The Primary Research on the Gut Microbes in KKAY Mice

Yanqin Li · Liang Han · Ming Xu ·
Junjie Guo · Mali Feng · Xiaojiong Wang

Received: 3 January 2013 / Accepted: 12 April 2013 / Published online: 28 April 2013
© Association of Microbiologists of India 2013

Abstract In the first part of this paper, gut microbial difference of two genotypes mice was researched. The gut microbial community of type 2 diabetes animal model KKAY mice and normal C57BL/6J mice had clear distinctions in DGGE (denaturing gradient gel electrophoresis) profiles. The pairwise similarity coefficient (C_s) was only 26–44 % between KKAY and C57BL/6J, but C_s was 82–100 % among same genotypes mice. Thirteen dominant bands were cloned from DGGE profiles to exhibit difference on gut microbial structure further. In the second part of this paper, the influence of hypoglycemic drug Pioglitazone on the gut microbes in KKAY mice was researched by gut microbial diversity analysis and principal component analysis (PCA). The results showed that Pioglitazone reduced the gut microbial diversity slightly and changed gut microbial structure of KKAY mice to that of normal C57BL/6J mice.

Keywords Gut microbes · KKAY mice · Type 2 diabetes · Genotypes · Pioglitazone

Introduction

KKAY mice, due to gene mutation, were the spontaneous animal model of hyperglycemia and hyperlipidemia, and their symptoms are similar to that of type 2 diabetes. Type 2 diabetes was caused by metabolic disorders. The gut

microbes participated directly and indirectly in the metabolism of the host. Yadav et al. [1] discovered that *Lactobacillus* could lower plasma glucose and LDL (low-density lipoprotein), and alleviate the type 2 diabetes effectively. Disordered microbial community would aggravate the metabolic abnormalities, which could accelerate the development of hyperglycemia and led to other complications of the patient. Some reports indicated that *Rikenella* and *Odoribacter* present in ceca of db/db mice which were a model for diabetic dyslipidemia [2]. *Barnesiella* were increased in fasting hyperglycaemia and high plasma concentration of pro-inflammatory cytokines mice [3].

The human gut is colonized with an enormous variety and amount of microorganisms, dramatically affecting human physiology and pathology [4]. The entire cohort harbors more than 1,000 prevalent bacterial species. The gene set of gut microbes is about 150 times overwhelmingly larger than the human gene complement [5]. So some scholars believe that gut microbes can be pictured as a microbial organ or an environmental factor, influencing and regulating the host [6, 7]. If we only focus on the blood glucose and ignore gut microbes of the patient, we might miss the optimal scheme of treatment for type 2 diabetes. Therefore the microbes, which were associated with diabetes, were identified by researching gut microbial differences of two genotypes mice and the influence of drug on gut microbes in our paper.

Materials and Methods

Animals

KKAY and C57BL/6J mice (female, 10 weeks) were bought from Institute of Laboratory Animal Sciences,

Y. Li (✉) · L. Han · M. Xu
Institute of Biotechnology, Shanxi University,
92 Wucheng Road, Taiyuan 030006, China
e-mail: yanqin@sxu.edu.cn

J. Guo · M. Feng · X. Wang
Shanxi Hospital of Traditional Chinese Medicine,
Taiyuan 030012, China

CAMS&PUMC. They were maintained in the animal room of Shanxi Hospital of Traditional Chinese Medicine under specific pathogen free conditions (SPF) and allowed free access to food and water. The 12 KKAY mice were randomly divided into two groups (six in each group), drug group (Pioglitazone by gavage) and control group (equal distilled water by gavage), and there were six C57BL/6J mice as a normal group (equal distilled water by gavage). Each animal was contained in its own cage. All procedures were approved by the Institutional Animal Care and Use Committee of the Shanxi Hospital of Traditional Chinese Medicine.

Sample Collection and the Extraction of Total DNA

Fresh faecal samples were collected separately into sterile centrifuge tubes and were immediately stored at -20°C . In accordance with the method [8], the genomic DNA of gut microbes were extracted.

16S rDNA V3 Region PCR Amplification and DGGE

V3 region of 16S rDNA of gut microbes was amplified twice from diluted DNA using universal primers. The first PCR used Touchdown protocol described by Korbie and Mattick [9]. The second PCR used Reconditioning PCR with the 1st PCR products as a template [10].

Fig. 1 The partial DGGE profile of 16S rDNA V3 region from gut microbes D1–D3 drug group, C1–C3 control group, N1–N3 normal group. A–M are cloned bands in KKAY mice or C57BL/6J mice

Fig. 2 Phylogenetic tree of cloned bands on DGGE profiles. Phylogenetic tree intuitively demonstrated relationship among gut microbes. The tree was constructed using neighbor-joining method with Jukes-Cantor corrections for distance values and 1,000 bootstrap replicates

DGGE was performed with Bio-Rad Dcode system (Bio-Rad, Hercules, CA, USA). The gradient of denaturing gel was from 32 to 58 %, PCR product was 200 ng on every lane and the electrophoresis lasted for 4 h (200 V, 60°C). The results of electrophoresis were tested by silver staining method [11] and photographed with UVI (UVitec, Cambridge, UK) system.

Similarity Analysis of Different Samples

Samples similarity was analyzed by pairwise similarity coefficient (C_s): $C_s = 2j/(a + b) \times 100\%$. Two completely different DGGE profiles had a C_s value of 0 %, and two identical profiles had a C_s value of 100 % [12].

Gut Microbial Diversity Analysis by Optical Density

The relative intensities of bands could be used to infer the relative abundance of population [13]. The diversity of gut microbes was described by Shannon–Weaver index (diversity index, H'),

Table 1 The results of comparing with 16S rDNA sequences available in GenBank and RDP database

DGGE band	Clone number	Closest relative (GenBank accession number)	Similarity (%)	Accession number	Phylum	Taxon
A	A1, A6	<i>Rikenella microfus</i> strain Q-1(NR025910)	96	JX103599	Bacteroidetes	<i>Rikenella</i>
	A2	<i>Bacteroides acidifaciens</i> strain A40 (NR028607)	96	JX103600	Bacteroidetes	<i>Bacteroides</i>
	A3	<i>Rikenella microfus</i> strain Q-1(NR025910)	95	JX103601	Bacteroidetes	<i>Rikenella</i>
	A4	<i>Bacteroides acidifaciens</i> strain A40 (NR028607)	98	JX103602	Bacteroidetes	<i>Bacteroides</i>
	A5	<i>Odoribacter splanchnicus</i> DSM 20712 (CP002544)	91	JX103603	Bacteroidetes	<i>Odoribacter</i>
B	B1, B6, B8	Uncultured bacterium clone WT_dss_B5_D07 (JQ084421)	100	JX103604	Bacteroidetes	Bacteroidales
C	C1	Uncultured bacterium clone (JF255812)	99	JX103605	Bacteroidetes	<i>Barnesiella</i>
	C2	Uncultured bacterium clone (JF255812)	100	JX103606	Bacteroidetes	<i>Barnesiella</i>
	C3	Uncultured bacterium clone (JF255806)	99	JX103607	Bacteroidetes	<i>Barnesiella</i>
	C4	<i>Desulfovibrio</i> sp. ABHU2SB (AF056090)	99	JX103608	Proteobacteria	<i>Desulfovibrio</i>
	C5	Uncultured bacterium clone RMAM2931 (HQ321795)	97	JX103609	Bacteroidetes	<i>Barnesiella</i>
	C6	Uncultured bacterium clone 16sms93-3e03 (JF259484)	100	JX103610	Bacteroidetes	<i>Barnesiella</i>
D	D2, D4, D6	Uncultured bacterium clone U000000884 (FJ032747)	100	JX103611	Bacteroidetes	<i>Barnesiella</i>
E	E2	Uncultured bacterium clone 16sms132-3h08 (JF247107)	100	JX103612	Firmicutes	Lachnospiraceae
	E4	Uncultured bacterium clone K80N3_93f02 (EU456778)	100	JX103613	Bacteroidetes	<i>Barnesiella</i>
	E5	<i>Lactobacillus animalis</i> KCTC 3501 strain NBRC 15882 (NR041610)	100	JX103614	Firmicutes	<i>Lactobacillus</i>
F	F1	<i>Facklamia languida</i> strain 1144-97 (NR026487)	96	JX103615	Firmicutes	<i>Facklamia</i>
	F3	<i>Facklamia sourekii</i> strain SS 1019 (NR029351)	91	JX103616	Firmicutes	<i>Facklamia</i>
	F4	Uncultured <i>Aerosphaera</i> sp. clone (JN834458)	96	JX103617	Firmicutes	<i>Aerosphaera</i>
G	G3	<i>Prevotella loescheii</i> strain NCTC 11321 (NR043216)	92	JX103618	Bacteroidetes	<i>Prevotella</i>
	G1, G6	<i>Anaerotruncus colihominis</i> DSM 17241 (NR027588)	96	JX103619	Firmicutes	<i>Anaerotruncus</i>
H	H1	<i>Alistipes putredinis</i> strain ATCC 29800 (NR025909)	93	JX103620	Bacteroidetes	<i>Alistipes</i>
	H2	<i>Alistipes finegoldii</i> DSM 17242 strain CIP 107999 (NR043064)	92	JX103621	Bacteroidetes	<i>Alistipes</i>
	H3	<i>Alistipes putredinis</i> strain ATCC 29800 (NR025909)	94	JX103622	Bacteroidetes	<i>Alistipes</i>
I	I2	<i>Prevotella loescheii</i> strain NCTC 11321 (NR043216)	92	JX103623	Bacteroidetes	<i>Prevotella</i>
	I4	<i>Prevotella loescheii</i> strain NCTC 11321 (NR043216)	91	JX103624	Bacteroidetes	<i>Prevotella</i>
	I7	<i>Prevotella loescheii</i> strain NCTC 11321 (NR043216)	91	JX103625	Bacteroidetes	<i>Prevotella</i>
J	J4	Uncultured Firmicutes bacterium clone TF3-41 (GU959648)	100	JX103626	Firmicutes	<i>Acetivibrio</i>
	J5	<i>Pseudomonas</i> sp. FI 1007 (JQ691542)	99	JX103627	Proteobacteria	<i>Pseudomonas</i>
	J6	<i>Desulfovibrio</i> sp. ABHU2SB (AF056090)	98	JX103628	Proteobacteria	<i>Desulfovibrio</i>
K	K3	Microbacteriaceae bacterium LC058 (JQ014422)	100	JX103629	Actinobacteria	Microbacteriaceae
	K5	<i>Prevotella loescheii</i> strain NCTC 11321 (NR043216)	92	JX103630	Bacteroidetes	<i>Prevotella</i>
	K6	Uncultured bacterium clone 16sms238-3c11 (JF255578)	99	JX103631	Bacteroidetes	Porphyromonadaceae

Table 1 continued

DGGE band	Clone number	Closest relative (GenBank accession number)	Similarity (%)	Accession number	Phylum	Taxon
L	L3	<i>Lactobacillus animalis</i> KCTC 3501 strain NBRC 15882 (NR041610)	100	JX103632	Firmicutes	<i>Lactobacillus</i>
	L4	Uncultured bacterium clone S1_3_2052 (JQ171585)	100	JX103633	Firmicutes	<i>Acetivibrio</i>
	L6	<i>Acinetobacter lwoffii</i> DSM 2403 (NR026209)	100	JX103634	Proteobacteria	<i>Acinetobacter</i>
M	M1, M2, M3	<i>Lactobacillus animalis</i> KCTC 3501 strain NBRC 15882 (NR041610)	99	JX103635	Firmicutes	<i>Lactobacillus</i>

The sequences compared results in GenBank had 15 uncultured bacteria which were unclassified. All sequences were newly compared in RDP database. These uncultured bacteria were classified through taxonomy with phylogeny in RDP database and the others were the same with compared results in GenBank. Taxon represented the lowest taxonomic unit in compared results

$$H' = -\sum p_i \ln p_i = -\sum (n_i/N) \ln (n_i/N),$$

where n_i was the peak area of band i , N was the sum of all bands' peak areas on this lane [14].

Principal Component Analysis of DGGE Profile

Two-dimensional matrix of relative intensities and the location of bands on DGGE profile were analyzed by Quantity One[®] v4.4, and principal component analysis (PCA) was performed using the Matlab7.0 program.

Clone and Sequencing of DGGE Bands

The DNA of DGGE band was eluted in 100 μ l sterile water. The eluted DNA was re-amplified using same conditions as described above. The PCR products were cloned (pMD18-T vector, Takara Bio). The positive clones of each band were sequenced (Sangon Biotech, Shanghai) and the results were compared with GenBank database by BLAST.

Nucleotide Sequence Accession Number

The partial sequences of 16S rRNA genes from key DGGE bands obtained in this study were deposited in GenBank with the following accession numbers: JX103599–JX103635.

Results

Analysis of DGGE Profiles

Figure 1 presented the partial 16S rDNA V3 PCR-DGGE profiles on 10 days before drug and 70 days after drug. The profiles of KKAY and C57BL/6J mice were clearly distinguished. Band A, B, F, G, H, L and M are dominant or unique in KKAY mice. Band C, D, E, J and K are dominant

Fig. 3 The gut microbial diversity among groups in different times (10d* 10 days before drug, 10d–70d 10–70 days after drug)

or unique in C57BL/6J mice. Band I was the common band of the two kinds of mice.

Similarity Analysis of Different Samples

Among KKAY mice $C_s = 82$ –100 %, C57BL/6J mice $C_s = 83$ –94 %, and the different genotypes mice $C_s = 26$ –44 %. Much less similarities were showed between different genotypes, perhaps because genotypes led to diversity in physiology and metabolism, which then led to differences in gut microbial community [15].

Sequencing of Fragments and Phylogenetic Analysis

Thirteen dominant bands (marked A–M at Fig. 1) were cloned from DGGE profiles and 3–6 clones were

Fig. 4 PCA of the DGGE profiles of samples in different periods. *D1–D3* drug group, *C1–C3* control group, *N1–N3* normal group

sequenced for every band. Forty-five sequences were obtained in total, and among which 24 belonged to KKAY mice, 18 to C57BL/6J mice, 3 to common. All sequences fell into four Phylum (Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria), compared by GenBank and RDP database (Table 1).

The results of phylogenetic analysis (Fig. 2) [16] by neighbor-joining method by MEGA 5.05, were the same as that of compared. Among all sequences, 27 belonged to Bacteroidetes, 13 to Firmicutes, 4 to Proteobacteria and 1 to Actinobacteria. Therefore, the dominant gut microbes of the two genotypes fell into Bacteroidetes and Firmicutes.

Gut Microbial Diversity Analysis

Calculate the diversity index H' of each sample, and use the average of each group at same period to evaluate the group gut microbial diversity. The H' of normal group was slightly higher than that of control group and drug group at the beginning. The former remained stable, but the latter gradually declined. Unexpectedly, the microbial diversity of the drug group was lower than the control group (Fig. 3). We inferred that the gut microbes were influenced not only by metabolic abnormalities of KKAY mice itself, but also the drug. The interference of drug on gut microbes resulted in further enrichment of some bacteria, but others lost, or Pioglitazone had the antibacterial effect, resulting in gut microbial diversity decreasing.

Principal Component Analysis

In order to better exhibit changes of gut microbial community structure at different periods, PCA of DGGE profiles were performed by digital matrixes (Fig. 4). The drug group gradually moved close to the normal group. And 70 days after drug, two groups merged along the PC1 axis. In the meantime, control group moved away along the PC1 axis and separated itself. So Pioglitazone could make gut microbes structure close to normal, somewhat improve microbial structure in gut.

Discussion

In our research, the difference of gut microbial structure between KKAY mice and C57BL/6J mice infer that gut microbes is an important factor for type 2 diabetes, and there is an obvious correlation between gut microbes and host genotypes. The difference in structure of gut microbes is extremely important for exploring their physiological functions. The 16S rDNA fragments of *Bacteroides*, *Facklamia*, *Alistipes*, *Rikenella*, *Odoribacter* and *Anaerotruncus* are discovered from intestine of KKAY mice. The

fragments of *Barnesiella*, *Desulfovibrio*, *Pseudomonas*, *Lachnospiraceae*, *Porphyromonadaceae* and *Microbacteriaceae* are obtained from sample of C57BL/6J mice. The two species of mice share common gut microbes, including *Lactobacillus*, *Acetivibrio* and *Prevotella*. *Bacteroides* is a prominent group in KKAY mice. Its capacity for digesting indigestible dietary polysaccharides is prodigious. The type strain (ATCC 29148) of *Bacteroides thetaiotaomicron* only has 6.3 Mb genome sequence, but these genes can code 226 predicted glycoside hydrolases, 163 digesting starch proteins and 15 polysaccharide lyases [6]. Band B from KKAY, is similar with a series of uncultured bacteria which are beneficial for mice to express TLR4 on intestinal mucosa [8]. TLR4 triggers the activation of kinase cascades and stimulates signaling pathways of inflammation in cells for prompted insulin resistance [17]. In our experiment, only one bacterium, from C57BL/6J, belongs to Actinobacteria. At present, there are few reports about Actinobacteria in mice intestine. Marine actinobacteria can produce α -glucosidase enzyme inhibitor as clinical tool for treating diabetes [18]. The difference of gut microbes from two genotypes mice is not accidental. Some of them closely linked with the type 2 diabetes. We concluded that effect of gut microbes on diabetes had two critical respects. First, gut microbes ferment indigestible polysaccharides for blood glucose. Second, they promote the release of inflammatory factors which are an early factor in the triggering of insulin resistance [19].

Acknowledgments This work has been supported by the National Natural Science Foundation of China (30771310) and the Program of Science and Technology of Shanxi province, China (20120311006-1).

References

1. Yadav H, Jain S, Sinha PR (2007) Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition* 23:62–68
2. Harris K, Kassis A, Major G, Chou CJ (2012) Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? *J Obes* 2012:1–14
3. Le Roy T, Llopis M, Lepage P, Bruneau A et al (2012) Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* 0:1–8
4. Ventura M, O'Flaherty S, Claesson MJ, Turrone F et al (2009) Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat Rev Microbiol* 7:61–71
5. Qin J, Li R, Rase J, Arumugam M et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464:59–65
6. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. *Science* 307:1915–1920
7. Bäckhed F, Ding H, Wang T, Hooper LV et al (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 101:15718–15723

8. Wang Y, Devkota S, Musch MW, Jabri B et al (2010) Regional mucosa-associated microbiota determine physiological expression of TLR2 and TLR4 in murine colon. *PLoS ONE* 5:e13607
9. Korbie DJ, Mattick JS (2008) Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat Protoc* 3:1452–1456
10. Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by ‘reconditioning PCR’. *Nucleic Acids Res* 30:2083–2088
11. Kusumakar AL, Savita Malik Y, Minakshi Prasad G (2007) Detection of human rotavirus in hospitalized diarrheic children in central India. *Indian J Microbiol* 47:373–376
12. Diserud OH, Odegaard F (2007) A multiple-site similarity measure. *Biol Lett* 3:20–22
13. Lyautey E, Lacoste B, Ten-Hage L, Rols JL, Garabetian F (2005) Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: methodological settings and fingerprints interpretation. *Water Res* 39:380–388
14. Sundar SK, Palavesam A, Parthipan B (2011) AM fungal diversity in selected medicinal plants of Kanyakumari District, Tamil Nadu, India. *Indian J Microbiol* 51:259–265
15. Spor A, Koren O, Ley R (2011) Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat Rev Microbiol* 9:279–290
16. Shi L, Cai Y, Yang H, Xing P, Li P et al (2009) Phylogenetic diversity and specificity of bacteria associated with *Microcystis aeruginosa* and other cyanobacteria. *J Environ Sci* 21:1581–1590
17. Evans JL, Maddux BA, Goldfine ID (2005) The molecular basis for oxidative stress-induced insulin resistance. *Antioxid Redox Signal* 7:1040–1052
18. Ganesan S, Raja S, Sampathkumar P et al (2011) Isolation and screening of α -glucosidase enzyme inhibitor producing marine actinobacteria. *Afr J Microbiol Res* 5:3437–3445
19. Cani PD, Amar J, Iglesias MA et al (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56:1761–1772