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Association between thyroid cancer and CTLA-4 gene polymorphisms

Liping Sun[1*], Tianfeng Niu[2], Yi Zhang[3]

[1] Endocrine Department, National Medicine Gezhouba Central Hospital, The Third Clinical Medical College of China Three Gorges University, Yichang, 443000, China

[2] General Surgery, National Medicine Gezhouba Central Hospital, The Third Clinical Medical College of China Three Gorges University, Yichang, 443000, China

[3] ICU, National Medicine Gezhouba Central Hospital, The Third Clinical Medical College of China Three Gorges University, Yichang, 443000, China

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ABSTRACT

The study aimed to explore the relationship between cytotoxic T lymphocyte associate protein-4 (CTLA-4) gene polymorphisms and the onset of thyroid cancer. 200 patients with thyroid cancer were included in the disease group and 200 healthy people were selected as the control group (all of them were admitted to Huashan Hospital (East) of Fudan University). The peripheral blood was collected in both groups, and the polymorphic regions at CTLA-4 gene loci rs3087243 (G>A), rs606231417 (C>T) and rs1553657430 (C>A) were amplified via polymerase chain reaction (PCR). The expression level of the CTLA-4 gene was detected via RT-qPCR. Moreover, the associations of clinical indexes with CTLA-4 genotypes were analyzed. The G allele frequency at CTLA-4 gene locus rs3087243 was raised in the disease group (p=0.000). The frequencies of the GG genotype at rs3087243, TT genotype at rs606231417 and CA genotype at rs1553657430were decreased in the control group (p<0.001, p<0.001, p=0.002). The GA+AAfrequency at rs3087243 and CC+CTfrequency at rs606231417 in the disease group were lower than those in the control group. The linkage disequilibrium was higher at rs606231417 and rs1553657430(D'=0.431). Moreover, the CTLA-4 gene expression was remarkably raised in patients with CC genotype at rs1553657430than that in other genotype patients (p<0.05). The genotype at rs606231417was significantly associated with the calcitonin level in thyroid cancer patients (p=0.039), while the genotype at rs3087243 was significantly associated with the thyroid-stimulating hormone level in thyroid cancer patients (p=0.002).CTLA-4 gene polymorphisms have a significant association with the progression of thyroid cancer, which may be a susceptible factor for it.

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Introduction

Thyroid cancer accounts for about 0.5-1% of all malignant tumors in the human body, which is a lowly malignant tumor with a relatively good prognosis(1, 2). The number of female patients with thyroid cancer is about 3 times that of male patients, and the risk of onset increases in postmenopausal women which may be related to the body's disorders of sex hormone levels and the endocrine system (3). The onset of thyroid cancer may be related to radiation, familial inheritance and genetic mutation (4). In addition, immune factors, such as cytokine levels also have an important impact on the development of the disease (5).

Gene polymorphism is an important factor regulating susceptibility to various diseases. The differences in the same allele of different individuals may be the driving factor for changing various pathological processes in the body (6, 7). Gene polymorphism can also affect the growth and development of malignant tumor cells in different individuals. Studies have demonstrated that the prognosis of thyroid cancer is related to MALAT1 rs619586 (8).TheTERT rs10069690 (9), and IL-6 gene polymorphism (10) are related to the thyroid cancer risk. The cytotoxic T lymphocyte associate protein-4 (CTLA-4) gene (a regulatory molecule for immune checkpoint) polymorphism may have a strong association with susceptibility and development of thyroid cancer, immune-related cancer.

In this paper, therefore, we analyzed the polymorphisms (allele and genotype distribution) at CTLA-4 gene loci rs3087243 (G>A), rs606231417 (C>T) and rs1553657430 (C>A), combined the analysis of CTLA-4 gene haplotype and linkage disequilibrium, as well as the CTLA-4 expression and the content of free thyroxine, calcitonin and thyroid-stimulating hormone in patients, so as to explore the association of susceptibility and development process of thyroid cancer with CTLA-4gene polymorphisms.

Materials and Methods

General data

In Huashan Hospital (East) of Fudan University, 200 patients were diagnosed with thyroid cancer (disease group)in the last 3 years, while 200 healthy people (control group) were collected as the objects of study, in the last 3 years. General data had no statistical differences between the two groups (p>0.05). Our study was approved by the Ethics Committee of Huashan Hospital (East) of Fudan University.

* Corresponding author. Email: shachenjiu9881489@163.com

Diagnostic criteria for thyroid cancer patients in the disease group: 1) single or multiple masses in the thyroid gland of the neck, with a hard texture and poor mobility, 2) such symptoms as diarrhea, facial flushing, and hypocalcemia, 3) thyroid cancer indicated in the B-mode ultrasound and radionuclide scanning and 4) thyroid cancer diagnosed via a pathological biopsy.

Sample collection and treatment

Collected 10 mL peripheral blood from both groups, 3000 rpm centrifuged, 8 min. The middle-layer nucleated cells were separated into 1.5 mL centrifuge tubes and used for extraction of genomic deoxyribonucleic acid (DNA) later.

DNA extraction

The genomic DNA was extracted according to the Blood Genomic DNA Extraction Kit(Thermo Fisher) instructions, specifically as follows: 300 μL protease K solution and buffer were added to the sample. Mixed mixture and incubated at 65°C 8min. Then added absolute alcohol into the samples. Finally, it centrifugated and solvents the genomic DNA. The DNA purity was determined using a spectrophotometer.

PCR and CTLA-4gene polymorphism analysis

The CTLA-4genepolymorphic regions loci rs3087243, rs606231417and rs1553657430 were amplified via PCR. The primers of polymorphic loci are as follows: CTLA-4gene locus rs3087243: forward (5'→3'): GCCCTGCACTCTCCTGTTTTT, reverse (5'→3'): GGTTGCCGCACAGACTTCA. rs606231417: forward (5'→3'): CATGATGGGGAATGAGTTGACC, reverse (5'→3'): TCAGTCCTTGGATAGTGAGGTTC. rs1553657430: forward (5'→3'): CATGGTGTCGCCAGCTTTC, reverse (5'→3'): GGTAATCTAGGAAGCCCACTGTA. The products were sent to Jiangxi Biotechnology Co., Ltd. for sequencing, and analyzedCTLA-4gene polymorphic region loci in both groups.

CTLA-4gene expression detection

The CTLA-4gene expression was measured via RT-qPCR in two groups. The gene primers were synthesized by Sangon (Shanghai): CTLA-4: forward (5'→3'): AGTGGGCTTCCTAGATTACCC, reverse (5'→3'): GTCCCGTGTCAACAGCTCTC.

Clinical indexes analysis

The serum-free thyroxine, calcitonin and thyroid-stimulating hormone were detected in the biochemical laboratory. Drawn 5 mL peripheral blood from patients using pro-coagulation tubes, and centrifuged at 3000 rpm, for 5 min. Then the upper-layer serum was taken and detected using a full-automatic biochemical analyzer(normally used after daily routine quality control).

Statistical analysis

We used SPSS 22.0 software for statistical analysis. Enumeration data were compared using the χ^2 test. Haplotype analysis was conducted at the SHEsis website. $p<0.05$ suggested the significant difference.

Results

Allele distribution of CLA-4

The allele distribution at CTLA-4 gene locus rs3087243 was different between the two groups ($p<0.001$), and the G allele frequency was higher in the disease group (Table 1).

Genotype distribution of CTLA-4

The genotype distribution atCTLA-4 gene loci rs3087243 ($p<0.001$), rs606231417 ($p<0.001$) and rs1553657430 ($p=0.002$) were significantly different in the two groups. The frequencies of the GG genotype at rs3087243, TT genotype at rs606231417 and CA genotype at rs1553657430 were higher in the disease group (Table 2).

Table 1. Allele distribution at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430.

Locus	Allele	Control group	Disease group	Odd ratio (OR)	95% confidence interval (CI)	χ^2	*p*
rs3087243	G	201 (0.502)	272 (0.680)	0.47	0.35-0.63	26.07	0.000
	A	199 (0.497)	128 (0.320)				
rs606231417	C	195 (0.487)	170 (0.425)	0.77	0.58-1.02	3.14	0.071
	T	205 (0.512)	230 (0.575)				
rs1553657430	C	212 (0.530)	210 (0.525)	1.02	0.77-1.34	0.021	0.882
	A	188 (0.470)	190 (0.475)				

Table 2. Genotype distribution at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430.

Locus	Genotype	Control group	Disease group	OR	95%CI	χ^2	*p*
rs3087243	GG	55 (0.275)	101 (0.505)	0.89	0.64-1.41	23.83	0.000
	GA	91 (0.455)	70 (0.350)				
	AA	54 (0.270)	29 (0.145)				
rs606231417	CC	44 (0.220)	55 (0.275)	1.02	0.72-1.65	24.12	0.000
	CT	107 (0.535)	60 (0.300)				
	TT	49 (0.245)	85 (0.425)				
rs1553657430	CC	73 (0.365)	55 (0.275)	1.42	1.24-1.54	11.91	0.002
	CA	66 (0.330)	100 (0.500)				
	AA	61 (0.305)	45 (0.225)				

Table 3. Analysis of polymorphisms at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430 in both groups.

	Locus	Genotype	Control group	Disease group	χ^2	*p*
Dominant model	rs3087243	GG+GA	146 (0.730)	171 (0.855)	2.89	0.236
		AA	54 (0.270)	29 (0.145)		
	rs606231417	CC+CT	151 (0.755)	115 (0.575)	8.52	0.024
		TT	49 (0.245)	85 (0.425)		
	rs1553657430	CC+CA	139 (0.695)	155 (0.775)	3.96	0.138
		AA	61 (0.305)	45 (0.225)		
Recessive model	rs3087243	GG	55 (0.275)	101 (0.505)	14.24	0.000
		GA+AA	145 (0.725)	99 (0.495)		
	rs606231417	CC	44 (0.220)	55 (0.275)	1.8	0.407
		CT+TT	156 (0.780)	145 (0.725)		
	rs1553657430	CC	73 (0.365)	55 (0.275)	2.74	0.254
		CA+AA	127 (0.635)	145 (0.725)		
Heterozygous model	rs3087243	GG	55 (0.275)	101 (0.505)	2.91	0.233
		GA	91 (0.455)	70 (0.350)		
	rs606231417	CC	44 (0.220)	55 (0.275)	1.55	0.461
		CT	107 (0.535)	60 (0.300)		
	rs1553657430	CC	73 (0.365)	55 (0.275)	3.46	0.177
		CA	66 (0.330)	100 (0.500)		
Homozygous model	rs3087243	GG	55 (0.275)	101 (0.505)	1.92	0.383
		AA	54 (0.270)	29 (0.145)		
	rs606231417	CC	44 (0.220)	55 (0.275)	3.55	0.169
		TT	49 (0.245)	85 (0.425)		
	rs1553657430	CC	73 (0.365)	55 (0.275)	2.58	0.275
		AA	61 (0.305)	45 (0.225)		

Table 4. Haplotype analysis of CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430.

Haplotype	Control group	Disease group	OR	95%CI	χ^2	*p*
ACA	42.87 (0.107)	31.72 (0.079)	0.717	0.443~1.161	1.84	0.175
ACC	57.71 (0.144)	22.19 (0.055)	0.348	0.209~0.581	17.543	0.000
ATA	41.18 (0.103)	35.48 (0.089)	0.848	0.529~1.360	0.469	0.493
ATC	57.24 (0.143)	38.62 (0.097)	0.64	0.415~0.988	4.111	0.043
GCA	54.83 (0.137)	61.87 (0.155)	1.152	0.777~1.707	0.497	0.481
GCC	39.59 (0.099)	54.22 (0.136)	1.428	0.924~2.207	2.587	0.108
GTA	49.12 (0.123)	60.94 (0.152)	1.284	0.857~1.924	1.472	0.225
GTC	57.47 (0.144)	94.97 (0.237)	1.856	1.292~2.666	11.401	0.001

Table 5. Linkage disequilibrium analysis of CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430.

D'	rs3087243	rs606231417	rs1553657430
rs3087243	-	0.002	0.084
rs606231417	0.002	-	0.431
rs1553657430	0.084	0.431	-

Polymorphismsanalysisat CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430 in both groups

According to the analysis of polymorphisms(Table 3), the distributions of the recessive model at rs3087243(p<0.001) and dominant model at rs606231417 (p=0.024) had differences in two control groups, in which the GA+AAfrequency at rs3087243 and CC+CTfrequency at rs606231417were lower in the disease group.

Haplotype analysis of CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430

The results of haplotype analysis revealed that the distributionsofACC (p<0.001), ATC (p=0.043) and GTC (p=0.001)haplotypes at rs3087243, rs606231417 and rs1553657430 were different between in two groups, in which the linkage disequilibrium was higher at rs606231417 and rs1553657430(D'=0.431) (Tables 4 and 5).

Associationsof genotypes at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430 with gene expression

As shown in Figures 1-3, the CTLA-4 gene expression was remarkably higher in patients with CC genotype

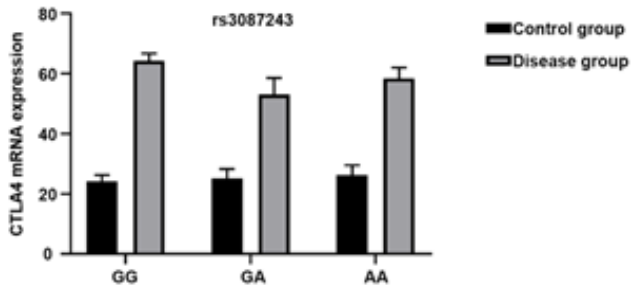

Figure 1. Association between genotypes at CTLA-4 gene locus rs3087243 and gene expression

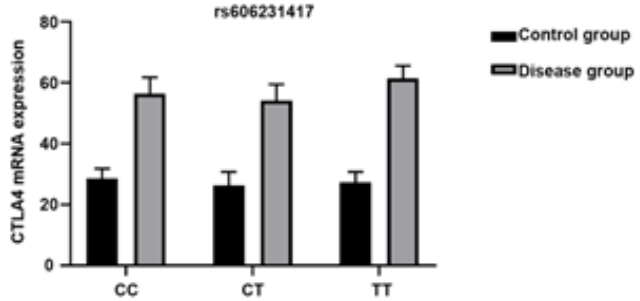

Figure 2. Association between genotypes at CTLA-4 gene locus rs606231417 and gene expression.

at rs1553657430 than in patients with other genotypes (p<0.05).

Associationsof genotypes at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430 with clinical indexes

The genotype at rs606231417 was significantly associated with the calcitonin level in thyroid cancer patients (p=0.039), while the genotype at rs3087243 was significantly associated with the thyroid-stimulating hormone level in thyroid cancer patients (p=0.002) (Table 6).

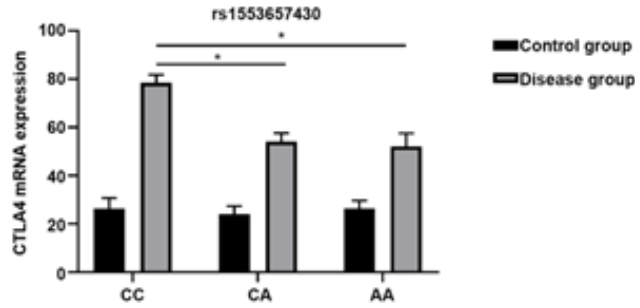

Figure 3. Association between genotypes at CTLA-4 gene locus rs1553657430 and gene expression.

Discussion

Thyroid cancer is the most common disease in the neck, which has a great impact on people's normal work and life. Papillary carcinoma, the most common histological type of thyroid cancer, has a relatively low-grade malignancy and a good prognosis, mainly manifested as local compression symptoms (11), while the undifferentiated thyroid cancer has a higher grade of malignancy and a higher mortality rate, with a poorer prognosis(12). In addition to mutation and changes in expressions of oncogenes and tumor suppressor genes, the changes in the components of the immune system also contribute to the development of the disease (13). The thyroid cancer cells are mainly killed by cytotoxic T cells, NK cells and various cytokines in the immune system(14). However, with the development of the disease, tumor cells will avoid the killing effect of immune cells via altering the expression of autoantigens, so the immune system of thyroid cancer patients fails to inhibit tumor cells (15).

CTLA-4 is the first immune checkpoint discovered, and tumor cells evade the killing of T cells by binding to CTLA-4(16, 17). There have been a number of targeted drugs for CTLA-4 currently, which can well reactivate the immune system in cancer patients (18).CTLA-4 gene polymorphisms could affect the occurrence and development of various diseases, such as Graves' ophthalmopathy (19-23) and systemic lupus erythematosus (20), by affecting its expression level or disease susceptibility. In thyroid cancer, CTLA-4 is also one of the reasons for inhibiting cancer cells from being killed by T cells, which is closely related to the progression of tumors. Therefore, whether CTLA-4 is associated with susceptibility to thyroid cancer was verified in this study. First, according to the analysis of polymorphisms at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430 in genomic DNA in peripheral blood mononuclear cells in thyroid cancer patients and healthy people, the allele distribution at CTLA-4 gene locus rs3087243was different between two groups (p<0.001), and the G allele frequency was higher in the disease group.

Table 6. Associations of genotypes at CTLA-4 gene loci rs3087243, rs606231417 and rs1553657430 with clinical **indexes.**

Locus	Genotype	Calcitonin (ng/L)		Free thyroxine (pmol/L)		Thyroid-stimulating hormone (ulU/mL)	
		Disease group	*p*	Disease group	*p*	Disease group	*p*
rs3087243	GG	52.31±4.24	0.281	32.12±4.24	0.074	4.22±0.88	0.002
	GA	56.13±3.24		35.25±3.12		8.33±0.57	
	AA	51.24±5.35		36.51±3.84		4.76±1.01	
rs606231417	CC	62.35±2.24	0.039	28.34±2.11	0.192	4.16±0.98	0.212
	CT	84.24±5.25		30.41±2.56		4.74±1.21	
	TT	56.34±4.23		32.12±3.34		5.01±0.71	
rs1553657430	CC	57.35±4.11	0.221	30.18±2.15	0.112	4.44±0.84	0.641
	CA	59.35±5.19		31.47±2.14		5.08±1.11	
	AA	62.35±4.65		32.14±4.21		4.39±0.54	

The genotype distribution atCTLA-4 gene loci rs3087243 (p<0.001), rs606231417 (p<0.001) and rs1553657430 (p=0.002) were different in the two groups. The frequencies of the GG genotype at rs3087243, TT genotype at rs606231417 and CA genotype at rs1553657430in the disease group were higher than those in the control group. These findings suggest that CTLA-4 gene polymorphisms can indeed regulate the susceptibility of thyroid cancer, and the risk of thyroid cancer is significantly increased in people with GGgenotype at rs3087243, TT genotype at rs606231417 and CA genotype at rs1553657430. Therefore, CTLA-4 gene polymorphisms can be detected for the initial screening of different populations, and then the populations with specific genotypes can be examined, so as to prevent the occurrence of thyroid cancer.

The different genotypes at the same locus were analyzed in this study, and we found that the distributions of the recessive model at rs3087243(p<0.001) and dominant model at rs606231417 (p=0.024) had differences between the two groups, in which the GA+AAfrequency at rs3087243 and CC+CTfrequency at rs606231417 were lower in the disease group. Besides, according to the combined analysis (haplotype analysis) of CTLA-4 gene loci, the distributionsofACC (p<0.001), ATC (p=0.043) and GTC (p=0.001) haplotype at rs3087243, rs606231417 and rs1553657430 also were different in two groups, and the linkage disequilibrium was higher at rs606231417 and rs1553657430(D'=0.431) in the disease group. The above results showed that the CTLA-4 gene polymorphism effect of thyroid cancer may not be caused by a single genotype at a single locus, but may be influenced by multiple loci or multiple genotypes. Such a conclusion has important implications for clarifying the pathogenesis of thyroid cancer.

Then the CTLA-4 expression was analyzed. We revealed that the CTLA-4 gene expression was remarkably higher in patients with CC genotype at rs1553657430than other genotypes patients (p<0.05), indicating that CTLA-4 gene polymorphisms can affect the CTLA-4 mRNA level, thereby affecting the activity of the immune checkpoint. Moreover, it was found through combining with clinical data that the genotype at rs606231417 was significantly associated with the calcitonin level in thyroid cancer patients (p=0.039), while the genotype at rs3087243 was significantly associated with the thyroid-stimulating hormone level in thyroid cancer patients (p=0.002). The above results suggest thatCTLA-4 gene polymorphisms are associated with not only the susceptibility to thyroid cancer but also the progression of the disease, which needs further research.

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