Relationship between intestinal flora, inflammation, BDNF gene polymorphism and generalized anxiety disorder
A clinical investigation

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Abstract
Introduction: Understanding factors related to generalized anxiety disorder pathogenesis is critical for elucidating the mechanism and preventing its establishment. Intestinal flora and hereditary factors such as brain-derived neurotrophic factor (BDNF) gene polymorphism may have a role in the development of generalized anxiety disorder. This work explored the relationship between intestinal flora, inflammatory changes and BDNF gene polymorphisms and the occurrence of generalized anxiety disorder.

Methods: Forty-eight patients with generalized anxiety disorder and 57 healthy people were included in the study. As the disease group and control group, the polymorphisms of rs10767664 and rs7124442 of the BDNF gene, differences in the distribution of intestinal flora, and changes in inflammatory and immune indicators were analyzed.

Results: The distribution of BDNF gene alleles, genotypes and haplotypes in the disease group were different from those in the control group. The levels of TNF-α (P = .000), interleukin-4 (P = .000), interleukin-10 (P = .043) and IgG (P = .008) in patients with generalized anxiety disorder in the disease group were different from those in the control group. The distribution of gut microbes in patients with generalized anxiety disorder in the disease group was different from that in the control group.

Conclusion: The onset of generalized anxiety disorder is related to BDNF gene polymorphism, and is accompanied by changes in intestinal flora and inflammatory immune status in the body.

Abbreviations: BDNF = brain-derived neurotrophic factor, DNA = deoxyribonucleic acid, GAD = generalized anxiety disorder, IL = interleukin, PCR = polymerase chain reaction.

Keywords: BDNF, gene polymorphism, generalized anxiety disorder, intestinal flora

1. Introduction

Generalized anxiety disorder (GAD) belongs to anxiety neurosis, patients of which display varying degrees of worry and anxiety toward various things. GAD is characterized by excessive agitation of autonomic nerve and excessive response to external things, accompanied with muscular tension.[1-3] GAD has a prevalence of over 5%, affecting women more than men, and is often diagnosed in children and adolescents.[4] GAD patients often develop other anxiety disorders such as phobia and depressive disorder, worsening their condition.[3] Though the etiology is not fully elucidated, GAD seems to be associated with altered secretion of 5-HT and norepinephrine, and the reduction of cerebral blood flow caused by cerebrovascular change and the alteration of blood viscosity. In addition, cerebral metabolism and inflammation level are also involved.[6,7] However, the development of GAD is more concerned with genetics, like gene polymorphism, with distinct features of
family aggregation.\cite{8,9} Discovering factors that are related to GAD pathogenesis is meaningful to elucidate the mechanism and prevent the oncome of this disease.

Intestinal flora affects many diseases through acting locally or systemically, and their alteration is one of the main causes of diseases.\cite{10,11} The potential pathological change such as inflammation and stress status of many diseases might be attributed to microbiota change.\cite{12,13} Brain-derived neurotrophic factor (BDNF) is a neurotrophin that can improve neural function.\cite{14}

Previous studies proved that hereditary variabilities such as BDNF gene polymorphism is related to the development of mental diseases.\cite{15,16}

Therefore, intestinal flora, inflammation, immunological reaction and rs10767664 and rs7124442 polymorphisms in BDNF gene of 48 GAD patients and 57 healthy people were analyzed in this study, aiming to determine the susceptibility factor of the disease and provide important evidence for elucidating the pathogenesis of GAD.

2. Methods

2.1. Patients and ethical statement

Forty-eight GAD patients were treated in our hospital from January 2019 to January 2020, and 57 healthy people were included in the study and were set as disease and control groups, respectively. GAD patients in the disease group met the diagnostic criteria of Chinese Classification and Diagnostic Criteria for Mental Disorders, third edition. The disease group included 20 male and 28 female GAD patients, and the median age was 41.24 ± 3.82 years old. The control group was composed of 24 male, and 33 female participants averaged 40.91 ± 4.4 years old, and no significant difference in age and gender distribution was found between the 2 groups (\(P > .05\)). The study was carried out following the Helsinki Declaration and authorized by the Medical Ethics Committee of Shandong Mental Health Center. General materials and clinical information of participants in the disease and control groups were collected and written informed consent was obtained. The study profile is shown in Figure 1.

2.2. Sampling and deoxyribonucleic acid (DNA) extraction

Approximately 5 mL of peripheral venous blood was collected from all participants and fully mixed with an anticoagulant. Mononuclear cells were purified by Percoll, and their genomic DNA was extracted by a DNA extraction kit from Thermo Fisher.

2.3. Polymorphism analysis of rs10767664 and rs7124442 of BDNF gene

The polymorphic region of rs10767664 and rs7124442 site of BDNF gene was amplified by polymerase chain reaction, and the polymorphism of loci of BDNF gene was analyzed by sequencing. Primer used to analyze the polymorphism of rs10767664 and rs7124442 in the BDNF gene was designed on the Primer 3 website (http://primer3.ut.ee/) and synthesized by Shanghai Bioengineering Co., LTD. Primer sequences are listed in Table 1.

2.4. Intestinal flora analysis

Fresh feces (5g) of participants in the disease and control groups were collected and frozen in a liquid nitrogen tank. Bacterial DNA was extracted from fecal samples by DNA extraction kit following the manufacturer’s instructions. Fecal microbiota was analyzed by 16s rRNA next-generation sequencing, and the microbial flora diversity was determined by LefSe analysis. The Chao 1 index was calculated and compared.

2.5. Analysis of inflammatory and immune indexes

Peripheral blood was collected from all participants, and serum was obtained after centrifugation for 10 minutes. The serum concentration of TNF-\(\alpha\), interleukin (IL)-1\(\beta\), IL-4, IL-10, IgG, and IgM was detected by enzyme-linked immunosorbent assay (BD) following the producer’s instructions.

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**Figure 1.** Study profile.
2.6. Statistical analysis

SPSS 2.0 was used for statistical analysis. The Student t test was used to compare quantitative data, and the enumeration data were analyzed by Chi-squared test. Haplotype was estimated with the online program SHEsis. P value < .05 was considered statistically significant.

3. Results

3.1. Distribution of rs10767664 and rs7124442 allele of BDNF gene

The distribution of rs10767664 and rs7124442 allele of the BDNF gene is shown in Table 2. A significant difference was observed in the distribution of the rs10767664 allele of the BDNF gene between GAD patients and healthy people (P = .004), and the frequency of the T allele in GAD patients was higher.

3.2. Genotype distribution of rs10767664 and rs7124442 of BDNF gene

As shown in Table 3, the genotype distribution of BDNF gene rs10767664 (P = .000) and rs7124442 (P = .011) in GAD patients were different from those in control group. The frequencies of TT genotype of rs10767664 and CT genotype of rs7124442 in the disease group were higher than those in the control group.

3.3. Model of rs10767664 and rs7124442 site of BDNF gene

GAD patients showed different distribution of rs10767664 recessive model (P = .002) and rs7124442 dominant model (P = .040) of BDNF gene with participants in control group (Table 4). The frequency of TT + TA in recessive model of rs10767664 locus of BDNF gene was lower, and the frequency of CC + CT in the dominant model of rs7124442 locus was higher in the disease group.

3.4. Haplotype analysis

As shown in Table 5, the distribution of AC (P = .004) and TC (P = .019) haplotypes of BDNF gene rs10767664 and rs7124442 sites in GAD patients were different from those in the control group, and the frequency of AC haplotype was lower, whereas the TC haplotype frequency was higher in the disease group.

Table 1

| Primer type | Primer sequences |
|-------------|------------------|
| rs10767664 site | Sense TTCCCATCTATTCTTGTCGGG |
|              | Anti-sense AGGTAGGAGTAACTAGGAAGCT |
| rs7124442 site | Sense CCCTTGCAATCCTTTAAGTTTGT |
|              | Anti-sense AGGAATGCTTGGAATCTGCT |

Table 2

| Site           | Control group | Disease group | OR value | 95% CI | ?2   | P     |
|----------------|---------------|---------------|----------|--------|------|-------|
| rs10767664     | T             | 59 (0.518)    | 68 (0.708) | 0.44   | 0.24-0.78 | 7.93 | .004 |
| A              | 55 (0.482)    | 28 (0.292)    |          |        |      |       |
| rs7124442      | C             | 59 (0.518)    | 49 (0.510) | 0.97   | 0.56-1.67 | 0.01 | .918 |
| T              | 55 (0.482)    | 47 (0.490)    |          |        |      |       |

Table 3

| Site           | Genotype | Control group | Disease group | OR value | 95% CI | ?2   | P     |
|----------------|----------|---------------|---------------|----------|--------|------|-------|
| rs10767664     | TT       | 13 (0.228)    | 28 (0.583)    | 0.44     | 0.24-0.78 | 15.11 | .000 |
| rs7124442      | CC       | 17 (0.298)    | 7 (0.146)     | 0.97     | 0.56-1.67 | 8.98  | .011 |
|                | CT       | 25 (0.439)    | 35 (0.729)    | 0.97     | 0.56-1.67 |       |       |
|                | TT       | 15 (0.263)    | 6 (0.125)     | 0.97     | 0.56-1.67 |       |       |

Table 4

| Site           | Genotype | Control group | Disease group | OR value | 95% CI | ?2   | P     |
|----------------|----------|---------------|---------------|----------|--------|------|-------|
| Dominant model | rs10767664 | TT+TA         | 46 (0.807)    | 40 (0.833) | 0.44   | 0.24-0.78 | 3.26  | .196 |
|               | rs7124442 | CC+CT         | 42 (0.737)    | 42 (0.875) | 0.97   | 0.56-1.67 | 6.43  | .040 |
| Recessive model| rs10767664 | TT           | 13 (0.228)    | 28 (0.583) | 12.13  | .002  |       |       |
|               | rs7124442 | CC+CT         | 40 (0.702)    | 41 (0.854) | 0.97   | 0.56-1.67 | 2.31  | .119 |
| Heterozygous model | rs10767664 | TT+TA       | 13 (0.228)    | 28 (0.583) | 8.98   | .011  |       |       |
|                | rs7124442 | CC+CT         | 25 (0.459)    | 35 (0.729) | 0.97   | 0.56-1.67 | 3.92  | .141 |

Table 5

| Haplotype | Control group | Disease group | OR value | 95% CI | ?2   | P     |
|-----------|---------------|---------------|----------|--------|------|-------|
| AC        | 30.64 (0.269) | 10.53 (0.110) | 0.335   | 0.156-0.719 | 8.362 | .004 |
| AT        | 24.36 (0.214) | 17.47 (0.182) | 0.818   | 0.412-1.624 | 0.33  | .566 |
| TC        | 28.36 (0.249) | 38.47 (0.401) | 2.019   | 1.120-3.638 | 5.542 | .019 |
| TT        | 30.64 (0.269) | 29.53 (0.308) | 1.209   | 0.664-2.202 | 0.386 | .535 |

Table 6

| n  | TNF-a  | IL-1ß | IL-4 | IL-10 |
|----|--------|-------|------|-------|
| 57 | 8.13±1.30 | 7.24±0.69 | 8.84±0.92 | 4.83±0.38 |
| 48 | 18.24±1.74 | 7.01±1.24 | 17.62±1.63 | 5.22±0.27 |
| t  |        | 21.24 | 2.91 | 18.42 | 7.28 |
| P  |        | 0.000 | 0.438 | 0.000 | 0.043 |

Table 7

| n | IgG | IgM |
|---|-----|-----|
| 57 | 64.35±4.39 | 43.35±3.84 |
| 48 | 89.24±8.47 | 45.39±5.40 |
| t | 9.34 | 3.82 |
| P | 0.008 | 0.277 |
3.5. Comparison of inflammatory levels between disease group and control group

The levels of TNF-α ($P = .000$), IL-4 ($P = .000$), IL-10 ($P = .043$), and IgG ($P = .008$) in GAD patients were significantly different from those in the control group, and the overall inflammatory immune level of disease group was higher compared to the control group as shown in Tables 6 and 7.

3.6. Comparison of intestinal flora composition between control and disease group

The abundance of Paraprevotella, Euryarchaeota, Caldivirga, Porphyromonadaceae, and Desulfovibrionales was higher in GAD patients than in the control group. However, the Lactobacillus, Vagococcus, Paludibacter, and Barnesiella were more abundant in the control than in the disease group, as shown in Figures 2 and 3.

3.7. Correlational analysis of microbiota

As depicted in Figure 4, there was a high positive correlation between Bifidobacterium and Rumen cocci ($P = .002$, $r = 0.44$), and a high negative correlation with Escherichia coli ($P = .000$, $r = -0.62$). At the same time, E coli and Bacteroides were positively correlated ($P = .032$, $r = 0.26$).

3.8. Comparison of intestinal flora diversity between disease and control group

As shown in Figure 5, the Chao1 index representing intestinal flora diversity is significantly lower in the disease group than in the control group ($P < .05$).

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**Figure 2.** LDA score of microbiota of control and disease group.
4. Discussion

GAD patients suffer from different levels of plant nerve dysfunction, and based on that; they display overstrain and excessive anxiety. GAD is a chronic disease with a longer course, and the patients’ conditions worsen if no effective treatment is performed. The diagnosis rate of GAD is low worldwide, which might be attributed to the less attention it receives from the public. GAD has a closer relationship with genetic factors. Previous research proved that the occurrence of GAD is related to the polymorphism of several genes, such as rs4680 of catechol-O-methyltransferase gene, C677T of methylene-tetrahydrofolate reductase gene rs324981 of NPSR1 gene. These studies indicated that alteration of gene polymorphism might play an important role in the development of GAD. Therefore, a deep understanding of the relationship between gene polymorphism and GAD development is important for elucidating the mechanism of this disease. Meanwhile, discovering more GAD-related polymorphism genes is beneficial to GAD prevention and screening susceptible populations.

BDNF is a neurotrophin vital for the restoration and regeneration of neurologic function. BDNF functions through targeting and binding to its receptor after being released. Its polymorphism is a critical susceptibility factor associated with diseases, especially nerve system diseases. According to previous reports, BDNF gene polymorphism is relevant to the occurrence of many diseases, including major depressive disorder and bipolar disorder. In our study, we compared the difference between rs10767664 and rs7124442 polymorphism of BDNF gene between 48 GAD patients and 57 healthy people and found that the distribution of rs10767664 allele of BDNF gene in GAD patients was significantly different from that in the control group and the frequency of T allele in GAD patients was higher. The genotype distribution of the rs10767664 and rs7124442 sites of the BDNF gene in GAD patients was different from those in the control group. The frequencies of TT genotype and CT genotype of rs10767664 and rs7124442 of the BDNF gene in the disease group were higher than those in the control group. The distribution of rs10767664 recessive model and rs7124442 dominant model of BDNF gene in GAD patients were different from those in the control group. The frequency of TT + TA in the recessive model of rs10767664 locus of BDNF gene was lower than that of CC + CT of rs7124442 dominant locus model in the disease group. The distribution of AC and TC haplotypes of BDNF gene rs10767664 and rs7124442 sites in patients with generalized anxiety disorder were different from those in the control group, and the frequency of AC haplotype was lower, and TC haplotype frequency was higher in the disease group compared to the control group. These results suggested that BDNF gene polymorphism is related to the outcome of GAD, and it modulates the disease in certain ways. Meanwhile, these data also provide important support for screening the potential susceptible populations of GAD and making early intervention measures for the highly susceptible people. Body checks should be taken regularly by people with certain genotypes in rs10767664 and rs7124442 site of BDNF gene, which would reduce the severity of GAD through early diagnosis and treatment.

Intestinal flora is the sum of various microorganisms that reside in the digestive tract, consisting of enormous species and number of bacteria groups. Intestinal flora is involved in various physiology and pathology.
processes, such as modulating inflammatory responses, changing the immune status, and influencing the progression of tumors.[31,32] By comparing the microbiota of 48 GAD patients and 57 healthy humans, we found the abundance of Paraprevotella, Euryarchaeota, Caldivirga, Porphyromonadaceae, and Desulfovibrionales in GAD patients than the control groups. However, abundant Lactobacillus, Vagococcus, Paludibacter, and Barnesiella were reported in the control group compared to the disease group. Bifidobacterium has a high positive correlation with Rumen cocci, and a high negative correlation with E coli ($P = .000, r = –0.62$). Meanwhile, E coli is positively correlated with Bacteroides. The Chao1 index, which symbolizes intestinal flora diversity, was significantly lower in the disease group than in the control group. These results indicated that the composition of intestinal flora in GAD patients is significantly different from that in healthy humans, and it might be the leading cause of GAD. The remarkable change in the abundance of some microbial species might promote the progression of GAD. Inhibitors of the corresponding species or probiotics might modulate the distribution of microbiota in patients and indirectly suppress GAD’s development. The shortcoming of this study is that we included 48 GAD patients and 57 healthy humans because of the condition limitations, and the insufficiency of research objects might be an obstacle to the accuracy of results and conclusions. However, our research can still provide new directions for the following explorations and provide a theoretical basis for investigating GAD’s pathogenesis.

5. Conclusion
In conclusion, BDNF gene rs10767664 and rs7124442 polymorphisms are associated with the pathogenesis of GAD, which is accompanied by the increased abundance of Paraprevotella, Euryarchaeota, Caldivirga, Porphyromonadaceae, and Desulfovibrionales, and the reduced abundance of Lactobacillus, Vagococcus, Paludibacter, and Barnesiella, as well as the elevated inflammatory immune status.

Author contributions
Study concept and design: YC; analysis and interpretation of data: YW; drafting of the manuscript: WZ and YB; critical revision of the manuscript for important intellectual content: YC and JL; statistical analysis: YW.
References

[1] DeMartini J, Patel G, Fancher TL. Generalized anxiety disorder. Ann Intern Med 2019;170:ITC49–64.
[2] Maron E, Nutt D. Biological markers of generalized anxiety disorder. Dialogues Clin Neurosci 2017;19:147–58.
[3] Stein MB, Sareen J. Clinical practice. Generalized anxiety disorder. N Engl J Med 2015;373:2059–68.
[4] Kozel FA. Clinical repetitive transcranial magnetic stimulation for post-traumatic stress disorder, generalized anxiety disorder, and bipolar disorder. Psychiatr Clin North Am 2018;41:433–46.
[5] Goodwin H, Yiend J, Hirsch CR. Generalized anxiety disorder, worry and attention to threat: a systematic review. Clin Psychol Rev 2017;54:107–22.
[6] Cuijpers P, Gentili C, Banos RM, Garcia-Campayo J, Botella C, Cristea IA. Relative effects of cognitive and behavioral therapies on generalized anxiety disorder, social anxiety disorder and panic disorder: a meta-analysis. J Anxiety Disord 2016;43:79–89.
[7] Schanzer B, Rivas-Grajaes AM, Khan A, Mathew SJ. Novel investigational therapeutics for generalized anxiety disorder (GAD). Expert Opin Investig Drugs 2019;28:1003–12.
[8] Gottschalk MG, Domschke K. Genetics of generalized anxiety disorder and related traits. Dialogues Clin Neurosci 2017;19:159–68.
[9] Ciuculete DM, Bostrom AE, Tuananen AK, et al. Changes in methylation within the STK32B promoter are associated with an increased risk for generalized anxiety disorder in adolescents. J Psychiatr Res 2018;102:44–51.
[10] Kataoka K. The intestinal microbiota and its role in human health and disease. J Med Invest 2016;63:27–37.
[11] Cibelli S, Ianiro G, Giorgio V, et al. The role of diet on gut microbiota composition. Eur Rev Med Pharmacol Sci 2016;20:4742–9.
[12] Knip M, Silhander H. The role of the intestinal microbiota in type 1 diabetes mellitus. Nat Rev Endocrinol 2016;12:154–67.
[13] Spielman LJ, Gibson DL, Klegersis A. Unhealthy gut, unhealthy brain: the role of the intestinal microbiota in neurodegenerative diseases. Neurochem Int 2018;120:149–63.
[14] Shen T, You Y, Joseph C, et al. BDNF polymorphism: a review of its diagnostic and clinical relevance in neurodegenerative disorders. Aging Dis 2018;9:523–36.
[15] Zhao M, Chen L, Yang J, et al. BDNF Val66Met polymorphism, life stress and depression: a meta-analysis of gene-environment interaction. J Affect Disord 2018;227:226–35.
[16] Wang Q, Liu J, Guo Y, Dong G, Zou W, Chen Z. Association between BDNF G196A (Val66Met) polymorphism and cognitive impairment in patients with Parkinson’s disease: a meta-analysis. Braz J Med Biol Res 2019;52:e8443.
[17] Newman MG, Shin KE, Zueilig AR. Developmental risk factors in generalized anxiety disorder and panic disorder. J Affect Disord 2016;206:94–102.
[18] Mossman SA, Luft MJ, Schroeder HK, et al. The Generalized Anxiety Disorder 7-item scale in adolescents with generalized anxiety disorder: signal detection and validation. Ann Clin Psychiatry 2017;29:227–34A.
[19] Sagliano L, Atipaldi D, De Vita D, D’Olimpio F, Trojano L. Non-invasive brain stimulation in generalized anxiety disorder: a systematic review. Prog Neuropsychopharmacol Biol Psychiatry 2019;93:31–8.
[20] Fonzo GA, Erkin A. Affective neuroimaging in generalized anxiety disorder: an integrated review. Dialogues Clin Neurosci 2017;19:169–79.
[21] Altunoz U, Kokurcan A, Kirici S, Bastug G, Ozel-Kizil ET. Clinical characteristics of generalized anxiety disorder: older vs. young adults. Nord J Psychiatry 2018;72:97–102.
[22] Chang HA, Fang WH, Wan FJ, et al. Age-specific associations among functional COMT Val(158)Met polymorphism, resting parasympathetic nervous control and generalized anxiety disorder. Psychoneuroendocrinology 2019;106:57–64.
[23] Sarawathy KN, Ansari SN, Kaur G, Joshi PC, Chandel S. Association of vitamin B12 mediated hyperhomocysteinemia with depression and anxiety disorder: a cross-sectional study among Bhil indigenous population of India. Clin Nutr ESPEN 2019;30:199–203.
[24] He Q, Shen Z, Ren L, et al. Association of NPSR1 rs324981 polymorphism and treatment response to antidepressants in Chinese Han population with generalized anxiety disorder. Biochem Biophys Res Commun 2018;504:137–42.
[25] Kojima M, Mizui T. BDNF propeptide: a novel modulator of synaptic plasticity. Vitam Horm 2017;104:19–28.
[26] Mandolini GM, Lazzaretto M, Pigioni A, Delvecchio G, Soares JC, Brambilla P. The impact of BDNF Val66Met polymorphism on cognition in Bipolar Disorder: a review: Special Section on "Translational and Neuroscience Studies in Affective Disorders" Section Editor, Maria Nobile MD, PhD. This Section of JAD focuses on the relevance of translational and neuroscience studies in providing a better understanding of the neural basis of affective disorders. The main aim is to briefly summaries relevant research findings in clinical neuroscience with particular regards to specific innovative topics in mood and anxiety disorders. J Affect Disord 2019;243:332–8.
[27] Calderararo MA, McKee M, Leisnner-Segal S, et al. Val66Met polymorphism association with serum BDNF and inflammatory biomarkers in major depression. World J Biol Psychiatry 2018;19:402–9.
[28] Li M, Chang H, Xiao X. BDNF Val66Met polymorphism and bipolar disorder in European populations: a risk association in case-control, family-based and GWAS studies. Neurosci Biobehav Rev 2016;68:218–33.
[29] Zhang M, Yang XJ. Effects of a high fat diet on intestinal microbiota and gastrointestinal diseases. World J Gastroenterol 2016;22:8905–9.
[30] Lin P. Importance of the intestinal microbiota in ocular inflammatory diseases: a review. Clin Exp Ophthalmol 2019;47:418–22.
[31] Goubet AG, Daillere R, Routy B, Derosa L, Sivelage M, Zitvogel L. The impact of the intestinal microbiota in therapeutic responses against cancer. C R Biol 2018;341:284–9.
[32] Nishida A, Inoue R, Inatomi O, Bamba S, Naito Y, Andoh A, Gut microbiota in the pathogenesis of inflammatory bowel disease. Clin J Gastroenterol 2018;11:1–10.