Title: Association analysis between type 2 diabetes and the gut microbiota: a community-based cross-sectional study in Japan

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Running head: Association between type 2 diabetes and the gut microbiota

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Abstract: There are a few studies on the association between type 2 diabetes (T2D) and the gut microbiota but no community-based cross-sectional study. In the Iwaki Health Promotion Project (Hirosaki, Japan), we surveyed medical information and lifestyle and analyzed the gut microbiota using the terminal restriction fragment length polymorphism technique or next-generation sequencing. Based on these data, multivariable logistic regression analysis was performed with or without T2D as the dependent variable. Consequently, it was suggested that the *Lactobacillales* population in the gut microbiota was positively associated with T2D, consistent with the results of case-control studies.

INTRODUCTION

Type 2 diabetes (T2D) is caused by insulin resistance or impaired insulin secretion. T2D is related to genetic factors and lifestyle, such as overeating or lack of exercise. In Japan, the sum of patients with T2D and potential diabetics was 20 million in 2016 (The National Health and Nutrition Survey in Japan, 2018). The prevalence is higher in males and with increasing years.

Research on the association between various diseases and the gut microbiota has advanced [1] and become increasingly important. Also, case-control studies on the association between T2D and the gut microbiota have been reported [2-10]. These studies commonly indicated an increased *Lactobacillales* population in the fecal microbiota in T2D, so *Lactobacillales* have the potential to be a novel target for prevention and therapy of T2D.

Community-based health examination surveys (in Hirosaki City, Aomori, Japan) have been performed for many years, called "the Iwaki Health Promotion Project" (UMIN ID: UMIN000040459) (11). In this project, about ten hundreds of people participate every year, and data from more than 2000 items related to vital and medical information, dietary habits, and lifestyle are collected. These also include data on the gut microbiota obtained using the terminal restriction fragment length polymorphism (T-RFLP) technique [12, 13] or next-generation sequencing (NGS) [14].

There has been no community-based cross-sectional study on the association between T2D and the gut microbiota. Therefore, in this study, multivariable logistic regression analysis was performed with or without T2D as the dependent variable using Iwaki Health Promotion Project data.

MATERIALS AND METHODS

The Iwaki Health Promotion Project

This survey was intended for the Iwaki Health Promotion Project participants in 2012, 2014, and 2016. The number of subjects by sex and age is shown in Table 1, in which those with healed T2D were excluded because of the difficulty of attributing to either of the binary variables. Subjects were inquired about sex, age, medical history, drug intake, smoking, alcohol drinking, exercise, etc. Weight and height were measured to calculate the body mass index (BMI). The results of inquiries to the subjects with or without T2D are shown in Table 2. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine. Written informed consent was obtained from all participants.

Microflora analysis

Fecal DNA was prepared as described previously [15]. Feces were collected into a 4 M guanidine thiocyanate solution, followed by bead-beating and extraction using magnetic beads. The fecal microbiota was analyzed by T-RFLP [12, 13] or NGS [14], targeting the 16S rRNA gene.

In silico T-RFLP using bacterial 16S rRNA gene sequences obtained by random cloning analysis of the fecal microbiota provided correspondence between operational taxonomic units (OTUs) in T-RFLP and phylogenetic bacterial groups [12, 13]. This study classified these OTUs into eight bacterial functional groups (B-1–B-8; see Table 2) based on the organic acid-producing activity, which refers to the report of Kettle et al. [16]. Conversion equations are given below.

$$B1 = OTU366 \times 0.952 + OTU469 \times 0.97 + OTU853$$

$$B2 = OTU317$$

B3 = OTU106 + OTU168 + OTU338 + OTU369 × 0.33 + OTU494 × 0.84 + OTU650 + OTU657 × 0.08 + OTU754 × 0.52 + OTU920 × 0.80 + OTU940 × 0.28 + OTU955 × 0.92

B4 = OTU110 × 0.04 + OTU124

B5 = OTU332 + OTU520 + OTU657 × 0.92

B6 = OTU369 × 0.33 + OTU494 × 0.14 + OTU505 × 0.5 + OTU517 + OTU640 + OTU749 + OTU754 × 0.10 + OTU940 × 0.24 + OTU955 × 0.08

B7 = OTU110 × 0.96 + OTU369 × 0.17 + OTU469 × 0.03

B8 = OTU369 × 0.17 + OTU494 × 0.02 + OTU505 × 0.5 + OTU754 × 0.38 + OTU920 × 0.20 + OTU940 × 0.48 + OTU990

Multivariable logistic regression analysis

Multivariable logistic regression analysis (forward selection method) was performed with or without T2D as the dependent variable. The number of subjects with or without T2D is shown by sex in Table 2. The functional bacterial groups in Table 3 in T-RFLP or seven orders, including *Bacteroidales*, *Bifidobacteriales*, *Clostridiales*, *Coriobacteriales*, *Enterobacteriales*, *Lactobacillales*, and *Selenomonadales*, in NGS were the independent variables. The sequences derived from these seven orders accounted for >90% of the total. Age, BMI, smoking, alcohol drinking, and exercise were used as moderators in the regression analysis. In 2016, when subjects were inquired about drug intake, α-glucosidase inhibitor (α-GI), a diabetes drug, was also used as a moderator. JUSE-StatWorks/V5E (The Institute of JUSE, Tokyo, Japan) was used for statistical analysis. The significance level was set at $p < 0.05$.

RESULTS

In Table 2, the T2D ratio in subjects was 3.6% to 4.2% in females and 7.2% to 7.7% in males, which is almost the same level as the ratio of outpatients with T2D to the population (>20 years old in 2016) in Aomori Prefecture: 3.1% in females and 5.4% in males. Moreover, differences between subjects with and without T2D in age, BMI and habits of life were shown.

This study used multivariable logistic regression to assess the association between T2D and the gut microbiota (OTUs) in the Iwaki Health Promotion Project participant population for 3 years. A summary of the results is presented in Table 4. The functional bacterial group B-5 (*Lactobacillales*, refer to Table 3) was commonly extracted as a significant variable in males and females in case of 2012 and 2014, in which T-RFLP analysis was performed, with odds ratios [OR; 95% confidence intervals (95% CIs)] was 1.062 (1.025–1.100), 1.068 (1.019–1.20), 1.085 (1.032–1.142), and 1.069 (1.020–1.121), respectively. In case of 2016, in which NGS analysis was performed, *Lactobacillales* was a significant variable in males with ORs (95% CIs) of 1.174 (1.019–1.351), but it was an insignificant variable in females, with p-value of a slightly over 10% (p = 0.109). The functional bacterial group B-4 (*Bifidobacterium* spp.) was also a significant variable in some cases, that is, males and females in 2012 and males in 2014 and 2016, with ORs (95% CIs) of 1.150 (1.064–1.244), 1.075 (1.007–1.146), 1.053 (1.013–1.095), and 1.074 (1.024–1.125), respectively. As for the rest, the functional bacterial group B-3 (*Clostridium* clusters IV, XI, XIVa, and XVIII) in females in 2014, *Bifidobacterium* and *Coriobacteriales* in males in 2016, and *Bacteroidales* in females in 2016 were significant variables.

DISCUSSION

Although previous studies on the association between T2D and the gut microbiota were performed in various countries (Austria, Iran, Denmark, Japan, and Sweden) with various techniques (NGS, quantitative polymerase chain reaction, and T-RFLP), a positive association with *Lactobacillales* was indicated in any studies, suggesting a considerably high certainty of the results. In this study, a multivariable logistic regression analysis was performed to assess the association between T2D and the gut microbiota in a cross-sectional study intended for people in Hirosaki, Japan. Similar results to previous studies were obtained.

This and previous studies suggested the causal relationship between T2D and *Lactobacillales*, but the cause or result is unknown. First, whether *Lactobacillales* are innate bacteria or bacteria originating from foods, such as yogurt, was discussed. Sato et al. [6] and Adachi et al. [9] considered that these bacteria are innate because the number of subjects who consumed yogurt was not significantly different between the control and T2D groups and significantly fewer in the latter group. Furthermore, the number of probiotic bacteria taken from supplements or foods was ~10^{10} cells/day. These bacteria cannot colonize and proliferate in the gut to remain a minor population. Therefore, the idea that these bacteria are innate is deemed to be appropriate.

Morita et al. [17] indicated that lactate and pyruvate, produced in the small intestinal in a bacteria-dependent manner, enhanced immune responses by inducing dendrite protrusion of intestinal $CX3CR1^{+}$ mononuclear cells. These findings speculated a possible causal relationship as follows: an excess of bacteria, including lactic acid bacteria in the small intestine, induces the overstimulation of immune responses, followed by chronic inflammation, resulting in the T2D onset (18, 19).

If that is the case, why do bacteria such as lactic acid bacteria proliferate abnormally in the small intestine? Usually, gastric acid strongly prevents bacterial entry

to the small intestine from the oral cavity so that almost no bacterial proliferation occurs. Therefore, reduced secretion of gastric acid by aging or by taking the medicine for gastric hyperacidity that is induced by stress, disturbed eating habit and so on, may result in the proliferation of these bacteria in the small intestine followed by their subsequent increase in the large intestine [20-23]. Yuan et al. [24] and Ciardullo et al. [25] have reported that regular and prolonged use of proton pump inhibitors (PPIs) was associated with a higher risk of type 2 diabetes. In the Iwaki Health Promotion Project in 2016, we also found that the ratio of subjects prescribed PPIs was significantly greater in population with T2D than in that without T2D (Table 2). These findings seem to provide partial support to the above-mentioned speculation.

On the one hand, some studies suggested that α-GI increases populations of *Bifidobacterium* spp. and *Lactobacillales* in the fecal microbiota [6, 9, 10]. In this project in 2016, the number of subjects with T2D who did or did not take α-GI was 12 (10 males and 2 females) and 45 (23 males and 22 females), respectively. *Bifidobacterium* spp. population in the fecal microbiota in males was higher in subjects with T2D taking α-GI than those not taking α-GI [mean ± standard deviation (SD), 24.3 ± 7.9 vs. 9.3 ± 11.0; $p = 0.003$, Wilcoxon rank-sum test]. Similarly, in *Lactobacillales* in males, the population was higher in subjects with T2D taking α-GI (mean ± SD, 29.4 ± 14.6 vs. 8.5 ± 14.9; $p < 0.001$). No significant difference was observed in females. From these facts, here we accessed using α-GI as an additional moderator for the regression analysis in 2016, in which subjects were inquired about drug intake.

On the other hand, α-GI is not involved in the other studies that indicate the positive association between T2D and *Lactobacillales* [5, 7, 8]. Furthermore, the *Lactobacillales* population in the fecal microbiota also increased in a mouse model of insulin resistance [26]. Considering these findings, α-GI is deemed to have only limited effectiveness in this and previous studies on the association between T2D and the gut

microbiota.

Taken together, multivariable logistic regression analysis was performed in the community-based cross-sectional study to assess the association between T2D and the gut microbiota. It was suggested that the *Lactobacillales* population was positively related to T2D, as indicated in previous studies. It is expected that future analyses will be performed with more subjects, and studies on the elucidation of the causal relationship are making progress. As such, novel techniques for prevention and therapy targeted to the bacteria are being developed.

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AUTHER CONTRIBUTIONS

K.N. played a role in the investigation, conceptualization and writing original draft. T.H. played a role in the data curation, formal analysis and investigation. J.M., T.M. and Y.T. played a role in the project administration and reviewing. S.N. played a role in the project administration and supervision.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data presented in this study are available under the agreement with Innovation Center for Health Promotion, Hirosaki University.

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Table 1. The number of subjects by sex and age each year

Year		2012		2014		2016	
No. of subjects		936		1,103		1,088	
Sex		Male	Female	Male	Female	Male	Female
Age	～20	16	19	27	43	24	34
	30	51	68	73	84	81	96
	40	56	86	78	101	74	111
	50	73	131	76	130	82	125
	60	95	166	103	196	106	177
	70	45	104	48	107	43	95
	80～	12	14	14	23	17	23
Sum		348	588	419	684	427	661

The subjects with healed T2D are excluded.

Table 2. Results of inquiries to subjects with or without T2D by sex each year

	Year	2012			2014			2016		
		With T2D	Without T2D	*p*	With T2D	Without T2D	*p*	With T2D	Without T2D	*p*
Male	No. of subjects, n (%)	25 (7.2)	323 (92.8)		31 (7.4)	388 (92.6)		33 (7.7)	394 (92.3)	
	Age	67 (77-62)	55 (64-42.5)	0.000	66, 77-63	52 (64-39)	0.000	67 (76-63)	52 (64-38)	0.000
	BMI	24.2 (26.3-22.5)	23.2 (25.3-21.2)	0.000	23.9, 25.6-22.9	23.3 (25.0-21.5)	0.062	23.8 (25.7-22.2)	23.6 (25.5-21.7)	0.594
	No. of subjecs, n (%) With smoking habit	3 (12.0)	107 (33.1)	0.027	6 (19.4)	125 (32.2)	0.162	4 (12.1)	117 (29.7)	0.042
	with drinking habit	18 (72.0)	226 (70.0)	1.000	18 (58.1)	269 (69.3)	0.228	21 (63.6)	275 (69.8)	0.440
	With exercise habit	13 (52.0)	95 (29.4)	0.025	12 (38.7)	122 (31.4)	0.426	10 (30.3)	111 (28.2)	0.841
	Prescribed PPIs	ND	ND		ND	ND		6 (18.2)	18 (4.6)	0.007
Female	No. of subjects, n (%)	21 (3.6)	567 (96.4)		29 (4.2)	655 (95.8)		24 (3.6)	637 (96.4)	
	Age	69.5 (72.5-64)	58 (67-46)	0.000	70, 74-64	58 (66-42.5)	0.000	68 (72.3-57)	57 (66-43)	0.000
	BMI	24.4 (25.9-22.8)	22.1 (24.1-20.0)	0.000	24.0 (27.6-22.5)	21.7 (23.9-19.6)	0.000	23.8 (26.6-22.0)	21.9 (24.2-19.8)	0.016
	No. of subjecs, n (%) With smoking habit	1 (4.8)	33 (5.8)	1.000	2 (6.9)	51 (7.8)	1.000	0 (0.0)	64 (10.1)[a]	0.156
	with drinking habit	1 (4.8)	137 (24.2)	0.037	2 (6.9)	179 (27.3)	0.016	5 (20.8)	176 (27.8)[b]	0.642
	With exercise habit	11 (52.4)	168 (29.6)	0.032	15 (51.7)	213 (32.5)	0.043	7 (29.2)	155 (24.3)	0.629
	Prescribed PPIs*	ND	ND		ND	ND		6 (25.0)	44 (6.9)	0.007

Age and BMI are expressed as median (interquartile range). The number of subjects in [a] and [b] are 636 and 634, respectively.

P values were calculated by using the Wilcoxon rank sum test and the Fisher's exact test.

*The number of subjects that were prescribed PPIs within three months before the date of inquiry. PPIs, proton pump inhibitors; ND, no data

Table 3. Classification of OTUs in T-RFLP into the bacterial functional groups

Bacterial functional groups	Phylogenetic bacterial groups	Organic acid producing activity	OTUs in T-RFLP *
B-1	*Bacteroides*	Acetate, Propionate, Succinate	366, 469 (97), 853
B-2	*Prevotella*	Acetate, Propionate, Succinate	317
B-3	*Clostridium* cluster IV, XI, XIVa, XVIII	Acetate, Succinate	106, 168, 338, 369 (33), 494 (84), 657 (8), 754 (52), 920 (80), 940 (28), 955 (92)
B-4	*Bifidobacterium*	Acetate, Lactate	110 (4), 124
B-5	*Lactobacillales* (*Enterococcus*, *Lactobacillus*, *Streptococcus*)	Lactate	332, 520, 657 (92)
B-6	*Clostridium* cluster IV, XI, XIVa, XVIII	Butyrate, Lactate, Formate	369 (33), 494 (14), 505 (50), 517, 640, 749, 754 (10), 940 (24), 955 (8)
B-7	*Clostridium* cluster IX	Acetate, Propionate	110 (96), 369 (17), 469 (3)
B-8	*Clostridium* cluster XIVa	Acetate, Butyrate, Formate Lactate, Acetate → Butyrate	369 (17), 494 (2), 505 (50), 754 (38), 920 (20), 940 (48), 990

* In silico T-RFLP using the 16S rRNA gene sequences obtained by the random cloning analysis of feacal microbiota provides a correspondence between the OTUs in T-RFLP and the phylogenetic bacterial groups (11, 12). In this research, these OTUs were further classified into the 8 bacterial functional groups (B-1 to B-8) on the basis of the organic acid producing activity. Even if the clones from the bacterial 16S rRNA gene belong to the same OTU, they are allocated to the different functional group in some cases because of the different organic acid producing activity. Numbers in parenthese indicate the allocation ratios (%), which were calculated from the number of clones belonging to each functional group.

Table 4. Multivariable logistic regression analysis for the presence of T2D

Year	Sex	Variables	B	SE	*P*		OR	95% CI	
								Lower	Upper
2012	Male	B-4 (*Bifidobacterium*)	0.140	0.040	0.00045	*	1.150	1.064	1.244
		B-5 (*Lactobacillales*)	0.060	0.018	0.00075	*	1.062	1.025	1.100
		Constant	-15.195	2.810	0.00000				
	Female	B-5 (*Lactobacillales*)	0.066	0.024	0.00603	*	1.068	1.019	1.120
		B-4 (*Bifidobacterium*)	0.072	0.033	0.03159	*	1.075	1.007	1.146
		B-6 (*Clostridium*)	0.057	0.032	0.07192		1.059	0.994	1.127
		Constant	-16.395	2.974	0.00000				
2014	Male	B-5 (*Lactobacillales*)	0.082	0.026	0.00163	*	1.085	1.032	1.142
		B-4 (*Bifidobacterium*)	0.052	0.020	0.00788	*	1.053	1.013	1.095
		Constant	-11.749	2.397	0.00000				
	Female	B-3 (*Clostridium*)	-0.094	0.029	0.00128	*	0.910	0.860	0.964
		B-5 (*Lactobacillales*)	0.067	0.024	0.00498	*	1.069	1.020	1.121
		B-8 (*Clostridium*)	-0.053	0.033	0.10939		0.948	0.889	1.012
		B-4 (*Bifidobacterium*)	0.024	0.018	0.19678		1.024	0.989	1.061
		Constant	-11.465	2.269	0.00000				

continued

2016	Male	*Bifidobacteriales*	0.071	0.024	0.00382	*	1.074	1.024	1.125
		Coriobacteriales	0.160	0.072	0.02529	*	1.174	1.019	1.351
		Lactobacillales	0.036	0.018	0.04820	*	1.037	1.001	1.074
		Constant	-8.968	1.352	0.00000				
	Female	*Bacteroidales*	-0.063	0.025	0.01043	*	0.939	0.894	0.986
		Lactobacillales	0.033	0.021	0.10876		1.034	0.992	1.077
		Constant	-4.960	1.609	0.00206				

Refer to Table 2 with respect to the variables of B-3, B-4, B-5, B-6 and B-8.

Age, BMI, the habits of smoking, alcohol drinking and exercise, and the medication of α-GI (only in the case of 2016) were used as the moderator variable.

The variables with $p < 0.2$ were presented. * indicate $p < 0.05$.

SE, standard error; OR, odds ratio; CI, confidence interval.