Int J Clin Exp Med 2015;8(3):3300-3308
www.ijcem.com /ISSN:1940-5901/IJCEM0005757

Original Article

Association between glutathione S-transferases M1 and T1 gene polymorphisms and esophageal cancer prognosis

Ge-Nan Li[1*], Xue-Min Li[2*], Yang-Qi Liu[1], Zhi-Qin Bao[2], Li-Xia Yang[2], Xiao Wang[2], Min-Wei Li[2], Jin-Lin Du[1], Ting-Ting Chen[1], Zhi-Gang Huang[1]

[1]*Department of Epidemiology, School of Public Health, Guangdong Medical University, Dongguan 523808, China;* [2]*People's Hospital of Ci County, Ci County 056500, China.* [*]*Equal contributors.*

Received January 10, 2015; Accepted March 5, 2015; Epub March 15, 2015; Published March 30, 2015

Abstract: Objective: To investigate the independent factors affecting the prognosis of patients after resection of esophageal cancer, and to inquire into the relationship between GSTM1, GSTT1 gene polymorphisms and esophageal cancer prognosis. Methods: The clinical data of 273 patients with esophageal cancer were retrospectively analyzed. The patients were followed-up after their surgery, and the gene polymorphisms of GSTM1 and GSTT1 in each individual were detected by polymerase chain reaction (PCR). The clinical features along with the gene polymorphisms of GSTM1 and GSTT1 associated with the prognosis of patients were analyzed by using the method of univariate analysis and Cox proportional hazard model. The cumulative survival rate was estimated by Kaplan-Meier methods, and the survival curves were compared by using the log-rank test. Results: The overall cumulative survival rate of first year, third year and fifth year is 94.6%, 58.5% and 17.8%, respectively. The median survival time (MST) is 38.7 months. The results of univariate analysis showed that: infiltration depth, length of tumor, the number of lymph node metastasis, the region of lymph node metastasis and the genetic polymorphism of GSTM1 and GSTT1 gene loci were associated with the survival of postoperative patients. Cox multivariate analysis further indicated that the length of tumor, the number of lymph node metastasis and the combined genotype (1) [GSTM1 (+/+) or (+/-) & GSTT1 (-/-)] were the independent prognostic factors. The length of tumor, the number of lymph node metastasis were the risk factors for the prognosis, and the combined genotype (1) had protective effect on survival when compared with reference [GSTM1 (+/+) or (+/-) & GSTT1 (+/+) or (+/-)]. Conclusion: The length of tumor, the number of lymph node metastasis were confirmed as the independent prognostic factors of esophageal carcinoma, and the null genotypes for GSTT1 (-/-) might be a protective factor for survival and GSTM1 (-/-) might be a potential negative prognostic factor in patients with esophageal cancer.

Keywords: Esophageal carcinoma, GSTM1, GSTT1, prognostic factors

Introduction

Esophageal cancer is one of the most common cancers worldwide. As "Chinese Cancer Registry Annual Report" shows, in nearly 40 years, the mortality rate of esophageal cancer always ranks in the top five cancer death list [1]. Prognosis of esophageal cancer is poor, the 5-year survival rate of patients with advanced esophageal cancer is only about 10% [2]. It is well-known that the main factors affecting the prognosis of esophageal cancer are the TNM staging and treatment of tumor, however, for the esophageal cancer patients with the same clinical features, even received the same treatment, a big difference may also exist in their disease progress and prognosis, which indicates that besides clinical features, the individual genetic susceptibility may also be an important factor affecting the prognosis of patients [3]. Therefore, identifying the genetic variants associated with the prognosis of patients with esophageal cancer to enhance the predictive value of the clinical prognosis is a hot research in oncology at home and abroad.

Glutathione transferases, a super family of dimeric phase II metabolic enzymes play a vital

Table 1. Clinical characteristics of patients with esophageal cancer

Characteristics		No. cases	%
Gender	Male	176	64.47
	Female	97	35.53
Age group	≤ 44 years old	8	2.93
	45-59 years old	162	59.34
	60-74 years old	100	36.63
	≥ 75years old	3	1.1
Tissue type	Adenocarcinoma	13	4.76
	Squamous carcinoma	241	88.28
	The others	19	6.96
Tumor grade	Period I	32	11.72
	Period II- Period III	178	65.2
	Above period III	63	23.08
Tumor length	<3 cm	7	2.56
	3-5 cm	73	26.74
	>5 cm	75	27.47
Infiltration depth	Tis Carcinoma in situ	104	38.1
	T1 Tumor invades mucous layer or submucosa	14	5.13
	T2 Tumor invades muscular	96	35.16
	T3 Tumor invades esophageal adventitia	128	46.89
	T4 Tumor invades adjacent organs	49	17.95
Number of lymph node metastasis	<1	186	68.13
	1-3	63	23.08
	>3	24	8.79
Region of lymph node metastasis	0	186	68.13
	1	48	17.58
	2	28	10.26
	3	11	4.03

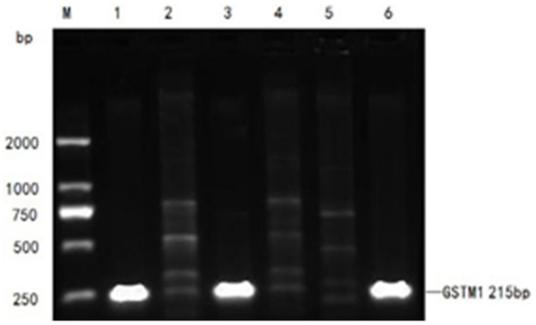

Figure 1. Represents PCR analysis of GSTM1 gene resolved on 1.5% agarose gel electrophoresis. M is a 100 bp DNA marker. A 215-bp product indicates the presence of at least one GSTM1 non-null allele (samples 1, 3 and 6). Absence of GSTM1 product indicates homozygous null genotype of that gene (samples 2, 4 and 5).

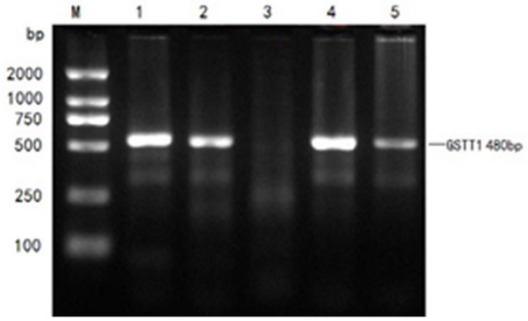

Figure 2. Represents PCR analysis of GSTT1 gene resolved on 1.5% agarose gel electrophoresis. M is a 100 bp DNA marker. A 480-bp product indicates the presence of at least one GSTT1 non-null allele (samples 1, 2, 4 and 5). Absence of GSTT1 product indicates homozygous null genotype of that gene (sample 3).

role in biotransformation of many substances. As members of GSTs family, Glutathione S-transferases M1 (GSTM1) and Glutathione S-transferases T1 (GSTT1) both have the func-

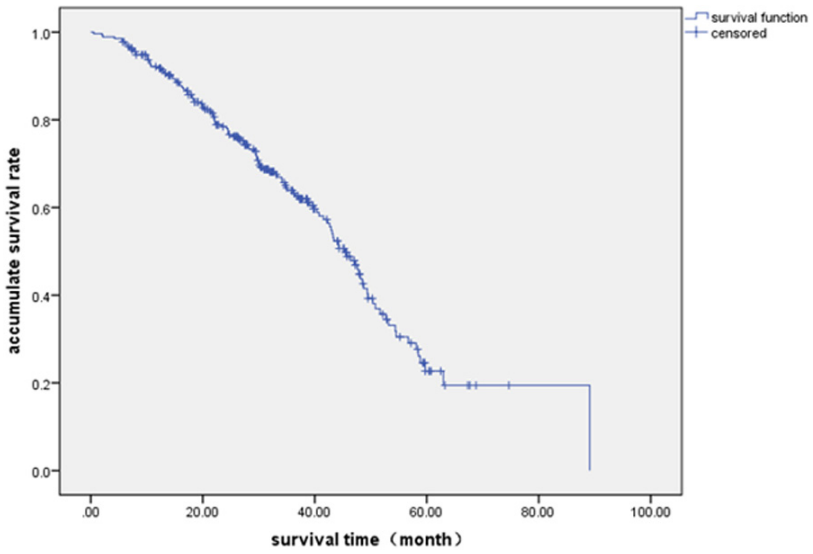

Figure 3. The overall survival curves of patients with esophageal cancer.

tions of detoxifying the exogenous chemicals [4]. It is well-known that the GSTM1 and GSTT1 genes are polymorphic in humans, and the most common variants of both genes are homozygous deletion (null genotype). The null genotype of GSTM1 and GSTT1 genes have been suggested to be associated with the loss of the enzyme activity, which may result in the susceptibility to cancers [5]. At present, the relationship between GSTM1 and GSTT1 and esophageal cancer susceptibility has attracted much attention, but their effects on the prognosis of esophageal cancer have not yet been reported.

We present herein the results of survival analysis, in which the clinical features of patients with esophageal cancer along with the polymorphisms of GSTM1 and GSTT1 were all as candidate factors to identify the independent prognostic factors and to evaluate their prognostic value. This paper will shed some light on the exactly relationship between GSTM1, GSTT1 null genotype and the postoperative prognosis of esophageal cancer for the first time and provide the references for individualized treatment and prognosis judgment for esophageal cancer as well.

Materials and methods

Study subjects

Patients in this study were recruited from People's Hospital of Ci county , Ci County, Hebei Province, China, who had their surgical resection at the hospital between October 2003 and December 2009. A total of 499 esophageal cancer cases were chosen primarily. Among them, follow-up information of 130 patients was not adequate. Ninety six patients sufferred also the stomach adenocarcinoma according to the pathological diagnosis by senior pathologists. They were excluded from the study. Finally, 273 cases with esophageal cancer were enrolled in further analyses. None of the 273 cases were with family history of esophageal cancer. Data on patients' clinicopathological features such as tissue types, tumor grade, infiltration depth, length of tumor, the number of lymph node metastasis, the region of lymph node metastasis were gathered from their pathological report. The study was approved by the institutional review board of Guangdong Medical University.

Genotyping

Genomic DNA was extracted from patients' tumor tissues by using the AxyPrep genomic DNA miniprep kits (Axygen Bioscience, USA). The extracted DNA was stored at 4°C until analysis. Genotyping of GSTM1 and GSTT1 were performed by polymerase chain reaction. The sequences of primers used to amplify DNA fragment of GSTM1 gene were as follow: P1: 5'-GAACTCCCTGAAAAGCTAAAGC-3' and P2: 5'-CTTGGGCTCAAATATACGGTGG-3' (Invitrogen™/ Life Technologies, USA). Each amplification reaction was performed in a total volume of 25 μL, containing 2 × Taq PCR MasterMix (TIANGEN Biotech Beijing Co.LTD) 12.5 μL, 1 U Taq polymerase, 20 pmol/L of each primer and 50 ng of genomic DNA, processing started with 94°C for 3 min and 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. This was followed by a final extension at 72°C for 5 min. GSTT1 genotypes were also identified by PCR using the primers with sequences P3: 5'-TTCC-

Table 2. Univariate analysis of prognosis of patients with esophageal cancer

Characteristics		No. cases	MST (Months)	X^2	*P*
Gender	Male	176	37.47	1.672	0.196
	Female	97	44.37		
Age	≤ 44 years old	8	50.30	2.079	0.556
	45-59 years old	162	38.73		
	60-74 years old	100	37.67		
	≥ 75 years old	3	.		
Tissue type	Adenocarcinoma	13	.	1.289	0.525
	Squamous carcinoma	241	38.53		
	The others	19	38.70		
Tumor grade	Period I	32	37.47	3.57	0.168
	Period II- Period III	178	37.83		
	Above period III	63	48.00		
Infiltration depth	Tis Carcinoma in situ	7	31.23	13.769	0.008
	T1 Tumor invades mucous layer or submucosa	73	35.87		
	T2 Tumor invades muscular	75	48.00		
	T3 Tumor invades esophageal adventitia	104	45.03		
	T4 Tumor invades adjacent organs	14	32.20		
Tumor length	< 3 cm	96	37.50	6.498	0.039
	3-5 cm	128	38.93		
	> 5 cm	49	48.00		
Number of lymph node metastasis	< 1	186	37.47	9.104	0.011
	1-3	63	52.83		
	> 3	24	63.27		
Region of lymph node metastasis	0	186	37.47	10.154	0.017
	1	48	52.83		
	2	28	67.33		
	3	11	63.27		
GSTM1	(+/+) or (+/-)	115	52.83	40.211	<0.001
	(-/-)	158	31.70		
GSTT1	(+/+) or (+/-)	127	37.00	8.176	0.004
	(-/-)	146	45.23		

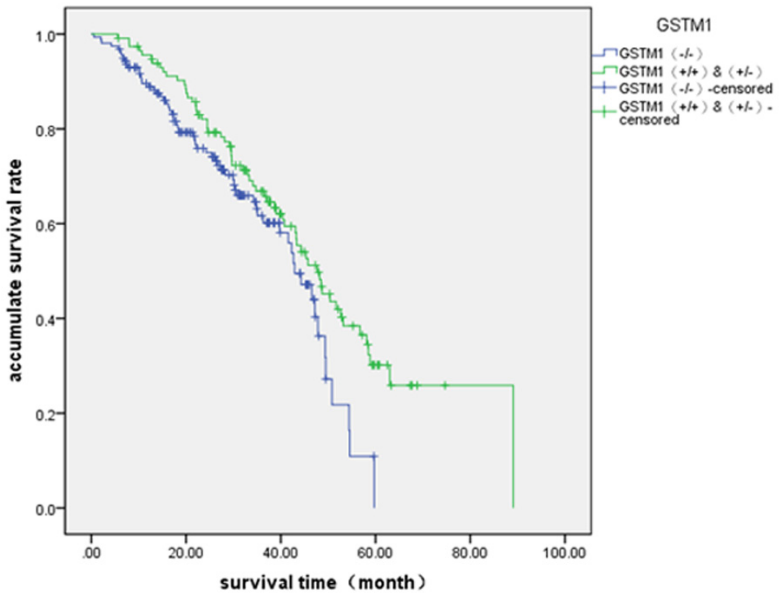

Figure 4. Comparison of GSTM1 gene polymorphism survival curves.

TTACTGGTCCTCACATCTC-3' and P4: 5'-TCACCGGATCATGGCCAGCA-3' (Invitrogen™/Life Technologies, USA). The reaction system and PCR condition of GSTT1 were the same as mentioned above. The PCR products were separated by 1.5% agarose gel electrophoresis.

GSTM1 Genotype of patients were identified by the results of PCR amplification. If the DNA fragment was about 215 bp, GSTM1 Genotype was considered as the non-null genotype (+/+ or +/-), and if there was no PCR products observed, GSTM1 genotype was identified as

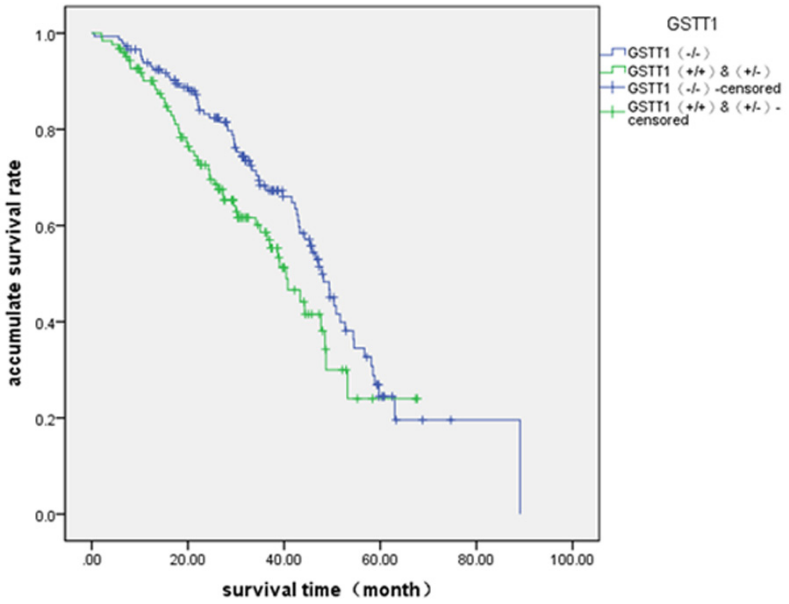

Figure 5. Comparison of GSTT1 gene polymorphism survival curves.

Table 3. Combined genotype categorical variable coding

Jointed genotype	Frequency	(1)	(2)	(3)
1 = GSTM1 (+/+) or (+/-) GSTT1 (+/+) or (+/-)	63	0	0	0
2 = GSTM1 (+/+) or (+/-) GSTT1 (-/-)	52	1	0	0
3 = GSTM1 (-/-) GSTT1 (+/+) or (+/-)	62	0	1	0
4 = GSTM1 (-/-) GSTT1 (-/-)	96	0	0	1

the null genotype (-/-). GSTT1 Genotype of patients were analyzed by the the results of PCR amplification. If the DNA fragment was about 480bp, GSTT1 Genotype was considered as the non-null genotype (+/+ or +/-), and if there was no PCR products observed, GSTT1 genotype was identified as the null genotype (-/-).

Follow-up

The follow-up department of our hospital was responsible for postoperative follow-up of all patients. The follow up data of all postoperative patients were obtained by reviewing records of clinical reexamination or by directly contacting the patient or their family by interview or by telephone. Follow up was stopped upon patient's death or on October 2012. 34 cases were lost to follow up and defined as censored cases. The follow-up rate was 88.9%. The mean follow-up was 35.77 ± 20.25 months (range, 0.53-89.1 months). Overall survival time was calculated as the time between surgery and death or last follow-up.

Statistical analysis

All statistical analyses were done with the statistical software package SPSS 15.0 (SPSS Inc., Chicago, Illinois). Quantitative data were described as mean ± standard deviation, and a *t*-test was used for comparison. The clinical features along with the gene polymorphisms of GSTM1 and GSTT1 associated with the prognosis of patients were performed using univariate and multivariate Cox regression analysis. The cumulative survival rate was estimated using the Kaplan-Meier method, and compared by means of the log-rank test. A *P* value of less than 0.05 was considered statistically significant.

Results

The general clinical data

The median age of patients with esophageal cancer in this study was 57 years old (Range 35-77 years), the male to female ratio of cases was 2.5:1, general condition of the patients is seen in **Table 1**.

GSTM1, GSTT1 polymorphism detection results

Use the fragment 215 bp of GSTM1 gene order amplified by primer P1, P2, as shown in **Figure 1**, samples 1, 3 and 6 are respectively the GSTM1 genotype (+/+) or (+/-), samples 2, 4 and 5 are the GSTM1 genotype (-/-); Use the fragment 480 bp of GSTT1 gene order amplified by primer P3, P4, as shown in **Figure 2**, samples 1, 2, 4 and 5 are respectively the GSTT1 genotype (+/+) or (+/-), sample 3 is the GSTT1 genotype (-/-).

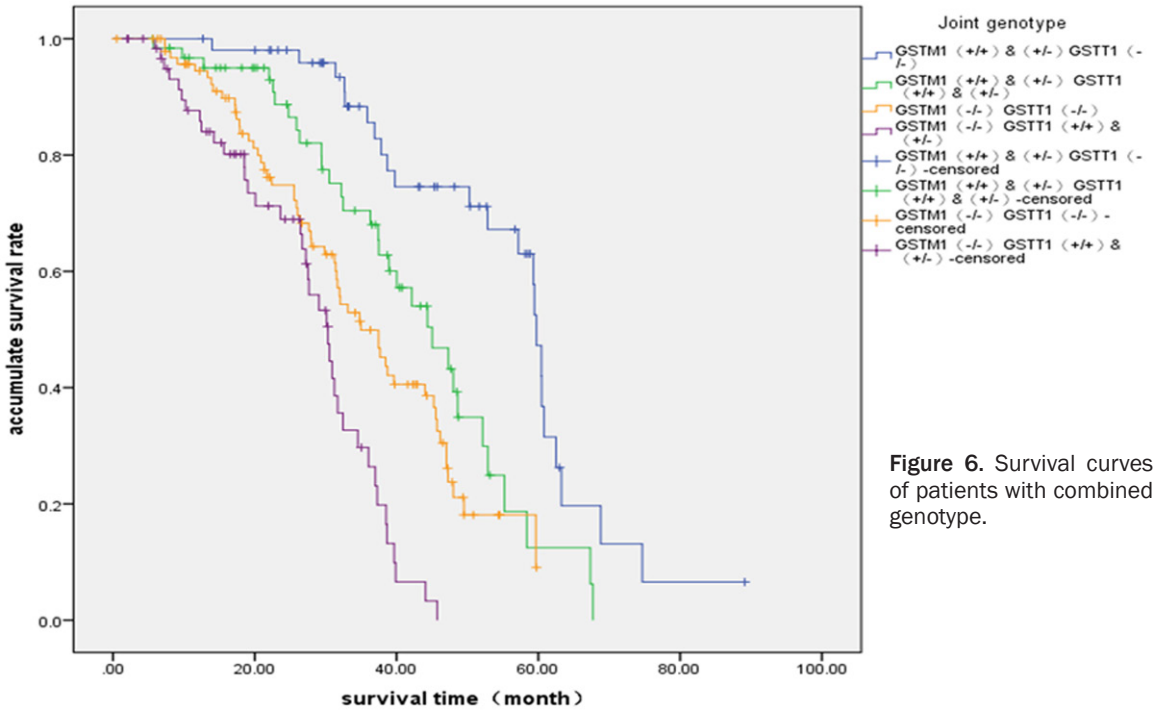

Figure 6. Survival curves of patients with combined genotype.

Table 4. Survival conditions of esophageal cancer patient with combined genotype

Combined genotype		Total number	MST (Months)	95% CI		X^2	*P*
GSTM1	GSTT1			Lower	Upper		
(+/+) or (+/-)	(+/+) or (+/-)	63	45.033	36.575	53.492	65.43	< 0.001
(+/+) or (+/-)	(-/-)	52	59.733	58.300	61.167		
(-/-)	(+/+) or (+/-)	62	30.333	26.458	34.209		
(-/-)	(-/-)	96	34.967	28.867	41.067		

Distribution of GSTM1, GSTT1 gene polymorphism in different clinical characteristics

Compare respectively the distribution of GSTM1, GSTT1 gene polymorphism in Gender, Age group, Tissue type, Tumor grade, Tumor length, infiltration depth, Number of lymph node metastasis and Region of lymph node metastasis. There was not statistically significant in these studies, table leaves.

Survival condition

For 273 cases of patients enrolled, the number of censored data was 149 cases, while 124 cases for comprehensive data. Median time to progress (MTTP) of all patients was 27.3 months (Range 0.53-89.10 months), the median survival time (MST) was 38.7 months (95% CI: 35.43-41.97), the cumulative survival rate of first year, third year and fifth year is respective 94.6%, 58.5% and 17.8%. Survival curve is shown in **Figure 3**.

Univariate analysis of prognostic of the whole group of patients

Univariate analysis shows that: Infiltration depth, tumor length, the number of lymph node metastasis the region of lymph node metastasis and GSTM1, GSTT1 gene polymorphism are the factors affecting the survival of patients after surgery ($P<0.05$); No significant differences were observed in gender, age, tissue type and tumor grade have relationship with prognosis ($P>0.05$), the result is shown in **Table 2**, the comparison of GSTM1, GSTT1 gene polymorphisms survival curves is shown in **Figures 4** and **5**.

Survival conditions of patients with GSTM1, GSTT1 joint genotype

Do further analysis of the relationship between joint genotypic polymorphisms of GSTM1 & GSTT1 and prognosis. Regard GSTM1 (+/+) or (+/-) & GSTT1 (+/+) or (+/-) as the reference

Table 5. Cox regression analysis of prognosis of patients with esophageal cancer

Prognostic factors	*β*	Wald	*P*	*RR*	Value of *RR* 95.0% CI	
					Lower	Upper
Tumor length	0.358	6.647	0.010	1.431	1.090	1.879
Number of lymph node metastasis	0.529	16.316	< 0.001	1.697	1.313	2.193
Combined genotype		15.649	0.001			
Combined genotype (1)	-0.681	6.365	0.012	0.506	0.298	0.859
Combined genotype (2)	0.555	3.809	0.051	1.742	0.998	3.041
Combined genotype (3)	-0.001	0.000	0.997	0.999	0.627	1.593

groups. Specific dummy variable settings are shown in **Table 3**, survival curve is shown in **Figure 6**, median survival time of Jointed-gene is shown in **Table 4**.

Multivariate analysis

Take the statistically significant variables of univariate analysis into Cox proportional hazard model, the result showed that : infiltration depth, length of tumor, the number of lymph node metastasis, the region of lymph node metastasis and the genetic polymorphism of GSTM1 and GSTT1 gene loci were associated with the survival of postoperative patients. The length of tumor, the number of lymph node metastasis and the combined genotype (1) [GSTM1 (+/+) or (+/-) & GSTT1 (-/-)] were the independent prognostic factors. The length of tumor, the number of lymph node metastasis were the risk factors for the prognosis. The combined genotype(1) had protective effect on survival when compared with reference [GSTM1 (+/+) or (+/-) & GSTT1 (+/+) or (+/-)],while the combined genotype (2), (3) did not achieve statistical significance. The result is shown in **Table 5**.

Discussion

It was indicated from our research that the overall survival rate of the postoperative patients in the 1-yr 3-yr and 5-yr is 94.6%, 58.5% and 17.8%, respectively. Compared with Li's result [6], the 1 year survival rate is a little higher (94.6% vs 85.18%), but 5 years survival rate is almost an half of it (17.8% vs 37.08%), the cause of which may be related to the differences in the clinical features, recurrence, metastasis, treatment and rehabilitation conditions.

The previous studies on prognostic factors of patients with esophageal cancer after surgery have showed that the main factors affecting the prognosis of patients are the tumor immersion depth, metastasis of lymph gland, histological differentiation, tumor location, clinical stages and so on [7-9]. In this study, we found by using univariate analysis that the infiltration depth, tumor length, number of lymph node metastasis and region of lymph node metastasis were the risk factors affecting the survival of patients. In addition, it was showed that patients with homozygous null genotype of GSTM1 (-/-) had a relatively poor survival, compared with GSTM1 non-null genotype (+/+ or +/-). But GSTT1 genotype is just the opposite: while patients with homozygous null genotype of GSTT1 (-/-) had a better survival than GSTT1 non-null genotype (+/+ or +/-), which was indicated that homozygous null genotype of GSTM1 (-/-) could be a risk factor affecting survival of patients, and null genotype of GSTT1 (-/-) may seems to play a protective role in these populations.

Cox multivariate analysis results showed that the number of metastatic lymph nodes and the length of tumor were the main factors affecting the prognosis of esophageal cancer. Zuo et al. [10] have also reported similar results. His research indicated that the main factors influencing the prognosis of postoperative are: lymph node metastasis, low protein preoperatively, differentiation degree of cancer lesions, the length of the cancer lesions, by way of surgery, cancer cells infiltrating depth, clinical staging and preoperative radiation and chemotherapy.

In the research of surgical operation treatment and prognosis of esophageal carcinoma in patients over 70 years, Wang. Et al [11] observed that the maximum diameter of the tumor tissues had a significant impact on the survival, and the longer the maximum tumor diameter is, the greater the grade malignancy of tumor is, and the worse the prognosis is. For the effect of lympa nodes on the prognosis,

several studies emphasized the importance of the number and the range of metastatic lymph nodes. Li. et al [12] reported that for the non-surgical patients with thoracic esophageal carcinoma, metastatic lymph volume is also one of the factors affecting the survival of patients. Chen. et al [13] reported that the patients with lymph node metastasis accounted for 74.1% of those sufferred from the recurrence and metastasis of esophageal cancer. Thus, lymph node metastasis maybe one of the key factors associated with the prognosis patients with esophageal cancer. We found that the factor of the number of metastatic lymph nodes entered In the Cox regression model, instead of the factor of the number of region of metastatic lymph nodes, the reason of which may be related to the problem of collinearity between the two factors.

In addition, it was shown from Cox multivariate analysis that neither GSTT1 gene polymorphism nor GSTM1 was associated with the prognosis of patients with esophageal cancer. We did further research about the relationship between the combined genotype polymorphism of two genes and the prognosis, and the results showed that the combined genotype (1) [GSTM1 (+/+), (+/-) & GSTT1 (-/-)] was the protective factor of the survival when compared with reference [GSTM1 (+/+) or (+/-) & GSTT1 (+/+) or (+/-)], *RR* = 0.506 (0.298~0.859), which indicated that the patients with non homozygous deletion of GSTM1 [(+/+) or (+/-)] and homozygous deletion of GSTT1 (-/-) would have a better survival situation, and the combined genotype (2) [GSTM1 (-/-) & GSTT1 (+/+) or (+/-)] might be a potential risk factor (*RR* = 1.742) (0.998~3.041). According to our results, it is not true that the patients with two homozygous deletion genetypes have the worst survival situations, but homozygous deletion of GSTT1 (-/-) seems to have a protective effect, and the homozygous deletion of GSTM1 (-/-) seems to undertake a potential risky action, the reason of which needs to be further elucidated.

It is wellknown that many factors such as :the individual clinical features, histological type, pathological stage,recurrence-metastasis and so on are found to be associated with the prognosis of esophageal carcinoma, but as to the relation between the prognosis and the functional genetic variations of some genes is not clarified yet. Our study only provided the limited evidences in this field, and we should increase our sample size and select multiple cancer-related genes polymorphisms to do further research, in order to provide references for individualized treatment and prognosis judgment for esophageal cancer in the future.

Acknowledgements

This work was partly supported by the Science and Technology Innovation Foundation of Guangdong Provincial Education Department, China (No. 2012KJCX0058), the Science and Technology Plan Foundation in the Field of Social Development of Guangdong Province, China (No. 2013B021800065), Fund of Dongguan Science and Technology Development, China (No. 2013108101052) and the Construction Fund for Key Subjects of Guangdong Medical University (No. XZ1105).

Disclosure of conflict of interest

None.

Address correspondence to: Zhi-Gang Huang, Department of Epidemiology, School of Public Health, Guangdong Medical University, Dongguan 523808, China. E-mail: hzg71@126.com

References

[1] He J, Zhao P, Chen WQ. Chinese cancer registration report 2012 [M]. Beijing: Military Science Publishing House; 2012.

[2] Sun JM, Li Q, Gu HY, Chen YJ, Wei JS, Zhu Q, Chen L. Interleukin 10 rs1800872 T>G polym、phism was associated with an increased risk of esophageal cancer in a Chinese population.Asian Pac J Cancer Prev 2013; 14: 3443-3447.

[3] Harirchi I, Kolahdoozan S, Hajizadeh S, Safari F, Sedighi Z, Nahvijou A, Mir MR, Mousavi SM, Zendehdel K. Esophageal cancer in Iran; a population-based study regarding adequacy of cancer surgery and overall survival.Eur J Surg Oncol 2014; 40: 352-357.

[4] Gao CM, Takezaki T, Sugimura H, et al. The relationship between CYP2E1, GSTT1 and GSTM1 genotypes and esophageal cancer. Bulletin of Chinese Cancer 2001; 10: 346-349.

[5] Li YM. Study on the characteristics of lymph node metastasis and influencing factors in thoracic esophageal carcinoma. Shijiazhuang, Hebei Medical University 2010: 6.

[6] Liu W, Chen Y, Hao XS. Lymph node metastases from carcinoma of the thoracic esophagus

and gastric cardia: a random sampling report. Chinese J Clin Oncol 2008; 35: 1253-1260.
[7] Yang XL, Ying XL, Guo GY. Multivariate analysis of recurrence and metastasis of colon cancer after radical resection. Prac J Clin Med 2011; 08: 104-106
[8] Cui JL, Hou ZC, Liu Y. Relation of tumor length and invasion and lymph node metastasis and relevant risk factors on survival of esophageal squamous cell carcinoma patients. Cancer Res Prev Treat 2014; 41: 214-220.
[9] Zuo CT, Mao NQ, Liu DS, et al. Analysis of prognosis of Cox in elderly patients with esophageal squamous cell carcinoma after regression. J Chinese Med Abstr Oncol 2011; 03: 139-141.
[10] Wang ZG, Wei JL, Shi WC, et al. Analysis of surgical operation treatment and prognosis of esophageal carcinoma in patients over 70 years. Guangdong Medical Journal 2011; 32: 207-209.
[11] Li M, Peng X, Lin ZX, et al. Influence of volume of lymph node metastases on survival in thoracic esophageal squamous cell carcinoma. Chinese Journal of Cancer Prevention and Treatment 2010; 17: 1845-1848.
[12] Chen JH,Sang M, Chen YH, et al. Recurrence pattern and prognosis of esophageal cancer following tumor resection. Chinese Journal of Oncology 1998; 20: 293-295.