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[Original Article]

PREDICTION OF RECURRENCE FOR NON-SMALL CELL LUNG CANCER BY COMBINED ANALYSIS OF MOLECULAR MARKERS AND ^{18}F 2-FLUORO-2-DEOXY-D-GLUCOSE POSITRON EMISSION TOMOGRAPHY

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Abstract : [Purpose] Numerous biomarkers have been reported to reflect prognosis in patients with non-small cell lung cancer, but most of them remain controversial in terms of the clinical benefits. The aim of this study is to establish a novel procedure in combined analyses of molecular markers and biomedical image for precise prediction for patient prognosis of non-small cell lung cancer. [Experimental design] Molecular markers related to cell cycle and proliferation and ^{18}F 2-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) were retrospectively analyzed for their utility as prognostic parameters in 54 patients with non-small cell lung cancer. Expression of ten representative molecular markers (Glut-1, proliferating cell nuclear antigen, Ki-67, cyclin B1, cyclin D1, cyclin E, E2F-1, p21, p27, and p53) were immunohistochemically analyzed using tissue microarray. The maximum standardized uptake value (SUVmax) on FDG-PET was analyzed as a semiquantitative value of FDG uptake of the primary tumor. [Results] Several molecular markers were significantly correlated with some of clinicopathological parameters, whereas none of each marker were correlated with recurrence or survival. Hierarchical clustering analysis in combination of immunohistochemical analysis of molecular expressions and SUVmax divided them into three subgroups significantly different in two-year recurrent-free survival (Cluster A, 56.3% ; B, 100% ; C 93.8%). These clustering subgroups were also significantly correlated with disease recurrence (p=0.0282). [Conclusions] Hierarchical clustering analysis, based on molecular markers and FDG accumulation, could be an efficient tool for prediction of recurrence and survival in patients with non-small cell lung cancer.

Key words : lung cancer, molecular markers, FDG-PET, recurrence

INTRODUCTION

Lung cancer has the highest mortality due to cancer in the majority of developed countries[1)]. Despite advances in diagnostic and therapeutic modalities, disease recurs in 50% of patients after complete resection and the prognosis is so poor that overall 5-year survival is only 15%[2)]. Furthermore, approximately one-third of patients even in stage I develop disease recurrence[3,4)]. More effective biomarkers are needed for prediction of patient prognosis as well as for molecular targeted therapy in non-small lung cancer.

During the past decades, many molecular markers involved in cell cycle regulation, apoptosis, and angiogenesis have been identified for prediction of clinical outcome in non-small cell lung cancer[5,6)]. From a viewpoint of clinical usefulness, a major mo-

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lecular marker in patients with non-small cell lung cancer is altered p53[7,8]. The p53 protein is a regulator of cell cycle, apoptosis, and DNA repair. Immunohistochemistry can detect missense mutant p53 proteins. Several reports have suggested that p53 alteration is a prognostic factor for patients with lung cancer[2,7-10], whereas other reports have not supported this conclusion[5]. Accordingly, the prognostic value of a single molecular marker, such as p53, in patients with non-small cell lung cancer remains controversial[6,9]. A balance between stimulators and inhibitors of cell proliferation maintains growth homeostasis in normal cells. Cell cycle progression is promoted by cyclins and cyclin-dependent kinases (CDKs). Cyclin overexpression may cause failure of checkpoint arrests and may lead to uncontrolled proliferation. Cyclin-dependent kinase inhibitors (CDKIs) are negative regulators of cyclins. The major CDKIs are p21 and p27 ; they inhibit all CDKs and are related to prognosis in non-small cell lung cancer. The loss of CDKIs leads to tumor progression and poorer prognosis. Transcription factor E2F-1 acts as a growth-promoting factor and plays a key role in G1-to-S phase transition by responding to numerous upstream signals. E2F-1 overexpression could be a significant prognostic factor for non-small cell lung cancer patients. PCNA is the subunit of DNA polymerase δ that is responsible for its proofreading activity, and has a marked role in maintaining the fidelity of mammalian DNA replication. Overexpression of PCNA may therefore potentially influence cell cycle progression. Ki-67, another nuclear antigen that is expressed only in proliferating cells, is commonly used as a marker to evaluate proliferation of tumor cells. Expression of PCNA and Ki-67 is associated with poorer prognosis. These molecular markers could be prognostic factors for patients with lung cancer, but consensus on this matter has yet to be reached.

Recently, positron emission tomography imaging with ^{18}F 2-fluoro-2-deoxy-D-glucose (FDG-PET) has become an important non-invasive imaging technique for evaluating focal pulmonary abnormalities (e.g., solitary pulmonary nodules), staging of lung cancer, and detecting recurrent neoplasms. Interestingly, the primary tumor standardized uptake value (SUV), which is a widely used semi-quantitative parameter of FDG accumulation, has been studied as a potential prognostic factor[11-14]. Berghmans *et al.*[12] reported that FDG-PET could be a useful tool for prediction of recurrence after pulmonary resection[13,14]. They also refer to a SUV cut-off value to predict recurrence[12]. As many reports have suggested, it is difficult to establish a SUV cut-off value for prediction of recurrence[11-14]. In the population of patients with NSCLC, Glut-1 is the major glucose transporter expressed. Overexpression of Glut-1 correlated with high FDG uptake and lesser differentiation, positive lymph node metastasis[15-17]. Glut-1 expression is a predictor for prognosis in patients with resected NSCLC[18].

These findings led to the speculation that analyses limited to a single factor or to a few factors might not be able to predict recurrence or survival in patients with non-small cell lung cancer. In the present study, a novel strategy for prediction of clinical outcome was investigated using combined analysis of molecular markers and FDG-PET status.

MATERIALS AND METHODS

1. Patients

Patients' characteristics and clinical backgrounds are shown in Table 1. Retrospectively, 54 patients who underwent complete resection for non-small cell lung cancer in 2004 and 2005 were analyzed. Thirty-three patients were treated in Fukushima Medical University Hospital, and 21 patients were in Southern Touhoku Hospital. We have obtained informed consent from patients for the study samples. The sample consisted of 37 men (68.5%) and 17 women (31.5%) with a mean age of 69.7 years (SD=8.6 years, range 53-83 years). The mean size of the primary lesion was 3.3±1.6 cm (range ; 1.0-10.0 cm).

Histological diagnosis was adenocarcinoma in 24 patients, squamous cell carcinoma in 27, large cell carcinoma in 2, and adenosquamous cell carcinoma in 1. Twenty-six tumors were well differentiated, 9 were moderately differentiated, and 12 were poorly differentiated. Pathologic staging revealed stage I disease in 37 patients, stage II in 9, and stage III in 8. Lymph node metastases were detected in 14 patients (35%). Survival was measured in months from the day of surgery until death or recurrence. The mean follow-up period was 17 months (range ; 5-36 months).

2. Tissue Microarray (TMA) Construction

Formalin-fixed paraffin-embedded archival tissue blocks of lung cancer were used for TMA analysis. Tissue blocks were constructed using a manual microarray builder, (KIN-1, Azumaya-Ikakiki, Tokyo, Japan), which allows assembly of recipient blocks incorporating 48 pieces of a 2-mm diameter

Table 1. Patients' Characteristics and Clinical Background

Age	69.7±7.7 y
Tumor size	3.33±1.6 cm (1.0–10.0 cm)
SUVmax	10.6±7.0
T factor	
T1	30
T2	8
T3	3
T4	3
N factor	
Negative	40
Positive	14
p-Stage	
IA	23
IB	14
IIA	5
IIB	4
IIIA	4
IIIB	4
Histology	
Adeno	24
SCC	27
Other	3
Differentiation	
Well	26
Mod	9
Poor	12
Unknown	7

SUV : standardized uptake value
Adeno : adenocarcinoma
SCC : squamous cell carcinoma

tissue core in a 6×8 orientation. Under microscopy of hematoxylin and eosin-stained sections, areas containing viable tumor were marked on the paraffin wax tissue blocks, and two tissue cores were randomly selected and punched out from the tumor of one donor-paraffin block. With a microtome, 5-μm sections were cut from the TMA blocks to generate TMA slides for molecular analyses.

3. Immunohistochemistry

Sections (3 μm thick) were cut from blocks and mounted on glass slides precoated with 0.05% poly-L-lysin solution. Sections were dewaxed in xylene and dehydrated through graded alcohol solutions. Endogenous peroxidase activity was quenched by 20-min incubation with a 0.3% (v/v) solution of hydrogen peroxidase in 100% methanol. Pretreatment was in accordance with the protocol attached to each antibody. After incubation with 5% dry skim milk in phosphate buffered saline for 30 min at room temperature, the sections were incubated overnight at 4°C with primary antibody. The primary antibody was subsequently detected with a biotinylated secondary antibody by the avidin-biotin complex method, using 3.3´-diaminobenzidine as a chromogen. As the primary antibody, mouse monoclonal antibodies specific for p53 (DO-7, Abcam, Cambridge, MA, 1/100 dilution), cyclin E (E12, Abcam, 1/50 dilution), cyclin D1 (DSC-6, Abcam, 1/200 dilution), p21 (DSC60.2, Abcam, 1/50 dilution), PCNA (PC-10, Abcam, 1/3,000 dilution), cyclin B1 (GNS-1, BD Pharmingen, San Diego, CA, 1/50 dilution), p27 (DSC-72.F6, Neomarkers, Fremont, CA, 1/100 dilution), Ki-67 (MIB-1, Dako, Carpinteria, CA, 1/100 dilution), and E2F-1 (KH-95, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1/50 dilution) were used[5,6,10]. A polyclonal antibody specific for Glut-1 (Dako) was used at a 1/500 dilution. Sections were washed several times in phosphate buffered saline after each step. Sections were counterstained with Mayer's hematoxylin before they were dehydrated through graded alcohol solutions and mounted.

Molecular expression was analyzed by immunohistochemistry of paraffin sections, using each antibody to determine the percentage of labeled cells/overall tumor cells. All slides were evaluated by the same two pathologists. All immunoreactive cells were regarded as positive, irrespective of staining intensity. The entire field of the cancer tissue was examined, and the percentage of positively stained cancer cells was estimated. Immunostained sections were scored using an empirical 4-point system (0=0% ; 1=1-10% ; 2=11-50% ; and 3=51-100%) based on the frequency of positively stained cells. Score 3 was determined as positive. For low expression of molecules, score 2 and 3 were determined as positive in E2F1, cyclin E, p21, p27. We have investigated the correlation between the expression of each molecule and clinicopathological parameters.

4. Whole-body FDG-PET

FDG-PET was performed using a conventional full-ring, high-resolution dedicated positron emission tomographic scanner (Discovery LS/Advance Nxi, GE Medical Systems). Pyrogen-free ^{18}F FDG (0.1 mCi/kg) was administered to patients intravenously. Region of interest (ROI) analysis tools, shipped with the scanners, were used to calculate the ^{18}F FDG concentration within the primary tumor mass. For the purposes of this study, only uptake

in the primary tumor site was analyzed. FDG uptake was quantified by calculating SUV in the images acquired from 90 min after injection. To minimize partial-volume effects, the maximum SUV within an ROI (SUVmax) was used.

FDG-PET results were scored on the basis of SUVmax as follows : 0, SUVmax <5 ; 1, SUVmax 5-10 ; 2, SUVmax 10.1-15.0 ; and 3, SUVmax >15.1. This 4-scale system was adopted to evaluate the FDG accumulation under the assumption that there is no true cutoff point but rather a transition zone in which prognosis gradually worsens[12,13].

5. Statistical Methods

Correlations between each molecular marker or SUVmax and clinicopathological parameters were statistically analyzed using the χ^2 or Student's *t* test. Survival curves were assessed by Kaplan-Meier analysis. The log-rank test was used to analyze the univariate significance for survival. A significant difference was recognized if the p value from a two-tailed test was less than 0.05. Statistical analyses were performed using SPSS v.1.1 software (Chicago, IL).

Hierarchical clustering analysis is a technique that is frequently used in microarray studies to detect patterns of gene expression. Unsupervised hierarchical clustering analysis based on immunostaining data and FDG uptake was performed using the Cluster program, and the results were visualized using the TreeView program as previously reported[19,20]. Cases with similar antibody expression profiles were placed next to each other.

RESULTS

Correlations of molecular markers and SUVmax with clinicopathological parameters

Correlations between molecular markers and clinicopathological parameters are shown in Table 2. No correlations were found between molecular markers and age, TNM stage, or lymph node metastasis. A positive correlation was found between expression of Glut-1 and pathological stage. Well differentiated cancer was low expression of Ki-67,

Table 2. Molecular Markers and Clinicopathological Parameters

	Age	T factor	N factor	p-Stage	Differentiation	Histlogy	Rec	SUVmax
Glut-1	*	*	*	● $p=0.0326$	*	△ $p=0.0007$	*	● $p=0.0350$
Ki-67	*	*	*	*	○ $p=0.0344$	▼ $p=0.0129$	*	*
E2F-1	*	*	*	*	*	*	*	*
PCNA	*	○ $p=0.0443$	*	*	*	*	*	*
Cyclin B1	*	*	*	*	*	*	*	*
Cyclin D1	*	*	*	*	● $p=0.0307$	▽ $p=0.0491$	*	*
Cyclin E	*	*	*	*	*	▽ $p=0.0358$	*	○ $p=0.0191$
p53	*	*	*	*	*	▼ $p=0.0053$	*	● $p=0.0041$
p21	*	*	*	*	*	*	*	*
p27	*	*	*	*	*	*	*	*
SUVmax	*	● $p=0.0079$	*	*	*	△ $p<0.0001$	*	

Rec : Recurrence SUVmax : the maximum standardized uptake value within an ROI * : not significant
● : high expression of molecules correlated with larger size of tumor, advanced stage, poorly differentiated or higher SUVmax
○ : low expression of molecules correlated with smaller size of tumor, not advanced stage, well differentiated or lower SUVmax
▲ : high expression of molecules in adenocarcinoma
▼ : low expression of molecules in adenocarcinoma
△ : high expression of molecules in SqCC
▽ : low expression of molecules in SqCC

and in poorly differentiated cancer was high expression of cyclin D1. Histological type was correlated with various molecular markers.

SUVmax was correlated with tumor size and histology. SUVmax was also related to expression of several molecular markers (Glut-1, p53, and Cyclin E) and histological type.

There was no significant relationship between disease recurrence and molecular markers or SUVmax.

Correlations of patient groups determined by hierarchical clustering analysis with clinical parameters and prognosis

There was no significant impact on disease recurrence as mentioned above. However, we found surprising impact on recurrence after surgery by our hierarchical clustering analysis using the combination of molecular markers and PET SUV.

Fifty-four cases were categorized into three cluster subgroups (Clusters A, B and C ; Fig. 1) by using hierarchical clustering analysis containing 11 parameters (10 molecular markers and SUVmax).

Relationships of the clustering subgroups with expression of molecular markers were analyzed (Table 3). Cluster A contained many cases with overexpression of the selected molecular markers, including cyclin B1, and D1, Ki-67, and Glut-1, and strong FDG accumulation. Cluster B was categorized by high FDG accumulation but low expression of the markers except for Glut-1 and p21. Cluster C was characterized by low FDG accumulation, high expression of cyclin proteins and low expression of the other markers.

Clusters were correlated with the T factor of TNM stage, histology, differentiation, and tumor size. No correlations were detected between clusters and clinical stage or lymph node metastasis (Ta-

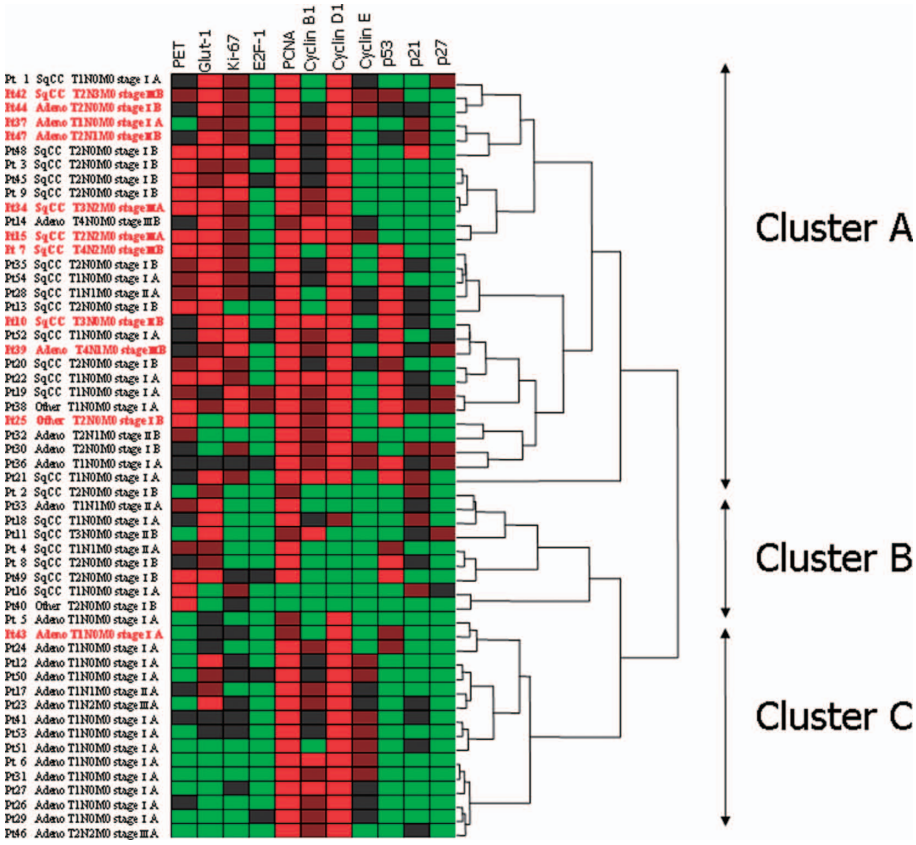


Fig. 1.
Hierarchical clustering analysis using 10 biomarkers and SUVmax of FDG-PET in analysis of lung cancer. Immunostained sections were scored using the following empirical 4-scale system : green cube, 0 = 0% ; black cube, 1 = 1-10% ; brown cube, 2 = 11-50% ; and red cube, 3 = 51-100%.
FDG-PET results were divided into four levels as follows : green cube, 0≤SUVmax<5.0 ; black cube, 5.1≤SUVmax<10.0 ; brown cube, 10.1≤SUVmax<15.0 ; and red cube, 15.1≤SUVmax.
Patients are sorted into one of three groups through hierarchical clustering analysis. Clustered data are displayed with antibodies on the horizontal axis and cases on the vertical axis. A red patient number indicates recurrent cases.

Table 3. Clusters and Molecular Markers

	Cluster A	Cluster B	Cluster C	CA-CB	CA-CC	CB-CC
Glut-1	●	●	○	*	$P<0.0001$	$P=0.0088$
Ki-67	●	○	○	$P<0.0001$	$P<0.0001$	*
E2F-1	*	*	*	*	*	*
PCNA	●	○	*	$P=0.0068$	*	*
Cyclin B1	●	○	●	$P=0.0058$	*	$P=0.0168$
Cyclin D1	●	○	●	$P<0.0001$	*	$P<0.0001$
Cyclin E	*	○	●	$P=0.0386$	$P=0.0191$	$P=0.0003$
p53	●	*	○	*	$P=0.0009$	*
p21	●	●	○	*	$P=0.0406$	$P=0.0085$
p27	●	*	○	*	$P=0.0333$	*
SUVmax	●	●	○	*	$P<0.0001$	P<0.0001

CA～CB : Compare Cluster A and Cluster B, CA～CC : Compare Cluster A and Cluster C
CB～CC : Compare Cluster B and Cluster C, * : not significant
● : high expression of molecules
○ : low expression of molecules

ble 4).

Cluster A consisted of 19 squamous cell carcinomas (69%) and 8 adenocarcinomas (31%). Cluster B consisted of 1 adenocarcinoma (11%) and 7 squamous cell carcinomas (90%). In Cluster B, 3 patients (33%) had lymph node metastasis and 6 had moderate or poorly differentiated carcinoma. Although Cluster B contained patients at high risk for recurrence according to a clinicopathological factors, no recurrent disease was observed in fact. This result clearly showed that the patients grouping in Cluster B have much better outcome if it was estimated as high-risk group by using clinical parameters. This Clustering method probably can predict accurately the disease recurrence than the conventional clinical factors. Cluster C consisted of 1 squamous cell carcinoma (6%) and 15 adenocarcinomas (94%). In Cluster C most patients (15/16) had well-differentiated carcinoma, eight patients were bronchioloalveolar carcinoma, and the tumors were smaller than those of patients in other clusters.

Clustering subgroups and patients with recurrence are shown in Table 4. Ten of 11 cases of recurrence (90.9%) were in Cluster A. Cluster A included 3 patients who had recurrence of stage I lung cancer. One patient with recurrence was in Cluster C. No patients with recurrence were found in Cluster B. Kaplan-Meier survival curves are shown in Fig. 2. Disease-free survival was 56.3% for Cluster A, 100% for Cluster B, and 93.8% for Cluster C. Recurrence-free survival was significantly longer in Cluster C than Cluster A ($p=0.0282$). A similar tendency was observed in overall 2-year survival in spite of no statistical significance.

DISCUSSION

Huge number of biomarker including several molecular markers for predicting patients' outcome after complete resection for patients with NSCLC is under analyzing in the world. However, clinically useful biomarker was still not established. In this study, we found the novel prognostic biomarker by using unique clustering analysis containing molecular marker and FDG-PET accumulation index. By our study, we could not found the usefulness of individual prognostic impact of these markers. However, if we apply our novel clustering analysis, we could more accurately predict the patients' recurrence than conventional clinic-pathological parameters.

To date, numerous studies of biomarkers aimed at prediction of outcome for patients with non-small cell lung cancer have been reported as mentioned above[5,6]. Cell-cycle related molecular markers such as cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors, which were also analyzed in the present study, have been extensively investigated in patients with lung cancer[5,6]. However, only a few studies have shown a distinct value of predicting outcome in patients with non-small cell lung cancer using a single molecule[5]. p53 alteration is thought to be a single molecular marker that is most likely a major prognostic factor of non-small cell lung cancer, but studies of this marker have produced conflicting results[2,5,9]. Because of the limitations of studies using a single molecular marker, several studies us-

Table 4. Clusters and Clinicopathological Parameters

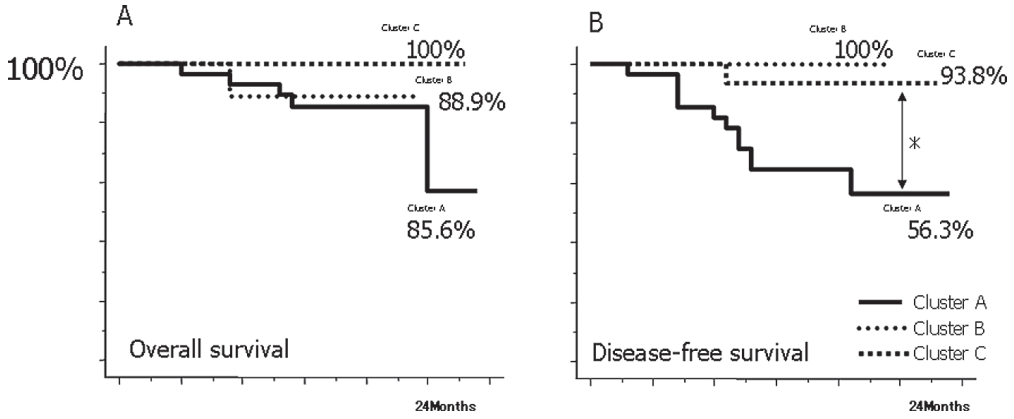
	Cluster A (N=29)	Cluster B (N=9)	Cluster C (N=16)	P-value
Age	69.3±7.7	69.3±8.9	68.3±7.6	N.S.
Tumor size	3.73±1.3 cm	3.76±2.7 cm	2.28±0.7 cm	$p<0.05$
SUVmax	13.9±6.6	11.7±6.2	3.8±2.0	$p=0.0001$
T factor				
p-T1	10	5	15	
p-T2	14	3	1	
p-T3	2	1	0	
p-T4	3	0	0	$p=0.0135$
N factor				
p-N (−)	21	6	13	
p-N (+)	8	3	3	N.S.
p-stage				
Stage I	19	5	13	
Stage II	4	4	1	
Stage III	6	0	2	N.S.
Histology				
Adeno	8	1	15	
SCC	19	7	1	$p=0.0001$
Differentiation				
well	11	0	15	
mod	5	2	1	
poor	9	4	0	$p=0.0007$
Recurrence				
Rec (−)	19	9	15	
Rec (+)	10	0	1	$p=0.0199$

N.S. : not significant
Adeno : adenocarcinoma
SCC : squamous cell carcinoma

ing two or more molecular markers have recently been reported[19,21-22]. Esposito *et al.* reported that a group of patients with non-small cell lung cancer who were negative for both CDK inhibitors p21 and p16 had a significantly shorter overall survival[21]. Their study suggested that analysis of several molecular markers could be more effective than that of a single marker in screening for high-risk subgroups. This was the basis for subsequent approaches involving comprehensive analysis of numerous molecular markers. Analyzing another set of molecular markers might be more effective.

FDG-PET imaging, which was used as the clinical prognostic examination in the present study, is useful for confirmation of clinical diagnosis or for making decisions on treatment strategy. SUVmax, which was used as a semi-quantitative parameter of FDG-PET in the present study, enables quantification of tumor glucose consumption. A recent systematic review and meta-analysis suggested that SUVmax of the primary tumor has a prognostic value in non-small cell lung cancer. However, as other authors have mentioned, it is difficult to set a cut-off value for discrimination of patients with good prognosis from those with poor prognosis[11]. Thus, the clinical benefits of SUVmax of FDG-PET for prediction of recurrence remain controversial. This study may resolve this problem by considering at the same time as the other markers.

On the basis of the issues outlined above, molecular markers and FDG-PET of patients with lung cancer were analyzed together, and hierarchical clustering was performed to analyze the present data. Hierarchical clustering analysis of SUVmax and of expression of cell cycle-related molecules resulted in creation of three different patient subgroups. These subgroups were independent of tumor stage, but they were significantly correlated with recur-



No. of patient at risk

	0 M	6 M	12 M	18 M	24 M
Cluster A (*N*=28)	28	27	26	12	5
Cluster B (*N*=9)	9	9	8	5	0
Cluster C (*N*= 16)	16	16	15	5	1

No. of patient at risk

	0 M	6 M	12 M	18 M	24 M
Cluster A (*N*=28)	28	27	21	10	4
Cluster B (*N*=9)	9	9	8	5	0
Cluster C (*N*=16)	16	16	16	5	1

Fig. 2.
Kaplan-Meier survival curves by lung cancer cluster subgroups for overall survival (A) and recurrence-free survival (B). There was a significant difference in recurrence-free survival between clusters. Survival analysis did not reveal any significant differences.
* : p=0.0282.

rence. Patients in Cluster C contained many well differentiated adenocarcinomas. Therefore, patients in Cluster C were good prognosis. In Cluster A and B the disease was advanced and contained poorly differentiated carcinomas, those in Cluster A and B appeared likely to have a poor prognosis. However, no recurrent disease was observed at least in Cluster B. This Clustering method was able to accurately predict the recurrence than clinical factors. This classification scheme might be superior to conventional clinicopathological parameters for prediction of outcome in non-small cell lung cancer.

This study was retrospective exploratory research and consisted of only a small number of cases. A large-scale prospective study is required to confirm and validate the present findings. Modifications to our method could involve limiting the analysis to patients of the same tumor stage or the same tumor histology. Analyzing another or broader set of molecular markers might also be more effective. Apart from molecular markers, mutations of genes such as K-ras and EGFR, known to be important clinical data in non-small cell lung cancer, could also be useful parameters for prediction of prognosis. On the other hand, if we might reduce the number of makers, we can also reduce efforts and costs in order to put into practical use. By try the appropriate combination from the function of the molecules, our method will be modified and continue to improve.

CONCLUSION

Many patients with NSCLC finally developed to disease recurrence after complete resection. Molecular markers might be useful parameters for predicting prognosis of non-small cell lung cancer. However the usefulness in prediction of recurrence was limited by using single biomarker. This study clearly showed that analysis of molecular markers and clinical data concurrently could have a great value in identification of patients with high risk of recurrence after surgery. This novel method could be a useful tool for selection of those patients who need further therapy after surgical resection.

CONTRIBUTION

H Suzuki participated as principle investigator of the study. AY contributed to all of the study design and manuscript. AY, MH, JO, NO, SM and MG participated in the design and coordination of the

study, data acquisition and analysis and helped draft the manuscript. H Sakuma helped for pathological data analysis and evaluation. MH, JO, NO, SM and YT participated in the clinical data acquisition and evaluation and helped draft the manuscript. All authors have given final approval of the manuscript for publication.

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