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RNASET2, *GPR174*, and *PTPN22* gene polymorphisms are related to the risk of liver damage associated with the hyperthyroidism in patients with Graves' disease

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Objectives: This study was designed to unveil the association of *GPR174* rs3827440, *PTPN22* rs3789604, and *RNASET2* rs9355610 with the onset of liver damage (LD) among the Graves' disease (GD) patients.

Methods: A total of 120 GD patients were divided into the none-LD and LD groups. Several indicators were detected for assessing liver functions, and genotypes of single nucleotide polymorphisms (SNPs) were identified. Logistic regression was introduced for investigating the relationship between risk SNPs and LD-associated hyperthyroidism in GD patients.

Results: Significant differences were identified between LD and none-LD groups regarding genotype distributions of rs3827440, rs3789604, and rs9355610. Results from logistic regression indicted that among the GD patients, C carriers of *PTPN22* rs3789604 were associated with a higher risk of LD-associated hyperthyroidism, while C carriers of rs3827440 (*GPR174*) and G carriers of rs9355610 (*RNASET2*) were associated with a reduced risk of LD-associated hyperthyroidism.

Conclusions: The C allele of rs3789604 (*PTPN22*) was a significant risk factor for LD-associated hyperthyroidism in GD patients, whereas C allele of *GPR174* rs3827440 and G allele of *RNASET2* rs9355610 appeared to be a protective factor for this disease.

KEYWORDS
gene polymorphism, *GPR174*, liver damage, *PTPN22*, *RNASET2*

1 | INTRODUCTION

Graves' disease (GD) is the most usual cause of hyperthyroidism, and its prevalence is high at 0.25%~1.09% in China and up to 1.3% in the US.[1,2] The GD patients produce auto-antibodies which bound to the thyroid-stimulating hormone receptor (TSHR), leading to hyperthyroidism and diffuse hyperplasia of the thyroid gland.[3-5] Approximately 70%-80% of untreated GD patients are estimated to have biochemical liver damage (LD).[5] LD will add difficulties to the selection of appropriate GD treatments, particularly for assessing the anti-thyroid drugs (ATDs).[5] What's more, a large number of

studies have confirmed that metabolites of ATDs, particularly propylthiouracil, might contribute to severe hepatotoxicity within GD patients.[6]

To date, some evidence has revealed that GD is a multi-factorial disease, which may result from interactions between susceptible genes and environmental hazards.[3,7] As suggested by family and twin studies, about 79% of predisposition to GD could be attributed to genetic factors.[3] Thus, identifying susceptible genes and loci that are related to hepatic dysfunctions may be critical to discovering novel interventions for GD. Recent studies have screened out a series of genes as potential thyroid disease susceptibility loci,

including *Ribonuclease T2* (*RNASET2*), G protein-coupled receptor 174 (*GPR174*), and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*).[4,8,9]

RNASET2 is discovered as the member of the Rh/T2/S family and is able to encode extra-cellular RNases.[10] The abnormal *RNASET2* expression is probably due to its sub-cellular distribution and the presence of splice variants.[11] Previous studies have implied that RNASET2 may participate in the immune response involved in GD.[12] Investigations on Chinese Han populations have dug out evidence indicating that *RNASET2* polymorphisms especially the loci rs9355610 is significantly associated with the susceptibility to GD.[4] *GPR174* is an Xq21 hypothetical purinergic receptor, and it is extensively expressed in immune and thyroid tissues.[9,13] Furthermore, T allele of *GPR174* rs3827440 has been suggested to be associated with a higher risk of GD in both Chinese and Polish populations.[14] *PTPN22* is located on chromosome 1 (1p13.1–lp13.3) and it encodes human lymphoid tyrosine phosphatase (LYP).[8,15] More importantly, *PTPN22* has been discovered to have significant associations with a series of autoimmune diseases, such as GD, rheumatoid arthritis (RA), systemic lupus erythematosus, and type 1 diabetes.[8,16] Aside from that, *PTPN22* is highly expressed in immune and hematopoietic cells, and it has a negative impact on both T-cell activation and signal transduction of Csk proteins.[3,16] As suggested by Ichimura et al., rs3789604 was a functional SNP of *R620W* within *PTPN22*, and it was related to GD among the Japanese population.[17] Besides, it has been reported that the gain-of-function variants of *PTPN22* might be associated with the rejection in liver transplantation, suggesting that *PTPN22* could also function to regulate the liver damages derived from hyperthyroidism of GD.[18]

Since few studies have clarified the association between genetic risk factors and LD-associated hyperthyroidism in GD patients, this prospective cohort study was specifically designed in order to provide solid evidence for such a relationship. Additionally, investigation of gene polymorphism within *RNASET2*, *GPR174*, and *PTPN22* may contribute to discovering new interventions for LD-associated hyperthyroidism in GD patients.

2 | MATERIALS AND METHODS

2.1 | Subjects

Altogether 120 GD patients were recruited from the First Affiliated Hospital of Nanchang University from March 2014 to April 2015. They were further divided into the none-LD group (n=79) and the LD group (n=41). All the participants were individuals of Han ethnicities, and they all have signed the informed consents. This study was approved by the ethics committee of the First Affiliated Hospital of Nanchang University.

2.2 | Diagnostic criteria of GD

GD was diagnosed based on the following criteria: (1) symptoms or signs of high metabolism were observed; (2) diffuse enlargement

of thyroid gland was existent; (3) laboratory examination showed increased free triiodothyronine (FT_3) and free thyroxine (FT_4), declined thyroid stimulating hormone (TSH) and positive thyroid stimulating hormone receptor (TSHR) antibody; (4) potential symptoms of exophthalmos and other invasive diseases, such as pretibial mucous edema, were present; (5) nodular goiter toxicity, highly functional thyroid adenoma and other diseases that could also cause hyperthyroidism were excluded.

2.3 | Diagnostic criteria of LD in GD patients

The diagnosis of GD-induced LD was based on the following diagnostic criteria: (1) with untreated GD hyperthyroidism; (2) presence of at least one indicator of LD including increase in alanine aminotransferase (ALT) and aspartate transaminase (AST), total bilirubin (TBIL), gamma glutamyltranspeptidase (GGT), and alkaline phosphatase (ALP), declined total protein (TP), serum albumin (ALB), and symptoms of hepatomegaly and jaundice.

2.4 | Collection of specimens

Venous blood samples were collected from patients prior to the therapy. The anticoagulant citrate dextrose (ACD) solution was used for anti-coagulation. The extraction kit for DNA (Teck biological technology limited corporation, Beijing, China) was applied to extract the genome of subjects. Nanodrop2000 (Thermo Scientific, Waltham, MA, USA) was applied for detecting the concentration and purity of DNA, and the purity of sample DNA (A_{260}/A_{280}) was between 1.8 and 2.0. The automatic biochemical analyzer (Sinothinker Technology, Shenzhen, China) was used to evaluate several GD and LD indicators for assistance of diagnosis and patient characterization. Genomic DNA was extracted from samples based on the instructions of DNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) and then preserved in an −20°C refrigerator.

2.5 | Genotyping

SNPs related to GD were chosen and investigated in this study. Three SNPs (rs3827440 within *GPR174*, rs3789604 within *PTPN22* and rs9355610 within *RNASET2*) were included according to their association with GD pathogenicity. Samples were sent to Microread Genetics Go, Ltd (Beijing, China) for direct sequencing and genotyping. They used *Taq*Man probes synthesized by American ABI corporation, and genotyping was conducted based on the Fluidigm EP1 platform of Fluidigm 96×96 Dynamic Array. The results were shown in Figure 1.

2.6 | Statistical analysis

All statistics in this study were analyzed by SPSS 21.00 statistical software. Mann-Whitney test was performed in order to analyze continuous data that were expressed as the mean±SD between two groups. Chi-square test was carried out in order to analyze categorical data. Logistic regression which incorporated the OR and the corresponding
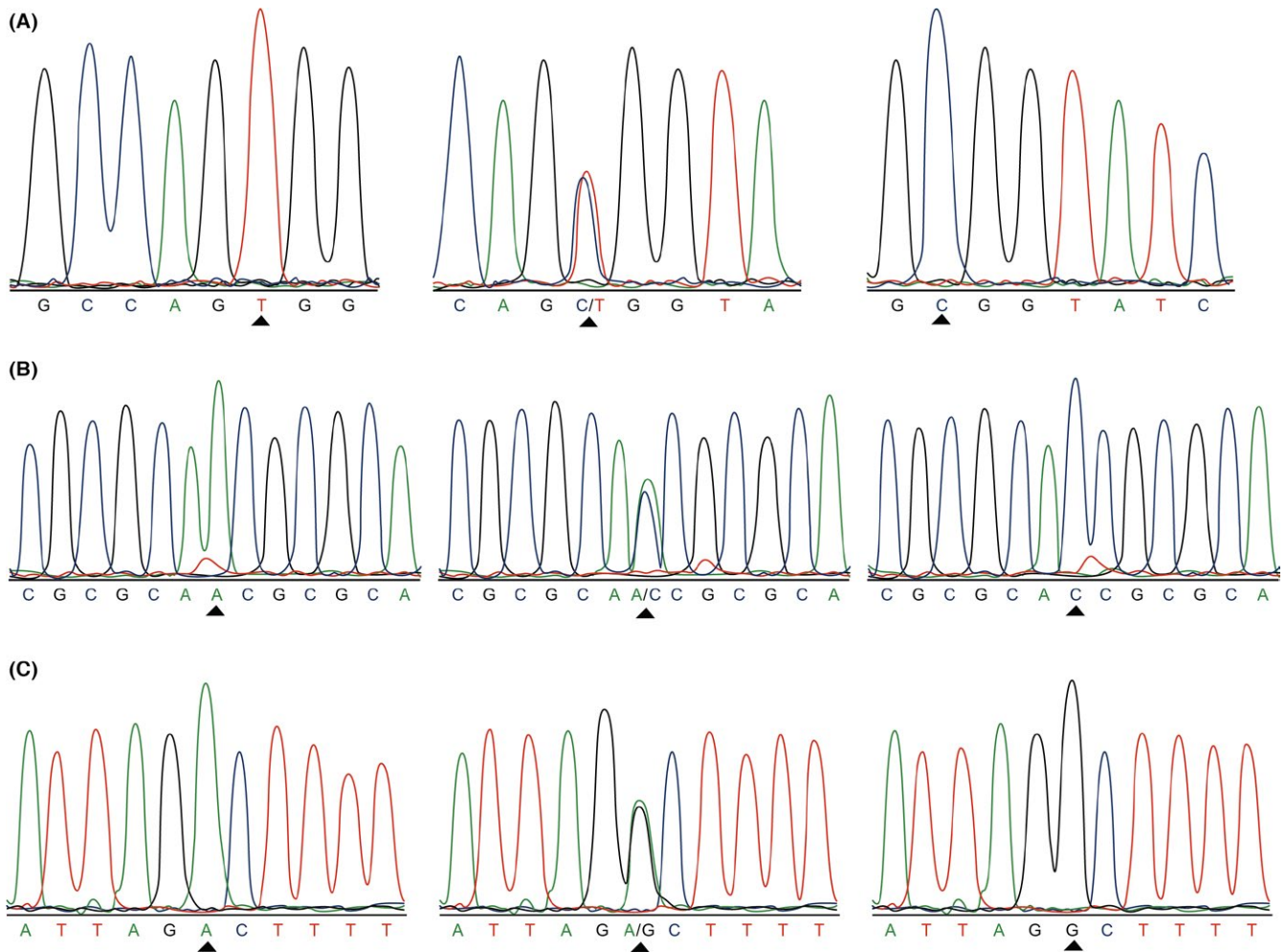
FIGURE 1 Direct sequencing and genotyping for *GPR174*, *PTPN22* and *RNASET2* among the GD patients with LD. (A) Direct sequencing results of rs3827440 polymorphisms in *GPR174*: T, C/T and C. (B) Direct sequencing results of rs3789604 polymorphisms (A/C) in *PTPN22*: A, A/C and C. (C) Direct sequencing results of rs9355610 polymorphisms (A/G) in *RNASET2*: A, A/G and G

95% confidence interval was specifically used to assess the influence of risk loci for LD associated with the hyperthyroidism in GD patients. The power calculation was performed with the software of ps power. *P*-value of less than .05 provided evidence for statistical significance.

3 | RESULTS

3.1 | Characteristics of study participants

The sample size was found to be robust after power calculation with the software of ps power. As shown in Table 1, 120 GD patients were enrolled, including 41 LD cases. The LD group consisted of 11 males and 30 females with an average age of (38.1±10.9) years old. The none-LD group included 20 males and 59 females with the average age of (41.6±11.4) years old. In addition, gender, age, as well as the levels of GD indicators, including FT_3, FT_4 and TSH, showed little discrepancy between the LD and non-LD groups (*P*>.05). As for LD indicators, ALT, AST, GGT, TBIL and ALP presented significantly increased levels in LD patients, while TP and ALB displayed declined levels in the LD group in comparison with those in the non-LD group (*P*<.05).

3.2 | Association between risk SNPs and LD in GD patients

The detailed genotype and allele frequency distribution of *GPR174* rs3827440, *PTPN22* rs3789604, and *RNASET2* rs9355610 were shown in Table 2, and the approach of logistic regression was applied to identify the risk factors for the onset of LD-associated hyperthyroidism among GD patients. After adjusting age, gender and other confounding factors, the results of logistic regression suggested that the frequency of rs3827440 C allele in LD patients was significantly lower than that in the non-LD group (TC vs TT: OR=0.13; 95% CI: 0.04-0.48; *P*=.002; CC vs TT: OR=0.12; 95% CI: 0.02-0.69; *P*=.018; TC+CC vs TT: OR=0.24; 95% CI: 0.08-0.67, *P*=.007; C vs T: OR=0.31; 95% CI: 0.14-0.68; *P*=.004), which provided an evidence that C allele of rs3827440 might act as a protective factor in preventing GD patients from the onset of LD. As for rs3789604 of *PTPN22*, it was indicated in the form of raw ORs that the C allele might increase the risk of LD in GD patients (AC vs AA: OR=1.36, 95% CI: 0.57-3.25, *P*=.483; CC vs AA: OR=8.81; 95% CI: 2.49-31.25, *P*<.001; AC+CC vs AA: OR=2.30, 95% CI: 1.06-4.97, *P*=.033; C vs A: OR=2.79, 95%

TABLE 1 The basic characteristics of GD patients in none-LD group and LD group

Group	None-LD group (n=79)	LD group (n=41)	P value
Age	41.6±11.4	38.1±10.9	.328*
Gender			
Male	20	11	.858**
Female	59	30	
ALT (0~40 U/L)	25.2±6.5	68.4±35.7	<.001*
AST (29~35 U/L)	30.0±7.0	50.5±19.1	<.001*
GGT (3~50 U/L)	37.0±20.1	62.9±27.2	<.001*
TBIL (3.4~17.1 µmol/L)	14.7±5.3	19.9±8.2	<.001*
ALP (40~150 U/L)	127.3±58.7	171.2±57.1	<.001*
TP (60~80 g/L)	60.4±1.8	54.8±5.2	<.001*
ALB (35~55 g/L)	38.7±2.1	33.7±2.9	<.001*
FT_3 (3~9 pmol/L)	14.3±6.8	16.8±8.9	.089*
FT_4 (9~25 pmol/L)	52.6±20.7	59.6±18.9	.073*
TSH (0.04-2.5 mIU/L)	0.016±0.003	0.015±0.004	.126*

GD, Graves' disease; none-LD group, GD patients without LD were included; LD group, GD patients with LD were included; ALT, Alanine aminotransferase; AST, Aspartate transaminase; GGT: Gamma-glutamyl transferase; TBIL, Total bilirubin; ALP, Alkaline phosphatase; TP, Total protein; ALB, Serum albumin; FT_3, Free triiodothyronine; FT_4, Free thyroxine; TSH, thyroid stimulating hormone.

*Represents that P value was related to none-LD and LD group and was analyzed by Mann-Whitney test.

**Represents that P value was related to none-LD and LD group and was analyzed by Chi-square test.

CI: 1.57-4.94, P<.001). In addition, the adjusted ORs also attained a similar trend as the raw ORs (AC vs AA: OR=1.80, 95% CI: 0.57-5.69, P=.315; CC vs AA: OR=35.15, 95% CI: 5.41-228.56, P<.001; AC+CC vs AA: OR=3.09, 95% CI: 1.13-8.47, P=.028; C vs A: OR=5.82, 95% CI: 2.66-12.77, P<.001). Under all genetic models, it was also revealed that the G allele of *RNASET2* rs9355610 might play a protective role against LD in GD patients (GA vs GG: OR=0.10, 95% CI: 0.03-0.31, P<.001; AA vs GG: OR=0.09, 95% CI: 0.02-0.52, P=.007; GA+AA vs GG: OR=0.17, 95% CI: 0.07-0.42, P<.001; A vs G: OR=0.25, 95% CI: 0.13-0.50, P<.001).

4 | DISCUSSION

GD is an autoimmune disorder where autoantibodies target antigens expressed in thyroid tissues and hence hyperthyroidism is promoted.[14,19,20] Consensus has been reached that several genetic and environmental factors would affect GD.[2,14] On the other hand, there has been evidence suggesting that part of untreated GD patients tend to suffer from LD.[5] Thus, we attempted to investigate the relation of genetic polymorphisms and the onset of LD-associated hyperthyroidism among GD patients.

It has been suspected that GD may be affected by various susceptible genes,[3] and sequencing enabled us to discover that different genotypes were identified in rs3827440 (*GPR174*), rs3789604 (*PTPN22*) and rs9355610 (*RNASET2*). More importantly, the frequency of *GPR174* rs3827440 C/T polymorphism, *PTPN22* rs3789604 A/C polymorphism and *RNASET2* rs9355610 G/A polymorphism significantly differed among the LD and non-LD groups. For rs3827440, T allele carriers were associated with a higher risk of hyperthyroid LD compared with C allele carriers. Similarly, C allele carriers of rs3789604 may experience a higher risk of hyperthyroid LD when compared with the A allele carriers, whereas G allele carriers of rs9355610 were related with an increased risk of hyperthyroid LD in comparison to the A allele carriers. As suggested by recent studies, lysophosphatidylserine (LysoPS), a ligand of *GPR174*, had an important role in regulating the immune system.[21,22] Moreover, LysoPS was able to stimulate intracellular cyclic adenosine monophosphate (cAMP) by interacting with *GPR174*.[22] The elevated cAMP could inhibit the production of T-helper 1 (Th1) cytokines, and stabilize or enhance the level of T-helper 2 (Th2) cytokines.[23] Graves's disease was a Th2-related disorder, and increased cAMP may contribute to the polarization of Th2 and hence facilitation of GD-derived LD pathogenesis.[9] Another research revealed that *GPR174* was extensively expressed in immune-related tissues, and was moderately expressed in thyroid tissues.[9] Besides that, variants of *PTPN22* have been considered as a significant risk factor for GD, since they triggered irregular antigen presentation and T-cell activation in immunological synapses.[24]

In this study, C allele of rs3789604 was a significant risk factor for LD, while C allele of rs3827440 and G allele of rs9355610 appeared to be a protective factor for LD-associated hyperthyroidism in GD. Several susceptible genes that are related with GD have been discovered in different ethnic populations.[3] *PTPN22* rs2476601 was initially discovered as a susceptible locus for autoimmune diabetes. Studies on Asian populations have suggested that rs2476601 may play a significant role in GD.[25-28] However, genome wide association study (GWAS) has provided contradictory results that SNPs of *PTPN22* were not significantly associated with the autoimmune diseases among Asians.[3]

So far, a large number of researches on GD were devoted to unveiling the correlation between Graves' disease and rs2476601.[29,30] Some studies indicated that rs2476601 rarely influenced the onset of GD in the Chinese Han population,[3] while others provided evidence that rs3789604 also had an association with GD.[31] Meanwhile, rs3789604 was strongly associated with GD among a Japanese population and such an association was confirmed by a study on the Chinese population.[25,26] Since this study investigated GD-derived LD, it was reasonable to believe that the aforementioned SNPs were associated with GD-derived LD.

Several limitations in this study should be addressed carefully. This study only indicated that the distribution of *GPR174* rs3827440 polymorphisms significantly differed among the three groups. However, whether this trend was related with GD-derived LD remained vague, hence studies on the mechanisms underlying the correlation between genotypes and GD-derived LD were encouraged in the future.

TABLE 2 The genotype distribution of three SNPs in none-LD group and LD group

Gene	Genotype	LD group (n=41)	None-LD group (n=79)	P value[a]	Raw OR (95% CI)	P value[b]	Adjusted OR (95% CI)
GPR174	rs3827440						
	TT	23	34	Ref.	Ref.	Ref.	Ref.
	TC	12	28	.296	0.63 (0.27-1.50)	.002	0.13 (0.04-0.48)
	CC	6	17	.230	0.52 (0.18-1.53)	.018	0.12 (0.02-0.69)
	TC+CC	18	45	.276	0.59 (0.28-1.27)	.007	0.24 (0.08-0.67)
	T	58	96	Ref.	Ref.	Ref.	Ref.
	C	24	62	.127	0.64 (0.36-1.50)	.004	0.31 (0.14-0.68)
PTPN22	rs3789604						
	AA	16	47	Ref.	Ref.	Ref.	Ref.
	AC	13	28	.483	1.36 (0.57-3.25)	.315	1.80 (0.57-5.69)
	CC	12	4	<.001	8.81 (2.49-31.25)	<.001	35.15 (5.41-228.56)
	AC+CC	25	32	.033	2.30 (1.06-4.97)	.028	3.09 (1.13-8.47)
	A	45	122	Ref.	Ref.	Ref.	Ref.
	C	37	36	<.001	2.79 (1.57-4.94)	<.001	5.82 (2.66-12.77)
RNASET2	rs9355610						
	GG	27	20	Ref.	Ref.	Ref.	Ref.
	GA	12	41	<.001	0.22 (0.09-0.52)	<.001	0.10 (0.03-0.31)
	AA	2	18	<.001	0.08 (0.02-0.40)	.007	0.09 (0.02-0.52)
	GA+AA	14	59	<.001	0.18 (0.08-0.40)	<.001	0.17 (0.07-0.42)
	G	66	81	Ref.	Ref.	Ref.	Ref.
	A	16	77	<.001	0.26 (0.14-0.48)	<.001	0.25 (0.13-0.50)

GD, Graves' disease; LD, Liver damage; OR, Odds ratios; CI, Confidence interval; Ref., Reference.
[a]P value for raw OR.
[b]P value for adjusted OR.

In summary, *GPR174* rs3827440, *PTPN22* rs3789604, and *RNASET2* rs9355610 were significantly associated with altered GD-derived LD risk. Ongoing researches should be further devoted to this so that potential interventions for LD resulted from GD may be discovered.

5 | CONCLUSION

The C allele of rs3789604 (*PTPN22*) was a significant risk factor for LD associated with the hyperthyroidism in GD patients, whereas C allele of rs3827440 (*GPR174*) and G allele of rs9355610 (*RNASET2*) appeared to be a protective factor for this disease. The result of this research would provide several new loci for the diagnosis and targeted therapy of LD associated with the hyperthyroidism in GD in clinical application.

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