Clinical target volume (CTV) in postoperation radiotherapy of esophageal squamous cell carcinoma could benefit from the detection of telomere length in lymph node

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ABSTRACT

▶ *Original article*

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Background: This study evaluated the relation between telomere length in lymph node (LN) and prognosis of esophageal squamous cell carcinoma (ESCC). **Materials and Methods:** LNs collected from 50 patients were assessed by pathological examination and quantitative reverse transcription polymerase chain reaction (qRT-PCR), which was used for detecting telomere length. The relation between clinical factors and the number of lymph node metastasis (LNM) identified were analyzed by the $\chi 2$ test. The comparison of the pattern of LNM identified by pathological examination and detection of telomere length was assessed by Wilcoxon signed-rank test. Overall survival was assessed using the Kaplan-Meier method, and Cox proportional hazard regression analysis was used to evaluate the relationship between survival and the number of LNM. **Results:** The best threshold values, which could define the positive metastasis by detecting the telomere length, were 1.50, using the critical value method of statistic. Length of tumor, depth of tumor invasion and differentiation of tumor correlated closely with LNM were identified by detecting telomere length. The rates of LNM identified by detecting telomere length were 34.4%, 22.4%, 22.9%, 5.0% in 108, 107, 7, and 3 LN station, respectively. The number of LNM identified by detecting telomere length was more closely related to the prognosis of ESCC than that of pathological examination (HR: 1.23 VERSUS 1.04). **Conclusion:** The change of telomere length in LN was closely related to the prognosis of ESCC. Delineation of clinical target volume (CTV) may benefit from the detection of telomere length in regional LN.

Keywords: *Telomere length, lymph node, the clinical target volume, esophageal squamous cell carcinoma.*

INTRODUCTION

The rate of lymph node metastasis (LNM) is high, and LNM is a major independent risk factor of recurrent in esophageal squamous cell carcinoma (ESCC) [1-3]. Postoperative irradiation on the area of LNM plays an important role in promoting the overall survival (OS) rate of patients with ESCC.

The delineation of clinical target volume (CTV) in postoperative radiotherapy of ESCC was based on the pattern of LNM pathologically [4], and the region with high frequency metastasis were recommended to be included in treatment planning. Some reports revealed that the pathologically negative LN was also closely

correlated with the prognosis of ESCC because false negative was identified in the method of pathological detection on LNM [5-7]. This report showed that detecting the expression of cancer genes by quantitative reverse transcription polymerase chain reaction (qRT-PCR) could deal with this problem of false negative.

Telomere length in tumor tissue was significantly shorter than those in non-tumor tissue, and closely associated with the LNM and prognosis [8-11]. But the relation between the alteration of telomere length in regional LN and patient prognosis has not yet been reported. If the relationship was identified, detection of telomere length in LN could decrease the rate of false negative and increase the accuracy of CTV delineation in radiotherapy treatment planning.

Though this study was carried out on a small scale, but only the middle thoracic squamous cell carcinoma was included which the patient underwent surgical resection without neoadjuvant chemo-radiation. The relation between alteration of telomere length in regional LN and prognosis of ESCC was investigated.

MATERIALS AND METHODS

Patients and characteristics

Fifty patients with ESCC at the department of oncology in LinYi People's Hospital, affiliated to the Shandong University were enrolled between January 2008 and February 2009. All patients underwent curative esophagectomy with systemic lymphadenectomy. Only the patients with middle thoracic and stage II/III (UICC 2002) tumors were included. The patients who underwent preoperative radiotherapy or chemotherapy were excluded. The maximum follow-up time was 5 years. All LNs were obtained from the department of pathology and analyzed by two pathologists independently. This study had been approved by the institutional review board of our hospital, and the informed consent of every patient was obtained. The main clinical and pathological information of the patients were recorded.

Histopathological assessment of LN

The lymph nodes were packed up and marked corresponding to its site by surgeon after operation, and two pathologists evaluated the LN statue of pathological positive after they were stained by haematoxylin-eosin (HE).

Telomere length measure

DNA was extracted from 8 tissue sections (around 10 mm of each LN tissue) by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany).

Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as an internal control to normalize the starting amount of DNA. Relative telomere length was estimated from the ratio of the copy number of telomere to that of GAPDH, and each sample was amplified for three times by the method of qRT-PCR. The mean value was used for analyses. The primers used in this study were showed as follows: for GAPDH, forward: 5`-CCCCACACACATGCACTTACC-3` and reverse: 5`-CCTAGTCCCAGGGCTTTGATT-3`; for telomere DNA, forward: 5`-CGGTTTGTTTGGGTTTGGGTT TGGGTTTGGGTTTGGGTT-3` and reverse: 5`-GGCTGCCTTACCCTTACCCTTACCCTTACCCTTAC CCT-3`.

The reagents in the reaction were showed as follows: 5μl of template DNA, 12.5 μl SYBR Green PCR Master Mix (Applied Bio-systems) and 7.5 μl of each 10 mM primers.

The thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 54 °C for 2 min for the telomere amplification, and 95°C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for GAPDH amplification.

ABI Prism 7000 Sequence Detector and ABI Prism 7000 SDS software (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR. R^2 for each standard curve was higher than 0.95. Because the average Ct of DNA could represent copy numbers of DNAs in the result analysis of qRT-PCR and the DNA length of GAPDH was fixed in all kinds of cells, the ratio between telomere and GAPDH average Ct was used to estimate the relative DNA length of telomere.

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Naming and number of LN stations

To accurately describe the pattern of LNM, the terminology of the regional LN of esophageal cancer was defined by the Japanese Society for Esophageal Diseases.

Endpoint and statistical analysis

All patients were followed up every 3 months in the first 2 years and every 6 months thereafter. OS was observed from the day of operation to the research endpoint of 5 years after that or the death of patients. The relationship between clinical factors and the LNM was evaluated using the $\chi2$ test. For comparing relative telomere length between pathologically positive LNs and negative LNs, the unpaired t-test with unequal variances was performed. Wilcoxon signed-rank test was used to evaluate the difference between patterns of positive LN rates identified by pathological examination and telomere length. Survival analysis was conducted with Kaplan-Meier method, and Cox proportional hazards models were used for analysis of the relation between the positive LN defined by telomere length and survival. *P*-value of less than 0.05 was considered statistically significant. The data were analyzed by SPSS Version 17.0 (SPSS Inc., IBM Company).

RESULTS

Relative telomere length in LN and definition of positive LN detecting telomere length

The relative telomere length was 0.97 in pathologically positive LN and 1.77 in pathologically negative LN (table 1). The difference was significant (P < 0.001). The dot-plot of the relative telomere length in pathologically positive LN and pathologically negative LN are shown in figure 1.

Table 1. Relative Telomere Length in Lymph Node with pathological positive and negative.

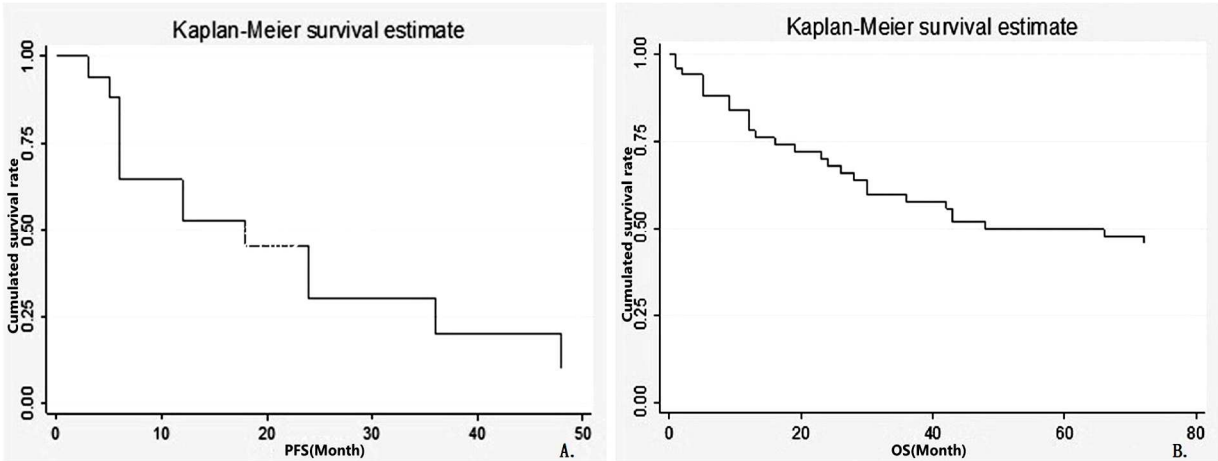| pathological conditions	n	Mean±SD	P^1
Positive	65	0.236±0.971	0.00
Negative	627	0.311±1.774	

Figure 1. A: The progression-free survival (PFS) of the patients; B: The overall survival (OS) of the patients.

We selected a cut-off value to define positive telomere length. The cut-off value of positive telomere length was calculated by "minimum *P value*" approach using X-tile software (version 3.6.1, Yale University, New Haven, CT). As a result of strictly statistical evaluation using by X-tile, 1.50 was identified as the optimal cut-off value corresponding to the best P value. The main clinical and pathological variables of the patients are shown in table 2.

Table 2. The main clinical and pathological variables of the patients with LNM based on pathology and telomere length.

Clinicopathological features		Total number of LN	LNM(+) Telomere length	P^1	LNM(+) pathology	P^2
Age (year)	< 60	220	66	0.114	16	0.325
	60≤	482	152		46	
Sex	Men	640	203	0.221	60	0.109
	Women	62	15		2	
Length of tumor (cm)	4≥	330	78	0	19	0.013
	6-4	200	73		20	
	6≤	172	67		23	
Differentiation	Well	280	62	0	21	0.459
	moderate	350	116		35	
	Poor	72	40		8	
Depth of tumor invasion	T1-T2	159	31	0	5	0.004
	T3-T4	543	187		57	
Postoperative therapy	Yes	164	46	0.427	18	0.269
	No	538	172		44	

Abbreviation: LN: Lymph Node; LNM: Lymph Node Metastases. [1] From a chi-square test.

The rates of positive LN detecting by pathological examination and telomere length

The rate of positive LN detecting telomere length was significantly higher than that of pathological examination ($P<0.001$). The results are shown in table 3. Our previous study (1266 cases) had found the rates of LNM on 108, 107, 7, 3, 2, 109 station were 27.9%, 21.1%, 13.8%, 11.5%, 5.0%, and 4.7%, respectively [17, 18]. The rates of the same site in this study were 28.1%, 21.8%, 17.1%, 3.1%, 7.8% and 3.1%, respectively, detecting by pathological examination, and were 34.4%, 22.4%, 22.9%, 5.0%, 6.4%, and 1.3%, respectively, under the detection of telomere length. The result was shown in table 4. For the same location, the rate was similar and significant difference was not confirmed using by the Wilcoxon signed-rank test. ($P= 0.24$).

Table 3. The LNM rate determined by pathological or telomere length detection.

	telomere length（+)	telomere length (-)	P^1
Pathology（+)	64	1	0.000
Pathology（-)	154	473	

Abbreviation: [1] From a chi-square test, χ2=149.0537.

Table 4. The LNM rate of pathological positive and telomere length positive.

The location of LN	LNM（+) pathology[1]	LNM（+) Telomere length[1]	LNM(+) Previously
108	%28.1	%34.4	%27.9
107	%21.8	%22.4	%21.1
7	%17.1	%22.9	%13.8
3	%3.1	%5.0	%11.5
2	%7.8	%6.4	%5.0
109	%3.1	%1.3	%4.7

Abbreviation: [1] From Wilcoxon signed-rank test, P=0.24.

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Correlation between the number of positive LN detecting telomere length and survival

At the end of follow-up, survival information were obtained in 46 of 50 cases, 4 patients were lost to follow up because of wrong phone number. Minimum time of follow-up was 2 years, while the maximum was 5 years. OS at 1 and 2 years were 86.0% (43/50) and 60% (30/50), respectively. The median survival time was 43 months (range, 1.0–60.0 months). The OS and PFS were showed in figure 1 by the Kaplan–Meier method. As determined by Cox proportional hazards model, the number of positive LN detecting telomere length were more significantly correlated than that of pathological examination in ESCC (HR: 1.23 VERSUS 1.04). The result was showed in table 5.

Table 5. The relationship between OS and the number of the lymph node of pathological positive and telomere length positive.

t	Haz. Ratio	Std. Err.	Z	P	[%95	CI]
X1	1.23	0.13	2.00	0.045	1.00	1.52
X2	1.05	0.02	2.06	0.04	1.00	1.09

Abbreviation: X1: the number of telomere length positive, X2: the number of pathological positive, 95%CI: confidence Interval.

DISCUSSION

The long-term survival of patients with thoracic ESCC is poor, and the major reason was the high rate of LNM [12, 13]. Postoperative radiotherapy of high-risk regional LN in ESCC is very important, and it can decrease the rate of local recurrence of tumor [14]. Though it improved long-term survival of esophageal cancer, there was considerable controversy about the selection of high-risk regional LN [15].

The incidence of thoracic esophageal cancer accounts for the majority (about 50%) of cases of esophageal carcinoma. The cases in this study were all identified from middle thoracic esophageal cancer and all the cases were squamous cell carcinoma in order to avoid the confounder induced by the tumor site or histological type. The proportion of LNM in each LN stations was similar to our previous large-scale study (1266 cases). For instance, the rates of 108, 107, 109 station were 21.1%, 27.9%, 4.7%, respectively [16, 17].

The delineation of CTV was based on pathological identification of regional LNM in clinic currently. But micrometastases was a huge challenge leading to the false negative of pathological examination, it was reported that the proportion of micrometastases in regional LN was high and closely correlated with the development of ESCC [18, 19]. It had been proved that tumor micrometastases could be identified in negative regional LN by detecting the expression of GUCY2C [20]. Pimpec-Barthes identified lung tumor micrometastases in mediastinal LN by detecting the expression level of CK19 mRNA [21]. Goydos assessed tumor micrometastases in the sentinel LN by detecting tyrosine kinase [22]. Molecular biology was a major method for the diagnosis of tumor micrometastases [23].

Obvious differences were existed between telomere length of tumor tissue and non-tumor tissue, and it was correlated to outcome of patients [24]. It had been proved that telomere length was obviously foreshortened in prostate and colorectal cancers [25, 26]. Some researchers showed that telomere length was an important factor for prognosis. It had been reported that the change of telomere length in esophageal and adjacent cancer tissues were both closely related to prognosis [27, 28]. In conclusion, detection of telomere length might be an effective method for identifying micrometastases.

The number and pattern of LNM influenced the delineation of CTV and the outcome of ESCC [29, 30], so it was necessary to assess the pattern of LNM defined by detecting the tumor micrometastases. In this study, the relative telomere length was detected using the methods published in other studies on LNM of ESCC [30-32].

The number of positive LN by detecting telomerase length was 154, while only 64 were

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identified by pathological examination. It could be inferred that the false negative rate of pathological examination might be high. So it was necessary to investigate the correlation between the number of positive LN defined by detecting telomerase length and the prognosis of ESCC, which might influence the delineation of CTV.

This study demonstrated that the length, differentiation of tumor and the depth of tumor invasion were all significantly correlated with the number of positive LN by detecting telomerase length (*P/6.667* for each). But significant results were only identified between the tumor length, depth of tumor invasion and the number of pathologically positive LN (*P*=0.013, and 0.004, respectively). The reason might be that it was more sensitive to identify micrometastases using the method of detecting telomerase length than pathological examination in regional LN.

The median survival time was about 43 months, which was longer than that of Tanaka's result (26 months) [33], but shorter than that of Greenstein's report (72 months) [34]. The possible reason was that their studies had different proportions of phase II patients and included some patients, which were not middle thoracic cancers.

The Cox proportional hazard regression model used in this study revealed, the correlation between the number of positive LN defined by detecting telomerase length and OS was more significant than that between the number of pathologically positive LN and OS (HR: 1.23 VERSUS 1.04). This result showed that the number of positive LN defined by detecting telomerase length had a more significant relationship with the outcome of ESCC, and false negative of pathological examinations might be the reason which was in accordance with the published report [23].

This hypothesis was also supported by another result of this study. There were obvious differences between the rate of LNM defined respectively by pathological examination and molecular biological approaches. These two methods had positive LN at rates of 22.4% VERSUS 21.8%, 34.4% VERSUS 28.1%, and 22.9% VERSUS 17.1% in 107, 108 and 7 station, respectively. 107, 108, and 7 stations were the highest frequency regions of LNM were in accordance with the published report, and similar pattern of LNM was identified by molecular biological approaches used in this study. It showed that the detecting of telomerase length was an effective method to identify micrometastases.

In current, involved nodal irradiation was applied in postoperative radiotherapy, and only the LN station with high frequency of metastasis was included in CTV delineation. The result of this study showed that we could delineate the CTV more accurately on the basis of detecting telomerase length, which could identify micrometastases on regional LN effectively. The margin of CTV may be enlarged in 107, 108, and 7 stations based on the result of the new method of LNM detection in this study, and a better OS might be obtained in future from it.

First limitation of this study was that it was a small cohort. A larger cohort should confirm these preliminary findings. Only the relation between the total number of positive LN defined by detecting telomerase length in LN and OS was investigated, and the hypothesis will be strongly supported if the relation between the number of each LN station and OS was investigated. Second, it is difficult to ascertain definitely the delineation of CTV based on detecting telomerase length have superiority over that of pathological examination until a stage I clinical testing is performed.

In summary, the detection of the telomere length in regional LN can decrease the false negative rate of pathological examination and present a new method to delineate CTV more accurately.

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Conflict of interest: *Declared none.*

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