Thorax Online First, published on April 4, 2008 as 10.1136/thx.2007.090324

Title Page

Analysis of Cell Cycle-related Proteins in Mediastinal Lymph Nodes of N2-NSCLC Patients Obtained by EBUS-TBNA : Relevance to Chemotherapy Response

Sherif Mohamed[1,3], **Kazuhiro Yasufuku**[1], **Takahiro Nakajima**[1], **Kenzo Hiroshima**[2], **Rieko Kubo**[1], **Akira Iyoda**[1], **Shigetoshi Yoshida**[1], **Makoto Suzuki**[1], **Yasuo Sekine**[1], **Kiyoshi Shibuya**[1], **Atef Farouk**[3], **Takehiko Fujisawa**[1]

[1]Department of Thoracic Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan. [2]Department of Diagnostic Pathology, Graduate School of Medicine, Chiba University, Japan. [3]Department of Pulmonology, Faculty of Medicine, Assiut University, Assiut, Egypt.

Correspondance and reprint request to:

Kazuhiro Yasufuku MD, PhD, FCCP
Department of Thoracic Surgery.
Graduate School of Medicine. Chiba University.
1-8-1 Inohana, Chuo-Ku. Chiba 260-8670, Japan
Tel : +81-43-222-7171 ext. 5464
Fax : +81-43-226-2172
E-mail : kyasufuku@faculty.chiba-u.jp

There are no sources of actual or potential conflict of interest for any authors in the preparation or submission of this manuscript.

Key words :

Cell cycle

Lymph nodes

NSCLC

EBUS-TBNA

Chemotherapy

Abstract

Background

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is an accurate tool for lymph node staging of non-small lung cancer (NSCLC). The majority of NSCLC patients require systemic chemotherapy during their treatment, with relatively poor responses. If one could predict chemotherapy response, ideally at the time of initial bronchoscopic examination, one could maximize therapeutic benefit while limiting toxicity. Thus, we aimed to investigate the feasibility of EBUS-TBNA for obtaining tissue samples from mediastinal lymph nodes that can be utilized for immunohistochemical analysis; and to stratify, molecularly-based, pN2-NSCLC patients into chemoresponsive and chemoresistant subgroups who might benefit from chemotherapy-tailorment.

Methods

We examined the expression of six cell cycle-related proteins (pRb, cyclin D1, $p16^{INK4A}$, p53, $p21^{Waf1}$, Ki-67), in mediastinal lymph node specimens obtained by EBUS-TBNA, by immunohistochemistry in 36 pN2-NSCLC patients. We investigated their predictive role(s) for platinum-based chemotherapy response.

Results

Immunostaining was feasible in all studied specimens. Univariate analysis revealed that p53 and $p21^{Waf1}$ expressions were significantly related to the chemotherapy response (p=0.002, p=0.011, respectively). Multivariate logistic regression analysis revealed that only p53 overexpression was associated with poor response to chemotherapy (p=0.021).

Conclusions

These results suggest that EBUS-TBNA is a feasible tool for obtaining mediastinal nodal tissue samples amenable for immunohistochemical analysis. Immunostaining of p53 in EBUS-TBNA guided specimens may be useful in predicting response to chemotherapy in N2-NSCLC patients, and help selection of those patients who might benefit from certain chemotherapeutic strategies.

Abbreviations :

CP-EBUS=convex probe endobronchial ultrasound
CT=computed tomography
EBUS-TBNA=endobronchial ultrasound guided transbronchial needle aspiration
ERCC1=excision repair cross-complementing group 1
IHC=immunohistochemistry
LI=labelling index
LN=lymph node
NER=nucleotide excision repair
NSCLC=non-small cell lung cancer
PET=positron emission tomography
PRb=retinoblastoma protein

Introduction

Lung cancer is one of the most common causes of death. While surgery is the standard approach to early stage non-small cell lung cancer (NSCLC), radiotherapy plus or minus chemotherapy represent the main treatment option in locally advanced disease (30% of patients), and chemotherapy remains the only available treatment for those with metastatic disease (50% of patients).[1] Moreover, NSCLC is often found to be intrinsically resistant to both chemo- and radiotherapy at the start of treatment and still, the basis behind treatment resistance either as primary or secondary, remains a challenge.[2] If one could predict chemotherapy response, based on assessment of biological tumor markers, one could maximize therapeutic benefit while limiting toxicity. This assessment would be ideal if performed at the time of initial bronchoscopy, so that it allows patients the option of pursuing alternative regimens earlier in the course of their treatment.

Direct real-time endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) using the convex probe endobronchial ultrasound (CP-EBUS) is a relatively new minimally-invasive and accurate technique for preoperative staging of NSCLC patients.[3-6] Moreover, we have recently reported that EBUS-TBNA has a high sensitivity and specificity compared to computed tomography (CT) and positron emission tomography (PET), and as a single procedure for mediastinal lymph node (LN) staging, it allows tissue diagnosis.[7] Tissues obtained by EBUS-TBNA allow further analyses to be carried out e.g genetic analysis; and may help directing NSCLC patients to molecularely-based different therapies.[8]

Many studies have reported the predictive value(s) of one or more of cell cycle proteins, for chemotherapy response in lung cancer [2,9,10], still with controversial results [2]. Taking into consideration that patients with stage IIIA N2-NSCLC represent heterogenous prognostic groups, we examined the expression of the Rb pathway (pRb, cyclin D1, $p16^{INK4A}$), p53 pathway (p53, $p21^{Waf1}$) proteins and Ki-67 labelling indices (LI), by immunohistochemistry in mediastinal lymph node specimens obtained by EBUS-TBNA, in pathologically-proven N2-NSCLCs. We investigated their predictive role(s) for platinum-based chemotherapy response. The main objectives of this study were to investigate the feasibility of EBUS-TBNA for obtaining nodal tissue samples that can be utilized for immunohistochemical analysis; and to stratify, molecularly-based, pN2-NSCLC patients into chemoresponsive and chemoresistant subgroups who might benefit from chemotherapy-tailorment.

Materials and Methods

Patients and Tissue Samples

From July 2004 to April 2006, 67 patients were diagnosed histologically as metastatic lung cancer of hilar and/or mediastinal lymph nodes in samples obtained by EBUS-TBNA. Rapid on-site cytologic examination was conducted for all patients during the procedure. Of these patients, evaluation of the histological cores obtained in 36 patients revealed the pathological diagnosis of stage IIIA N2-NSCLC and were enrolled in this study. The presence of both nodal tissue and cancer tissue was confirmed in all specimens by a pathologist. The pathological diagnoses were made according to the World Health Organization classification of lung tumors.[11] The primary tumor and lymph node status were classified according to the International TNM staging system .[12] Twenty-eight patients out of the 36 patients received platinum-based combination chemotherapy. Additional inclusion criteria included (1) No past history of either malignancy at the lung or elsewhere in the body; (2) No evidence of distant metastatic disease; (3) The patients received neither chemotherapy nor radiotherapy before performing EBUS-TBNA; (4) For those patients who received chemotherapy; the regimens consisted of platinum-based doublets, then the patients underwent post-chemotherapy complete radiologic re-staging; to evaluate response to therapy. The study was approved by our institutional review board.

EBUS-TBNA

EBUS-TBNA was performed on an outpatient basis under conscious sedation, using a flexible ultrasonic puncture bronchoscope (CP-EBUS, XBF-UC260F-OL8, Olympus, Tokyo, Japan), as described previously.[3-5,7,8] Histological samples were obtained by EBUS-TBNA, as previously reported.[5,7,8] Briefly, the dedicated 22-gauge needle equipped with an internal sheath is used. After the initial puncture, the internal sheath is used to clean out the internal lumen clogged with the bronchial tissue. The internal sheath is removed and negative pressure is applied by a syringe. The needle is moved back and forth inside the lymph node. Finally, the needle is retrieved and the internal sheath is used once again to push out the histological core.

Immunohistochemistry

Immunohistochemical (IHC) analysis of the specimens was performed for the expression of pRb, cyclin D1, $p16^{INK4A}$, p53, and $p21^{Waf1}$ proteins, Ki-67 LI was calculated for Ki-67 expression. All immunohistochemical assays were carried out on 10% formalin-fixed, paraffin-embedded tissue sections cut to 3- 4 µm thickness, and mounted on scilanized glass slides (Dako, Glostrup, Denmark). All sections were then dewaxed in xylene, rehydrated through a graded alcohol series, and washed in phosphate buffered saline; PBS [0.01 M sodium phosphate (pH 7.2), 0.15 M NaCl]. This buffer was used for all subsequent washes and for the dilution of the antibodies. Antigen retrieval was achieved via heating after immersion of the tissue slides in citrate buffer, pH 6.0. Tissue sections for cyclin D1, $p16^{INK4A}$, p53, and $p21^{Waf1}$ were heated at 100ºC, for 5 times in a microwave, each for 3 minutes, while those for Ki-67 and pRb were heated in an autoclave at 121ºC for 15 minutes. Then all the tissue sections were processed with the streptavidin-biotin technique (Histofine Kit; Nichirei, Tokyo, Japan). Mouse monoclonal antibodies (Dako, Glostrup, Denmark) specific for cyclin D1 (DSC-6), p53 (DO-7) and Ki-67 (MIB-1) were used at a dilution of 1:40 and 1:800 for cyclin D1 and p53 respectively and pre-diluted for Ki-67. Monoclonal antibodies (Santa Cruz Biotechnology, Inc. Heidelberg, Germany) and (EMD Biosciences, Inc. San Diego, CA) specific for $p16^{INK4A}$ (F-12; sc-1661) and $p21^{Waf1}$ (Ab-1), were used at a dilution of 1:50 and 1:20 respectively. The monoclonal antibody DO-7,

reacts with both wild type and mutant p53 proteins. On the other hand, a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc. Heidelberg, Germany) specific for pRb (C-15; sc-50) was used at a dilution of 1:50. All the primary antibodies were incubated overnight at 4ºC. 3,3'-Diaminobenzidine was used as the final chromogen, and haematoxylin as the nuclear counterstain. Positive tissue controls were included in each experiment and consisted of tissues previously shown to stain specifically for the target antigen after exposure to primary antibody.

Evaluation of the Immunostaining Results

Without any knowledge of the patients' clinico-pathologic features or chemotherapy response, all slides were evaluated. Two independent observers (K.H. and S.M.) evaluated the staining pattern of the six proteins separately and scored the protein expression in each specimen by scanning the entire section and estimating the percentage of positive tumour cells. Nuclear coloration was recognized as the primary standard for demonstrating a positive reaction for pRb, $p16^{INK4A}$, p53, $p21^{Waf1}$, and Ki-67 [13,14], irrespective of staining intensity; whereas for cyclin D1, cytoplasmic staining was recognized as the primary standard for positive reaction.[14] A cut-off value of >10 % tumor cells with positively stained nuclei in the entire section was considered as a positive expression for pRb, $p16^{INK4A}$, p53, and $p21^{Waf1}$, while a cut-off value of >10 % tumor cells with positive cytoplasmic staining was considered as a positive reaction for cyclin D1.[14] Calculation of the Ki-67 LI was performed by counting > 1000 positively stained tumour nuclei in randomly selected high-power field (10~100) from different representative parts of the tumour. Ki-67 LI values were defined as high (over-expression) if they were more than 20% and low if they were less than 20%.[15] An abnormal expression was defined for positive expression of cyclin D1, p53, and high Ki-67 labelling indices, whereas it was defined as that for negative expression (inactivation) of pRb, $p16^{INK4A}$ and $p21^{Waf1}$.

Response to Chemotherapy Evaluation

For those patients who received chemotherapy; they received scheduled CT examination to measure the target tumor size. The responses to chemotherapy were evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[16] The response rate (RR) was defined as the number of chemotherapy-responders (complete response + partial response) divided by the total number of patients. On the other hand, progressive disease (PD) rate was defined as the number of patients with progressive disease, divided by the total number of patients.[16] Response to chemotherapy was reviewed without knowledge of the immunostaining results.

Statistical Analysis

The associations between categorical immunohistochemical/clinico-pathological parameters, as well as immunohistochemical parameters/chemotherapy response, were analysed by the use of the χ^2 test or Fisher's exact test. The clinico-pathologic features were age, gender, histopathologic type, and number of involved mediastinal lymph nodes stations. To simultaneously examine the impact of more than one factor on response to chemotherapy, multivariate logistic regression analysis was performed.[17] Statistical analysis was carried out using the SPSS statistical software program package (SPSS version 12.0 for Windows, SPSS Inc., Chicago, IL). The criterion of significance chosen was P <0.05, and all tests were two-tailed.

Results

Patient Characteristics and Chemotherapy Response

The clinico-pathological features, IHC and chemotherapy response results of the patients are listed in Table 1. Response to chemotherapy revealed; one Complete Response (CR), twelve Partial Response (PR), ten Stable Disease (SD), and five Progressive Disease (PD). The overall clinical RR was 46.4%.

Immunostaining Results

Remarkably, all the examined cases; (36/36; 100.0%) showed an abnormal expression of at least one of the studied cell cycle proteins. Our immunostaining results revealed altered expression of pRb, cyclin D1, $p16^{INK4A}$, p53, and $p21^{Waf1}$ in; 36.1%, 30.6%, 47.2%, 52.8%, and 75.0% of nodal biopsies, respectively. As regards to Ki-67 LI, 23/36 (63.9%) cases showed Ki-67 LI values higher than 20% (table 1). Interestingly, histological cores obtained by EBUS-TBNA consisted mainly of tumor cells, blood constituents, and minimal amounts of lymph node tissue [Fig. 1(A)]. Expression of pRb, $p16^{INK4A}$, p53, $p21^{Waf1}$ and Ki-67 was present mainly in the nuclei of the tumor cells, whereas cyclin D1 was displayed mainly in the cytoplasm [Fig. 1(B)]. Some cells displayed additional cytoplasmic (in case of pRb), or nuclear (in case of cyclin D1) immunostaining, respectively.

Table 1 Characteristics of pN2-NSCLC patients*

Characteristic	No (%)
Overall	**(n=36)**
Age (mean±SD, years)	66.8 ± 9.4
< 66.8 years	14 (38.9)
> 66.8 years	22 (61.1)
Gender	
Female	5 (13.9)
Male	31 (86.1)
Histopathology	
Adenoca	19 (52.8)
SCC	17 (47.2)
Mediastinal LN stations	
Single	5 (13.9)
Multiple	31 (86.1)
Protein expressions	
pRb	
Negative	13 (36.1)
Positive	23 (63.9)
Cyclin D1	
Negative	25 (69.4)
Positive	11 (30.6)
p16	
Negative	17 (47.2)
Positive	19 (52.8)
p53	
Negative	17 (47.2)
Positive	19 (52.8)
p21	
Negative	27 (75.0)
Positive	9 (25.0)
Ki-67	
LI <20%	13 (36.1)
LI >20%	23 (63.9)
Treatment response	**(n=28)**
CR	1 (3.5)
PR	12 (42.9)
SD	10 (35.7)
PD	5 (17.9)

*Adenoca, Adenocarcinoma ; SCC, squamous cell carcinoma ; LN, lymph nodes ; LI, labeling index ; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease

Relationship between immunohistochemical parameters and clinico-pathological features

We investigated the relationships among the immunohistochemical, and clinico-pathologic parameters, as well as their possible inter-relationship(s). Interestingly, we found no statistically significant relation between the expressions of any two proteins within the Rb pathway, nor between that of p53 and $p21^{Waf1}$. Also, no significant relation was found between any two proteins belonging to two different pathways. As regards to the clinico-pathological inter-relationships, we found that only histopathological type was significantly related to both age and number of involved mediastinal LN stations. Patients with squamous cell carcinoma who were older than the mean age of 66.8 years, were significantly more than those with adenocarcinomas and older than the mean age (14/22, 63.7% versus 8/22, 36.3%; p=0.013), respectively. All patients with squamous cell carcinoma histopathology had multiple mediastinal LNs (17/17, 100%), in comparison to those with adenocarcinoma with multiple stations nodal affection (14/19, 73.7%); p=0.047[data not shown]. On the other hand, some relevant clinico-pathologic/IHC relationships were obtained (table 2). Patients'gender was significantly related to both pRb (p=0.047) and p53 (p=0.047) expressions. Histopathology was significantly associated with both cyclin D1 (p=0.042) and $p16^{INK4A}$ (p=0.007) expressions. Lastly, the number of involved mediastinal LN stations was related to $p16^{INK4A}$ expression (p=0.016).

Table 2 Immunohistochemical/Clinicopathologic relationships*

IHC	Age			Gender			Histopathology			MLN stations		
Parameters (%)	**<66.8**	**>66.8**	***P*†**	**F**	**M**	***P***	**AC**	**SCC**	***P***	**Single**	**Multiple**	***P***
pRb												
Negative (36.1)	3	10	0.143	4	9	0.047	5	8	0.196	0	13	0.136
Positive (63.9)	11	12		1	22		14	9		5	18	
Total	14	22		5	31		19	17		5	31	
Cyclin D1												
Negative (69.4)	11	14	0.467	3	22	1.000	16	9	0.042	4	21	0.664
Positive (30.6)	3	8		2	9		3	8		1	10	
Total	14	22		5	31		19	17		5	31	
p16												
Negative (47.2)	9	8	0.102	3	14	0.650	13	4	0.007	5	12	0.016
Positive (52.8)	5	14		2	17		6	13		0	19	
Total	14	22		5	31		19	17		5	31	
p53												
Negative (47.2)	8	9	0.342	0	17	0.047	9	8	0.985	3	14	0.650
Positive (52.8)	6	13		5	14		10	9		2	17	
Total	14	22		5	31		19	17		5	31	
p21												
Negative (75.0)	11	16	1.000	4	23	1.000	16	11	0.255	4	23	1.000
Positive (25.0)	3	6		1	8		3	6		1	8	
Total	14	22		5	31		19	17		5	31	
Ki-67 LI												
LI <20% (36.1)	4	9	0.452	1	12	0.634	9	4	0.137	1	12	0.634
LI >20% (63.9)	10	13		4	19		10	13		4	19	
Total	14	22		5	31		19	17		5	31	

*IHC, immunohistochemistry; F, female; M, male; AC, adenocarcinoma ; SCC, squamous cell carcinoma ; MLN, mediastinal lymph nodes ; LI, labeling index

† *P* χ^2 test or Fisher's exact test

Predictive Values for Chemotherapy Response

We then analyzed the relationship between both the clinico-pathologic and IHC parameters, and the response to chemotherapy in patients who received chemotherapy (28 cases), as well. Our results revealed that none of the clinico-pathologic parameters was significantly associated with the response to chemotherapy. As regards to the IHC parameters, univariate analysis revealed that only p53 and $p21^{Waf1}$ expressions were significantly related to chemotherapy response. Twelve of fifteen chemotherapy non-responders had p53 overexpression, with response rates of 20% and 76.9% for patients with p53-positive and p53-negative expression, respectively, (estimated risk=0.288, 95% confidence interval (CI); 0.104 – 0.803; p=0.002). For $p21^{Waf1}$ expression, 14/20 non-responders had $p21^{Waf1}$ inactivation, with response rates of 30% and 87.5% for patients with p21-negative and p21-positive expression, respectively, (estimated risk=0.061, 95% CI; 0.006– 0.613; p=0.011). Moreover, only p53 expression was significantly related to response to chemotherapy (p=0.044), upon the use of PD rate, as another tool to test for the IHC parameters/chemotherapy response relationship (table 3)

Depending on the results of univariate analysis, a multivariate logistic regression analysis was performed for the association between p53 and $p21^{Waf1}$ expressions and response to chemotherapy (table 4). Only p53 expression was significantly associated with response to chemotherapy (odds ratio 0.095, 95% confidence interval 0.013–0.705, p=0.021).

Table 3 Relationships between chemotherapy response and various immunohistochemical parameters (Univariate analysis)*

IHC Parameters	Responders (CR+PR)	Non-responders (SD+PD)	Response rate (%)	Risk value	95% confidence interval	$P^{\dagger}$	PD rate (%)	$P^{\dagger}$
pRb								
Negative	3	5	37.5	0.692	0.204 – 2.353	0.686	37.5	0.123
Positive	10	10	50.0				10.0	
Total	13	15						
Cyclin D1								
Negative	10	11	47.6	0.865	0.236 – 3.174	1.000	14.3	0.574
Positive	3	4	42.9				28.6	
Total	13	15						
p16								
Negative	4	10	28.6	0.222	0.045 – 1.094	0.061	21.4	1.000
Positive	9	5	64.3				14.3	
Total	13	15						
p53								
Negative	10	3	76.9	0.288	0.104 – 0.803	**0.002**	0.0	**0.044**
Positive	3	12	20.0				33.3	
Total	13	15						
p21								
Negative	6	14	30.0	0.061	0.006 – 0.613	**0.011**	20.0	0.654
Positive	7	1	87.5				12.5	
Total	13	15						
Ki-67 LI								
LI <20%	5	6	45.5	1.026	0.565 - 1.862	0.937	18.2	1.000
LI >20%	8	9	47.0				17.6	
Total	13	15						

* IHC, immunohistochemical ; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease
† *P* χ^2 test or Fisher's exact test

Table 4 Multivariate logistic regression analysis*

Predictive factors (– Vs + expression)	Odds ratio	95 % confidence interval	*p*
p53	0.095	0.013 – 0.705	**0.021**
p21	0.082	0.006 – 1.056	0.055

* – , negative ; + , positive

Discussion

EBUS-TBNA is a relatively new technique for the evaluation of mediastinal and hilar lymph node metastasis in patients with lung cancer.[3-7] One major advantage of EBUS-TBNA is its ability to collect samples rich in tumor cells, which can be confirmed by pathology.[5,7] Recently, we have reported the feasibility of EBUS-TBNA for genetic evaluations of tumor cells within the mediastinal LNs of NSCLC patients.[8] Remarkably, the use of EBUS-TBNA technique (mentioned above) is helpful in minimizing the non-target normal cell contamination within the samples, occasionally seen in transbronchial lung biopsies and/or genetic analyses.[8] In the current study, compared to the amount of tumor cells, there was relatively little representation of normal or reactive tissue within the samples. An aberrant expression of at least one of the six cell cycle proteins was found in all included patients. Moreover, the percentages of aberrant expressions of these proteins were relatively similar to those published in the literature [13,14,18]; notably with recruited larger population numbers. Furthermore, we were able to obtain some statistical relevances. The histopathological type was significantly related to age, number of involved mediastinal LN stations, cyclin D1 and $p16^{INK4A}$ expressions. Prior reports observed that different histopathologic subtypes of NSCLC could have distinct biologic behaviors.[19] On the otherhand, gender was related to both pRb and p53 expressions, and number of LN stations was related to $p16^{INK4A}$ expression. These findings are in concordance with those studies found significant clinical/IHC relevances [14,20], while not with others.[18] These controversies could be explained on the bases of differences between patient populations, sample sizes, methodology as well as tumors biologic heterogeneity[18]; the later is particularly a characteristic feature for pN2-NSCLCs.[14] Then, our results revealed lack of significant cell cycle markers inter-relationships, also observed by others.[14,18]

Given the low response rates and high side effect profiles of chemotherapy, the ability to utilize molecular marker(s) assessment for patients with NSCLC, whose tumors may be resistant to a particular treatment regimen would avoid unnecessary toxicities and reduce medical costs. This assessment would be ideal if performed at the time of initial bronchoscopy, so that it allows a better selection of patients who may benefit from specific; either neoadjuvant or adjuvant; chemotherapy regimens. Our results showed that only p53 expression was significantly related to response to chemotherapy. The expression of p53 is normally not detectable by IHC. However, the mutant p53 proteins have an extended half-life, thus accumulate in tumor cells and result in the apparent p53 overexpression on IHC.[21] Radiotherapy and most chemotherapeutic agents directly target DNA [2], and in response to such therapies, p53 functions as a coordinator of the DNA repair process, cell cycle arrest, and apoptosis.[22] Notably, p53 participates in the main DNA repair systems operative in cells (reviewed in details in Ref [2]). Given the high frequency of p53 mutations in lung cancer, a role of p53 as a predictive marker for treatment responses has been strongly suggested. In response to DNA damage at least some of the p53 mutants show less capacity to bind and initiate transcription from their target genes, e.g., $p21^{WAF1}$, *Mdm2*, *Bax*, *and cyclin G*, and thus some of the p53-mediated effects are blunted.[23] Several bodies of evidence had linked p53 status with the response to therapy, observing that the vast majority of chemotherapeutic agents were more effective in killing human tumors with wild-type as compared to mutant p53.[24,25] Indeed, given the important function of nucleotide excision repair (NER) in the repair of DNA damages induced by among all platinum-based chemotherapy, it has been shown that increased NER activity in NSCLC cell lines or tumors is associated with increased failure of chemotherapy responses.[26] Furthermore, resistance to cisplatin was associated with increased activity of excision repair cross-complementing group 1 (ERCC1), and notably, such polymorphism was measurable at mRNA level and thus could act as a predictive marker for

therapy outcome.[27] Our results support these p53-related roles, as the presence of p53 overexpression was associated with poor response to platinum-based chemotherapy.

As regards to our previous work [14], such discrepancy between survival and chemotherapy response results, can be explained in two ways. First, the presence of aggressive tumor features and consequently shortened survival does not necessarily lead to treatment resistance; because from one side chemo- and radiotherapy may target different pathways, and from the other side a shorter survival is not always linked to treatment resistance.[2] Second, an argumentation between protein expressions in both primary and metastatic sites could exist. Primary and metastatic tumor cells may exhibit different characteristics and the former cells may undergo selection in the course of metastasis.[28] On the other hand, conserved mutations were observed between the primary and metastatic sites.[29] Our results could have important clinical and therapeutic implications. Being a minimally-invasive procedure that can be done repeatedly under local anaethesia, EBUS-TBNA represents a very useful tool for initial assessment as well as follow up of N2-NSCLC patients. Thus, EBUS-TBNA can help identify patients who may benefit from induction chemotherapy or adjuvant chemoradiotherapy [30], for N2-NSCLC candidates with or without surgical potential, respectively. Furthermore, EBUS-TBNA may become a useful tool for molecular assessment post-induction chemotherapy, that may help directing N2-NSCLC patients to different therapeutic strategies.[8,31] From the therapeutic point of view, recent studies have employed manipulations to mutant p53, via either its functional correction, or elimination, aiming to improve the ongoing therapies or even highlighting new anticancer strategies .[32,33] Our study has two possible limitations; the relatively small patients number and, the fact that it is being a retrospective study. Therefore, further prospective and larger studies evaluating molecular markers in EBUS-TBNA-guided biopsies are needed.

Conclusion

Our results suggest that EBUS-TBNA is a feasible tool for obtaining mediastinal nodal tissue samples amenable for immunohistochemical analysis. Immunostaining of p53 in specimens obtained by EBUS-TBNA may be useful in predicting response to chemotherapy in N2-NSCLC patients which may lead to better selection of N2-NSCLC patients who might benefit from certain chemotherapeutic strategies.

Figure legend

Figure 1: A representative example of mediastinal lymph node tissue sample obtained by EBUS-TBNA in pN2-NSCLC adenocarcinoma (original magnification, x 20) (A) Note that the main constituents are tumor cells, blood constituents, and small amount of lymphocytes and histiocytes (H&E) (B) Immunohistochemical staining for Do7 showing overexpression of p53 protein.

Competing interest

There are no sources of actual or potential conflict of interest for any authors in the preparation or submission of this manuscript.

Aknowledgment : We thank Prof. Hammad El-Shahhat and Dr. Gamal Agamy; Department of Pulmonology; and Dr. Eman Mahmoud, Department of Pathology, Assiut University; for their aids to this work.

Funding : Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan No. 16591384 and 19591611, to **Kazuhiro Yasufuku**

This study was approaved by Chiba University Institutional Review Board (approval No. 119)

References

1. Spira A, Ettinger DS. Multidisciplinary management of lung cancer. *N Eng J Med* 2004;**350**:379-392.
2. Viktorsson K, De Petris L, Lewensohn R. The role of p53 in treatment responses of lung cancer. *Biochemical and Biophysical Research Com* 2005;**331**:868−880.
3. 3. Yasufuku K, Chhajed PN, Sekine Y, *et al*. Endobronchial ultrasound using a new convex probe: a preliminary study on surgically resected specimens. *Oncol Rep* 2004;**11**:293-6.
4. Yasufuku K, Chiyo M, Sekine Y, *et al*. Real-time endobronchial ultrasound-guided transbronchial needle aspiration of mediastinal and hilar lymph nodes. *Chest* 2004;**126**:122-8.
5. Yasufuku K, Chiyo M, Koh E, *et al*. Endobronchial ultrasound guided transbronchial needle aspiration for staging of lung cancer. *Lung Cancer* 2005;**50**:347-354.
6. Herth FJ, Eberhardt R, Vilmann P, *et al*. Real-time endobronchial ultrasound guided transbronchial needle aspiration for sampling mediastinal lymph nodes. *Thorax* 2006;**61**:795-8.
7. Yasufuku K, Nakajima T, Motoori K, *et al*. Comparison of endobronchial ultrasound, positron emission tomography, and CT for lymph node staging of lung cancer. *Chest* 2006;**130**:710-718.
8. Nakajima T, Yasufuku K, Suzuki M, *et al*. Assessment of epidermal growth factor receptor mutation by endobronchial ultrasound-guided transbronchial needle aspiration. *Chest* 2007;**132**:597-602.
9. Harada T, Ogura S, Yamazaki K, *et al*. Predictive value of expression of P53, Bcl-2 and lung resistance-related protein for response to chemotherapy in non-small cell lung cancers. *Cancer Sci* 2003;**94**:394-9.
10. Gautschi O, Hugli B, Ziegler A, *et al.* Cyclin D1 (CCND1) A870G gene polymorphism modulates smoking-induced lung cancer risk and response to platinum-based chemotherapy in non-small cell lung cancer (NSCLC) patients. *Lung Cancer* 2006;**51**:303-11.
11. Travis WD, Brambilla E, Müller-Hermelink HK, Harris CC. World Health Organization Classification of Tumours. Pathology and Genetics. Tumours of the Lung, Pleura, Thymus and Heart. 4th ed. IARC Press: Lyon 2004:10.
12. Mountain CF, Dresler CM. Regional lymph node classification for lung cancer staging. *Chest* 1997;**111**:1718−23.
13. Burke L, Flieder DB, Guinee DG, *et al*. Prognostic Implications of Molecular and Immunohistochemical Profiles of the Rb and p53 Cell Cycle Regulatory Pathways in Primary Non–Small Cell Lung Carcinoma. *Clin Cancer Res* 2005;**11**:232–241.
14. Mohamed S, Yasufuku K, Hiroshima K, *et al*. Prognostic Implications of Cell Cycle-related Proteins in Primary Resectable Pathologic N2 Nonsmall Cell Lung Cancer. *Cancer* 2007;**109**:2506-14.
15. Shiba M, Kohno H, Kakizawa K, *et al*. Ki-67 immunostaining and other prognostic factors including tobacco smoking in patients with resected non-small cell lung carcinoma. *Cancer* 2000; **89**:1457-65.
16. Therasse P, Arbuck SG, Eisenhauer EA, *et al*. New guidelines to evaluate the response to

treatment in solid tumors. *J Natl Cancer Inst* 2000;**92**:205-16.
17. Cox DR, Snell EJ. Analysis of binary data (2nd ed.) London: Chapman and Hall 1989.
18. Geradts J, Fong KM, Zimmerman PV, *et al*. Correlation of abnormal RB, p16ink4a, and p53 expression with 3p loss of heterozygosity, other genetic abnormalities, and clinical features in 103 primary non-small cell lung cancers. *Clin Cancer Res* 1999;**5**:791– 800.
19. Mangili F, Cigala C, Arrigoni G, *et al*. Cell loss and proliferation in non-small cell lung carcinoma: correlation with histological subtype. *Eur J Histochem* 1998;**42**:287-95.
20. Esposito V, Baldi A, Tonini G, *et al*. Analysis of cell cycle regulator proteins in non-small cell lung cancer. *J Clin Pathol* 2004;**57**:58-63.
21. Tominaga O, Hamelin R, Remvikos Y, *et al*. P53 from basic research to clinical applications. *Crit Rev Oncog* 1992;**3**:257–82.
22. El-Deiry WS. The role of p53 in chemosensitivity and radiosensitivity. *Oncogene* 2003;**22**:7486–95.
23. de Vries A, Flores ER, Miranda B, *et al*. Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. *Proc Natl Acad Sci USA* 2002;**99**:2948−53.
24. O'Connor PM, Jackman J, Bae I, *et al*. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Res* 1997;**57**:4285-300.
25. Schmitt CA, Fridman JS, Yang M, *et al*. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* 2002;**109**:335-46.
26. Tsai CM, Chang KT, Li L, *et al*. Interrelationships between cellular nucleotide excision repair, cisplatin cytotoxicity, HER-2/neu gene expression, and epidermal growth factor receptor level in non-small cell lung cancer cells. *Jpn J Cancer Res* 2000;**91**:213−22.
27. Isla D, Sarries C, Rosell R, *et al*. Single nucleotide polymorphisms and outcome in docetaxel-cisplatin-treated advanced non-small-cell lung cancer. *Ann Oncol* 2004;**15**:1194−203.
28. Okamoto A, Hussain SP, Hagiwara K, *et al*. Mutations in the p16INK4/MTS1/CDKN2, p15INK4B/MTS2, and p18 genes in primary and metastatic lung cancer. *Cancer Res* 1995;**55**: 1448–51.
29. Schlegel U, Rosenfeld MR, Volkenandt M, *et al*. p53 gene mutations in primary lung tumors are conserved in brain metastases. *J Neurooncol* 1992;**14**:93-100.
30. Bepler G. Using translational research to tailor the use of chemotherapy in the treatment of NSCLC [supplement] *Lung Cancer* 2005; **50**(Suppl 1):P13.
31. Goldstraw P. Selection of patients for surgery after induction chemotherapy for N2 non–small-cell lung cancer [editorial] *J Cl Oncol* 2006;**24**:3317-18.
32. Bykov VJ, Issaeva N, Shilov A, *et al*. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* 2002;**8**:282-8.
33. Fujiwara T, Tanaka N, Kanazawa S, *et al*. Multicenter phase I study of repeated intratumoral delivery of adenoviral p53 in patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2006; **24**:1689-99.