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DISEASES OF THE ESOPHAGUS

Original article

Clinical study on lymph node metastatic recurrence in patients with N0 esophageal squamous cell cancer

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SUMMARY. **Esophageal squamous cell cancer (ESCC) is one of the most common malignancies treated by thoracic surgeons. It is aggressive and generally associated with a poor prognosis. One of the most important prognostic factors is the presence of the lymph node metastasis (LNM). The purpose of the present study was to investigate the risk factor with lymph node metastatic recurrence in patients with N0 esophageal squamous cancer after Ivor Lewis esophagectomy based on the detection of mucin1 (MUC1) mRNA. The subjects were 82 patients with pN0 ESCC who underwent Ivor Lewis esophagectomy with two-field lymph node dissection from January 2001 to January 2005. All lymph nodes (501 stations) obtained from these patients were reevaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) to detect MUC1mRNA. The diagnosis of lymph node micrometastasis (LNMM) was based on the detection of MUC1 mRNA. The Kaplan–Meier method was used to calculate the survival rate and lymph nodal metastatic rate. Log-rank test was performed to compare the recurrence rate, and Cox regression multivariate analysis was performed to determine independent prognostic factors. The overall 3-year survival rates of 82 patients were 78.0%, and the first recurrence exhibiting lymph nodal metastasis was recognized in 37 patients (45.1%) in the first 3 years after operation. Lymph node metastatic rate in patients in the first 3 years after operation was significantly associated with the T status ($P < 0.05$). MUC1 mRNA was identified in at least one lymph node station from 23 (28.1%) patients. Also, lymph node metastatic rate of the patients with LNMM was significantly higher than that of the patients without LNMM ($P < 0.01$).The results of multivariate analysis confirmed that LNMM and T status in patients with N0 ESCC were independent risk factors for 3-year lymph node metastatic recurrence after Ivor Lewis esophagectomy. Adjunctive therapy might be beneficial in controlling the locoregional recurrence and elevated healing rates for certain patients.**

KEY WORDS: **esophageal squamous cell cancer, lymph node metastasis, mucin1.**

INTRODUCTION

Esophageal cancer is one of the most common intrathoracic malignancies in China. Up to now, radical resection has remained the most effective means of cure for esophageal cancer. However, the long-term outcome after routine esophagectomy is far from satisfactory with 5-year survival rates at 20–30%.[1,2] The 5-year survival rate after complete resection of pathologic N0 (pN0) disease can be as high as 70%, but some patients still have a poor prognosis and suffer tumor relapse.[3,4] One of the most important prognostic factors is the presence of the lymph node metastasis (LNM). Some investigators suggest that the early postoperative tumor relapse in patients with stage I–II disease is correlated with the micrometastases to lymph nodes not detected by conventional histopathologic examination at the time of surgical resection.[5,6] Therefore, investigation of the genes and corresponding gene products involved in LNM and lymph node micrometastasis (LNMM) has been an important research field.

Mucin 1 (MUC1), a cell surface glycoprotein, is a specific marker for epithelial tissues. MUC1 is highly and frequently expressed in epithelial tissues, and its expression is preserved in malignancies originating from epithelial tissues.[7] However, MUC1 does not appear to be expressed in normal lymph nodes. Thus,

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if MUC1 is detected in regional lymph nodes from patients, this could indicate LNM.[8]

This study was therefore designed to investigate the correlation between clinical characteristics and lymph node metastatic recurrence in patients with N0 ESCC after Ivor Lewis esophagectomy based on the detection of MUC1mRNA.

PATIENTS AND METHODS

Patients

A total of 82 patients with mid-thoracic esophageal cancer who successfully underwent complete resection of esophageal cancer without LNM confirmed by histologic examination were enrolled in this study. All patients underwent subtotal esophagectomy with regional lymph node dissection[9] via a right thoracoabdominal approach (Ivor Lewis esophagectomy[10]) at the Department of Thoracic Surgery, Provincial Hospital Affiliated to Shandong University between January 2001 and January 2005. Subsequent reconstruction was performed by an esophagogastric anastomosis with a gastric tube. None of the patients had received preoperative and postoperative adjuvant therapy before tumor relapse. Histologic examination confirmed that there was no residual tumor in any of the patients, and all of the cancer tissues studied were squamous cell carcinomas.

The study group was composed of 64 men and 18 women ranging in age from 43 years to 74 years on the basis of the TNM classification of the International Union against Cancer (UICC) in 1997. Patients were routinely examined during the first 3 years. The position and time of recurrent disease were recorded completely. The clinical characteristics of the 82 patients are listed in Table 1.

Samples

A total of 501 regional lymph node stations were removed. All the experimental specimens were cut in half longitudinally and handled with a fresh set of clean instruments to prevent cross-contamination of MUC1 mRNA. Each lymph node was labeled; one half was selected randomly to be immersed in 10% neutral buffered formalin immediately and it was then embedded in a paraffin block. Serial sections were made from the blocks obtained from each case at 3 μm intervals. It was stained by hematoxylin-eosin (HE) and the other half was wrapped quickly in foil and snap frozen in liquid nitrogen for 1 minute and kept at –80°C until RNA extraction. Ten paraesophageal lymph nodes from five patients with benign esophageal diseases during the same period were collected as negative controls. Ten lymph nodes from the five patients with ESCC proved to be metastatic by histopathology (pN1) were used as positive controls. The methods of collecting specimens were the same: RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR).

Total cell RNA was extracted from each specimen with Trizol reagent according to the protocol provided by the manufacturer. Then total RNA of each

Table 1 Clinical characteristics of the 82 patients with mid-thoracic esophageal cancer

Clinical characteristics	Patients	Lymph node metastasis Patients (rate %)	*P**	3-Year survival (%)	*P**
Gender			0.291		0.229
Male	64	31 (48.4)		75.0	
Female	18	6 (33.3)		88.9	
Age, years			0.276		0.756
≥50	66	28 (42.4)		78.8	
<50	16	9 (56.2)		75.0	
T status			0.028		0.515
T1	4	0 (0.0)		100	
T2	25	7 (28.0)		80.0	
T3	53	30 (56.6)		75.5	
Differentiation			0.136		0.129
Well	13	3 (23.1)		100	
Moderately	58	30 (51.7)		74.1	
Poorly	11	4 (36.4)		72.7	
Tumor length, cm			0.088		0.069
>5	9	4 (44.4)		66.7	
3–5	58	30 (51.7)		74.1	
<3	15	3 (20.0)		100	
Weight loss, kg			0.868		0.449
Positive	9	4 (44.4)		88.9	
Negative	73	33 (45.2)		76.7	
Mucin1 mRNA expression			0.000		0.000
Positive	23	17 (73.9)		47.8	
Negative	59	20 (33.9)		89.8	

*Log-rank test.

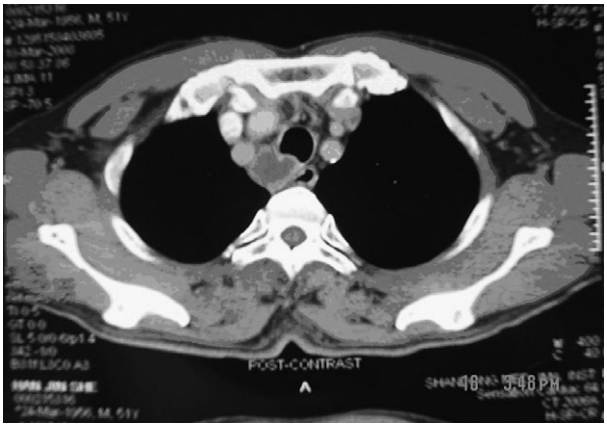

Fig. 1 Postoperative supraclavicular lymph node metastasis by computed tomographic scan.

specimen was reverse-transcribed into cDNA with an RT-PCR kit also as described by the manufacturer. Primers were designed according to previous reports[11] and synthesized by Takara Shuzo Co., Ltd. (Dalian, China), as follows: P1, 5′-CGTCGTGGACATTG ATGGTACC-3′, P2, 3′-GGTACCTCCTCT CACC TCCTCCAA-5′. Using these primers, a 287-bp fragment of cDNA was amplified and β-actin primers were as follows: P1, 5′-CACTGTGTTGGCGTA CAGGT-3′, P2, 3′-TCATCACCATTGGC AAT GAG-5′. Using these primers, a 154-bp fragment of cDNA was produced.

The PCR was performed and 30 cycles of amplification were performed at 94°C for 2 minutes, then at 50°C for 1 minute, then at 72°C for 1.5 minutes. The PCR product was evaluated using a standard 2% agarose gel electrophoresis technique with ethidium bromide staining, and it was analyzed by a figure gel image analytical system. Visualization of target bands of the 287-bp fragment demonstrated the existence of MUC1 mRNA. A 287-bp fragment of cDNA was amplified with the selected primers. The positive products were purified and the nucleotide sequence of the products was sequenced by Takara Shuzo Co., Ltd.

Follow-up

The patients were routinely examined every 3 to 6 months during the first 3 years and every 6 months or annually thereafter. During each follow-up visit, the patient underwent a thorough physical examination, chest roentgenography, ultrasonography of the neck and abdomen, chest computed tomography (CT), and endoscopic examination. Some patients even underwent positron emission tomography combined with CT examination (Figs. 1 and 2). Lymph node recurrence was clinically diagnosed when finding enlarged cervical-supraclavicular, mediastinal, or celiac lymph node compared with preoperative image, and some patients obtained histological confirmation by cervical-supraclavicular lymph node biopsy.

Statistical methods

Follow-up was complete for all the 82 patients. The time and site of postoperative lymph node recurrence was recorded. All statistic analyses were performed with SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). The Kaplan–Meier method was used to calculate the survival rate and lymph nodal metastatic rate. Log-rank test was performed to compare the recurrence difference. Cox regression multivariate analysis was performed to judge independent prognostic factors. Differences were considered significant when the *P*-value was less than 0.05.

RESULTS

Correlation between LNM and prognosis

Follow-up data were available for all the patients. According to the survival analysis, the overall 3-year survival rate of 82 patients was 78.0% (Fig. 3) and the first recurrence exhibiting lymph nodal metastasis was recognized in 37 patients (45.1%) in the first 3 years after operation (Fig. 4). The patients had first recurrence in cervical, supraclavicular, mediastinal, and celiac lymph nodes and five patients developed simultaneous lymph nodal metastasis and distant recurrence.

Correlations between LNM and clinical characteristics

Lymph node metastatic rate in patients in the first 3 years after operation was significantly associated with the T status at 0.0% in T1 cases, 28.0% in T2 cases, and 56.6% in T3 cases ($P < 0.05$). However,

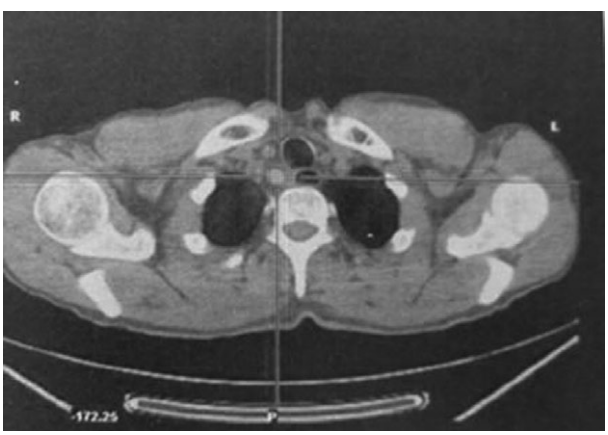

Fig. 2 Postoperative supraclavicular lymph node metastasis by positron emission tomography combined with computed tomography.

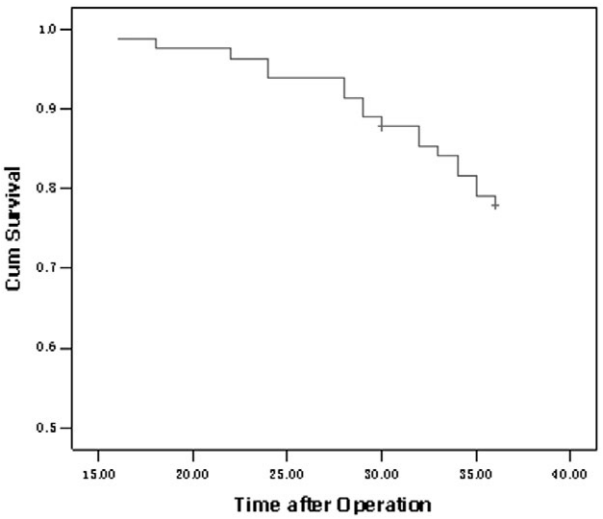

Fig. 3 **A** Kaplan–Meier analysis of the overall survival after operation.

no statistically significant correlations with gender, age, weight loss, tumor length, and histological differentiation were demonstrated for LNM ($P > 0.05$).

A total of 501 regional lymph node stations were examined. None of the lymph nodes were proved to be metastatic by HE. The sensitivity of MUC1 mRNA detection in diagnosing LNMM was calculated according to the results of RT-PCR assay. MUC1 mRNA was identified in at least one lymph node station from 23 patients (Fig. 5) and diagnostic sensitivity was 28.1% (23/82). In nine of the 10 positive-control lymph node stations from five patients, MUC1 mRNA was detected by RT-PCR with a specificity of 90% (9/10). The nucleotide sequence of MUC1 mRNA-positive products confirmed that the 287 bp fragment of cDNA was 100%

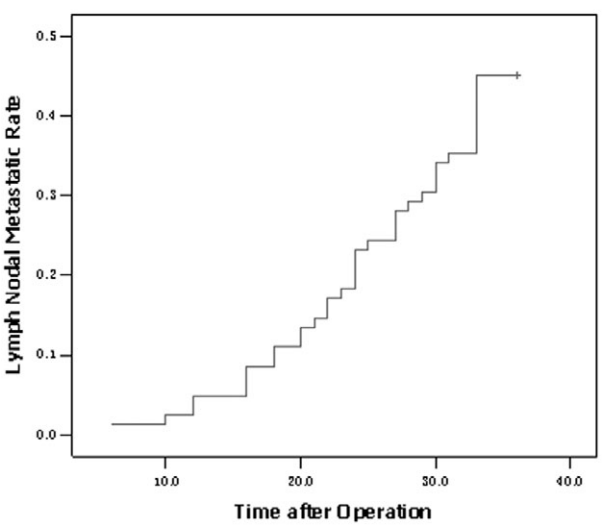

Fig. 4 **A** Kaplan–Meier analysis of lymph nodal metastatic rate after operation.

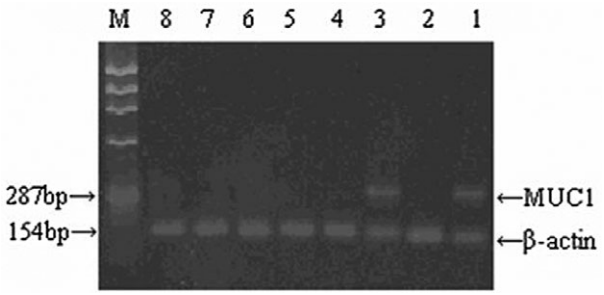

Fig. 5 Expression of Mucin1 mRNA detected by polymerase chain reaction (PCR). Lane 1: positive control; lane 2: negative control; lanes 3–8: regional lymph nodes (lane 3: MUC1 mRNA positive). M, molecular marker (bp).

homologous to the human MUC1 gene and none of the 10 negative-control lymph node stations had detectable MUC1 mRNA. Thus, LNMM could be confirmed on the basis of the detection of MUC1 mRNA.

Of the 23 patients with LNMM analyzed, 17 (73.9%) showed LNM in the first 3 years after operation. Of the 59 patients without LNMM analyzed, 20 (33.9%) showed LNM in the first 3 years after operation. Lymph node metastatic rate of the patients with LNMM was significantly higher than that of the patients without LNMM ($P < 0.01$) (Fig. 6).

Cox regression multivariate analysis was performed to analyze clinical characteristics data of all the patients (Table 1) and is depicted in Table 2. The results showed LNMM and T status in patients with N0 esophageal cancer were independent relevant factors for 3-year lymph node metastatic recurrence after Ivor Lewis esophagectomy.

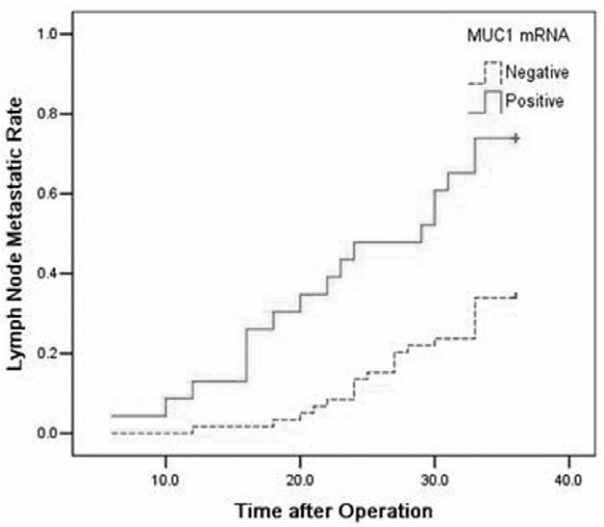

Fig. 6 Kaplan–Meier analysis of lymph nodal metastatic rate after operation in patients with positive and negative expression of MUC1 mRNA, respectively.

Table 2 Results of Cox regression multivariate survival analysis

	B	SE	Wald	*P*	HR	95.0% CI for Exp(B)
Age	–0.406	0.419	0.939	0.333	0.666	0.293–1.515
Gender	0.556	0.451	1.521	0.217	1.743	0.721–4.216
T status	0.883	0.422	4.387	0.036	2.419	1.058–5.530
MUC1 mRNA	1.248	0.357	12.212	0.000	3.482	1.730–7.011
Tumor length	4.691	75.255	0.004	0.950	108.932	0.000–1.242E + 066
Differentiation	–4.516	75.255	0.004	0.952	0.011	0.000–1.246E + 062
Weight loss	–0.381	0.541	0.495	0.482	0.683	0.237–1.974

B, regression coefficient; CI, confidence interval; HR, hazard ratio; SE, standard error; Wald, Wald value.

DISCUSSION

Esophageal cancer is one of the most common malignancies treated by thoracic surgeons, and most patients have an advanced stage of disease at the time of definitive diagnosis. It is aggressive and generally associated with poor prognosis.[12,13] Although the focus of pN0 disease is relatively local and lymph nodes are histologically negative, tumor relapse sometimes occurs within the early period after operation. The frequency of cervical, mediastinal, and celiac lymph node metastases in esophageal cancer is often high. Raja reported that the incidence of relapse after complete resection of pN0 disease could reach 30–50%.[14] However, after complete resection by a three-field lymph node dissection that Japanese surgeons had advocated, lymph node recurrence occurred in 11% patients with mid-thoracic esophageal squamous cell carcinoma.[15] Therefore, to control locoregional lymph node recurrence is one of the key factors to improve the overall survival of mid-thoracic esophageal cancer. We performed this study to investigate the risk factor with lymph node metastatic recurrence in patients with N0 ESCC after Ivor Lewis esophagectomy. The detection of such early tumor dissemination might be a promising approach to identify the specific patients who might benefit from adjuvant therapy.

The standard technique to evaluate LNM is histopathologic analysis of one or a few sections stained by HE and immunohistochemistry (IHC) assay from each lymph node; however, it has been reported that about 20% of LNMM cannot be detected by this method.[16] The term LNMM refers to a small metastatic lesion defined as a cluster of five or fewer tumor cells in a lymph node, which is not detected with conventional histological examination. Thus, LNMM results in underestimation of TNM staging for patients with esophageal cancer. However, recent efforts have been made to detect micrometastasis in lymph nodes at the molecular level using RT-PCR, which is more sensitive than the HE and IHC assay and serial sections.[17]

The MUC1 gene codes a core protein of polymorphic epithelium mucin, one of the specific markers for epithelial tissues.[7] MUC1 does not appear to be expressed in normal lymph nodes. LNMM could be diagnosed by the detection of MUC1 in regional lymph nodes of patients with esophageal cancer.[8] Salerno[18] used the RT-PCR assay for MUC1 mRNA to detect occult micrometastasis in the regional lymph nodes of patients with non-small cell lung cancer (NSCLC). Their results demonstrated that the RT-PCR assay was sensitive enough to detect one MUC1-positive NSCLC cell in 1×10^7 MUC1-negative cells.

The sensitivity of MUC1 mRNA detection in diagnosing LNMM was calculated according to the results of RT-PCR assay. MUC1 mRNA was identified in at least one lymph node station from 23 patients. In nine of the 10 positive-control lymph node stations from five patients, MUC1 mRNA was detected by RT-PCR with a specificity of 90% (9/10) and none of the 10 negative-control lymph node stations had detectable MUC1 mRNA. Thus, LNMM could be confirmed on the basis of the detection of MUC1 mRNA. The one of the 10 positive-control lymph nodes lacked detectable MUC1 mRNA, which probably indicated that there were no malignant cells in the half for RT-PCR assay, whereas the other half for histopathologic examination was invaded by malignant cells.

Owing to the higher sensitivity of the RT-PCR assay, we took multiple precautions to prevent false positive results in the current study. During specimen collection, we used a new, clean instrument to obtain and bisect each lymph node in an attempt to prevent cross-contamination between MUC1 mRNA-positive tissue and MUC1 mRNA-negative tissue. We prepared RNA in a clean manner to protect against ribonuclease activity. Moreover, the positive products of PCR were purified and sequenced, and the results confirmed that the 287 bp fragment of cDNA is 100% homologous to the human MUC1 gene. Because all of these precautions were taken with each specimen, it is likely that our results represent true findings.

Our previous study showed that 5-year survival rate of the patients of esophageal cancer with LNMM was lower than that of the patients without LNMM.[11] The findings of our study showed that 28.1% of

patients with esophageal cancer had LNMM. The first recurrence exhibiting lymph nodal metastasis was recognized in 37 patients (45.1%) in the first 3 years after operation and lymph node metastatic rate of the patients with LNMM was significantly higher than that of the patients without LNMM. Our results provide evidence that MUC1 mRNA expression (LNMM) was significantly associated with lymph node metastatic recurrence in pN0 esophageal cancer patients after surgery.

Although previous report[19] has shown that T status has a positive correlation with the risk of lymphatic metastasis in esophageal cancer, a significant correlation between T status and lymph node metastatic recurrence is not always found. These findings can be explained by difference in analytic method. Even in using the same analytic method, the result may differ depending on the selected site for assessment. In this study, we investigated the correlation between clinical characteristics and lymph node metastatic recurrence in patients with N0 ESCC after Ivor Lewis esophagectomy. The results showed that lymph node metastatic rate in patients in the first 3 years after operation was significantly associated with the T status and no statistically significant correlations with gender, age, weight loss, tumor length, and histological differentiation were demonstrated for LNM.

According to the survival analysis, the overall 3-year survival rate of 82 patients was 78.0%. Lymph node metastatic rate in patients in the first 3 years after operation was significantly associated with LNMM and T status in patients with N0 esophageal cancer. In our study, all of the patients successfully underwent esophagectomy with regional lymph node dissection. The tumor did not invade other organs, and both edges of resection were confirmed to be free of residual cancer cells by routine histological examination, ensuring complete resection. Furthermore, none of the patients had undergone preoperative and postoperative adjuvant therapy before tumor relapse. As a result, the comparability was increased and statistical bias was decreased, making the results of this study more objective. To eliminate the impact of mixed factors correlated with prognosis on statistical analysis, the Cox regression multivariate analysis was performed to determine the independent prognostic factors. The results of this analysis confirmed that LNMM and T in patients with N0 ESCC were independent relevant factors, respectively.

Surgical resection is the preferred modality for treating this malignancy and the latest National Comprehensive Cancer Network esophageal cancer guidelines (V.1.2009) pointed out that in patients with no residual disease at the surgical margins (R0 resection), no further treatment is necessary for those with squamous cell carcinoma. However, in our study, after complete resection by modified Ivor Lewis esophagectomy, cervical, supraclavicular, mediastinal, and celiac lymph node recurrence occurred in 45.1% of the patients with mid-thoracic esophageal squamous cell carcinoma. We recommend postoperative adjuvant therapy to patients with esophageal carcinoma after complete resection.

CONCLUSION

LNMM and T status in patients with N0 ESCC were independent risk factors for 3-year lymph node metastatic recurrence after Ivor Lewis esophagectomy. Adjunctive therapy might be beneficial in controlling the locoregional recurrence and elevated healing rates for certain patients.

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References

1 Zhang D W, Cheng G Y, Huang G J *et al*. Operable squamous esophageal carcinoma: current results from the East. World J Surg 1994; 18: 347–54.
2 Watson A. Operable squamous esophageal carcinoma: current results from the West. World J Surg 1994; 18: 361–6.
3 Visbal A L, Allen M S, Miller D L, Deschamps C, Trastek V F, Pairolero P C. Ivor Lewis esophagogastrectomy for esophageal cancer. Ann Thorac Surg 2001; 71: 1803–8.
4 Eloubeidi M A, Desmond R, Arguedas M R, Reed C E, Wilcox C M. Prognostic factors for the survival of patients with esophageal carcinoma in the U.S.: the importance of tumor length and lymph node status. Cancer 2002; 95: 1434–43.
5 Hosch S, Raus J, Scheunemann P *et al*. Malignant potential and cytogenetic characteristics of occult disseminated tumor cells in esophageal cancer. Cancer Res 2000; 60: 6836–40.
6 Godfrey T E, Raja S, Finkelstein S D, Gooding W E, Kelly L A, Luketich J D. Prognostic value of quantitative reverse transcription-polymerase chain reaction in lymph node-negative esophageal cancer patients. Clin Cancer Res 2001; 7: 4041–8.
7 Ho S B, Niehans G A, Lyftogt C *et al*. Heterogeneity of mucin gene expression in normal and neoplastic tissues. Cancer Res 1993; 53: 641–51.
8 Wang Z, Yin H, Zhang L, Lan X, Li H. Gene diagnosis and prognosis of mediastinal lymph node occult micrometastasis in non-small cell lung carcinoma. Zhonghua Zhong Liu Za Zhi 2002; 24: 247–9.
9 Korst R J, Rusch V W, Venkatraman E *et al*. Proposed revision of the staging classification for esophageal cancer. J Thorac Cardiovasc Surg 1998; 115: 660–70.
10 Chen G, Wang Z, Liu X Y, Liu F Y. Recurrence pattern of squamous cell carcinoma in the middle thoracic esophagus after modified Ivor Lewis esophagectomy. World J Surg 2007; 31: 1107–14.
11 Li S H, Wang Z, Liu X Y *et al*. Lymph node micrometastasis: a predictor of early tumor relapse after complete resection of histologically node-negative esophageal cancer. Surg Today 2007; 37: 1047–52.

12 Parkin D M. Global cancer statistics in the year 2000. Lancet Oncol 2001; 2: 533–43.
13 Li S H, Wang Z, Liu X Y. Metastasis-associated protein 1 (MTA1) overexpression is closely associated with shorter disease-free interval after complete resection of histologically node-negative esophageal cancer. World J Surg 2009; 33: 1876–81.
14 Raja S, Luketich J D, Kelly L A, Gooding W E, Finkelstein S D, Godfrey T E. Rapid, quantitative reverse transcriptase-polymerase chain reaction: application to intraoperative molecular detection of occult metastases in esophageal cancer. J Thorac Cardiovasc Surg 2002; 123: 475–82.
15 Kato H, Tachimori Y, Watanabe H, Igaki H, Nakanishi Y, Ochiai A. Recurrent esophageal carcinoma after esophagectomy with three-field lymph node dissection. J Surg Oncol 1996; 61: 267–72.
16 Gusterson B. Are micrometastase clinically relevant? Br J Hosp Med 1992; 47: 247–8.
17 Jiao X, Krasna M J. Clinical significance of micrometastasis in lung and esophageal cancer: a new paradigm in thoracic oncology. Ann Thorac Surg 2002; 74: 278–84.
18 Salerno C T, Frizelle S, Niehans G A *et al*. Detection of occult micrometastases in non-small cell lung carcinoma by reverse transcriptase-polymerase chain reaction. Chest 1998; 113: 1526–32.
19 Chen G, Wang Z, Liu X Y, Liu F Y. Adjuvant radiotherapy after modified Ivor-Lewis esophagectomy: can it prevent lymph node recurrence of the mid-thoracic esophageal carcinoma? Ann Thorac Surg 2009; 87: 1697–702.