Heliyon 6 (2020) e03223

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The serum concentration of βCGRP is novel marker for type 1 diabetes

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ARTICLE INFO

Keywords:
Clinical genetics
Pathology
Endocrinology
Laboratory medicine
T1D
βCGRP
CALCB

ABSTRACT

Objective: Dysregulation of neuropeptides, such as calcitonin gene-related peptide (CGRP) is thought to play significant roles in diabetes. In the present study, we investigated the importance of beta-calcitonin gene related peptide (βCGRP, *CALCB*) in the development of type 1 diabetes (T1D).

Methods: There were fifty-eight patients with T1D and 320 age and gender-matched healthy controls in Chinese Han population were included, the genotypes of *CALCB* were analyzed by direct sequencing and the clinico-pathologic parameters of patients were also evaluated.

Results: Among the SNPs genotyped, the C allele of *CALCB*_rs3829220 T (c.224G + 846) and rs382922 C (c.224G + 848) were positively associated with T1D (OR = 2.67 and 3.42, respectively). Serum βCGRP of patients is 1.58 times higher compare to normal control. Immunohistochemistry analysis revealed βCGRP expression in the infiltrating lymphocytes of pancreas from T1D mice as compared to normal control.

Conclusion: Our findings indicate that the presence of βCGRP is a probable molecular basis for the initial events triggered in T1D.

1. Introduction

Diabetes is ranked 3rd noncommunicable diseases after tumor and cardiovascular disease, which is more physical and mental health among adolescents with type 1 diabetes (T1D), but its trigger mechanisms haven't been able to clarify [1, 2, 3]. Diabetic neuropathy is a frequent and severe complication that involves the several clinical syndromes affecting motor, sensory and autonomic nerves. In case of diabetes-related autonomic neuropathy, dysfunction is observed in many systems including cardiovascular, gastrointestinal, genitourinary and neurovascular systems [2].

Calcitonin gene related peptide (CGRP) play an important transmission in the nerve-immune system. There are two subtypes, αCGRP and βCGRP, respectively by the expression of *CT/CALCA* and *CALCB*. αCGRP mainly distributed in the central nervous system, and βCGRP is mainly distributed in the peripheral nervous system and internal organs. βCGRP exists in the sensory nerve fibers within the pancreas, and it also exists in pancreatic endocrine cells, and participate in exocrine function regulation [4, 5, 6]. Under the inflammation stimulation, lymphoid cells can synthesize a small amount of βCGRP, but the immune cells lack the capacity of storage, therefore, it contributed to the lymphocyte gathered themselves together, and it maybe through this mechanism caused by its own components in pancreas antigen triggering the immune response to T1D [3, 4, 5]. Our preliminary study found, T1D displaying higher serum βCGRP and relations to target genotypes. Therefore, we hypothesized that βCGRP may also exert effects in T1D.

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https://doi.org/10.1016/j.heliyon.2020.e03223
Received 30 May 2019; Received in revised form 10 July 2019; Accepted 10 January 2020

Table 1. Clinical characteristics of the patients with T1D.

	T1D	Normal control	P
Gender (male/female)	36/22	198/122	0.0625
Age (years)	19.8 ± 10.2	20.2 ± 12.2	0.102
BMI (kg/m^2)	25.7 ± 3.16	24.8 ± 2.89	0.0636
TG (mmol/L)	2.01 ± 0.62	1.51 ± 0.48	0.0327
CHOL (mmol/L)	7.96 ± 2.42	4.96 ± 1.59	0.0081
GLU (mmol/L)	9.89 ± 4.08	5.19 ± 1.16	<0.0001

2. Materials and methods

2.1. Subjects

There were fifty-eight cases of patients with T1D from 1st Affiliated Hospital, 2nd Affiliated Hospital, Union Hospital, Maternal and Child Health Hospital, Fujian Medical University; Affiliated Hospital (Group) of Putian University; China. All of the cases (36 males/22 females) were enrolled as per the WHO diagnostic criteria. And there were 320 cases of normal controls serve as health check-up, among them, 198 cases were male and female 122 ones. Through the questionnaire, a detailed record of the cases and the control group of gender, age, blood pressure, smoking, height, weight, and the presence of diabetes complications were collected completely. This study was approved by the Ethics Committee of Fujian Medical University.

2.2. CALCB genotype and βCGRP

Blood was collected and DNA extracted using a Tiangen Genomic extraction kit (Beijing, China). Full-length *CALCB* was amplified, purified, and sequenced. Serum βCGRP from the patients with T1D and normal controls were measured using a specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocols.

2.3. Histology and immunohistochemistry

C57BL/6J mice were housed under a 12h/12h light-dark cycle at constant temperature (23 ± 1 °C) with free access to water. The animals were maintained on chow diet (Chow; 60% kJ provided by carbohydrates; 26% kJ protein^{-1}, and 14% kJ fat^{-1}). Low-dose chain urea with cephalosporins (STZ streptozotocin, Sigma Company, USA, 40 mg/kg [0.01 ml/g]) was used to make T1D mold on C57BL/6J mice at 8 weeks. Pancreas tissue were fixed in 4% formalin overnight, embedded in paraffin, sectioned at 4 mm and stained with hematoxylin and eosin (H&E) for pathology. The following antibodies were used: anti-insulin (ABclonal) and anti-βCGRP (Santa Cruz, USA).

2.4. Statistics

Statistical differences between groups were assessed by the nonparametric Mann-Whitney U-test for two groups and Kruskal-Wallis test for more than two groups. Spearman's rank correlation coefficient estimated the degree of association between two variables. Significance was calculated at $P < 0.05$ by GraphPad Prism 5 (La Jolla, CA).

3. Results

3.1. Clinical characteristics

It showed that serum glucose (GLU), total glyceride (TG), and total cholesterol (TC) were significant higher in the patients with T1D (Table 1). Medium serum βCGRP before therapy were significantly

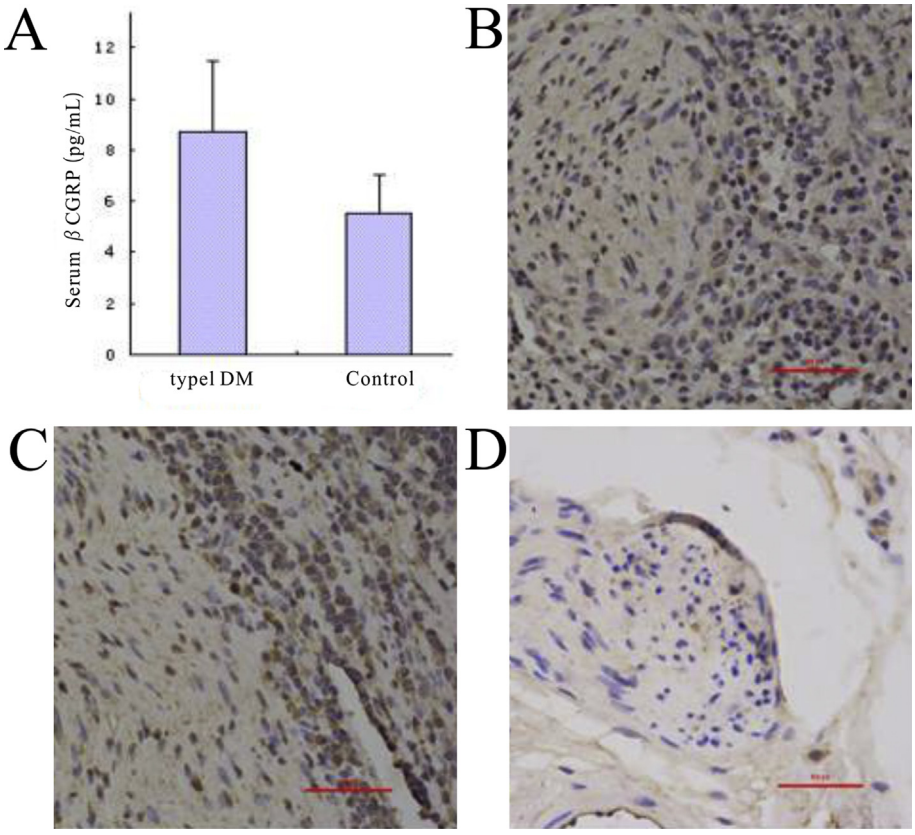

Figure 1. βCGRP higher expressed in T1D. A: Serum βCGRP in the patients with T1D is higher than normal controls; B, C: βCGRP strong express in the inflammatory cells around the nerve fibers in pancreatic tissues from T1DM mice. D: βCGRP weak expression was observed in the pancreatic tissue from control mice.

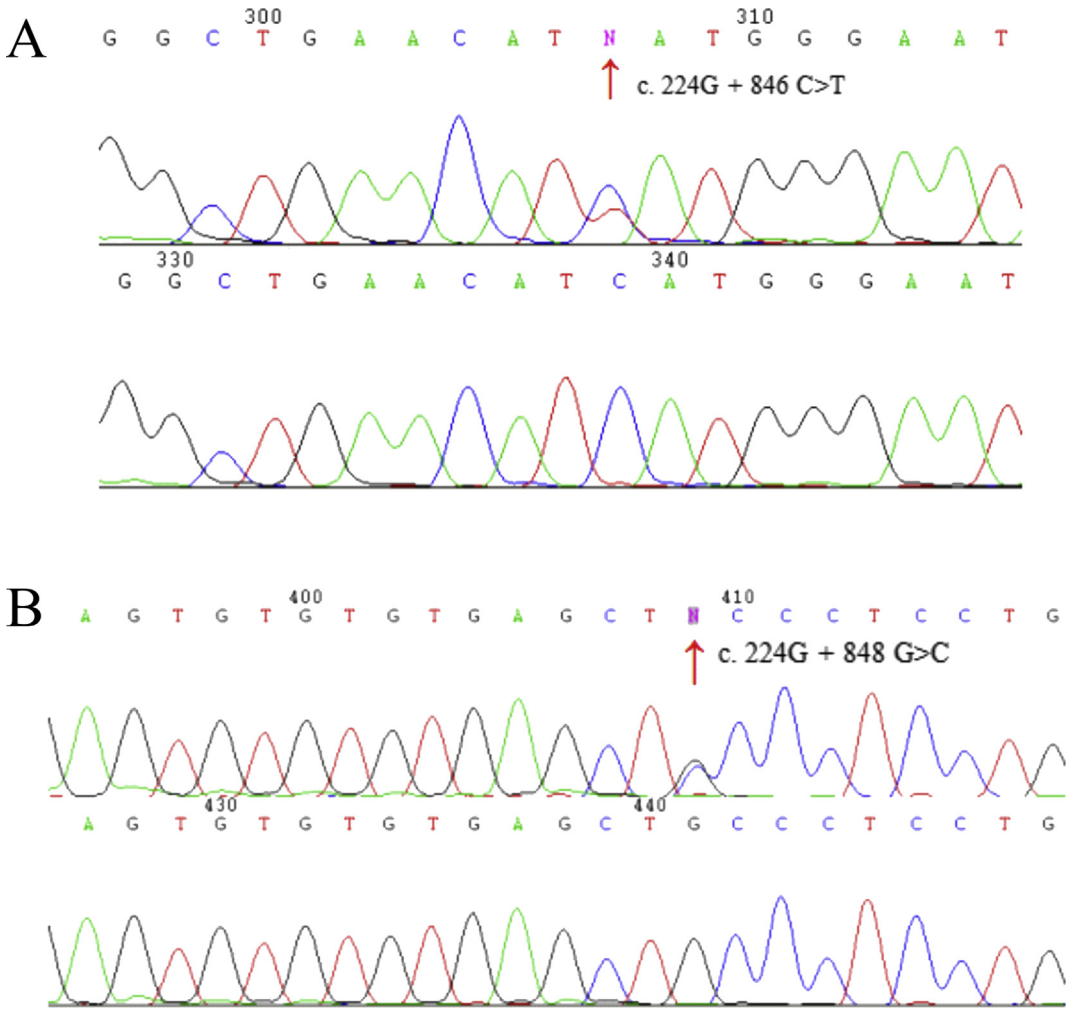

Figure 2. *CALCB* sequencing. A:*CALCB_* rs11603873 genotype; B : *CALCB_* rs79501047 A/G genotype.

higher in patients with T1D (n = 58, 8.71 pg mL^{-1} [5.23–11.35 pg mL^{-1}]) than that in healthy subjects (n = 320, 5.52 pg mL^{-1} [4.35–7.72 pg mL^{-1}], $P = 0.0215$) (Figure 1A). More excitingly, serum βCGRP was inversely associated with glucose (r = -0.8122, $P = 0.0153$) and HbA1c (r = -0.8852, $P < 0.0001$). Immunohistochemistry analysis revealed βCGRP strong express in the infiltrating lymphocytes of pancreas from T1D mice (Figure.1B, C) as compared to normal control (Figure 1D).

3.2. Alleles and genotypes in CALCB are associated with T1D

It showed that rs3829220 T (c.224G+846) (Figure 2A) genotype was positively associated with T1D (OR:2.67) compare to rs3829220 C, and rs382922 C (c.224G+848) (Figure 2B) genotype was 3.42 times to rs382922 G genotype (Table 2).

4. Discussion

CGRP is a potent microvascular dilator neuropeptide, considered to play an essential role in neurogenic vasodilatation and maintaining functional integrity in peripheral tissues, and it was known to down regulate the immune response and influence the key processes in autoimmunity [3, 4, 5, 6, 7]. It found CGRP-positive nerve fibers visible in pancreatic tissues, and it proved CGRP immunoreactive exists in the pancreas [3, 8, 9]. CGRP receptors on the pancreatic acinar cells which can accelerate the secretion of pancreatic amylase (increased 1.5 times), and it can make the base cAMP increases 25% [10, 11, 12, 13, 14].

Although people have found antibodies with T1D, but the sensitivity and specificity are not satisfactory [15, 16]. Recently, it found that pancreatic nerve endings defect is the "culprit" in type 1 diabetes on rats. This study confirmed immune system maybe shifts to overtly attacking nervous system in T1D [3, 17, 18, 19, 20]. In local inflammation, nervous system by peripheral neurotransmitter secretion regulation of the inflammatory response, which is distributed in the gut of intestinal nerve βCGRP play a major role, which can prevent the immune function after excessive activation of inflammatory cytokines to damage caused by pancreatic beta cells [10, 11, 12, 13, 21].

T1D is closely related to neural immune dysfunction through βCGRP direct influence CD4-positive T cells. Tests have shown that genetically modified NOD rat pancreatic beta cells of CGRP can prevent the occurrence of IDDM (Insulin-Dependent Diabetes Melitus), it related to the local immune inhibitory effect of CGRP. Growing evidence suggests that CGRP prevents excessive immune activation, inhibiting proinflammatory cytokine injury and maintaining a balance between pro- and anti-inflammatory in the pancreas [3]. CGRP reduced leukocyte infiltration into the pancreatic tissue and increased pancreatic blood flow before vascular damage, permitting removal of active digestive enzymes and

Table 2. The relationship between CALCB polymorphism and type 1 diabetes.

	rs3829220 (T:C)			rs382922 (C:G)		
	T	C	OR	A	G	OR
T1D	28	88	2.67	36	80	3.42
Control	294	346		388	252	

mediators of inflammation [3]. After screening for pancreatitis-associated neural immune-associated genes, the current study found several high frequency, among the SNPs genotyped, the C allele of *CALCB*_rs3829220 T and rs382922 C were positively associated with T1D (OR = 2.67 and 3.42, respectively). Immunohistochemistry analysis showed strong βCGRP expression in inflammatory cells around the nerve fibers in patients with T1D. βCGRP is synthesized by dorsal root ganglia, transported to the nerve endings along the axon, and stored in vesicles. The circular βCGRP expression pattern mainly comes from the sustained release of sensory nerves on the walls of blood vessels. T cells also synthesize a small amount of βCGRP, which supply the neurogenic βCGRP after long stimulation and induction. Therefore, lymphocyte aggregation can compensate for the decreased or absent expression of βCGRP in nerve fibers caused by inflammatory. Thus, nerves in the pancreatic tissue of patients with T1D were frequently encompassed by immune cells.

This study found that serum βCGRP is 1.58 times in T1DM, and *CALCB* polymorphism studies found that rs3829220 T and rs382922 C genotype significantly increased risk of T1D.

Declarations

Author contribution statement

L. Qicai, G. Feng: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Y. Chen, Y. Lin, J. Wang: Performed the experiments; Wrote the paper.

G. Xinxin, G. Yujia: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

D. Feng: Analyzed and interpreted the data.

Funding statement

This work was supported by a grant from Scientific Research Fund of Fujian Provincial Finance (no.BPB-DF2015) and Youth fund of Fujian Provincial Department of Health (2019-1-41 and 2019-1-46).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no.81871293 and no.81800070), Scientific Research Fund of Fujian Provincial Finance (no.BPB-DF2015) and Youth fund of Fujian Provincial Department of Health (2019-1-41 and 2019-1-46). These funding sources played a key supportive role for sample collection, molecular analysis of patient samples, bioinformatics analysis.

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