Prognostic Value of Genetically Diagnosed Lymph Node Micrometastasis in Non-Small Cell Lung Carcinoma Cases

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ABSTRACT

The predictive value of lymph node micrometastasis, detected by immunohistochemical or genetic methods, is well appreciated in terms of prognosis. However, a major problem is high false-positive rates, because most methods focus on cytokeratin, which is a component not only of carcinoma but also normal epithelial and nonepithelial cells. Mutant allele-specific amplification (MASA) can detect DNAs derived from cancer cells itself, reportedly with high sensitivity. It was, therefore, used with nested-PCR using p53 or K-ras mutation for analysis of lymph node micrometastasis in non-small cell lung carcinoma (NSCLC) patients in the present study, in comparison with the immunohistochemical method using an anti-cytokeratin reagent for the same samples. Lymph nodes from 31 NSCLC patients with p53 and K-ras mutated tumors (30 and 1, respectively) staged as pathological (p)-T1–4 N0–1 and M0 were examined. Genetic and immunohistochemical methods demonstrated positive reactions in 34 (15%) and 61 (27%) of 229 lymph nodes, respectively (9 cases, 29%, and 24 cases, 77%). The concordance with the two methods was 77%, but 13 (39%) of 34 genetically positive lymph nodes could not be detected by immunohistochemistry (IHC). Of 22 cases with p-N0 disease, 6 (27%) were genetically positive in hilar and/or mediastinal lymph nodes, and 4 (67%) of them died after cancer relapse. In contrast, none of the patients without micrometastasis died of cancer (P < 0.001, log rank analysis). Of the same p-N0 patients, 17 (77%) were positive by IHC, and 4 (24%) of them died of cancer, whereas 5 negative patients did not suffer cancer relapse. Survival did not significantly differ between cases positive and negative (P = 0.246) by IHC. According to the g-N (N factor restaged by a genetic method), patients with g-N1 and g-N2 disease had a shorter survival than those with g-N0 disease (P = 0.042 and P < 0.001, respectively). However, no significant difference was observed with grading by IHC. Thus, detection of micrometastasis in regional lymph nodes with the MASA method, in other words with a carcinoma-specific marker, is of greater prognostic significance for early stage NSCLC patients than immunohistochemical methods. This approach should facilitate selection of patients for whom postoperative adjuvant chemotherapy should be performed.

INTRODUCTION

Numbers of operable NSCLC3 cases are increasing because of improved detection of the disease with sophisticated diagnostic techniques. However, even after complete or potentially curative surgery, the long-time survival rate remains unsatisfactory (1). This is related to the fact that disseminated tumor cells, undetectable by routine diagnostic methods, may be present at the time of surgery. If they could be detected, prediction of relapse or prognosis would be facilitated, allowing additional appropriate treatment to be performed for high-risk patients. Micrometastasis to regional lymph nodes, not detected by routine histology, can now be identified by immunohistochemical or genetic methods, and this is reported to be useful for assessment of prognosis (2–12). High false-positive rates are a major problem, but the MASA genetic method has high sensitivity and specificity for carcinomas; one tumor cell containing genetic changes was detected in a background of thousands of normal cells (13–17). It has established prognostic value for colon cancer (2, 13), but data for lung cancer are limited. One reason is that it is difficult to collect enough cases of early stage carcinomas having genetic alteration for MASA analysis. The other is that amplification of the mutated DNA sequence in lymph nodes presented practical difficulties in the past.

In the present study, we therefore collected many NSCLCs with mutations and analyzed regional lymph node micrometastasis by a modified improved MASA method and assessed its prognostic potential. For comparison, the immunohistochemical method using a monoclonal anti-CK reagent was also performed for the same specimens.

MATERIALS AND METHODS

Patients, Clinicopathological Data, and Follow-Up. Fresh frozen samples from 151 NSCLCs (113 adenocarcinomas and 38 squamous cell carcinomas), which were resected consecutively from 1989 to 1993 at Cancer Institute Hospital, Tokyo, Japan, were screened for p53 mutations in exons 4–8 and 10 using single-strand conformation polymorphism, followed by sequencing, and for K-ras mutations of codons 12, 13, and 61 using the MASA method, as described previously (18–20). All patients analyzed had undergone a complete or potentially curative resection with lobectomy or pneumonectomy, combined with pulmonary hilar and mediastinal lymph node dissection. Histopathological classification and differentiation of the tumors were determined according to the 1981 WHO classification of lung tumors (21). The stage of the disease was based on the TNM staging system of the International Union against Cancer (UICC; Ref. 22). Of the 68 p53 and 12 K-ras mutated cases, 35 cases with p53 mutations and one case with a K-ras mutation were staged as pathological (p)-T1–4 N0 and N0, M0. Of those cases, 5 cases with p53 mutation were excluded because the PCR products with the MASA method for the positive controls could not be consistently detected. Finally, 31 cases staged as p-T1–4 N0 and N1, M0 with enough follow-up were eligible for analysis (Table 1). Location and type of mutations for each patient are shown in Table 2. None had received chemotherapy or radiotherapy before surgery, but 13 (41.9%) underwent postoperative adjuvant therapy. The patients comprised 24 men and 7 women with a mean age at surgery of 60 years (range, 26–76 years). Twenty-two had adenocarcinomas, and 9 had squamous cell carcinomas, with 8 well, 16 moderate, and 7 poorly differentiated lesions. Two hundred and twenty-nine lymph nodes (median, 8; range, 4–9 for each case) from the 31 patients were eligible for comparison of the micrometasis positivity between MASA and immunohistochemical methods and for evaluation of prognostic value (Table 3). One hundred and seventy lymph nodes of 22 p-N0 (no lymph node metastasis by conventional histological examination) cases were examined in both pulmonary hilar and mediastinal regions. For the remaining nine p-N1 patients, 59 lymph nodes from mediastinal regions were examined by both methods. Survival duration was calculated from the date of operation until the date of the last follow-up (censored) or the date of death. The end point was

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3 The abbreviations used are: NSCLC, non-small cell lung carcinoma; MASA, mutant allele-specific amplification; TNM, Tumor-Node-Metastasis; IHC, immunohistochemistry; CK, cytokeratin.

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for the second round of PCR (MASA method; Table 2; Refs. 13 and 16). MTA-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 mM dNTPs, and 1 U of AmpliTaq GOLD (Perkin-Elmer Corp., Foster City, CA), with denaturing at 95°C for 9 min, annealing and extension at 52–66°C for 30 s in each cycle, using a GeneAmp PCR System 9700 (Perkin-Elmer Corp.). Because of varied qualities of genomic DNAs from lymph nodes, PCR were performed for 45–50 cycles to obtain equivalent amplified products by saturation. After purification for removal of the primers and deoxyribonucleotide triphosphates, the amplified products were diluted 50-fold in 10 mM Tris (pH 8.1)–1 mM EDTA buffer, and 1 μl of aliquots of diluted products was subjected to a second round of PCR using nested primers (mutant allele-specific primers) under the same conditions, except for the temperature of annealing and extension (64–76°C) and the figures of PCR cycles (35–41 cycles), which differed with each set of primers. Primer sequences and PCR conditions of each primer set are shown in Table 2. The remaining one 4-μm section was examined by IHC.

Nest PCR for Genetic Analysis Using the MASA Method. DNAs used for nested-PCR were extracted from histologically negative lymph nodes. As positive controls, DNAs were extracted from primary tumors fixed in formalin and embedded in paraffin, without metastasis by conventional histological study, were analyzed for micrometastasis with serial sectioning. The first and last 4-μm sections were stained with H&E to confirm or refute the presence of overt lymph node metastasis by an experienced histopathologist (E. T.). Genomic DNA was extracted from the second and third 15-μm sections and used for genetic analysis (23). The remaining one 4-μm section was examined by IHC.

Sectioning of Lymph Nodes and DNA Preparation. Lymph nodes fixed in formalin and embedded in paraffin, without metastasis by conventional histological study, were analyzed for micrometastasis with serial sectioning. The first and last 4-μm sections were stained with H&E to confirm or refute the presence of overt lymph node metastasis by an experienced histopathologist (E. T.). Genomic DNA was extracted from the second and third 15-μm sections and used for genetic analysis (23). The remaining one 4-μm section was examined by IHC.

Immunohistochemical Staining. Paraffin-embedded tissue sections 4 μm thick were immunostained with the monoclonal anti-CK reagent, CAM5.2 (Becton Dickinson, San Jose, CA), using an immunoperoxidase method. This primary mouse monoclonal antibody is specific for CKs 8 and 18 of epithelial cells and is positive in both adenocarcinomas and squamous cell carcinomas of the lung (24). After exhaustion of endogenous peroxidase with hydrogen peroxide, deparaffinized sections were incubated with 0.1% trypsin for 45 min at room temperature to expose antigen sites. Possible nonspecific background staining was blocked with 10% normal goat serum for 10 min at room temperature. Then the sections were incubated with a primary antibody at room temperature for 1 h, and the antibody reaction was developed with secondary antibody reaction (23).

Statistical Analysis. To assess any correlations between the presence of micrometastasis and clinicopathological data, Fisher’s exact probability test, Mann-Whitney U test, and Student’s t test were used, with \( P < 0.05 \) indicating statistical significance.
Table 2. p53 and K-ras mutations of primary tumors, primer sequences, and PCR conditions

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<th>No.</th>
<th>Gene</th>
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<th>Codon</th>
<th>Base change</th>
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<th>MASA</th>
<th>Temperature of annealing and extension (°C)</th>
<th>PCR cycles</th>
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a | b | c | d | e | f
---|---|---|---|---|---
Histology: Ad, adenocarcinoma; Sq, squamous cell carcinoma.
Differentiation of the tumor: W, well differentiated; M, moderately differentiated; P, poorly differentiated.
Mutant allele-specific amplification method.
Immunohistochemistry.
–, negative by the method.
Underlines indicate relapse cases and initial site of relapse that was the cause of death.
controls); Lanes 1–9
Lane DW (positive controls);
diluted (1:10, 1:100, and 1:1000) with DNAs extracted from lymph nodes of other cases.
bottom first-round PCR; 
demonstrated positive reactions in 61 (26.6%) of 229 lymph nodes (24
method is shown in Fig. 2. The immunohistochemical approach
strongly in all adenocarcinomas but weakly in some cases of squa-
primary tumors were positive for staining with anti-CK regent,
clearly stained dark brown in their cytoplasm and cell membranes. All
lymph nodes (17 cases, 77.3%) with p-N0, and 14 (23.7%) of 59
among patients with p-T1–4 N1 M0 disease, 10 (16.9%) of 59 lymph nodes in the mediastinal region
were linked to a poor outcome.

Lymph Node Micrometastasis Detected by IHC and Correla-
tion between MASA and IHC Methods. CK-positive cells were
histologically, no lymph node metastases were reconfirmed, even
with retrospective examination of the H&E slides after obtaining
genetic and IHC results.

Lymph Node Micrometastasis Detected by the MASA Method.
Representative results of the MASA method are shown in Fig. 1, and
a summary of the results is presented in Tables 1 and 3. The genetic
method revealed 34 (14.8%) of 229 lymph nodes and 9 (29.0%) of 31
cases to be positive. Among 22 patients with p-T1–4 N1 M0 disease,
24 (14.1%) MASA-positive lymph nodes of a total of 170 in the hilar
and/or mediastinal regions were detected in 6 (27.3%) patients; mi-
crometastases in two patients were limited to pulmonary hilar lymph
nodes and 4 to mediastinal regions. Among nine cases with p-T1–4 N1
M0 disease, 10 (16.9%) of 59 lymph nodes in the mediastinal region
were MASA positive in 3 (33.3%) patients.

Lymph Node Micrometastasis Detected by IHC and Correla-
tion between MASA and IHC Methods. CK-positive cells were
clearly stained dark brown in their cytoplasm and cell membranes. All
primary tumors were positive for staining with anti-CK regent,
strongly in all adenocarcinomas but weakly in some cases of squa-
mous cell carcinomas. Lymph nodes were considered positive even if
only one CK-positive cell was detected within a lymph node. An
example of a CAM5.2-positive cell by the immunohistochemical
method is shown in Fig. 2. The immunohistochemical approach
demonstrated positive reactions in 61 (26.6%) of 229 lymph nodes (24
cases, 77.4%) among patients with p-N0 and p-N1; 47 (27.6%) of 170
lymph nodes (17 cases, 77.3%) with p-N0, and 14 (23.7%) of 59
lymph nodes (7 cases, 77.8%) with p-N1 patients (Table 3). Among 17
p-N0 patients with CK-positive reactions, positive lymph nodes were
limited to the pulmonary hilar region in eight cases and up to the
mediastinal region in nine cases. The concordance rate between the
two methods was 76.9%, but 13 (39%) of 34 genetically positive
lymph nodes were not positive with IHC.

Correlation between Patient Survival and Lymph Node Micro-
metastasis and the Significance of the Restaged N Factor. Among
22 p-N0 patients, 4 (66.7%) of 6 with micrometastasis detected by the
genetic method and none of those without micrometastasis died by
cancer relapse. Fig. 3A shows the survival curves of the p-N0 patients
with and without micrometastasis (P < 0.001, log rank analysis).
Among the same p-N0 patients, only 4 (23.5%) of 17 CK-positive
cases died by relapse, and although the remaining 13 (76.5%) patients
were censored, 5 CK-negative patients did not relapse. No prognostic
difference was observed between patients with and without micro-
metastasis decided by IHC (P = 0.246).

Fig. 3B shows survival curves according to the N factor assessed by
conventional histology. Survival duration between p-N0 and p-N1
patients did not differ (P = 0.856). However, when the N factor was
restaged according to the genetic results (g-N), patients with g-N1 and
g-N2 disease showed a shorter survival than those with g-N0
(P = 0.042 and P < 0.001, respectively; Fig. 3C). The 5-year
survivals were 100.0% for patients with g-N0, 75.0% for g-N1, and
42.9% for g-N2. When restaged by IHC (IHC-N), survival between
patients with IHC-N0 and IHC-N1 or IHC-N2 did not show any
statistically significant difference (P = 0.289 or P = 0.239, respec-
tively; Fig. 3D).

DISCUSSION

In the present study, a conventional MASA method was initially
applied to analyze mutations in DNAs extracted from formalin-fixed,
paraffin-embedded lymph nodes, but the results were sometimes
equivocal. Because the main reason was probably that DNAs
extracted from these samples were fragmented and not uniform, we first
amplified those DNA fragments that include the mutated region to
unify the quality of samples, and then MASA analysis was performed
using amplified DNA, in which targeted sequences could be reliably
detected. Furthermore, we used several positive and negative controls
for confirmation. As positive controls, primary tumor DNAs and
diluted DNAs were used. The dilution fold was determined on the
basis of initial experiments for several cases in which diluted tumor
DNAs at 10-, 100-, 1,000-, and 10,000-fold were amplified with the
modified MASA method. The results showed that in all cases a
MASA-specific product was constantly observed in the range from
10- to 1,000-fold but not always identified at 10,000-fold. By this
method, we demonstrated that ~30% of patients with histologically
negative lymph nodes indeed had micrometastases, and that these
were linked to a poor outcome.

Thus far, for detection of micrometastasis in lymph nodes the
immunohistochemical method with anti-CK regent has been used
frequently and reported to be effective for prediction of a poor
prognosis (3–5, 7–9). However, our results showed the modified
MASA method to be superior to IHC in terms of NSCLC relapse. In
regional lymph nodes, macrophages that phagocytize CKs derived
from degraded normal lung epithelial cells are presumably present,
and these could be misinterpreted and give rise to false-positives. In
fact, we could not recognize carcinoma cells histologically in CK-
positive lymph nodes. Furthermore, there have been reports that CK
expression may be detected in both normal and neoplastic nonepithelial
cells (11, 25, 26).

As a genetic approach to detect lymph node micrometastasis or
systematic tumor dissemination, reverse transcription-PCR analysis
for several markers, including CK19, has been described (10–12, 27).
Although having a high sensitivity, this is again plagued with the
problem of a high percentage of false-positive results, depending on
the number of PCR cycles, because of nonspecificity for cancer cells
(11, 12, 27). The modified MASA method applied here is not only
highly sensitive but also highly specific for carcinoma cells with
particular mutations, so that we could show clearly the correlation
between the micrometastasis and postoperative survival in lung car-

![Diagram](attachment:Diagram.png)
cinoma patients. As to mutated DNAs, they may be present in macrophages if they phagocytize carcinoma cells or carcinoma DNAs (28), but this would reflect the likelihood of metastasis.

Carcinoma relapse was found in contralateral lungs or other remote organs in our patients with micrometastasis. Two pathways of metastasis are probable; one is that carcinoma cells pass through the regional lymph nodes and reach blood vessels, and the other is that vascular invasion of carcinoma cells occurs at the primary site. In our study, all six patients with relapse had lymph node metastasis histologically and/or genetically, and in four patients of them metastasis extended to mediastinal region. However, none of the patients without such lymph node involvement showed recurrence. Therefore, the main root for systemic metastasis may be via lymphatic pathways, which strengthens the importance of micrometastasis in lymph nodes.

Fig. 2. An example of a CAM5.2-positive cell by immunohistochemical staining in a lymph node. An oval cell is diffusely positive by CAM5.2 in the cytoplasm. In a serial lymph node section stained with H&E, the cell was histologically interpretable as a macrophage or a histiocyte rather than a carcinoma cell. Several macrophages phagocytizing dust particles are also apparent. ×400.

Fig. 3. Kaplan-Meier survival curves with respect to disease-specific survival. A, cases with and without micrometastasis detected by the genetic method in pathological-N0 disease. A worse prognosis was observed in patients with micrometastasis (P < 0.001, log rank analysis). B, N0 and N1 disease decided by conventional histology (p-N), without any significant intergroup difference (P = 0.856). C, N0–2 disease restaged by the genetic method (g-N). A worse prognosis was observed in patients with g-N1 and g-N2 than those with g-N0 (P = 0.042 and P < 0.001, respectively). D, N0–2 disease restaged by IHC (IHC-N). No difference in survival is apparent between patients with IHC-N0 and IHC-N1 or IHC-N2 (P = 0.289 or P = 0.239, respectively).
However, in two relapsed patients, lymph node metastasis was limited to the hilar region, so that the second route cannot be ruled out. If lymph node or distant metastasis is within a “micro level,” undetectable by routine histological examination, intensive adjuvant therapy may prevent carcinoma recurrence. Therefore, a finding of micrometastasis in lymph nodes is important not only for prediction of prognosis but also for selection of patients for whom postoperative extensive adjuvant chemotherapy may be performed to advantage. Further analysis of a large number of patients with this method, possibly in a prospective fashion, is warranted to confirm our results. 

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REFERENCES
Prognostic Value of Genetically Diagnosed Lymph Node Micrometastasis in Non-Small Cell Lung Carcinoma Cases

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