Molecular Diagnosis of MACC1 Status in Lung Adenocarcinoma by Immunohistochemical Analysis

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Abstract. *Background: Recently, we reported that overexpression of metastasis-associated colon cancer-1 (MACC1) mRNA may be a useful marker for predicting postoperative recurrence in patients with lung adenocarcinoma following surgery. However, the biological significance of mRNA overexpression is difficult to determine and is not widely used because mRNA expression analysis is relatively expensive and time- and labor-intensive. On the other hand, immunohistochemical (IHC) staining is easy to perform, well-established, inexpensive, and is a useful method which can be routinely applied in solid tumor diagnosis in clinical laboratories. Patients and Methods: Tumor specimens were collected from 197 consecutive patients who underwent a complete resection for lung adenocarcinoma from 1998 to 2007. We analyzed the MACC1 status of the primary lung adenocarcinoma by IHC analysis. Results: The average postoperative observation period was 46.7 months. Forty (20.3%) of the 197 patients developed recurrences after surgery. Positive expression of MACC1 was identified in 129 (65.5%) patients. Furthermore, MACC1 IHC was positive in 33 (82.5%) out of the 40 patients and 96 (61.1%) out of the 157 patients, with and without recurrence, respectively (p=0.011). Both univariate and multivariate logistic regression models indicated that positive staining for MACC1 was an independent factor for tumor recurrence. Furthermore, positive staining for MACC1 was associated with poorer disease-free survival (DFS), according to the univariate survival analysis (p=0.080). Conclusion: Positive staining for MACC1 expression in resected specimens was associated with a poorer DFS. Therefore, positive staining of IHC for MACC1 may be a useful marker for predicting postoperative recurrence in patients with lung adenocarcinoma following surgery.*

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There are two important issues regarding surgery as a main method used to cure cancer. One is to define the treatment indications for patients who are unlikely to develop a recurrence. The other is the selection of the type of adjuvant chemotherapy for tumors with micrometastasis and to identify the patients who might benefit most from postoperative adjuvant chemotherapy. These criteria would not only precisely select the patients who require additional treatment, but would also prevent the induction of adverse events in patients who do not require treatment. Therefore, predictive biomarkers for recurrence are urgently needed to help in patient treatment decisions. Since non-small cell lung cancer (NSCLC) is a heterogeneous disease with significant variability in prognosis and individual response to treatment (2), it is important to evaluate the biological and molecular characteristics of each tumor in order to identify the factors related to recurrence following surgery. However, no useful markers that can predict clinical recurrence exist at present.

The *MACC1* gene was identified by differential display RT-PCR by analyzing the colon mucosa, primary tumors, and metastatic lesions of patients with colorectal cancer (CRCs) (3). MACC1 expression was reported to be a predictor of tumor growth, invasion, metastasis, and tumor recurrence in patients with colon cancer (4). Lung adenocarcinoma appears to be similar to CRC from the standpoint of histological type and carcinogenesis. We therefore hypothesized that MACC1 may also be a useful prognostic indicator of tumor recurrence in patients following lung adenocarcinoma resection, and that overexpression of *MACC1* mRNA may be a useful marker for predicting postoperative recurrence in patients with lung adenocarcinoma following surgery (1). However, the biological significance of mRNA overexpression is unclear and the evaluation of mRNA expression is not widely performed in the clinical setting due to its high cost and time- and labor-intense

performance. In addition, RNA extracted from tissues is often degraded and mixed with a significant amount of normal tissue. Furthermore, these methods do not take into account tumor heterogeneity. Since an antibody against MACC1 is commercially available, we decided to investigate the MACC1 expression by an IHC analysis to examine: the relationship between MACC1 expression and clinicopathological factors, the recurrence rate by MACC1 discrimination after surgery, and to evaluate the relationship between the MACC1 expression and *MACC1* mRNA overexpression.

Patients and Methods

Patients, clinical features, and follow-up. Tumor samples were obtained from 494 patients with primary lung adenocarcinoma who had undergone surgical resection between 1998 and 2007, in our department. Fifty-six patients had had an incomplete resection. The tumor samples from 59 patients were too small to extract sufficient amounts of DNA for the analyses. One hundred and sixty-eight had cancer overlapping other organs or had multiple lung tumors, and 13 patients died due to other reasons without recurrence. As a result, 297 patients were excluded from the analysis. Finally, a total of 197 patients were included in the present series.

The patients were followed-up every month within the first postoperative year and at approximately 2- to 4-month intervals thereafter. The evaluations included a physical examination, chest roentgenography, blood chemistry analyses, and the measurement of tumor markers. Chest and abdominal computed tomography, brain magnetic resonance imaging, and a bone scintiscan were performed every 6 months for 3 years following surgery. Additional examinations were performed if any symptoms or signs of recurrence were detected.

All of the patients were Japanese, including 107 males and 90 females in this series, with a mean age of 67.8 years (range: 23-91 years). No patients had received either chemotherapy or radiotherapy prior to the resection. There were 78 patients who had never smoked, 51 former smokers, and 68 current smokers. Former smokers were defined as those who had quit smoking at least 3 years before the time of surgery. The tumor stage was classified according to the new TNM (7th edition) classification for Lung Cancer (5). According to the pathological stage, 104 patients were at stage IA, 40 at IB, 13 at IIA, 8 with IIB, 25 at IIIA, 6 at IIIB, and 1 at IV. Twenty-nine (14.7%) patients received adjuvant chemotherapy: 22 received carboplatin plus paclitaxel, 5 received carboplatin plus gemcitabine, and 2 received tegafur-uracil. Follow-up was conducted for all patients and the median follow-up period was 46.7 months. One hundred and fifty-seven patients were alive and free of cancer at the last follow-up, 2 patients had died of other causes with recurrent cancer, 17 patients were alive with recurrent cancer, and 21 patients had died of cancer.

Immunohistochemical staining and evaluation of the MACC1 expression. The study was approved by the Institutional Review Board and informed consent for the use of the tumor specimens was obtained from either the patient or from the patient's legal guardian. IHC staining was conducted using serial sections from the same paraffin-embedded blocks, according to the previously described methods (6, 7). All specimens were stained with hematoxylin-eosin for the histological diagnosis. Briefly, the sections were placed in 0.01 mol/L citrate buffer (pH 6.0) and autoclaved at 121˚C for 10 min. They were treated with 3% H_2O_2 for 5 min to block the endogenous peroxidase activity. The primary antibodies used were a rabbit polyclonal antibody against human MACC1 (HPA020103; SIGMA, St, Louis, USA), diluted 1:100 in PBS and incubated for 18 hours at 4˚C. Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan) according to the manufacturer's instructions (8-10). The positive and the negative controls were a metastatic lung tumor arising from colon cancer specimens expressing MACC1, and the exclusion of the primary antibody, respectively. IHC was considered to be positive only when distinct cytoplasmic staining was evident (Figure 1). An average of 1,500 cells were evaluated per section. The stained specimens were then categorized into 6 degree classes according to the quantitative score (11). Initially, 4 degrees of the proportional score (PS) for the positive staining cells were assigned according to the frequency of positive tumor cells (0, none; 1, 1/100 to 1/4; 2, 1/4 to 1/2; and 3, >1/2). Thereafter, 4 degrees of the intensity score (IS) were assigned according to the intensity of the staining (0, none; 1, weak; 2, intermediate; and 3, strong). The proportional score and the intensity score were then added to obtain a total score (TS), which ranged from 0 to 6. According to the TS, the MACC1 expression of the tumor was categorized as negative when the score was 0-3 and as positive when the score was 4-6. The slides were independently examined by two of the investigators (H. S. and T. O.) who were blinded to the clinicopathological data. When a discrepancy was found between the two investigators, a consensus was reached *via* simultaneous examination using a double-headed microscope. The correlation of MACC1 protein and mRNA (described below) was also analyzed.

Detection of MACC1 mRNA. The expression of *MACC1* mRNA was analyzed in all samples by quantitative real-time PCR, performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) by using a Fast SYBR Green Master Mix (Applied Biosystems) as previously described (11). We quantified gene expression by comparing the levels of the target gene to the levels of beta-actin, used as an internal control. The quantification was based on a standard curve generated from human normal genomic DNA. The relative *MACC1* copy number was also normalized to human genomic DNA. PCR for each primer set was performed in triplicate, and the means were reported (1).

Statistical analysis. Statistical significance was evaluated using the Chi-square test or Fisher's exact test. The odds ratio and 95% confidence interval (95% CI) was calculated for each variable. The Kaplan-Meier method was used to estimate the probability of disease-free survival (DFS), and survival differences were analyzed by the log-rank test. A multivariate logistic regression analysis was used to evaluate independent associations. A multivariate analysis was then performed according to Cox's proportional hazards model. Differences were considered to be statistically significant for *p*-values of less than 0.05. The data were analyzed using the Stat View software package (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Detection of the MACC1 protein and mRNA expression, and patient clinicopathological factors. Forty (20.3%) out of the 197 patients had tumor recurrences following surgery. The positive expression of MACC1 was identified in 129 (65.5%) patients. There was no significant association between

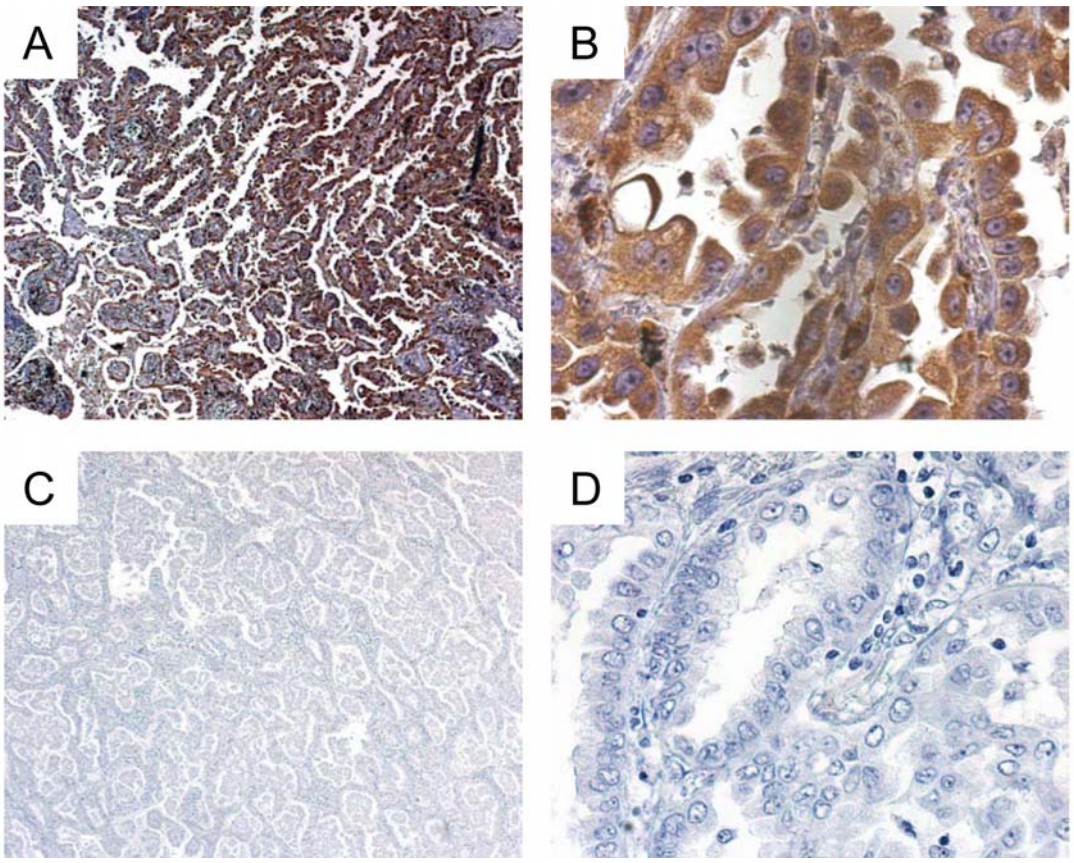

Figure 1. *Representative IHC staining. A: Positive staining for MACC1 with brown stained cytoplasm is shown at low power (original magnification ×40), the IS and PS were judged to be 3 and 3, respectively, in this sample. Therefore, the IHC of this tumor was assumed to be positive, with a score of 6. B: Representative IHC staining of tumors at high power (original magnification ×400). C: Negative staining for MACC1 is shown at low power. D: Negative staining for MACC1 is shown at high power.*

positive expression of MACC1 and any of the clinical factors (Table I). Overexpression of *MACC1* mRNA was present in the tumors of 92 (46.7%) patients. The correlation coefficient between TS by IHC staining and mRNA expression of MACC1 was 0.115 (p=0.109, 95% CI: -0.026, 0.250).

The relationship between MACC1 amplification and recurrence. The majority of the tumor recurrences were hematogenous metastases. Thirty-four and 7 cases had hematogenous (12 lung, 11 brain, 4 bone, 2 skin, and 1 adrenal metastasis) and locoregional (7 lymph node metastases and 7 pleural dissemination) recurrences, respectively. The numbers of recurrent sites overlapped.

The staining for MACC1 was positive in 33 (82.5%) out of 40 patients, and 66 (61.1%) out of 157 patients with and without recurrence, respectively (p=0.011) (Table II). The univariate and multivariate logistic regression models indicated MACC1 expression to be an independent predictor for recurrence, as were a pathological T and N status (Table III and Table IV).

Prognosis of patients according to the molecular parameters. The 2-year DFS rate for patients with negative and positive staining for MACC1 expression was 91.1% and 77.7%, respectively (Figure 2). Positive staining for MACC1 was associated with a poorer DFS according to a univariate survival analysis (p=0.022) (Table V). A multivariate analysis demonstrated the MACC1 expression was not independently associated with an increased risk for poor DFS (Table VI).

Discussion

The prognosis of NSCLC is unacceptable because the 5-year survival rate is only around 50%, even in patients who achieve complete surgical resection (12). This suggests that occult metastases are present at the time of surgical intervention. As a consequence, adjuvant chemotherapy has been shown to improve the prognosis of patients with resected NSCLC (13). However, the 5-year survival rate of patients with resected stage IB NSCLC reaches 74% without adjuvant chemotherapy

Table I. *Relationships between the MACC1 expression and clinicopathological characteristics.*

Variable	Category	No. of patients n=197	MACC1 expression	
			Positive (%) 129 (65.5)	Negative 68
Gender	Male	107	73 (68.2)	34
	Female	90	56 (62.2)	34
Age (years)	<69	97	61 (62.9)	36
	≥69	100	68 (68.0)	32
Stage*	I	144	89 (61.8)	55
	II-IV	53	39 (73.6)	14
pT	T1	115	74 (64.3)	41
	T2-4	82	55 (67.1)	27
pN	Negative	156	99 (63.5)	57
	Positive	41	30 (73.2)	11

*Pathological stage, pT: pathological T status, pN: pathological N status.

Table II. *Relationships between the MACC1 expression and cases with recurrence.*

Variable	MACC1 expression	
	Positive (%) 129 (65.5)	Negative 68
Cases with recurrence	33 (82.5)	7
Cases without recurrence	96 (61.1)	61

Table III. *A univariate analysis of the factors contributing to tumor recurrence.*

Variable	OR	95% CI	*p*-Value
Gender: Male	1.742	0.847-3.584	0.131
Age: <69 years	1.481	0.737-2.976	0.270
pT: 2-4	4.451	2.098-9.443	<0.001
pN: Positive	8.877	4.044-19.486	<0.001
MACC1: Positive	2.996	1.247-7.197	0.014

OR: Odds ratio, 95% CI: 95% confidence interval.

Table IV. *A multivariate analysis of the factors contributing to tumor recurrence.*

Variable	OR	95% CI	*p*-Value
Gender: Male	2.206	0.916-5.313	0.078
Age: <69 years	3.597	1.580-3.636	0.282
pT: 2-4	3.587	1.525-8.439	0.003
pN: Positive	7.249	3.071 -17.114	<0.001
MACC1: Positive	2.984	1.105-8.060	0.031

Table V. *A univariate analysis using a proportional hazard model for the disease free survival of the 197 lung adenocarcinoma patients.*

Variable	Characteristic		HR	95% CI	*p*-Value
	Unfavorable	Favorable			
Gender	Male	Female	1.686	0.881-3.236	0.115
Age (years)	<69	≥69	1.346	0.722-2.509	0.350
T status	T2-4	T1	3.953	2.008-7.813	<0.001
N status	Positive	Negative	7.042	3.759-13.333	<0.001
MACC1	Positive	Negative	2.591	1.145-5.848	0.022

HR: Hazard ratio.

Table VI. *A multivariate analysis using a proportional hazard model for the disease free survival of the 197 lung adenocarcinoma patients.*

Variable	Characteristic		HR	95% CI	*p*-Value
	Unfavorable	Favorable			
Gender	Male	Female	2.375	1.200-4.695	0.013
Age (years)	<69	≥69	1.267	0.677-2.370	0.460
T status	T2-4	T1	2.857	1.406-5.814	0.004
N status	Positive	Negative	5.495	2.825-10.753	<0.001
MACC1	Positive	Negative	2.083	0.917-4.717	0.080

(14). Not all patients with lung cancer require postoperative adjuvant chemotherapy after a complete resection. The current challenge is to identify factors that would predict tumor relapse despite apparently curative resection.

The present study demonstrated the potential clinical usefulness of examining MACC1 expression by IHC analysis. First, the incidence of positive staining for MACC1 was significantly higher in recurrent cases than in non-recurrent ones, suggesting that it represents a suitable biomarker to identify patients who would benefit most from adjuvant chemotherapy following a complete resection. Second, it allows for the identification of patients with a high risk of relapse, enabling tailored management and stricter follow-up, because no consensus exists regarding postoperative follow-up measures at the present time (15).

These findings include several limitations for interpretation: primarily the retrospective nature of the study and the fact that it was carried out at a single institution. We also failed to show a significant relationship in the expression level of MACC1 between IHC staining and quantitative RT-PCR. The reason for this finding still remains to be elucidated, although overexpression of *MACC1* mRNA also appears to be an indicator of postoperative recurrence (1). Further, the diagnostic sensitivity and specificity of MACC1 for recurrence were just 82.5% (3/40) and 38.9%

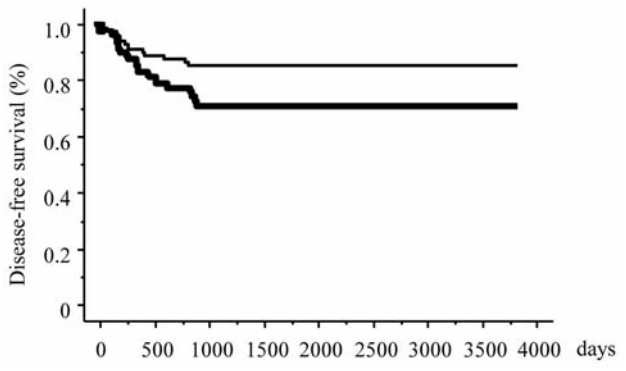

Figure 2. *Kaplan-Meier DFS curves stratified by MACC1 expression. The heavy and narrow lines show the positive and negative expression of the MACC1, respectively.*

(61/157), respectively. Thus, a number of biomarkers might be needed to more accurately select the patients (16). To overcome these limitations, a prospective investigation by biomarkers is necessary to elucidate the clinical usefulness of this evaluation.

In conclusion, the current results revealed that MACC1 expression may be a useful marker for predicting postoperative recurrence. This simple IHC approach may thus be useful for establishing a rapid and cost-effective method to identify lung adenocarcinoma patients who would benefit most from adjuvant chemotherapy following a complete resection.

Conflicts of Interest

We declare that we have no conflict of interest.

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