Validation of Intra-operative Detection of Paratracheal Lymph Node Metastasis Using Real-time RT–PCR Targeting Esophageal Squamous Cell Carcinoma

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**Background:** We previously reported that there was a significant correlation between paratracheal lymph node (LN) metastasis and cervical LN metastasis in thoracic esophageal squamous cell carcinoma (ESCC) patients. The purpose of this study was to establish an intra-operative detection method of LN micrometastasis (MM) of ESCC using hematoxylin–eosin (HE) staining, immunohistochemistry (IHC) and real-time RT–PCR with a Light Cycler technique, and to evaluate which method, or combination of methods, is most suitable for intra-operative detection of paratracheal LN MM.

**Methods:** Under informed consent, we obtained 33 dissected paratracheal LN samples from 22 operative patients with ESCC. Afterwards, one LN was separated into three parts by a sharp razor, and each part was checked for metastasis by HE staining, IHC with anti-cytokeratin antibody and real-time RT–PCR for SCC mRNA with a Light Cycler.

**Results:** It took 3 h for detection by real-time RT–PCR, while it took 2 h by IHC. The detection rates of MM by HE staining, IHC and real-time RT–PCR were 50.0, 33.3 and 83.3%, respectively. However, there was a case of false negative detection that was not detected by IHC or PCR.

**Conclusion:** The real-time RT–PCR method was useful for intra-operative detection of paratracheal LN metastasis. However, combination analysis of HE staining, IHC and real-time RT–PCR may be desirable because there was a case of false negative detection by IHC and real-time RT–PCR.

**Key words:** real-time RT–PCR – esophageal squamous cell carcinoma – lymph node micrometastasis – SCC

**INTRODUCTION**

In esophageal squamous cell carcinoma (ESCC), lymph node (LN) metastasis is a significant prognostic factor. Therefore, regional LN dissection is important for the best prognosis of the patient. Some authors have suggested that cervical LN metastases from thoracic ESCC should be regarded as part of the M component of the fifth edition of the TNM classification of UICC 1997, however, other authors have suggested that cervical LN metastasis should be included in the N component of this classification, and that radical esophagectomy with three fields dissection may help to improve the survival of patients with thoracic ESCC (1–5). However, there was no definitive indication for cervical LN dissection. A current report has shown, using immunohistochemistry (IHC), that there is a significant relationship between the micrometastasis (MM) of cervical LN and paratracheal LN in the thoracic ESCC (6). Some authors have shown that IHC can detect LN MM more frequently than hematoxylin–eosin (HE) staining and have suggested that IHC might detect the MM more sensitively and specifically than HE staining (7–9). Thus, we have intra-operatively diagnosed by means of IHC as well as by HE staining. On the other hand, PCR has been recognized as an improved detection method for MM. The PCR method has been used...
widely to detect MM in patients with a variety of malignant tumors, such as breast carcinoma (10,11), hepatocellular carcinoma (12) and gastrointestinal carcinomas (13–18). Previously, we reported that RT-nested PCR for SCC mRNA was useful for the detection of MM (19). Unfortunately, this method could not be used for the intra-operative diagnosis of LN metastasis due to the large amount of time required. Recently, a real-time RT–PCR method using the Light Cycler technique has been developed. This method is much quicker. Therefore, we applied it as an intra-operative diagnostic method for LN MM, and evaluated it in a comparative study of these three methods.

PATIENTS AND METHODS

PATIENTS AND LN SAMPLES

Under informed consent, 33 paratracheal LN samples were obtained from 22 intra-operative patients with ESCC. Of the 22 patients with ESCC, five had pT1 tumors, four had pT2 tumors, 11 had pT3 tumors and two had pT4 tumors. Patients were curatively operated on between January 1999 and June 2001 in our department by the same surgeon (M.I.). The standard surgical method used has been previously described (20).

To summarize, esophagectomy with systemic LN dissection was performed using a right thoracotomy, and reconstruction was carried out using an esophagogastric anastomosis with a gastric tube through the retro-sternal or intra-thoracic route. Our esophagectomy with systemic LN dissection method has been previously described (20).

Recently, a real-time RT–PCR method using the Light Cycler technique has been developed. This method is much quicker. Therefore, we applied it as an intra-operative diagnostic method for LN MM, and evaluated it in a comparative study of these three methods.

CELL LINE AND NORMAL PERIPHERAL BLOOD MONONUCLEOCYTES

The ESCC cell line, KYSE510, which was established from an ESCC in our laboratory (21), was used in this study. This cell line produces SCC antigen in its culture medium. Normal peripheral blood mononucleocytes (PBMN) were harvested from the blood of healthy volunteers using Lymphoprep (Nycomed Pharmacia, Oslo, Norway) gradient centrifugation according to the manufacturer’s instructions.

SERIAL DILUTION OF CARCINOMA CELLS

Serial dilution of KYSE 510 cells with normal PBMN was carried out to confirm the sensitivity of the real-time RT–PCR assay. Known numbers of KYSE510 cells were added to 1 × 10⁸ normal PBMN, to give a ratio ranging from 1:10⁶ to 1:10⁸, and then the total RNA was extracted. The procedure used has been previously described (19).

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total RNA was extracted from the cell lines, PBMN or LN using the acid guanidinium thiocyanate–phenol–chloroform extraction method. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and subjected to reverse transcription. Reverse transcription was performed using a First Strand cDNA Synthesis kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

OLIGONUCLEOTIDE PRIMERS

Primers were designed from the published sequence of human SCC (SCC A1: DDBJ/EMBL/GenBank; HSU19556) antigen cDNA. The primer sequences for SCC primers were as follows: 5′-GCAAATGCTCCAGAGAACAG-3′ and 5′-CGAG-CGAATAAGAAAGATG-3′. The primers produce a PCR fragment of 261 bp. The quality of RNA and cDNA synthesis was ascertained by amplification of the glyceraldehyde phosphate dehydrogenase (GAPDH) gene as the internal control. The primer sequences for the GAPDH primers were as follows: 5′-TGTTATCTTGGAAGGACTCATGAC-3′ and 5′-ATGCCAGTGCTTTCCGAGC-3′. The primers produced a PCR fragment of 189 bp.

REAL-TIME RT–PCR: LIGHT CYCLER TECHNIQUE

Real-time RT–PCR was performed with a Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) in Light Cycler capillaries using a commercially available master mix containing Taq DNA polymerase and SYBR-Green I deoxyribonucleoside triphosphates (Light Cycler DNA master SYBR-Green I, Roche Molecular Biochemicals). After the addition of primers (final concentration: 0.25 pM), MgCl₂ (4 mM) and template DNA to the master mix, 45 cycles of denaturation (94°C for 1 s), annealing (58°C for 10 s) and extension (72°C for 10 s) were performed. After the completion of PCR amplification, a melting curve analysis was performed.

IMMUNOHISTOCHEMISTRY

For IHC, segments of LN were embedded in a Tissue-Tek OCT compound. They were then sectioned into six slices at a thickness of 5 µm with a cryostat, mounted on glass slides, and air-dried at room temperature for 5 min. Endogenous peroxidase activity was inactivated by the incubation of the specimens with peroxidase containing 3% hydrogen peroxide for 10 min. After the specimens had been rinsed with phosphate buffered saline (TBS), they were immersed in peroxidase containing 3% hydrogen peroxide for 5 min to block any non-specific reaction. The solutions were then reacted with anti-cytookeratin antibodies (DAKO EPOS AntiCytokeratin/HRP) as the primary antibodies for 30 min at 37°C and reined in TBS. The specimens were then reacted with 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Nacalai Tesque, Kyoto, Japan) diluted in 0.1 M Tris buffer, pH 7.4, for 3 min at room temperature and were stained by hematoxylin.
Figure 1. Serial dilution of ESCC cells. (A) 10 cells/1 × 10^4 PBMN, (B) 10 cells/1 × 10^5 PBMN, (C) 10 cells/1 × 10^6 PBMN, (D) 10 cells/1 × 10^7 PBMN. The vertical axis represents the degree of amplification by SYBR-Green fluorescence and the horizontal axis represents the number of amplification cycles.

Figure 2. The typical amplification and melting curves of real-time RT–PCR for SCC mRNA. (A) Typical amplification curves. Curve (1) is for GAPDH and curve (2) is for SCC. (B) Typical melting curve. The figure shows a sharp peak with a melting temperature of SCC (Tm A) of ~87°C, and a melting temperature of GAPDH (Tm B) of ~89°C.
RESULTS

Sensitivity and Specificity

Serial dilution experiments showed that our real-time RT–PCR assay detected 10 KYSE510 cells per 10^7 of PBMN (Fig. 1). All five cell lines and seven tumor specimens of SCC showed positive amplification using the Light Cycler technique, and they showed a specific amplification curve within 40 cycles. We did not detect mRNA expression of SCC in 14 of the control LNs (Table 1) even when each PCR cycle was increased to 55 cycles. A non-specific amplification curve from the RNA templates without RT (negative control) was not obtained after 55 cycles. However, over 55 cycles a non-specific amplification curve from the RNA template without RT in a sample of normal LN and water was obtained (data not shown). Therefore, the amplification cycle was determined at 45 cycles in order to exclude the false positive result.

Real-time RT–PCR and IHC

The typical amplification and melting curves of real-time RT–PCR for SCC mRNA are shown in Fig. 2A and B. Figure 2B shows a sharp peak with a melting temperature (Tm) of ~87°C. The process of real-time RT–PCR is shown in Fig. 3, and the mean duration of the real-time RT–PCR using the Light Cycler technique among 22 cases was 3.11 h on average, ranging from 2.5 to 3.5 h with a standard deviation (SD) of 0.333. However, the mean duration of IHC among 22 cases was 2.04 h on average, ranging from 1.7 to 2.5 h with SD of 0.209 (Table 2), and was shorter than the mean duration of real-time RT–PCR (Fig. 3). In this study, the use of three intra-operative detection methods did not prolong the operation time, because the results of MM by each method were given to the surgeons at the time of the making of the gastric tube. Figure 4 shows a typical case of the LN metastasis by IHC using anti-cytokeratin antibodies.

Comparison between HE Staining, IHC and Real-time RT–PCR

The number of evaluated LNs, location and histology of primary tumor are shown in Table 2. We evaluated 1.77 LN per patient on average by HE staining and IHC, and 1.55 LN per patient on average by real-time RT–PCR. Positive rates from HE staining, IHC and real-time RT–PCR were 13.6% (3/22), 9.1% (2/22) and 22.7% (5/22), respectively (Tables 3 and 4).

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<th>Table 1. The positive rates of the expressions of SCC mRNA in esophageal cancer cell lines, primary tumor tissues and normal lymph nodes</th>
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<td>Esophageal SCC cell lines</td>
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<td>SCC tumor specimen</td>
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<td>Normal LNs</td>
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Figure 3. The process of operation and intra-operative detection methods. It took ~120 min to dissect LN and to complete RT, and ~60 min to complete real-time RT–PCR. It took ~45 min to dissect LN and make frozen sections, and required 75 min to complete IHC. Intra-operative detection of HE staining required 30 min.

Figure 4. Positive detection with intra-operative IHC using anti-cytokeratin antibodies of paratracheal LN. The red arrowheads indicate MM in paratracheal LN.
The positive rates of MM by the examination of a sample set containing two LNs or more was 40.0%, whereas the rate by the examination of a sample containing only one LN was 16.7%. Of the six patients with metastasis detected by at least one of the three methods, the detection rates of the metastasis by HE, IHC and real-time RT–PCR were 50% (3 of 6), 33% (2 of 6) and 83% (5 of 6), respectively (Tables 3 and 5). As a result, two cases only gave a positive result using real-time RT–PCR. We found one case of positive metastasis of paratracheal LN in an experiment using real-time RT–PCR and IHC staining, and in another experiment using real-time RT–PCR and HE staining, respectively (Table 3). However, there was one case where we found negative metastasis using real-time RT–PCR and IHC, but positive metastasis by HE staining (Table 3).

DISCUSSION

In this study we established an intra-operative detection method of LN MM of ESCC using real-time RT–PCR for SCC mRNA with the Light Cycler technique. The conventional method for intra-operative detection of LN metastasis in ESCC is frozen sectioning and HE staining. However, there is the possibility of overlooking MM in the examination of frozen HE sections. Recently, molecular biological methods using PCR analysis for the detection of LN MM have been developed. Some authors have reported these genetic diagnosis assays to be clinically useful for detecting LN MM in a variety of cancers (22–28). However, there were still several problems regarding RT–PCR. First, RT–PCR was time-consuming and relatively laborious. Second, the result of PCR assays could not exclude false positives. However, recent advances in PCR technology have allowed a significant reduction in the time required for amplification and detection of specific mRNA. The Light Cycler technique can also quantify cancer-specific mRNA with real-time monitoring of PCR products. With this technique we developed a rapid assay for detection of LN MM, and were able to reduce the frequency of false positive results, because we did not detect mRNA expression of the SCC in the control lymph nodes (Table 1).

Our reports indicate that real-time RT–PCR had the highest detection rates and positive rates of the three methods examined. However, if the MM detected by at least one of these
In conclusion, the real-time RT–PCR method was useful for intra-operative detection of paratracheal LN metastasis. However, a combination analysis of HE staining, IHC and real-time RT–PCR may be desirable because there was a false negative detection for all three methods. Based on the results of this study and intra-operative examination, we are now performing cervical LN dissection.

### References


