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Original Article

Correlation between TLR4 gene polymorphism and acute respiratory distress syndrome after esophageal cancer surgery

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Abstract: Background: One of the major postoperative complications of esophageal cancer is acute respiratory distress syndrome (ARDS), which poses a great threat to patients' lives. In this research, the cause of ARDS after esophageal cancer surgery was explained from the aspect of the single-nucleotide polymorphism at rs7873784, rs10759930 and rs10983755 of the Toll-like receptor 4 (TLR4) gene. Methods: A total of 75 patients complicated with ARDS after esophageal cancer surgery in our hospital were collected as the ARDS group and 150 patients without ARDS after surgery as the control group. Deoxyribonucleic acids (DNAs) in the peripheral blood of patients were extracted, and the polymorphism loci (rs7873784, rs10759930 and rs10983755) of the TLR4 gene were amplified through polymerase chain reaction (PCR) and sent to a company for sequencing. The concentration of serum TLR4 was detected by kits. Results: The frequencies of the G allele at rs7873784 (P=0.011) and C allele at rs10759930 (P=0.000) in the ARDS group were remarkably lower than those in the control group. Besides, the frequencies of GG genotype at rs7873784 (P=0.000) and CC and CT genotypes at rs10759930 (P=0.000) in the control group were notably higher than those in the ARDS group, while the frequency of AA genotype at rs10983755 (P=0.001) in the ARDS group was clearly lower than that in control group. The survival status of patients with complications of ARDS was notably correlated with CT genotype at rs10759930 of the TLR4 gene since patients with genotype CT were more likely to die (P=0.001). The GG genotype at rs10983755 of the TLR4 gene was remarkably related to the mean mechanical ventilation time (P=0.003) and the average length of intensive care unit (ICU) stay (P=0.018). The ARDS group had a lower frequency of GCG haplotype (P=0.009) and a higher frequency of GTA haplotype (P=0.001) than the control group. The linkage disequilibrium D' was 0.781 between rs7873784 and rs10759930 of the TLR4 gene, and two loci were linked to each other. In addition, the concentration of serum TRL4 in patients with genotype CC at rs7873784 (P=0.034), genotype CT at rs7873784 (P=0.000) and genotype GG at rs10983755 (P=0.000) of the TLR4 gene in the ARDS group was higher than that in the control group. Conclusion: The single-nucleotide polymorphisms at rs7873784, rs10759930 and rs10983755 of the TLR4 gene are significantly related to ARDS after esophageal cancer surgery.

Keywords: Toll-like receptor 4, single-nucleotide polymorphism, acute respiratory distress syndrome

Introduction

Advanced esophageal cancer is mainly treated by surgical resection combined with drug therapy and radiotherapy [1, 2]. Through surgery, esophageal cancer tissues and adjacent lymph nodes can be removed as much as possible, with good efficacy, but there will be postoperative complications. In addition to common surgical complications such as bacterial and fungal infection as well as hemorrhage at the wound site, esophageal obstruction and stenosis are major specific postoperative complications of esophageal cancer. However, the most prominent complication is acute respiratory distress syndrome (ARDS), which greatly threatens the patient's life and has a relatively high incidence [3]. At present, however, the cause of ARDS after esophageal cancer surgery remains unclear, and the high mortality of this complication requires us to explore its cause.

The degree of complexity of the immune system in the human body is proportional to its strong

function. The immune system is involved in anti-tumor reactions in the body before and after esophageal cancer surgery. Nevertheless, some molecules in the immune system also capture and kill normal tissues or cells due to dysfunction and imbalance in its anti-tumor process. The Toll-like receptor 4 (TLR4), as a vital molecule in immunity, mainly participates in the prevention of infection from pathogenic microorganisms or anti-tumor of effects [4, 5]. In the meantime, TLR4 can mediate the occurrence of various types of inflammation, killing pathogenic microorganisms or tumor cells while also promoting the release of inflammatory mediators such as IL-2, which causes damage to related tissues [6, 7]. The TLR4 level in esophageal cancer patients before operation is relatively high due to its participation in anti-tumor reaction. However, following the removal of the tumor, the high level of TLR4 in the patient's body may damage normal tissues such as lung tissues and vascular tissues, leading to such serious postoperative complications as ARDS. However, the single-nucleotide polymorphisms at rs7873784, rs-10759930 and rs10983755 of the TLR4 gene may influence the serum TLR4 level in patients and may function in the occurrence of ARDS after esophageal cancer surgery.

In this research, therefore, the cause of ARDS after esophageal cancer surgery was elaborated from the single-nucleotide polymorphisms at rs7873784, rs10759930 and rs10983755 of the TLR4 gene in combination with the TLR4 concentration in serum of patients and clinically related data, and the relationship between TLR4 gene polymorphism and the susceptibility to ARDS after esophageal cancer surgery was explored.

Data and methods

General data

This study was approved by the Ethics Committee of People's Hospital of Linyi Economic and Technological Development Zone, and all patients signed the informed consent. A total of 75 patients complicated with ARDS after esophageal cancer surgery in our hospital from 2017 to 2020 were collected as the ARDS group and 150 patients without ARDS after surgery were selected as the control group. The collected clinical data included the patient's name, ID number, gender, age, mechanical ventilation time (days of receiving the assisted respiratory therapy), intensive care unit (ICU) stay and survival and mortality status. In the control group, there were 45 males and 30 females with a mean age of (48.94±3.13) years old, while the ARDS group consisted of 80 males and 70 females with an average age of (48.94±3.13) years old. There were no significant differences in general data such as gender and age between the two groups (P<0.05).

Diagnostic criteria for ARDS were based on the consensus reached by the Expert Review Meeting of North American Respiratory Disease-European Society of Intensive Medicine, including acute onset accompanied by respiratory distress symptoms, hypoxemia, infiltration shadow in the lung shown in chest X-ray, PAWP <18 mHg, and PaO_2/FiO_2 <200.

Detection methods

Sample collection

A total of 5-10 mL of peripheral blood was collected from the ARDS group and control group, and was centrifuged at 3,500 rpm for 5 min within 1 h. Then serum in the upper layer and nucleated cells in the middle layer were separated into new centrifuge tubes, respectively. Serum in the upper layer was stored in liquid nitrogen for detection, and the genomic deoxyribonucleic acid (DNA) was extracted from the nucleated cells in the middle layer.

DNA extraction

The genomic DNA of the nucleated cells in the peripheral blood of patients was extracted using TIANGEN blood genome extraction kit. The specific operation steps are as follows. A total of 200 μL of Protein K solution was added to a 15 mL centrifuge tube, followed by addition of peripheral blood sample and 2.4 mL of Buffer GE. Then they were shaken, mixed evenly and placed at 65°C for 15 min. After that, the samples were mixed with 2 mL absolute ethyl alcohol, and transferred to the adsorption column. Thereafter, the adsorption column was added with 2 mL of buffer for 1 min of centrifugation at 4,000 rpm and with Buffer PW for centrifugation again. Subsequently, 250 μL of elution buffer was added to the adsorption column, and the obtained solution was the extracted genomic DNA, whose purity was examined via an ultraviolet spectro-

Table 1. Allele frequency distribution at rs7873784, rs10759930 and rs10983755 of TLR4 gene

	Allele	Control group	ARDS group	OR value	95% confidence interval (CI)	χ^2	*P*
rs7873784	C	152 (0.490)	93 (0.612)	1.63	1.10-2.43	6.04	0.011
	G	158 (0.510)	59 (0.388)				
rs10759930	C	170 (0.548)	56 (0.368)	0.48	0.32-0.71	13.21	0.000
	t	140 (0.452)	96 (0.632)				
rs10983755	A	147 (0.474)	70 (0.461)	0.94	0.64-1.39	0.07	0.782
	G	163 (0.526)	82 (0.539)				

photometer. OD_{260}/OD_{280}=1.8-2.0 represented a qualified sample, which could be selected for subsequent use.

Polymerase chain reaction (PCR) amplification and sequencing

The polymorphism loci (rs7873784, rs10759930 and rs10983755) of the TLR4 gene were amplified by PCR. The total PCR reaction system (20 μL) included 1 μL of primers, 0.5 μL of template DNAs, 4 μL of Taq enzymes (5 ×) and 13.5 μL of dH_2O. PCR reaction conditions: 95°C for 3 min, (95°C for 40 s, 63°C for 50 s and 72°C for 35 s) × 35 cycles and 72°C for 5 min. Primers at each locus: forward: (5'→3')'TTTGGACAGTTCCCACATTGA' and reverse: (5'→3')'AAGCATTCCCACCTTTGGG' at rs7873784, forward: (5'→3')'AGTTGATCTACCAAGCCTTGAGT' and reverse: (5'→3')'GCTGGTTGTCCCAAAATCACTTT' at rs10759930, and forward: (5'→3')'TGGCATGGCTTACACCACC' and reverse: (5'→3')'GAGGCCAATTTTGTCTCCACA' at rs10983755. PCR products were sent to Shanghai Sangon Biotech Co., Ltd. for sequencing to obtain DNA sequences at each locus, and the condition of single-nucleotide polymorphisms at each locus was observed through analysis.

Detection of serum TLR4

The serum samples stored in liquid nitrogen were taken out, and the serum TLR4 concentration was measured using a Thermo Fisher Scientific kit in accordance with the instructions of Thermo Fisher Scientific (Invitrogen Corporation, Carlsbad, CA, USA). The average sensitivity of the test was <0.8 pg/mL, and the coefficient of variation between batches was 7.1%.

Statistical analysis

IBM SPSS 22.0 software was adopted for statistical analysis. Comparisons of count data were achieved by the χ^2 test, and Hardy-Weinberg equilibrium analysis was carried out. The haplotype analysis was conducted on-line through SHEsis website, and Pearson method was employed for correlation analysis. *P*<0.05 suggested a statistically significant difference.

Results

Allele and genotype distribution in the TLR4 gene at rs7873784, rs10759930 and rs10983755

Allele distribution at rs7873784, rs10759930 and rs10983755 of TLR4 gene (**Table 1**) showed that the frequencies of the G allele at rs7873784 (*P*=0.011) and C allele (*P*=0.000) at rs10759930 in the ARDS group were markedly lower than those in the control group. Besides, the frequencies of GG genotype at rs7873784 (*P*=0.000) and CC and CT genotypes at rs10759930 in the control group were notably higher than those in the ARDS group (*P*=0.000), whereas the frequency of AA genotype at rs10983755 (*P*=0.001) in the ARDS group was clearly lower than that in the control group (**Table 2**).

Correlations of genotypes at rs7873784, rs10759930 and rs10983755 of the TLR4 gene with the survival status, mean mechanical ventilation time and average length of ICU stay

Correlations of genotypes at rs7873784, rs10759930 and rs10983755 of the TLR4 gene with the survival status, mean mechanical ventilation time and average length of ICU stay are present in **Table 3**. It was found that the survival status of patients with ARDS after esophageal cancer surgery was evidently associated with CT genotype at rs10759930 of TLR4 gene since patients with genotype CT were more likely to die (*P*=0.001). The GG genotype at rs10983755 of the TLR4 gene was remark-

Table 2. Genotype distribution of TLR4 gene at rs7873784, rs10759930 and rs10983755

	Genotype	Control group	ARDS group	OR value	95% CI	χ^2	*P*
rs7873784	CC	32 (0.206)	34 (0.447)	0.88	0.58-1.09	16.31	0.000
	CG	88 (0.568)	25 (0.329)				
	GG	35 (0.226)	17 (0.224)				
rs10759930	CC	46 (0.297)	14 (0.184)	0.91	0.68-1.32	15.59	0.000
	CT	78 (0.503)	28 (0.368)				
	TT	31 (0.200)	34 (0.447)				
rs10983755	AA	33 (0.213)	6 (0.079)	1.12	0.87-1.42	12.85	0.001
	AG	81 (0.523)	58 (0.763)				
	GG	41 (0.265)	12 (0.158)				

Table 3. Correlations of genotypes of TLR4 gene at rs7873784, rs10759930 and rs10983755 with the survival rate, average mechanical ventilation time and average length of ICU stay

	Genotype	n	Survival status			Mean mechanical ventilation time		Average length of ICU stay	
			Death (n)	Survival (n)	*P*	Day	*P*	Day	*P*
rs7873784	CC	34	4	30	0.231	19.88	0.876	24.14	0.751
	CG	25	2	23		17.26		19.23	
	GG	17	2	15		20.17		21.38	
rs10759930	CC	14	2	12	0.001	17.19	0.452	18.16	0.090
	CT	28	9	19		20.19		23.41	
	TT	34	2	32		18.74		21.26	
rs10983755	AA	6	1	5	0.415	15.18	0.003	16.51	0.018
	AG	58	5	53		20.17		21.15	
	GG	12	2	10		22.76		22.91	

Table 4. Haplotype analysis at rs7873784, rs10759930 and rs10983755

Haplotype	Control group	ARDS group	OR value	95% CI	χ^2	*P*
CCA	32.56 (0.105)	11.62 (0.076)	0.705	0.350-1.420	0.965	0.326
CCG	40.76 (0.131)	28.79 (0.189)	1.544	0.915-2.603	2.678	0.102
CTA	43.09 (0.139)	13.73 (0.090)	0.615	0.324-1.168	2.236	0.135
CTG	35.60 (0.115)	21.86 (0.144)	1.295	0.731-2.294	0.786	0.375
GCA	46.83 (0.151)	19.52 (0.128)	0.828	0.469-1.461	0.424	0.515
GCG	49.86 (0.161)	11.07 (0.073)	0.41	0.207-0.811	6.902	0.009
GTA	24.53 (0.079)	27.13 (0.178)	2.528	1.408-4.539	10.134	0.001
GTG	36.79 (0.119)	18.28 (0.120)	1.015	0.559-1.846	0.003	0.960

ably related to the mean mechanical ventilation time (*P*=0.003) and the average length of ICU stay (*P*=0.018).

Haplotype and linkage disequilibrium analyses at rs7873784, rs10759930 and rs10983755 of TLR4 gene

As shown in **Table 4**, the haplotype analysis at rs7873784, rs10759930 and rs10983755 of TLR4 gene manifested that the ARDS group had a lower GCG haplotype frequency (*P*=0.009) and a higher GTA haplotype frequency (*P*=0.001) than the control group. The linkage disequilibrium D' was 0.781 between rs7873-784 and rs10759930 of TLR4 gene, and the two loci were linked to each other (**Table 5**).

Relationships of serum TLR4 concentration with genotypes at rs7873784, rs10759930 and rs10983755 loci

Correlations of serum TLR4 concentration with genotypes at rs7873784 (**Figure 1**), rs10759-

Table 5. Linkage disequilibrium analysis at rs7873784, rs10759930 and rs10983755 of TLR4 gene

Polymorphism locus	rs10759930	rs10983755
rs7873784	0.781	0.048
rs10759930	-	0.051

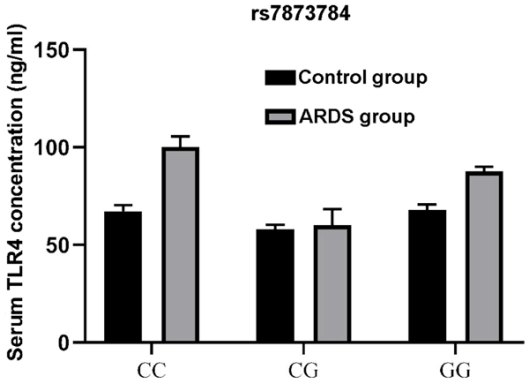

Figure 1. Correlation between serum TLR4 concentration and genotype at rs7873784.

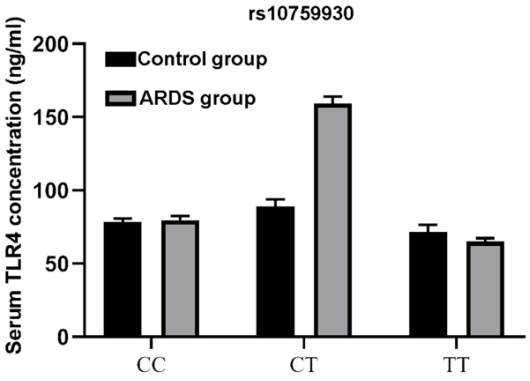

Figure 2. Correlation between serum TLR4 concentration and genotype at rs10759930.

930 (**Figure 2**) and rs10983755 (**Figure 3**) in the control group and ARDS group revealed that the concentration of serum TLR4 with CC genotype at rs7873784 (*P*=0.034), CT genotype at rs7873784 (*P*=0.000) and GG genotype at rs10983755 (*P*=0.000) in the ARDS group was higher than that in the control group.

Discussion

Esophageal cancer has become the 8th most common cancer type, whose mortality ranks 6th among all cancer types, and over 40,000 people die from this disease each year around

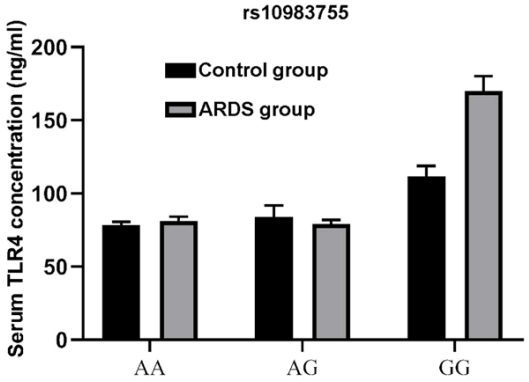

Figure 3. Correlation between serum TLR4 concentration and genotype at rs10983755.

the world [8, 9]. Although increasing progress has been made in treating many other solid tumors, the therapy for esophageal cancer patients is still a great challenge for researchers and clinicians. At present, there are only three basic treatment regimens for esophageal cancer patients, namely, surgical resection, radiotherapy and chemotherapy. The improvement of radiotherapy in recent years has alleviated the pain of esophageal cancer patients with certain efficacy. However, surgical resection of tumor tissues remains the major treatment method for esophageal cancer. Respiratory complications are the most serious risks in surgical treatment, of which ARDS is a major cause of postoperative morbidity and mortality [10, 11]. Exploring the cause of ARDS after esophageal cancer surgery is of significance for reducing postoperative deaths and improving the survival of esophageal cancer patients.

TLR4 is the first human Toll homologue identified, and it is a key molecule in congenital and adaptive immunity. Initially, TLR4 was considered to be expressed only in immune cells, but recent studies have demonstrated that it also exhibits a high expression in cells of certain cancers, including colon cancer [12, 13], pancreatic ductal adenocarcinoma [14], non-small cell lung cancer [15] and hepatocellular carcinoma [16]. According to some researchers, the high expression level of TLR4 is related to the poor prognosis of patients in some cancers [17]. In addition, TLR4 may also exert crucial effects on ARDS after esophageal cancer surgery due to its complex immunoregulation in cancer patients. The single-nucleotide poly-

morphism of TLR4 has an association with susceptibility to multiple diseases, including type 2 diabetes mellitus [18], periodontitis [19] and Alzheimer's disease [20]. Therefore, the polymorphisms at rs7873784, rs10759930 and rs10983755 were analyzed to investigate their correlations with ARDS after esophageal cancer surgery. Analysis of alleles at rs7873784, rs10759930 and rs10983755 of TLR4 gene in the two groups showed that the frequencies of allele G at rs7873784 and allele C at rs10759930 in the ARDS group were remarkably lower than those in control group, suggesting that these two alleles may be related to ARDS after esophageal cancer surgery.

In the meantime, it was discovered that the frequencies of GG genotype at rs7873784 (P=0.000) and CC and CT genotypes at rs10759930 in the control group were notably higher than those in the ARDS group (P=0.000), but the frequency of AA genotype at rs10983755 (P=0.001) in the ARDS group was clearly lower than that in control group. It can be seen that allele G at rs7873784 and allele C at rs10759930 are highly susceptible to ARDS after esophageal cancer surgery. The haplotype analysis at the three polymorphism loci revealed that patients with haplotype GCG were less likely to suffer from ARDS after esophageal cancer surgery (P=0.009), while the opposite result was obtained in patients with haplotype GTA (P<0.01).

The analysis of correlations of genotypes at rs7873784, rs10759930 and rs10983755 of TLR4 gene with the survival status, mean mechanical ventilation time and average length of ICU stay of patients in the ARDS group verified that there was an obvious relationship between CT genotype at rs10759930 of TLR4 gene and the survival status of patients with ARDS after esophageal cancer surgery, and patients with genotype CT were more likely to die (P=0.001). In addition, GG genotype at rs10983755 of TLR4 gene was correlated with the mean mechanical ventilation time (P=0.003) and the average length of ICU stay (P=0.018). The above results prove that the prognosis of esophageal cancer patients with CT genotype at rs10759930 and GG genotype at rs10983755 of the TLR4 gene was poorer after the occurrence of ARDS, with more serious damage to lung tissues and higher difficulty in rescuing. In clinical practice, more emphases should be laid on the postoperative nursing of such patients to reduce the death toll.

Finally, the genotypes at polymorphism loci of the TLR4 gene and the concentration of serum TLR4 were assessed. It was found that the concentration of serum TLR4 in ARDS patients with CC genotype at rs7873784 (P=0.034), CT genotype at rs7873784 (P=0.000) and GG genotype at rs10983755 (P=0.000) of TLR4 gene was higher than that in control group. The serum TLR4 level is a direct factor that affects the immune environment in the body and plays an inflammatory role, whose changes directly influence the severity of ARDS after esophageal cancer surgery. This study confirmed that higher TLR4 concentration in postoperative ARDS patients with CC genotype at rs7873784, CT genotype at rs7873784 and GG genotype at rs10983755 may indicate the occurrence of ARDS. Therefore, clinical personnel should monitor the TLR4 status in patients with the three genotypes in real time and pay attention to and timely respond to postoperative ARDS at any time.

Disclosure of conflict of interest

None.

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