. 65 patients passed away, and 36 patients survived at the last follow-up. The median PFS of the overall study population was 10 months (range: 1-73 months) and the median OS for all patients was 24 months (range: 4-86 months).

ATM, CHK2 and BRCA1 protein expression in NSCLC tumor tissues and their correlations with disease

It is reported that the alteration of chromatin remodeling can activate an ATM-mediated DNA signal pathway and thereby result in diverse ATM-dependent responses [12]. Then, we asked whether ATM, CHK2 and BRCA1 expressed in tumor tissues of NSCLC patients. In the trial, we performed a correlation study on ATM, CHK2 and BRCA1 protein expression by using IHC staining of tumor tissues from 101 NSCLC patients (**Figure 1**). As shown in **Table 2**, among the 101 cases of NSCLC, 52 patients showed positive protein expression of BRCA1, whereas 49 patients showed negative protein expression of BRCA1. The positive rate of BRCA1 was 51.5%. 55 patients presented positive ATM expression, and 46 patients present-

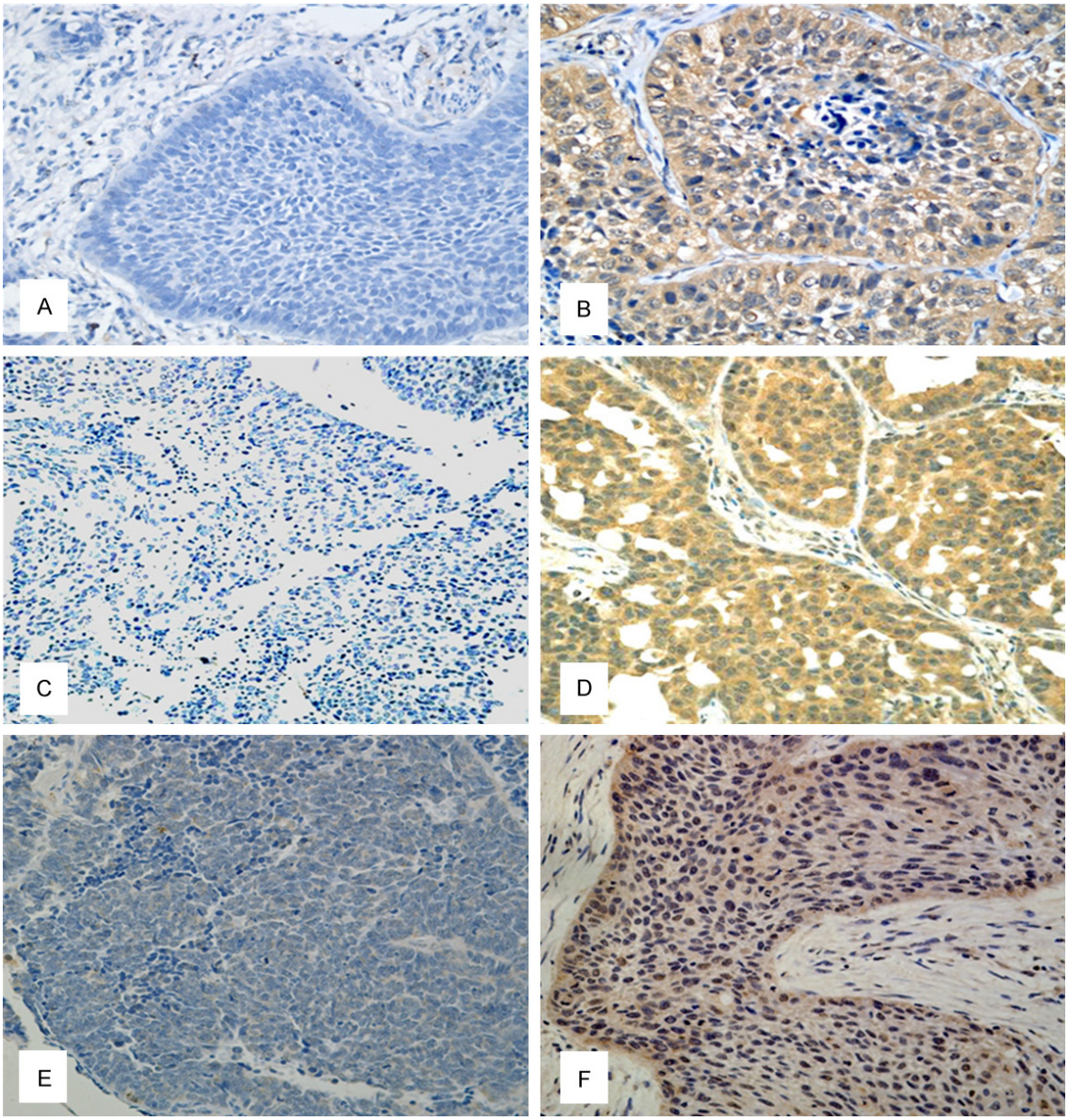

Figure 1. Immunohistochemistry analysis and expression of BRCA1, ATM, CHK2 in NSCLC. A, C, E: Immunohistochemistry analysis of tissue section counterstained with hematoxylin; B: Immunohistochemistry analysis of BRCA1 in lung tumor tissue (×400); D: Immunohistochemistry analysis of ATM in lung tumor tissue (×400); F: Immunohistochemistry analysis of CHK2 in lung tumor tissue (×400).

ed negative protein ATM, with a positive rate of 54.5%. 65 patients presented positive CHK2 expression, and 36 patients presented negative protein CHK2, with a positive rate of 64.3%.

The positive rate of ATM and BRCA1 had no relevance (P>0.05). Meanwhile, the positive rate of CHK2 and BRCA1 had no relevance (P>0.05). Interestingly, there was notable relevance exist in NSCLC between ATM and CHK2 protein expression (P=0.007, r=0.285, **Table 3**). These results highlighted the biological model among ATM, CHK2, BRCA1 and significant correlations were observed.

Correction between ATM, CHK2 and BRCA1 protein expression and clinical characteristics

In the previous section, we examined the expression of ATM, CHK2 and BRCA1. A certain amount of literatures indicate female carriers of ATM mutation have an increased risk of

Table 2. Relationship between BRCA1, ATM and CHK2 expression and clinical characteristics

		BRCA1				ATM				CHK2			
Characteristic	Number	-	+	x^2	*p*	-	+	x^2	*p*	-	+	x^2	*p*
Median age		49	52			46	55			36	65		
<60	50	28	22	2.221	0.136	25	25	1.749	0.186	23	27	6.696	0.01
<60	50	28	22	2.221	0.136	25	25	1.749	0.186	23	27	6.696	0.01
Gender													
Female	27	17	10	3.08	0.079	14	13	0.591	0.442	13	14	2.512	0.113
Male	74	32	42			32	42			23	51		
Smoking status													
Yes	61	25	36	3.498	0.061	27	34	0.102	0.749	20	41	0.548	0.459
No	40	24	16			19	21			16	24		
Performance stage													
0	76	36	40	0.162	0.688	35	41	0.032	0.858	28	48	0.192	0.661
1 and 2	25	13	12			11	14			8	17		
Histology													
Squamous	28	12	16	5.515	0.063	9	19	3.853	0.146	11	17	0.66	0.719
Adenocarcinoma	55	32	23			26	29			20	35		
Mixed NSCLC	18	5	13			11	7			5	13		
Stage													
I	7	3	4	0.956	0.812	2	5	2.085	0.555	3	4	1.266	0.737
II	47	25	22			21	36			16	31		
III	23	11	12			11	12			10	13		
IV	24	10	14			12	12			7	17		

Table 3. Correction between CHK2 and ATM protein expression

		CHK2			
Gene	Number of cases	-	+	*P* value	r coefficient
ATM					
-	46	21	25		
+	55	15	40		
Total	101			0.007	0.285

breast cancer, and ATM mutation carriers have a reduced life expectancy due to mortality from cancer and heart diseases [13]. Next we wanted to determine whether their expression related to clinical characteristics.

Table 2 indicated that no significant correction between ATM and BRCA1 protein expression and clinical characteristics, including age (<60 and ≥60 years of age), gender, smoking status, PS score, tissue morphologies, and clinical tumor stages of patients, was observed (P> 0.05). Interestingly, our data revealed a markedly relevance between CHK2 and patients' age (P=0.01). The patients whose age below 60 years got a lower CHK2 expression rate by comparison with patients older than 60 years.

PFS and OS of different kinds of NSCLC

Invasive adenocarcinoma patients who were <65 years or had tumors <2 cm in size may have improved survival outcomes after segmentectomy [14]. More than half of Chinese patients with lung cancer are diagnosed at an advanced stage. Chemotherapy is the main therapeutic to extend survival and to improve the quality of life in Chinese patients with advanced lung cancer [15]. The examination confirmed 27.7% was presented with squamous cell carcinoma, 54.5% with adenocarcinoma, and 17.8% cases mixed NSCLC. Our research found that median OS was 34 months (HR 0.349, 95% CI 0.163-0.746) in the squamous NSCLC and median OS was 14 months in the mixed NSCLC. A correction was observed between squamous and mixed. The squamous patients got a longer median OS than mixed NSCLC patients (34 *vs*. 14 months, P=0.007, **Table 4**). Thus, platinum-based chemotherapy has been considered the standard first-line therapy for patients with squamous NSCLC.

Table 4. Clinical characteristics and PFS and OS of NSCLC

Characteristic	Number	Median PFS (months)	HR (95% CI)	*P* value	Median OS (months)	HR (95% CI)	*P* value
Median age							
<60	50	8	1.343 (0.869, 2.074)	0.184	25	1.279 (0.785, 2.086)	0.324
≥60	51	10	Ref		31	Ref	
Gender							
Female	27	9	0.849 (0.518, 1.391)	0.515	24	1.119 (0.649, 1.927)	0.686
Male	74	8	Ref		25	Ref	
Smoking status							
Yes	61	9	1.075 (0.691, 1.672)	0.748	27	1.368 (0.836, 2.237)	0.212
No	40	8	Ref		23	Ref	
Performance stage							
0	76	8	0.791 (0.470, 1.330)	0.377	25	0.956 (0.535, 1.706)	0.879
1 and 2	25	7	Ref		30	Ref	
Histology							
Squamous	28	12	0.566 (0.288, 1.114)	0.099	34	0.349 (0.163, 0.746)	0.007
Adenocarcinoma	55	8	0.874 (0.510, 1.499)	0.626	27	0.574 (0.323, 1.021)	0.059
Mixed NSCLC	18	8	Ref		14	Ref	
Stage							
I	7	21	0.446 (0.060, 3.333)	0.432	31	0.233 (0.031, 1.740)	0.156
II	47	9	0.856 (0.507, 1.444)	0.56	25	0.693 (0.394, 1.217)	0.202
III	23	8	0.731 (0.394, 1.356)	0.321	25	0.682 (0.339, 1.374)	0.284
IV	24	8	Ref		18	Ref	

Table 5. ATM, CHK2 and BRCA1 protein expression and PFS and OS of NSCLC

Gene	N (N=101)	Median OS (months)	HR (95% CI)	*P* value	Median PFS (months)	HR (95% CI)	*P* value
BRCA1							
-	49	34	0.523 (0.317, 0.861)	0.011	11	0.876 (0.565, 1.385)	0.553
+	52	21	Ref		8	Ref	
ATM							
-	46	30	0.980 (0.601, 1.598)	0.937	8	0.982 (0.633, 1.525)	0.937
+	55	25	Ref		9	Ref	
CHK2							
-	36	36	0.582 (0.334, 1.013)	0.046	9	0.842 (0.530, 1.337)	0.465
+	65	25	Ref		8	Ref	

ATM, CHK2 and BRCA1 protein expression and PFS and OS of NSCLC

To determine whether ATM, CHK2 and BRCA1 protein expression had an effect on PFS and OS of NSCLC, we focused on this set of data. Cox proportional hazards regression analysis was guided to assess the effects of ATM, CHK2 and BRCA1 protein expression on PFS and OS of NSCLC patients. Firstly, a significant benefit was observed for patients with negative BRCA1 owned a longer median OS compared with those with positive BRCA1 (HR 0.523, 95% CI 0.317-0.861, P=0.011). In addition, patients with negative CHK2 owned a longer median OS compared with those with positive CHK2 (HR 0.582, 95% CI 0.334-1.013, P=0.046, **Table 5**). **Table 5** indicated that there was no significant correction between those genes and PFS. Taken together, these data confirmed that CHK2 and BRCA1 protein expression acted as independent factors associated with longer OS.

Discussion

To the best of our knowledge, this is the first study to investigate associations between clinical effect and the potential function of ATM, CHK2 and BRCA1 in NSCLC.

Platinum chemotherapy is a first-line therapy for non-small cell lung cancers [16]. But individualized cancer cell response to the drug compromises its efficacy. The analysis of DNA repair genes could improve our models for predicting. It is reported that ATM can induce activation of CHK2 by increasing phosphorylation of CHK2, and then subsequently phosphorylate BRCA1 through its SQ cluster domain [9]. We performed a prospective randomized clinical experiment, testing the hypothesis that individualized treatment would confer improved outcome.

ATM, a member of the PI3K-like protein kinases family of serine threonine kinases, plays an important role in the maintenance of genomic integrity [5]. Activated ATM phosphorylates a number of proteins involved in cellular homeostasis including CHK2. CHK2, a key substrate of ATM, phosphorylated at Thr68 results in activation. It could phosphorylate numbers of substrates such as p53, breast cancer1, BRCA1 [17]. ATM-CHK2 co-expression shows a trend and near significance, the data provides evidence that ATM and CHK2 expression has predictive significance in breast cancer [17]. In the current study, we investigated ATM-CHK2 pathway that may have predictive significance in NSCLC. We found that 55 patients showed positive ATM expression, on the contrary 65 patients showed positive CHK2 expression. According to accumulating evidence, ATM and CHK2 might to be biomarkers in NSCLC patients. But, we found that there was no relevance between ATM and BRCA1. This might be due to that the total number of enrolled patients remained not enough.

Emerging evidence indicated that BRCA1 expression level would confer improved PFS and OS, the multivariate analysis demonstrated that BRCA1 expression was an independent prognostic factor for survival.

In this study, we demonstrated that there was different median OS according to histology, and higher OS in squamous cell carcinomas. Previous studies showed that there was a significantly greater ORR (objective response rate) with carboplatin in the subset of patients with squamous cell histology, while the ORR was not significantly different between treatment groups in patients with non-squamous NSCLC, including adenocarcinoma, large-cell carcinoma and undifferentiated histology [18]. These results reminded us that some DNA repair genes might have predictive value only in squamous cell carcinomas. It suggested that analyses should be performed separately in squamous, adenocarcinoma and mixed NSCLC in future.

In addition, we found no significant correlation between BRCA1 protein expression and age, gender, smoking, status, histopathology, and clinical tumor stage, which was in parallel to the finding of other studies [19, 20]. ATM is a member of the PI3K-like protein kinases which can be affected by various factors. However, it's interesting to note that, although ATM can be affected by various factors, we did not observe association with any other factors in our research. Previous reports showed partial sleep deprivation in older adults (61-86 years) altered genes involved in signaling senescence including CHK2 ($P<0.001$) [21]. Consistent with the hypothesis that sleepless promoted biological aging, our study showed that there was a significant correlation between CHK2 and patients' age ($P=0.01$). The patients whose age over 60 years got a higher CHK2 expression rate by comparison with patients below 60 years. Maybe it's just because CHK2 was at increased risk for cellular senescence.

There is a tremendous amount of research that shows that NSCLC patients with negative BRCA1 expression are predictive of treatment efficacy [19, 20]. In this study, comparison between the negative expression group of BRCA1 with positive expression showed that the median OS of the patients were 34 months *vs*. 21 months, respectively ($P=0.011$). Additionally, comparison between the negative expression group of CHK2 with positive expression showed that the median OS of the patients were 36 months *vs*. 25 months, respectively ($P=0.046$), indicating a significant difference and impact on NSCLC patients' survival. Previous reports show that ATM polymorphisms are biomarkers for susceptibility to severe radiation-induced pneumonitis and non-small cell lung cancer [22, 23]. A small fraction of non-small cell lung cancers harbor BRCA1 mutation, with improved outcome to cisplatin. Experimental evidence suggests that ATM overexpression enhances resistance to cisplatin, but there is no significant correction between ATM and the median OS, it may due to that the

higher expression of ATM in younger patients can result in an enhanced radiosensitivity and therefore better survival [24]. Collectively, these results suggested that BRCA1 and CHK2 could be used as biomarkers and also reflected survival time in NSCLC patients respective.

In conclusion, the present study showed that protein expression of CHK2 and ATM in tumors showed an apparent correlation with each other. Protein expression of CHK2, ATM and BRCA1 in tumor tissues did not have a correction with clinical characteristics but CHK2. We further showed that the squamous patients got a longer median OS than mixed NSCLC patients.

It is obvious that most researchers have mainly focused on a subset of BRCA1-interacting protein. However, the other proteins maybe even more important for proper DNA repair. Our results reveal other influence factors critical to DNA repair that can be targeted for NSCLC. Thus, our research will be helpful for new molecular biomaker to improve the treatment efficacies and avoid unnecessary toxicities.

NSCLC patients with negative expression of BRCA1 or CHK2 showed longer survival compared to NSCLC patients presenting positive expression of BRCA1 or CHK2. While BRCA1 expression used as a predictor only was not enough sensitivity to platinum-based chemotherapy in NSCLC. Meanwhile, although CHK2 appeared to has prognostic significance, just CHK2 alone was unlikely to be a best predictive factor in NSCLC. Our results also provided a predictive two-gene model based on BRCA1 and CHK2. They got ready for future studies of BRCA1 and other DNA repair genes that could improve chemotherapeutic efficacy and NSCLC prognosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Linrun Wang and Yan Lou, The First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310000, Zhejiang, People's Republic of China. Tel: +86 571 87236675; Fax: +86 571 87236675; E-mail: linrunw@163.com (LRW); louyanly@126.com (YL)

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