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ORIGINAL PAPER

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Detection of disseminated lung cancer cells in regional lymph nodes by assay of CK_{19} reverse transcriptase polymerase chain reaction and its clinical significance[1]

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Abstract *Purpose:* To set up a molecular method (i.e. RT-PCR) that can be used to detect disseminated tumor cells (DTCs) in regional lymph nodes (LNs) in patients with lung cancer and to evaluate its clinical significance.*Methods*: Cytokeratin 19 (CK_{19}) was used as marker. Serial dilution study for LC-5 cells (a lung squamous cell line) was performed to detect sensitivity of the molecular protocol. Regional LNs ($n=261$) and primary lung cancer tissue ($n=40$) were obtained from 40 patients with lung cancer who underwent lobectomy or pneumonectomy. They were randomly categorized into two groups: group I (LN-based study, $n=20$) and group II (patient-based study, $n=20$). Each LN was halved. One half of a LN was subjected to histological examination (HE) and the other half was subjected to RT-PCR amplification of CK_{19} mRNA. The effect on survival was analyzed. The cumulative survival was calculated by the Kaplan–Meier method and compared by the log rank test. The Cox model analyzed the prognostic factors.*Results*: CK_{19} mRNA expressed in all tumor tissues as well as LC-5, PAa cells (a lung adenocarcinoma cell line), but not in normal control LNs. Serial dilution study for LC-5 cells demonstrated that CK_{19} mRNA was detectable at a concentration as low as 10 LC-5 cells in 1×10^7 LN cells. There was no significant difference between the detecting result of single LN and that of mixed LNs ($P>0.05$). In 18 of 40 patients, the metastasis in regional LNs was found by both HE and RT-PCR. Of 22 patients without pathologically involved nodes, six (27%) were found to express CK_{19} mRNA in regional LNs. According to the results of regional LNs in 40 patients by molecular assay, the presence of the CK_{19} product in LNs was related to tumor size ($\chi^2=5.76$, $P<0.025$) as well as cell differentiation of the tumor ($\chi^2=7.08$, $P<0.01$). Following a median observation time of 26 months (range, 4 to 60 months), patients with DTCs in nodes showed significant shorter disease-free survival duration than node-negative patients (log-rank test, $P=0.001$). The independence of this prognostic significance was demonstrated by a multivariate analysis (Cox regression model, $P=0.004$). The results diagnosed by HE had no significant effect on prognoses ($P=0.455$). *Conclusions*: Comparing with HE, RT-PCR can make more accurate assessment of metastatic status in LNs, which is helpful for screening the patients in whom the early subclinical metastasis exists and disclosing the intrinsic regulation of malignant metastasis. The presence of DTCs in LNs is an independent factor for prognosis. Molecular detection of DTCs in LNs is a supplement for current tumor staging in lung carcinoma.

Keywords Lung neoplasm · Lymphatic metastasis · Keratin · Polymerase chain reaction

Abbrevations RT-PCR: Reverse transcriptase-polymerase chain reaction · DTCs: Disseminated tumor cells · LNs: Lymph nodes · CK_{19}: Cytokeratin 19 · HE: Histological examination · p-status: The metastatic status of LNs by pathological examination · m-status: The metastatic status of LNs by molecular method · PAT: Postoperational adjuvant therapy · LM: Lymph nodal metastases · SCC: Squamous cell carcinoma · ADC: Adenocarcimoma · ASC: Adenosquamous carcinoma · Mod: Moderately differentiated

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Introduction

Distant metastasis is one of the main characteristic and chief cause of ineffective therapy and death in patients with solid tumor. Studying the mechanism of the procedure will greatly benefit the treatment of the disease. It has become one of the most important issues in cancer study to detect early and accurately the distant metastases. Initially, metastasis is looked on as a late event in the natural history of epithelial tumors. However, the unexpected poor prognosis of patients with apparently localized lung cancer indicated that micrometastasis occurs frequently before diagnosis of the primary tumor. It is due to the fact that the incidence of early metastasis of tumor cells is underestimated by current staging procedures. Accurate assessment of the metastatic status of lymph nodes (LNs) will show important clinical value. But disseminated tumor cells (DTCs) in regional LNs are often not detected by conventional histological examination (Pelkey et al. 1996). Clinical practice demonstrates that patients with regional LNs metastases are at a high risk for relapse and are generally considered to be the candidates for an adjuvant therapy. Nevertheless, not all patients without regional LNs metastases bear a good prognosis. Some of them die from local relapse or distant metastasis. It is generally accepted that 30–40% of them will develop distant metastasis within 5 years (Smith et al. 1995). This phenomenon indicates that the undetected micrometastasis, which exists preoperatively, is responsible for poor outcome after "radical resection".

This study aimed to set up a molecular protocol that could be used to detect DTCs in regional LNs in patients with resectable lung cancer to screen the subjects in whom the subclinical metastases existed early and to evaluate the clinical significance.

Materials and methods

Patients and follow-up

With informed consent, the LNs and corresponding tumor samples were collected from 40 consecutive patients with operable lung cancer who had been treated by lobectomy (36 cases) or pneumnectomy (4 cases) in combination with lymphadenectomy in the period from February to December 1998. The clinicopathologic parameters of all patients were shown in Table 1.There were no patients who had undergone neoadjuvant therapy before surgery.

After primary surgery, patients were reexamined every 3 months for 2 years and thereafter at 6-month intervals.The most recent follow-up examination had been performed at the end of 2003. The evaluations included physical examination, chest X-ray, bronchoscope, computed tomography, abdominal ultrasound and bone scan. For all patients, nine parameters including age, gender, pathological type, pN-status (the metastatic status of LNs by pathological examination), mN-status (the metastatic status of LNs by molecular method), T-extension, postoperational adjuvant therapy (PAT) and disease-free survival duration were analyzed.

Samples

Forty patients were categorized into two groups for collecting LNs with different methods. Group I: primary

Table 1 Clinical and pathological data of 40 patients and their correlation with lymph node metastasis

Items	Histological examination			RT-PCR		
	N	No. of LM	*P* value	*N*	No. of LM	*P* value
Age (years)			> 0.05			> 0.05
≤ 60	24	10		24	12	
> 60	16	8		16	12	
Gender			> 0.05			> 0.05
Male	30	13		30	18	
Female	10	5		10	6	
Smoking status			> 0.05			> 0.05
Smoker	25	11		25	13	
Nonsmoker	15	7		15	11	
Tumor type			> 0.05			> 0.05
Peripheral	26	11		26	15	
Central	14	7		14	9	
Tumor size (cm)			> 0.05			< 0.025
≤ 3.0	10	3		10	3	($\chi^2 = 5.76$)
> 3.0	30	15		30	21	
Histology			> 0.05			> 0.05
SCC	18	7		18	9	
ADC	12	6		12	8	
ASC	5	3		5	4	
Other	5	2		5	3	
Differentiation			> 0.05			< 0.01
Well	7	1		7	1	($\chi^2 = 7.08$)
Poor-mod	33	17		33	23	

lung cancer tissue ($n=20$) and regional LNs ($n=159$) were obtained from 20 patients who underwent operation. Each LN was cut into halves. One half of each LN was subjected to histological examination (HE). The other half was subjected to RT-PCR. Group II: Primary lung cancer tissue ($n=20$) and regional LNs ($n=20$) were obtained from 20 patients. Each LN was bisected. One half was fixed in 10% buffered formalin and subjected to HE, respectively. Other halves of each LN were pooled per patient and were subjected to RT-PCR subsequently. Regional LNs obtained from patients with operable tuberculosis and benign lung tumors were used as normal control (10 cases).

Primers

Oligonucleotide primers for β-actin and cytokeratin 19 (CK_{19}) were designed as reported by Nakajima et al. (1985) and Bader et al. (1988), respectively. The sequences of β-actin primer were as follows: Primer I (467–491) 5′-CCA AGG CCA ACC GCG AGA AGA TGA C-3′; Primer II(1589–1565) 5′-AGG GTA CAT GGT GGT GCC GCC AGA C-3′. The fragment sizes that were amplified from this primer were 589 bp which were used to rule out degraded RNA. Keratin 19 primers sequences were as follows: Primer I (757 – 786) 5′-TCC GCG ACT ACA GCC ACT ACT ACA CGA CC-3′; Primer II (1502–1473) 5′-CGC GAC TTG ATG TCC ATG AGC CGC TGG TAC-3′. The sizes for CK_{19} product were 745 bp. The products were different from those amplified from the corresponding genomic DNA templates because the primers were selected from two different exons with at least one intervening intron.

RNA extraction

Total cellular RNA was extracted using the acid guanidinium-phenol-chloroform technique. The detailed procedures have been described by Chomczynski and Sacch (1989). To remove any contaminating DNA, a sample of the extracted RNA preparation was treated with RNase-free DNase (Ge et al. 1998).

RT-PCR amplification

Total RNA (100 ng) was reverse transcribed in a 25 μl reaction mixture containing 50 mmol/l Tris–HCl, 50 mmol/l KCl, 10 mmol/l $MgCl_2$, deoxynucleotide triphosphates (500 μmol/l each), 25 pmol/l oligo deoxythymidine triphosphate and 200 U MMLV reverse transcriptase (GIBCO BRL, UK) for 1 h at 37°C and the reaction was terminated by heating at 95°C for 5 min. From this cDNA, 2 μl of solution was removed to be subsequently used for PCR amplification by adding each sample to 23 μl of a solution containing 10 mmol/l Tris–HCl, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, deoxynucleotide triphosphates (200 μmol/l each), downstream and upstream primers (20 pmol/l each) and 1 unit Taq polymerase (Sigma Co., USA). They were amplified in a thermal cycler (ABI Co., USA) for 40 cycles (45 s, 94°C; 45 s, 60°C; 60 s, 72°C) with an extra 5 min extension for the last cycle. After the amplification, the entire PCR reaction (25 μl) was stained with ethidium bromide and analyzed by 2% agarose gel electrophoresis.

Assessment of sensitivity

Sensitivity of RT-PCR assay was determined by performing serial dilutions of LC-5 cells (provided kindly from prof. Fan in this university) and preparing mixtures wit LN cells obtained from normal control LNs to represent 10^5, 10^4, 10^3, 10^2, 10^1, 1 LC-5 cell(s)/10^7 LN cells.

Statistical analysis

The results were analyzed with statistical software package SPSS 10.0 for windows. To assess any correlations between the presence of DTCs in LNs and clinicopathological data, Fisher's exact probability test or χ^2 test were used, with $P<0.05$ indicating significance. Survival curves were created by the Kaplan–Meier method, and the statistical significance of differences in disease-free survival was calculated by the log-rank test. Cox proportional hazards models were applied for multivariate analysis.

Results

Ck_{19} mRNA expressions in primary lung cancer tissue, normal LNs and LC-5, PAa cells

CK_{19} RT-PCR using total RNA from primary lung cancer tissue obtained from 40 patients included in this study demonstrated amplification on 745 bp fragment in all primary lung cancer tissue as well as LC-5 cells (a lung squamous cell line), PAa cells (a lung adenocarcinoma line). There was no evidence for detectable CK_{19} mRNA expression by the assay in any of the normal LNs (Fig. 1).

Sensitivity of the method

CK_{19} mRNA was detectable at a concentration as low as 10 LC-5 cells/10^7 LN cells. To study the detection sensitivity of 1 tumor cell/10^7 LN cells, we prepared four sample mixes at that dilution, isolated the RNA and performed RT-PCR. No CK_{19}mRNA was detectable in

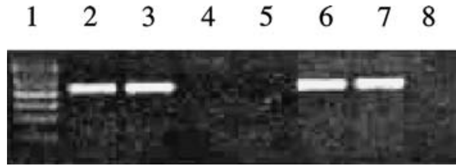


Fig. 1 Ck_{19} mRNA expression in primary lung cancer tissue, normal LNs and LC-5, PAa cells. *Lane 1* molecular marker; *lane 2, 3* lung cancer tissue; *lane 4, 5* normal lymph nodes; *lane 6, 7* LC-5 and PAa cells; *lane 8* control

LC-5 cell(s) 10^5 10^4 10^3 10^2 10^1 1 1 1 1

LN cells 10^7 10^7 10^7 10^7 10^7 10^7 10^7 10^7 10^7

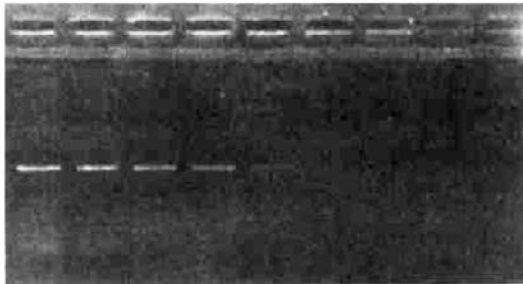


Fig. 2 Sensitivity of the method. The sensitivity of detection was determined by performing serial dilution of LC-5 cells and preparing mixture with normal LN cells. Total RNA was extracted from the mixture. Ck_{19} mRNA expression were studied on these RNA samples by RT-PCR. Results of agarose gel electrophoreses are shown

all four samples, indicating that these assays could not detect 1 tumor cell mixed into 10^7 LN cells (Fig 2).

Comparison of detectability of nodal metastases between RT-PCR and histological examination in LN-based study (Table 2 and Fig 3)

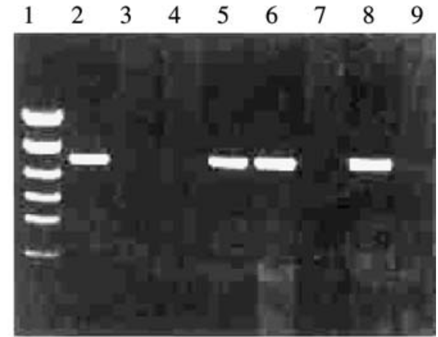
Comparison of detectability of nodal DTCs between LN-based study and patient-based study

Among 20 patients analyzed in group I, HE was done on each LN, which revealed LN metastasis in ten patients and these results were confirmed by RT-PCR. Of the other ten patients who were diagnosed to be devoid of metastasis by HE, four patients were found to be metastatic in regional LNs by the molecular assay. Of the 12 patients who were diagnosed to be devoid of metastasis by HE in group II ($n=20$), three patients were verified to be positive by CK_{19}RT-PCR.There was no significant difference between the detecting result of single LNs and that of pooled LNs ($P=0.27$, Fisher's exact probability test).

Table 2 Comparison between HE and CK_{19}RT-PCR detection of lung cancer metastases in regional lymph nodes

	Histological Examination	
	Positive	Negative
CK_{19} RT-PCR		
Positive	42	25
Negative	0	92

$\chi^2=25.00,\ P<0.005$



Genetic diagnosis — + + — + —

Histological diagnosis — + — — + —

Fig. 3 Detection of disseminated lung cancer cells in LNs by CK_{19} RT-PCR method (Case 20th). The number of involved lymph nodes was two by the histological examination (*lane 5* and *lane 8*) and three by the genetic diagnosis (*lane 5, 6* and *8*). RT-PCR amplification of CK_{19} mRNA for primary lung cancer tissue (*lane 2*) and normal control LN (*lane 3*) were used as positive and negative control, respectively

Clinicopathological correlation of m-status by RT-PCR

In 18 of 40 patients, the metastases in regional LNs were confirmed by both HE and RT-PCR. Of 22 patients uninvolved pathologically, six (27%) were found to express CK_{19} mRNA in regional nodes. The results obtained from two methods were significantly different (24/40 vs. 18/40, $\chi^2=6.0$, $P<0.025$). According to the results of nodal metastases in 40 patients by RT-PCR, the presence of the CK_{19} product in LNs was related to tumor size ($\chi^2=5.76$, $P<0.025$) and cell differentiation of the tumor ($\chi^2=7.08$, $P<0.01$) (Table 1). However, the results of routine HE did not show the above clinicopathological correlation ($P>0.05$).

Post-operative survival analysis

Of 40 patients analyzed, 38 were available for follow-up analysis. The remaining two patients were excluded because of lost follow-up ($n=1$) or cancer-unrelated death ($n=1$). The 5-year rate for follow-up and for survival was 95% and 32.5%, respectively. Following a median observation time of 26 months (range, 4 to 60), patients with nodal dissemination showed a significant shorter disease-free survival duration than node-negative

patients (univariate analysis, log-rank test, $P=0.001$, Table 3). The independence of this prognostic significance was demonstrated by a multivariate analysis (Cox regression model, $P=0.004$, Table 4). Figure 4 shows the survival curves of the patients with and without nodal DTCs.

Table 3 Univariate analysis of prognostic factors associated with postoperative disease-free survival duration in 40 patients with lung cancer

Variable	χ^2 value[a]	*P* value[a]	Stratification
Age	3.53	0.1714	< 40, 40–59, ≥60
Sex	1.64	0.2002	Male, female
PAT	0.18	0.6755	Yes, no
Grade	16.47	0.0003	Well, mod, poor
Type	10.34	0.0159	SCC, ADC, ASC, other
pN	5.83	0.0157	pN_0, $pN_{1\text{-}2}$
mN	15.90	0.0001	mN_0, $mN_{1\text{-}2}$
T extension	6.75	0.0805	T_1, T_2, T_3, T_4

Abbreviations: *PAT* postoperational adjuvant therapy, *SCC* squamous cell carcinoma; *ADC* adenocarcinoma, *ASC* adenosquamous carcinoma, *pN* the metastatic status of LNs by pathological examination, *mN* the metastatic status of LNs by molecular method

[a]By means the log-rank test

Table 4 Multivariate prognostic factors' analysis of the 40 patients by cox's proportional hazards regression model

Variable	B	SE	Wald	Sig.	Exp (B)	95.0% CI for Exp (B)	
						Lower	Upper
Grade	−1.222	0.577	6.450	0.040	0.269	0.203	0.365
Type	−0.346	0.768	2.296	0.513	0.724	0.519	0.953
pN	0.347	0.465	0.558	0.455	1.415	1.042	1.982
mN	−1.632	0.561	8.472	0.004	0.196	0.124	0.256

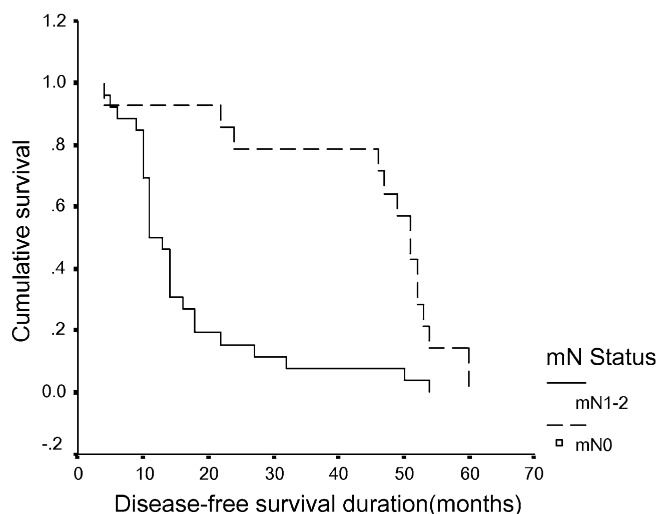


Fig. 4 Kaplan–Meier overall survivals plots for 40 patients with lung carcinoma according to the occult lymph nodes metastases

Discussion

Many patients with "localized" lung carcinoma die of postoperative distant metastases. It indicates early dissemination of cancer cells to other sites of the body such as peripheral blood, lymph nodes or bone marrow may be the intrinsic rule and an important biological characteristic of lung cancer and other solid tumor. Early screening of the subjects in whom the subclinical metastases exist helps for predicting recurrence , for accurately assessing prognosis and for tailoring postoperative adjuvant systemic therapy to raise postoperative 5-year survival rate. Currently, the DTCs from nonhematopoietic malignancies are usually neglected by conventional clinicopathological methods of stage. It is a meaningful task that all oncologists strive to set up more specific and sensitive methods to identify those patients in whom metastases exist early in peripheral blood, lymph nodes or bone marrow and so on.

RT-PCR technique for detecting DTCs is principally limited by lack of tumor-specific markers. Alternatively, to select a suitable "tissue-specific marker" is a hypothesis to detect DTCs with RT-PCR protocol. As a member of cytoskeleton in epithelial cells, CK_{19} expresses restrictively in epithelial tissues and corresponding tumor tissues derived therefrom. If CK_{19} transcript is detected in LN of patients with epithelial tumor, the presence of dissemination of cancer cells could be considered (Traweek et al. 1993). This study demonstrated that CK_{19} mRNA is limited in epithelial origin. PCR analysis of CK_{19} gene expression may be proved to be promising in the detection of epithelial tumor deposits in selected sites.

To conduct such a sensitive assay as RT-PCR with accuracy, special attention should be paid to the prevention of false-positive results. Interference of pseudogene is one of the main causes of the false-positive results in the detection of tumor dissemination by this molecular assay. We could find the influence of pseudogene through performing genomic DNA as well as non-reverse transcribed RNA control. By digesting the extracted RNA sample with RNase-free DNase, we could effectively eliminate the interference (Ge et al. 1998). Another important cause for the false-positive results is the contamination of LNs by cancer cells and normal epithelial cells. Thus special care was taken to prevent such a contamination when the LNs were sampled. In addition, contamination of carryover products from the previous PCR amplification seems to be a cause for the occurrence of false-positive results.

Nowadays, the lymph nodal metastatic status detected by conventional HE is still a chief basis for oncologists to formulate postoperative therapy regime. Because the metastatic status of single nodal section detected by conventional pathological examination is used to evaluate the metastatic information of the whole node, it is natural for the assay to underestimate the nodal metastasis (Pelkey et al. 1996). The detection

sensitivity studies using a serial dilution of LC-5 cell against control LN cells has revealed that it is a sensitive technique. The molecular assay is more sensitive than HE in the detection of patients with metastasis-positive LNs, indicating that it is an easy, practical and acceptable method.

In group I, where DTCs were analyzed on an LN basis using the 159 LNs from 20 patients, CK_{19} mRNA was found in 42 LN that were proved to be metastatic by HE. Of the other 117 LNs that were diagnosed as without metastases by HE, however, twenty-five LNs were found to express CK_{19} mRNA by RT-PCR, indicating that the later is more sensitive than HE. This result was consistent with that of Pimpec–Barthes (2005). Because it was cumbersome to prepare RNA from individual LNs obtained from patients for RT-PCR analysis on an LN basis, we attempted to study the issue on a patient basis by extracting RNA from a pooled sample of all the nodes obtained from a patient and subjecting the sample to RT-PCR analysis. In this study, three (25%) of the 12 patients with the histologically uninvolved LNs were found to be metastatic by RT-PCR, comparing with four (40%) of ten patients in LN basis study. There was no significant difference between the detecting result of single LN and that of mixed LNs ($P > 0.05$). Importantly, it can dramatically reduce the time and the cost of detection. When the results of metastases in regional LNs in patients with lung malignancy by RT-PCR were considered, the presence of CK_{19} product in LNs was associated with tumor size ($\chi^2 = 5.76$, $P < 0.025$) as well as grade ($\chi^2 = 7.08$, $P < 0.01$). Yet, the results of routine HE were not associated with any clinicopathological parameters ($P > 0.05$). The result is consistent with the previous study (Schoenfeld et al. 1996).

RT-PCR is the most recent technique to be applied in micrometastatic detection. The value of the assay should be assessed by clinical application, which can be determined only after a long-term follow-up of many patients. Currently, the studies on the clinical value of DTCs' detection in lung carcinoma focus chiefly on the results of immunohistochemical method (Wu et al. 2001; Zheng et al. 2002; Osaki et al. 2002, 2001; Gu et al. 2002; Ohta et al. 2001), where the results demonstrated that the occult metastases might have adverse effects on survival. Wu et al. (2001) studied 1438 regional LNs for occult micrometastases by IHC staining for cytokeratins in 103 pNo disease patients with peripheral lung adenocarcinoma of 2.0 cm or less in diameter. They found that the 5-year-survival rate (61.9%) of patients with micrometastases was significantly ($P = 0.0041$) lower than that of patients without micrometastases (86.3%). The study of Zheng et al. (2002) demonstrated that the survival of CK (+) and CK (-) patients were 32 months and 48 months, respectively ($P = 0.0178$). In contrast to above studies, some researchers insisted on that the presence of occult metastases had no influence on survival (Poncelet et al. 2001; Kawano et al. 2002).

With the mutant allele-specific amplification (MASA) method, Hashimoto et al. (2000) detected micrometastasis in regional lymph nodes from NSCLC patients with p53 and K-ras mutated tumors. They found that carcinoma-specific marker is of prognostic significance. No significant difference was observed by IHC; however, the fact that not all primary solid tumors express carcinoma-specific markers limits its clinical application. Currently, standard RT-PCR technique with tissue-specific markers were also utilized to detect DTCs in regional lymph nodes of patients with breast cancer (Masuda et al. 2000), oral cancer (Hamakawa et al. 2000), biliary tract carcinoma (Okami et al. 2000), esophageal cancer (Kassis et al. 1998; Luketich et al. 1998), colorectal cancer (Miyake et al. 2001; Bilchik et al. 2001) as well as melanoma (Palmieri et al. 2001). The results supported the concept that the molecular staging of lymph nodes is useful for prediction of clinical outcome and can provide a powerful and sensitive complement to routine histopathological analysis. Our study showed DTCs could be identified in regional lymph nodes determined to be free of tumor by hematoxylin and esoin staining. This result was consistent with that of the previous publications (Le Pimpec-Barthes et al. 2005; Salerno et al. 1998). Recently, Pimpec-Barthes et al. (2005) found that the two-year cancer-related death survival in non-small cell lung cancer patients with and without DTCs was significantly different. The prognostic value of molecularly diagnosed nodal metastases in lung cancer was also determined in present study. We can draw a conclusion that the metastatic status in regional nodes always be underestimated by conventional pathological examination. In contrast to HE, RT-PCR can make more accurate assessment of metastatic status in LNs.Recent studies showed that quantitative RT-PCR offers significant benefits over standard RT-PCR and identified node-negative patients at high risk for recurrence (Tokunaga et al. 2000; D'Cunha et al. 2002; Godfrey et al. 2001). Therefore this assay might be used to make comparisons of the possible differences in expression levels of the target gene in future. However, much further studies are needed to perform to determine its clinical value.

In the present study, of 22 patients uninvolved pathologically, six patients were up-staged according to the expression of CK19mRNA in regional nodes, indicating that the molecular evaluation of metastases in LNs is a supplement for current staging in lung carcinoma. There are still some defects in ongoing lung cancer staging system. One of the most important issues is molecular/cellular factors are not incorporated into the system. Therefore, it is difficult for oncologists to accurately comprehend the malignant dissemination in the body of host. Currently, data are sorely needed to assess the prognostic value of molecular methods and whether they yield improved staging information compared to conventional methods. In future, the prognostic information gained from dissemination cancer cells will improve as the understanding of molecular and immunological mechanisms involved in progressive malignant

behavior expands. Oncologists are struggling for tailoring "individualized" and "reasonable" treatment to every patients suffering from lung cancer, based on precise "molecular stage".

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