Association Between Promoter Polymorphisms of the *GRP78* Gene and Risk of Type 2 Diabetes in a Chinese Han Population

Shengyuan Liu,^{1,2,*} Keshen Li,^{1,*} Tao Li,³ Xingdong Xiong,³ Songpo Yao,⁴ Zhongwei Chen,² Changyi Wang,² and Bin Zhao⁵

There are large amounts of unfolding or misfolding protein accumulation in the endoplasmic reticulum in patients with type 2 diabetes (T2D), which in turn induces the expression of the glucose-regulated protein 78 (GRP78) that plays a key role in influencing insulin secretion and maintaining glucose homeostasis in pancreatic beta cells. The aim in the study is to analyze the potential association between single-nucleotide polymorphisms (SNPs) of *GRP78* and the risk of T2D. To assess the association between *GRP78* polymorphisms and T2D, a case-control study was conducted among 1058 consecutive unrelated subjects. Of the 1058 subjects, 523 of them were diagnosed with T2D and 535 of them were healthy controls. Four SNPs with $R^2 > 0.8$ and the minor allele frequency > 0.05 (rs391957, rs17840761, rs17840762, and rs11355458) in the *GRP78* gene promoter were analyzed. Overall, no associations of *GRP78* polymorphisms with T2D were observed in genotypic analyses. In addition, haplotypes combining those SNPs in the promoter in high linkage disequilibrium were also not associated with a T2D risk. However, the levels of fasting plasma glucose and HbA1c in patients with the –415AA/–180GG genotype were significantly lower than those of the patients with –415GG/–180deldel and –415AG/–180Gdel genotypes, and the level of fasting insulin in patients with the –415AA/–180GG genotype was significantly lower than that of the patients with –415GG/–180deldel. The study does not support a role for promoter polymorphisms of *GRP78* in T2D in a Chinese Han population, but it does provide a clue for association between low levels of fasting plasma glucose, HbA1c and fasting insulin, and the –415AA/–180GG model.

Introduction

TYPE 2 DIABETES (T2D) is recognized as a heterogeneous disorder with the common elements of insulin resistance (IR) and relative insulin deficiency affecting approximately 366 million people and causing 4.6 million deaths, and has been recognized as one of the most challenging health problems in the 21st century (IDF, 2012). Therefore, it is particularly urgent to gain an insight into the pathogenesis of T2D to discover different possibilities of preventive and effective treatment.

Despite distinct differences in the etiology of T2D, the pathophysiological manifestation that all etiologies lead to is characterized by IR and islet cell dysfunction. Recently, some evidence has revealed that the two manifestations were associated with a specific cellular response known as the en-

doplasmic reticulum (ER) stress response or the unfolded protein response that had an important function in synthesis, posttranslational modification and folding of a protein, and maintenance of the Ca²⁺ balance in a cell (Muoio and Newgard, 2004; Ozcan *et al.*, 2004). Due to the effect of external factors (high blood sugar or excessive saturated fatty acid), many unfolded or misfolded proteins are not processed and are accumulated in the ER of the pancreatic beta cells (β cells), which induces ER stress. To alleviate the situation, these proteins must be refolded or degraded by activating a ER stress response (Ron and Walter, 2007). In the stress response, the glucose-regulated protein 78 (GRP78), a major molecular chaperone in the lumen of the ER and referred as HSPA5 (Heat shock 70-kDa protein 5) or BiP (immunoglobulin heavy chain-binding protein), has been shown to modulate insulin sensitivity and maintain glucose

¹Key Laboratory of Neurodegenerative Disease and Aging Research, Affiliated Hospital of Guangdong Medical College, Zhanjiang, China.

²Department of Chronic Disease, Shenzhen Nanshan Center for Chronic Disease Control, Shenzhen, China.

³Key Laboratory for Medical Molecular Diagnostics of Guangdong Province, Guangdong Medical College, Dongguan, China.

⁴Department of Epidemiology and Statistics, College of Public Health, Jiamusi University, Jiamusi, China.

⁵Institute of Neurology, Affiliated Hospital of Guangdong Medical College, Zhanjiang, China.

*These authors contributed equally to this work.

homeostasis (Kammoun *et al.*, 2009) by binding to unfolded proteins and regulating the activation of ER stress transducers such as IRE1, PERK, and ATF6 (Ron and Walter, 2007). Based on this background, the expression of the GRP78 protein may be used extensively as a biological marker for the onset of the ER stress response in β cells. Meanwhile, the induction of GRP78 expression after ER stress may be determined by genetic variations in the promoter region of the *GRP78* gene, and therefore, these polymorphisms may provide a link between ER stress and T2D.

Several studies have investigated the associations of *GRP78* polymorphisms with diseases among populations. Kakiuchi *et al.* (2005) suggested that promoter polymorphisms of *GRP78* may affect the interindividual variability of the ER stress response and may confer a genetic risk factor for bipolar disorder. Hsu *et al.* (2008) reported that *GRP78* may increase susceptibility to Alzheimer's disease among Taiwanese. Zhu *et al.* (2011) demonstrated that the polymorphisms of *GRP78* constituted a risk factor for the development of advanced liver cirrhosis in one study, and was associated with reduced survival and a higher prevalence of early relapses in advanced patients with nonsmall cell lung cancer in another study. To date, whether the common polymorphisms in the *GRP78* gene are associated with T2D in the Han Chinese population is unclear.

The aim of this study is to explore the associations of *GRP78* polymorphisms with T2D in a Han Chinese population.

Materials and Methods

Study population and characteristics

Consecutive new patients with T2D as case series who had not been treated were enrolled in several hospitals from the Beijing and Harbin area of northern China. T2D was diagnosed based on the diagnostic criteria defined by WHO in 1999 (Alberti and Zimmet, 1998) and the American Diabetes Association in 2003 (Genuth *et al.*, 2003) (fasting plasma glucose ≥ 7.0 mM and/or 2-h plasma glucose ≥ 11.1 mM). Meanwhile, unrelated controls were recruited from healthy people who had undergone physical examination in the above hospitals. Subjects who had an evidence of history of cancers, cardiovascular and cerebrovascular diseases, mood disorders, type diabetes 1, and alcoholism were excluded. A written informed consent was obtained from each participant after the study was explained in detail. Ethical approval was obtained for the study from the relevant ethics committees.

The demographic, physical, and biochemical characteristics were extensively assessed from both the T2D subjects and the control subjects. The characteristics in our study included age, gender, smoking, drinking, height, weight, body mass index (BMI), waist/hip ratio, fasting glucose, fasting insulin, HbA1c, HOMA-IR, total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL).

Measurement methods

The height and weight were measured in light indoor clothes and without shoes, and the BMI was calculated as weight (kg)/height² (m²). The waist circumference was

measured in an upright position to the nearest 0.5 cm of the umbilical level among subjects according to WHO recommendation. The hip was recorded at the widest part of the hips, corresponding to the groin level for women and about 2–3 inches below the navel in men. The plasma concentration of glucose was analyzed by an automated glucose oxidase method. Plasma insulin was measured with ALPCO human Insulin ELISA Kit (ALPCO). HbA1c was evaluated by the ion-exchange high-pressure liquid chromatography (HPLC) method. Insulin sensitivity was assessed using the homeostatic model assessment (HOMA), in which the homeostasis model of insulin resistance (HOMA-IR) = [fasting insulin (μ U/mL)] $\times$ [fasting glucose (mM)] / 22.5 (μ U/mL = pmol $\div$ 6.965).

SNP selection and genotyping

Candidate SNPs in *GRP78* were selected as follows: (1) SNPs of the promoter region, (2) SNPs from public literatures and databases; (3) SNPs that previously were reported to be associated with a disease outcome (for instance, bipolar disorder, Alzheimer's disease, liver cirrhosis, and lung cancer) in epidemiological studies, and (4) SNPs with a minor allele frequency $> 5\%$. Finally, four tag SNPs (the promoter: rs391957, rs17840761, rs17840762, and rs11355458) were selected and genotyped.

Genomic DNA was extracted from peripheral blood leukocytes using the QIAGEN QIAamp DNA Mini Blood Kit. A total of 50 ng genomic DNA was amplified in a 100 μ L final volume PCR reaction containing 10 $\times$ buffer, 200 μ M each of dATP, dCTP, dGTP, dTTP, 1.5 mM MgCl₂, 10 pmol of each primer, and 0.5 unit *Taq* polymerase (Takara). Two sets of primers were designed: the primers for rs391957 (–415G/A), rs17840762 (–378C/T), and rs17840761 (–370C/T) were 5'-TCAGAGACTGGATGGAAGCTGG-3' (forward primer), 5'-TGGCTGCTATTCGTTTCTAACG-3' (reverse primer), and the primers for rs11355458 (–180del/G) were 5'-hex-CGGG GTCAGAAGTCGCAGGAGAGAT-3' and 5'-CGTTGGAGG CCGTTCATTGG-3' (Chen *et al.*, 2008). The amplification was performed at 95°C for 5 min with an initial denaturation, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension of 5 min at 72°C. 1 μ L of amplified PCR products was mixed with 1 μ L of formamide containing dextran blue dye, which was subjected to 3% agarose gels and visualized by staining with ethidium bromide. The SNPs were detected by the TaqMan Assay-by-Design service (Applied Biosystems). The details of the probe and reaction conditions were available upon request (<https://products.appliedbiosystems.com/ab/en/US/adirect/ab>). PCR was performed on the ABI PRISM 7900 HT Sequence Detection System by using the TaqMan Universal Master Mix without UNG (Applied Biosystems) and heated to 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

Statistical analysis

Before statistical analysis, normal distribution was evaluated using the Shapiro-Wilk test, and then variables were given a logarithmic transformation when necessary. The U-test for quantitative data and the Chi-square test for qualitative data were used to determine whether there were significant differences in relevant factors between cases and

controls. The Chi-square test was carried out to assess the deviations from the Hardy-Weinberg equilibrium (HWE) and frequencies of the genotype and allele of *GRP78* among cases and controls. Haplotype analyses were conducted using the Haploview version 3.2.0 (Whitehead Institute for Biomedical Research) (Barrett *et al.*, 2005). The most common haplotype served as the referent, which happened to be the wild-type allele for all three SNPs. Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were estimated to compare cases to controls in association with genotypes, alleles, and haplotypes. All statistical tests were two-sided with SPSS 11.0 and the statistical significance was taken as the *p* value less than 0.05.

Results

Baseline characteristics

The baseline characteristics of all participants in the study are summarized in Table 1. In 1058 participants, 523 were T2D patients and 535 were healthy controls. The T2D and control subjects were matched on age and gender. The mean age was 58.56 years (± 5.87 years; range, 30–78 years) for the T2D subjects and 58.94 years (± 6.83 years; range, 31–80 years) for the control subjects ($p=0.3248$). The gender (male/female) ratio was 1.29:1 in the T2D patients and the gender ratio was 1.24:1 in the controls ($p=0.7240$). Some factors were higher among the cases than among the controls like the BMI ($p=0.0128$), the waist/hip ratio ($p<0.0001$), fasting glucose ($p<0.0001$), fasting insulin ($p<0.0001$), HbA1c ($p<0.0001$) and HOMA-IR ($p<0.0001$), other factors like total cholesterol ($p=0.0006$) and HDLC ($p=0.0005$) were higher in controls.

Polymorphisms of *GRP78* gene and the risk of T2D

The genotype and allele frequencies of four SNPs in the study are shown in Table 2. The deviation from the Hardy-

Weinberg equilibrium for all the polymorphisms examined was not found in the distributions of genotypes in cases and controls (data not shown).

For $-415G/A$ and $-180del/G$, all individuals tested had identical polymorphic genotypes. Comparison of genotype distributions and allele frequencies between T2D subjects and control subjects by the χ^2 -test revealed that there were no statistically significant differences between cases and controls among genotypes, as well as the presence of single variant alleles. In addition, no significant differences were observed among haplotypes (Table 3).

The association of *GRP78* gene polymorphisms with biochemical parameters

The relationships of SNPs to fasting glucose, fasting insulin, HbA1c, and HOMA-IR were analyzed in the T2D subjects and control subjects. Among the examined SNPs (Table 4), patients with $-415AA/-180GG$ had lower levels of fasting plasma glucose ($P_{-415GA/-180delG}$ vs. $-415AA/-180GG=0.0001$, $P_{-415GG/-180deldel}$ vs. $-415AA/-180GG<0.0001$) and HbA1c ($P_{-415GA/-180delG}$ vs. $-415AA/-180GG<0.0001$, $P_{-415GG/-180deldel}$ vs. $-415AA/-180GG<0.0001$) as well as more HOMA-IR ($P_{-415GA/-180delG}$ vs. $-415AA/-180GG<0.0001$, $P_{-415GG/-180deldel}$ vs. $-415AA/-180GG<0.0001$) than the patients with $-415GG/-180deldel$ and $-415GA/-180delG$ had. In addition, patients with $-415AA/-180GG$ had a lower level of fasting insulin ($P_{-415GG/-180deldel}$ vs. $-415AA/-180GG=0.009$) than the patients with $-415GG/-180deldel$ had. No other SNPs were associated with biochemical parameters.

Discussion

ER stress-mediated apoptosis plays an important role in the destruction of β cells and contributes to the development of T2D. In the stress, ER chaperones can protect against T2D by inhibiting the accumulation of unfolded or misfolded proteins in β cells (Kammoun *et al.*, 2009).

TABLE 1. BASELINE CHARACTERISTICS OF SUBJECTS IN CASE GROUP AND CONTROL GROUP

Characteristics	Case group	Control group	p-Value
Total, N	523	535	
Mean age (years)	58.56 $\pm$ 5.87	58.94 $\pm$ 6.83	0.3248
Gender			0.7240
Male	295 (56.41%)	296 (55.33%)	
Female	228 (43.59%)	239 (44.67%)	
Smoking	76 (14.53%)	98 (18.32%)	0.0967
Drinking	64 (12.24%)	85 (15.89%)	0.0879
Family history of diabetes	204 (39.01%)	226 (42.24%)	0.2838
BMI (kg/m ²)	25.28 $\pm$ 4.21	24.64 $\pm$ 4.14	0.0128
Waist/hip ratio	0.89 $\pm$ 0.02	0.86 $\pm$ 0.01	<0.0001
Fasting glucose (mM)	8.93 $\pm$ 1.38	5.19 $\pm$ 0.81	<0.0001
Fasting insulin (pM) ^a	86.1 $\pm$ 4.37	53.6 $\pm$ 2.82	<0.0001
HbA1c (mmol/mol) [%]	73 $\pm$ 13 [8.8 $\pm$ 1.16]	34 $\pm$ 10 [5.3 $\pm$ 0.92]	<0.0001
HOMA-IR ^a	5.31 $\pm$ 0.46	2.08 $\pm$ 0.19	<0.0001
Total cholesterol (mM)	4.35 $\pm$ 0.79	4.49 $\pm$ 0.85	0.0006
Triglyceride (mM)	1.72 $\pm$ 0.98	1.64 $\pm$ 0.87	0.1603
HDL-C (mM)	1.08 $\pm$ 0.21	1.15 $\pm$ 0.30	0.0005
LDL-C (mM)	2.71 $\pm$ 0.78	2.78 $\pm$ 0.79	0.1474

Continuous data are expressed as the means $\pm$ SEM.

^aThe logarithms of these variables were used for the analysis.

BMI, body mass index; HDLC, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

TABLE 2. GENOTYPE AND ALLELE FREQUENCIES OF THE *GRP78* PROMOTER POLYMORPHISMS, AND THEIR ASSOCIATIONS WITH RISK OF TYPE 2 DIABETES

Reference SNP ID	Genotype and allele	Case group (N=523) n (%)	Control group (N=535) n (%)	OR (95%CI)	χ^2	p-Value
rs391957 (–415G/A)	GG	295 (56.41)	311 (58.13)	1		
	AG	204 (39.01)	205 (38.32)	1.049 (0.816–1.348)	0.14	0.7081
	AA	24 (4.59)	19 (3.55)	1.332 (0.715–2.482)	0.82	0.3659
	G	794 (75.91)	827 (77.29)	1		
	A	252 (24.09)	243 (22.71)	1.080 (0.883–1.321)	0.56	0.4529
rs17840761 (–370C/T)	CC	144 (27.53)	127 (23.74)	1		
	CT	244 (46.65)	253 (47.29)	0.851 (0.632–1.144)	1.15	0.2843
	TT	135 (25.81)	155 (28.97)	0.768 (0.551–1.071)	2.43	0.1191
	C	532 (50.86)	507 (47.38)	1		
	T	514 (49.14)	563 (52.62)	0.870 (0.734–1.032)	2.56	0.1097
rs17840762 (–378C/T)	CC	347 (66.35)	369 (68.97)	1		
	CT	147 (28.11)	144 (26.92)	1.086 (0.827–1.426)	0.35	0.5549
	TT	29 (5.54)	22 (4.11)	1.402 (0.790–2.487)	1.34	0.2463
	C	841 (80.40)	882 (82.43)	1		
	T	205 (19.60)	188 (17.57)	1.144 (0.918–1.424)	1.44	0.2303
rs11355458 (–180del/G)	dd	295 (56.41)	311 (58.13)	1		
	dG	204 (39.01)	205 (38.32)	1.049 (0.816–1.348)	0.14	0.7081
	GG	24 (4.59)	19 (3.55)	1.332 (0.715–2.482)	0.82	0.3659
	d	794 (75.91)	827 (77.29)	1		
	G	252 (24.09)	243 (22.71)	1.080 (0.883–1.321)	0.56	0.4529

OR, odds ratios; CI, confidence interval.

GRP78, one of the most important chaperones in ER, composed of the ATP enzyme domain and the unfolded protein domain, may play a key role in monitoring protein transport through the β cells. When unfolded or misfolded proteins increase in β cells, the expression of the GRP78 is induced and products interact with the unfolded/misfolded protein. When the correct protein conformation is achieved, the GRP78 disassociates with the unfolding protein and releases from the unfolded protein (Ma and Hendershot, 2004). Wang *et al.* (2009) have suggested that GRP78 was associated with the response of β cells to ER stress and may be important in the improvement of diabetes. Chien *et al.* (2010) have shown that GRP78 could detect and bind misfolded β cell peptide hA oligomers, effectively preventing hA into β -sheet-containing oligomers that was linked to β cell apoptosis and the pathogenesis of T2D. Laybutt *et al.* (2007) have demonstrated that an increased islet protein production of GRP78-associated protein was observed in human pancreas sections of T2D subjects.

Promoter variants of *GRP78* may exert functional effects on the activity itself, and thus, may lead to aberrant GRP78 expression. Using *in vitro* experimental approaches, Hsu *et al.* have shown that the basal *GRP78* transcriptional activity of the –415A/–180G allele was significantly lower compared

with the –415G/–180del alleles. However, in response to ER stress, GRP78 protein expression was markedly increased in the cells harboring the –415A/–180G allele, suggesting that *GRP78* promoter polymorphisms might affect the interindividual variability of the ER stress response. Based on these data, it is hypothesized in our study that *GRP78* promoter polymorphisms may be potentially associated with T2D. However, our study did not demonstrate a relationship of the *GRP78* polymorphisms to genotypes and haplotypes in the studied population. While we could not demonstrate the relevance for *GRP78* promoter genotypes on AD risk in the population, our data suggested that patients with –415AA/–180GG genotypes had significantly lower levels of fasting plasma glucose and HbA1c, and more HOMA-IR than the patients with –415GG/–180del and –415AG/–180Gdel genotypes had. The reason for this is unknown, but it is possible that the genotype with –415AA/–180GG stimulated by external factors (high blood sugar or excessive saturated fatty acid) yielded a higher activity and more GRP78 protein. The genotype-dependent increase of the GRP78 protein may inhibit the β cell apoptosis and maintain the glucose homeostasis (as the data were shown in our study). The findings are supported by the studies that the high activity of –415AA/–180GG

TABLE 3. HAPLOTYPE (–415/–378/–370/–180) FREQUENCIES IN THE PROMOTER REGION OF *GRP78* GENE IN CASE GROUP AND CONTROL GROUP

Haplotypes	Case group n (%)	Control group n (%)	OR (95%CI)	p
G-C-T-del	514 (49.14)	563 (52.62)	1	–
A-C-C-G	252 (24.09)	243 (22.71)	1.136 (0.918–1.406)	0.2408
G-T-C-del	205 (19.60)	188 (17.57)	1.194 (0.948–1.505)	0.1320
G-C-C-del	75 (7.17)	76 (7.10)	1.081 (0.769–1.520)	0.6543

TABLE 4. ASSOCIATION OF GRP78 –415G/A AND –180 DEL/G POLYMORPHISMS WITH BIOCHEMICAL PARAMETERS IN SUBJECTS WITH TYPE 2 DIABETES

Characteristics	Genotypes			P1	P2
	–415AA/–180GG	–415GA/–180delG	–415GG/–180deldel		
N	24	204	295		
Fasting glucose (mM)	7.74±1.26	8.87±1.34	9.07±1.37	0.0001	<0.0001
Fasting insulin (pM) ^a	84.1±4.29	85.9±4.38	86.4±4.32	0.058	0.009
HbA1c (%)	54±13 (7.1±1.18)	74±13 (8.9±1.21)	76±14 (9.1±1.27)	<0.0001	<0.0001
HOMA-IR ^a	4.49±0.43	5.26±0.48	5.41±0.47	<0.0001	<0.0001

Continuous data are expressed as the means±SEM.

^aThe logarithms of these variables were used for the analysis.

P1: P value for comparison of –415GA/–180delG vs. –415AA/–180GG.

P2: P value for comparison of –415GG/–180deldel vs. –415AA/–180GG.

genotypes of GRP78 alleviated the ER stress in lymphoblastoid cells treated with thapsigargin (Laybutt *et al.*, 2007; Hsu *et al.*, 2008). Moreover, the findings have the resonance with a study showing that GRP78 partially rescued high glucose-induced suppression of proinsulin levels and improved glucose-stimulated insulin secretion (GRP78 is essential for insulin biosynthesis, and enhancing the chaperone capacity can improve β cell function in the presence of prolonged hyperglycemia) (Zhang *et al.*, 2009).

So far, most studies on population-dependent variations in the allele frequency of GRP78 polymorphisms have been performed in Asia. Among Taiwanese healthy controls, similar –415A (–180G) and –370T allele frequencies were observed, ranging from 30.6% to 31.9% and 45.8% to 46.8%, respectively (Chen *et al.*, 2008; Hsu *et al.*, 2008; Lee *et al.*, 2008). These allele frequencies were lower than those frequencies (–415A [–180G]: 34.0% and –370T: 59.8%) in Japanese healthy controls (Sakurai *et al.*, 2007). In our healthy controls, the –415A (–180G) and –370T allele frequencies were 22.7% and 52.6%, which was different from Taiwanese healthy controls. Observed varieties demonstrated a different distribution pattern of GRP78 alleles among this Han Chinese population. Thus, similar investigations in ethnically different populations are recommended to clarify the role of GRP78 gene polymorphisms in susceptibility to T2D.

The strengths include (1) the large sample size and the well-defined homogeneous study population would make the associations more stable; (2) the confirmation of T2D and high response rates minimized the potential selection. The limitations include (1) the coverage of the GRP78 gene was limited because SNP selection was not based on complete sequencing data for the subjects; (2) this was a hospital-based case-control study, possibly leading to a selection bias; (3) our study was limited to Han subjects and it is uncertain whether these results were generalizable to other populations.

Conclusion

In conclusion, the study is the first report on the association of GRP78 with T2D. Our findings do not support the hypothesis that the genetic variants in GRP78 may contribute to the occurrence of T2D. However, the possibility that other alleles may exist to be a pathological cause of T2D development, and may be used to potentially evaluate individual susceptibility and explore the effective measures of control

and prevention for T2D cannot be excluded. Regardless, these results need further epidemiological studies to confirm the relationship of the molecular mechanism of GRP78 to risk of T2D.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Keshen Li, PhD

Key Laboratory of Neurodegenerative Disease and Aging Research

Affiliated Hospital of Guangdong Medical College

Zhanjiang 524001

China

E-mail: likeshen1971@126.com

Bin Zhao, MD

Institute of Neurology

Affiliated Hospital of Guangdong Medical College

Zhanjiang 524001

China

E-mail: zhaobine@163.net

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