Shared genetic susceptibilities for irritable bowel syndrome and depressive disorder in Chinese patients uncovered by pooled whole-exome sequencing

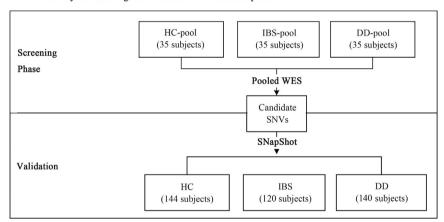Shiwei Zhu [a], Meibo He [a], Zuojing Liu [a], Zelian Qin [b], Zhiren Wang [c,*], Liping Duan [a,*]

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GRAPHICAL ABSTRACT

Aim: To analyze the same genetic variations in IBS and depression

ABSTRACT

Irritable bowel syndrome (IBS) is the most prevalent functional gastrointestinal disorder presenting a high comorbidity with depressive disorder (DD). Many studies have confirmed that these two disease share the similar pathophysiological process, but evidence of the genetic risks is limited. This study aimed to analyze the genetic susceptibilities for IBS and DD in Chinese patients. Pooled whole-exome sequencing (pooled-WES) was performed to identify the candidate variants in the group of diarrhea predominant IBS (IBS-D) patients, DD patients, and healthy controls (HC). Then, targeted sequencing was used to validate the candidate variants in three additional cohorts of IBS-D, DD, and HC. Four variants associated with both IBS-D and DD were identified through pooled-WES, and three of them were validated in targeted sequencing. *SYT8* rs3741231 G allele and *SSPO* rs12536873 TT genotype were associated with both IBS-D and DD. The genes of these variants are important in neurogenesis and neurotransmission. In addition, we found *COL6A1* rs13051496, a unique risk variation for IBS-D. It increased the IBS-D

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risk and had a positive correlation with the scores of abdominal bloating and dissatisfaction of bowel habits. Through the results of this study, it provides a genetic basis for the high comorbidity of IBS-D and DD.

Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal (GI) disorder, with a worldwide prevalence estimated at 12% [1]. In China, the prevalence of IBS has been reported at 6.5% [2], and its incidence is increasing. IBS is an important healthcare concern as it greatly affects patients' life quality and social functioning. Patients with IBS have a 3.6-fold increased risk of developing psychiatric disorders [3], the most common is depressive disorder (DD). Interestingly, 17–59% of patients with DD have been reported to meet IBS diagnostic criteria [4,5]. With the respect to clinical treatment, the antidepressants have notable relief effect on both IBS and DD patients [6,53]. All these evidences suggest that IBS and DD are epidemiologically linked and share the similar etiology.

However, the mechanisms of IBS and DD comorbidity remain undetermined. Studies suggested that these two diseases have similar risk factors. First of all, stress, it is the key factor responsible for both IBS and DD [7]. A history of physical or sexual abuse, or severe infections is associated with the development of IBS or DD as well [8]. In addition, there are overlaps in the pathophysiological features of these two diseases, such as brain–gut interaction, neurotransmitter dysregulation (hypothalamic–pituitary–adrenal axis or serotonin system), and immune system dysfunction [9,10,18]. Our previous study [11] suggested that diarrhea predominant IBS (IBS-D) and DD patients shared similar gut microbiota composition in the genus and family level. Slyepchenko et al. [12] also reported that both IBS and DD exhibit a trend of a higher relative abundance of *Bacteroidetes* and a lower abundance of *Firmicutes*.

Genetic factors increasing the risk of DD have been widely reported. For IBS, the genetic polymorphisms also associated with the incidence [13–16,54]. Among the IBS subtypes, hereditary factors seem to primely influence on IBS-D [13]. After a deep review [8–16,55] of genome-wide association studies (GWAS) and single nucleotide polymorphisms (SNPs) analysis of pure IBS-D patients or pure DD patients, we found that some variants were reported to be associated with both IBS-D and DD, such as *COMT* rs6267 and *SCL6A4 5-HTTLPR* [2,15]. Few studies have revealed a heritable component for IBS and DD comorbidity. For example, Kohen and colleagues found variants in gene *SLC6A4* are associated with comorbidity of IBS and DD [19]. One co-twin control study [20] suggested no association of genetic factors between IBS and DD comorbidity, but another twin's study [21] concluded differently. Their study suggested that genetic factors contribute to comorbidity of IBS and DD. It can be seen that the current evidence is debatable.

Whole-exome sequencing (WES) is powerful for dissecting the genetic basis of diseases as it targets all the protein-coding genes. Single nucleotide variations (SNVs) and insertions/deletions (InDels) are identified through WES. However, these techniques are costly, laborious, and time-consuming for most laboratories and population-based association studies. An alternative approach known as pooled sequencing (pooled-seq) involves pooling several individual DNA samples together and sequencing the pooled DNA. It can efficiently increase the sample size and sequencing depth in library preparation at a reduced cost and effort [22].

Thus, the aim of this study was to assess and validate the susceptibility SNVs in patients with IBS-D and patients with DD in Chinene. The findings of this study might provide some new insights into the genetic mechanisms underlying IBS, DD, and their comorbidity.

Materials and methods

Study design and subject recruitment

The study was performed at Peking University Third Hospital and Peking University Huilongguan Clinical Medical School. Patients and healthy volunteers aged 18–65 years old were filtered. IBS-D patients were diagnosed by Rome III criteria and underwent colonoscopy with biopsies in the distal ileum and sigmoid to rule out organic GI disorders. IBS-D patients also need to be excluded from psychiatric disorder. DD patients were diagnosed according to the Mini Mental State Examination and were included only if they had no GI-associated diseases. As confirmed through the same questionnaires with IBS-D and DD, age and gender matched healthy controls (HC) which had no previous or current GI symptoms and no psychiatric disorders were included. For all participants, GI symptom severity was evaluated by a validated questionnaire for IBS symptom severity scores (IBS-SSS). Depressive symptom severity was screened by the hospital anxiety and depressive scale (HAD); patients with significantly high HAD scores (>11) were assessed by the Hamilton anxiety and depression scale (HAMD). Visceral sensitivity was tested through a colon-rectal distension test using BAROSTAT (Distender Series II; G&J Electronics, Ontario, Canada) in some subjects. Blood samples were collected from all participants and subjected to DNA extraction and subsequent sequencing.

Overall, 155 eligible patients with IBS-D, 175 patients with DD; and 179 healthy individuals were enrolled. Among them, 35 subjects were randomly selected from each group and were analyzed in pooled-WES to filter the candidate SNVs. The rest of 120 IBS-D, 140 DD and 144 HC subjects were used for further validation.

All subjects provided written informed consent and the study protocol was approved by the Ethics Committee of the Peking University Health Science Center (No. 2013-12) and Beijing Huilongguan Hospital (No. 2016-42).

DNA extraction

DNA was isolated from approximately 3 mL of the peripheral blood following a phenol/chloroform protocol. Each DNA sample was quantified twice using the DNA quantification NanoDrop (Thermo Scientific, California, USA). Samples were accepted as suitable for the study if the average DNA concentration was at least 250 ng/μl and the coefficient of variation between the two rounds of quantification was smaller than 0.1.

Pooled-WES analysis

To minimize possible artifacts, "best practice" guidelines [23,24] for the sequencing of pooled samples were used. Each pool comprised DNA from 35 subjects, and was defined as either the IBS-pool, DD-pool, or HC-pool. Pooled-WES was performed based on the above patient's clinical features to optimize the results [33].

Pooled samples were sent to a sequencing provider (MICRO-READ, Beijing, China) for the preparation of population-specific libraries and subsequent Illumina HiSeq X-ten whole-exome sequencing. DNA was quantified using the Qubit system before library preparation. 50 ng DNA of each sample were used and fragmented using a Bioruptor Pico Sonication System (Diagenode, Liege, Belgium). Paired-end library preparation was based on the SureSelectXT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library (Agilent, PaloAlto, USA) for the pooled samples. Size selection and final library purification were performed using AMPureXP beads (Beckman Coulter, CA, USA) with an additional gel-based size selection for the pooled samples. The resulting insert sizes were 100–200 bp for the three pools. All libraries were sequenced on a HiSeq X-ten following the protocol [25].

Raw sequencing data were trimmed using in housing developed scripts (base quality threshold of 20) and trimmed reads were aligned to the human genome 19 (hg19) build by using Burrows-Wheeler aligner (BWA) v0.5.9 [26] without seeding. Duplicates and improper pairs (mapping quality less than 20) were filtered through Samtools v1.3 and Picard tools v2.1.1 [26,27]. Finally, all aligned bases were realigned around short insertions and deletions using Genomic analysis toolkit (GATK) v3.5 [28]. The cleaned and filtered alignment files were converted into a synchronized format (base quality more than 20) using PoPoolation2 [23].

Genetic variations were identified and quantified using Centre for Research and Industrial Staff Performance (CRISP) v0.7 [51]. The alignment files for each population were analyzed together, using a sample ploidy in the range of 50–70, a minimum base quality of 10, and a mapping quality of 20. All polymorphic sites were filtered for a minimum coverage of 12 for each sample and a minimum overall count of the minor allele of 50 for each sample. To correct for multiple testing, we enforced a false detection rate of 0.05 derived from an empirical null distribution as described by Endler et al [29]. To test for the associations of variants between the patients and healthy controls, we used the cmh-test.pl program of PoPoolation2 as described previously [29]. PoPoolation2 is a software tool dedicated to the comparison of allele frequencies between populations [23]. All the P-values were corrected by the false discovery rate (FDR). To test whether differentiating SNVs between the IBS-pool or DD-pool and HC-pool comparisons were enriched with potential molecular functions or other specific functional categories, gene ontology enrichment tests were performed in BLAST2GO and FunRich3.1.3. Annovar [30] was used to annotate variants with information from public databases, including gene reference hg19, SNP database, and the 1000 Genomes Project. Polymorphism phenotyping, sorting intolerant from tolerant (SIFT), and the likelihood ratio test (LRT) were used to evaluate the variants in terms of sequence conservation and chemical change [31]. The high-quality SNVs were prioritized following the principal steps (Fig. 1): (i) variants within exonic; (ii) nonsynonymous variants; (iii) different variants between HC and patients with a significance of $P_{cmh} \leq 0.05$; (iv) damage effect of the variants on amino acid (AA) or protein function according to SIFT, PolyPhen2, or LRT (Fig. 1).

SNaPshot analysis

The rest of subjects defined as IBS, DD, and HC group were used to validate the candidate SNVs through SNaPshot. Genomic DNA was diluted to a concentration of 10 ng/μL and stored at 4 °C for the subsequent identification of genetic variations. The amplification primers of the candidate genes are shown in supplementary Table 1. A multiplex SNaPshot assay (ABI PRISM, California, USA) was employed to determine the genotypes. Detailed for experimental processes were presented in http://www.gene99.com/scServ/9–244-173.html. Data were analyzed by GeneMapper 4.0 (ABI, California, USA). In order to guarantee the quality of the data, approximately 3% of the samples was randomly selected and regenotyped by direct sequencing.

Statistical analysis

Data conform to normal distribution presented as mean ± standard deviation was performed by t-test, otherwise, data presented as median (Q25, Q75) were performed by Mann–Whitney U test. Genetic association analyses and odds ratio (OR) calculations were performed for minor alleles based on genotypes using IBM SPSS 30.0 and PLINK 1.0.7 (http://pngu.mgh.harvard.edu/purcell/plink). A $\chi 2$ test was used in statistical analysis if the theoretical value was more than 5, otherwise the Fisher's exact test was used. And Hardy–Weinberg equilibrium (HWE) determinates in healthy controls. $P < 0.05$ was considered statistically significant. Both allele and genotype models (allele model, AM; dominant model, DM; recessive model, RM; homozygous model, HoM; heterozygous model, HeM) were used (Supplementary Table 2).

Results
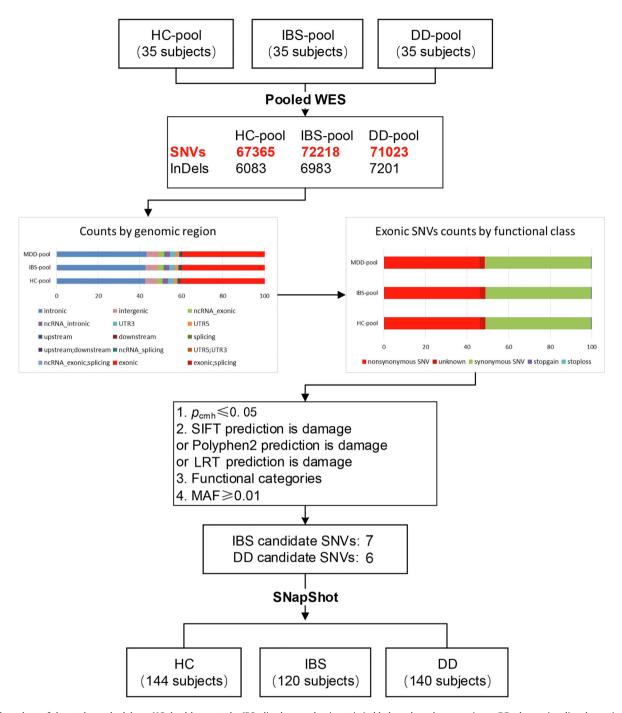
Subject identification and characteristics

No statistically significant differences were found for sex, age, or BMI (Table 1) in Pooled-WES or validation cohort. Subjects in the IBS-pool exhibited a significantly lower visceral pain threshold in initial sensation, initial defecation, and defecation urgency. And subjects in the DD-pool had significantly increased depressive symptom scores compared with the other two pools. The similar changes were found in validation cohort (Table 1). It suggested that IBS-D patients presented the highest visceral sensitivity, while DD patients had the most severe depression symptoms among all three cohorts. For the IBS-SSS survey of validation cohort, IBS patients had the highest scores of items "abdominal pain," "abdominal bloating," "dissatisfaction with bowel habits," and "disturbance in daily life" among the three groups. Interestingly, DD patients who took the IBS-SSS survey also had a higher score of item "disturbance in daily life" compared with HC, which might suggested that the mental disorder of those patients might have greatly influenced the judgment for bowel function (Table 1).

Candidate SNVs analysis through pooled-WES

4 to 12 million reads were generated in the three pools. The majority of paired reads were uniquely mapped onto a chromosome, with a rate up to 95.2% in almost all the target regions (coverage rate, over 99.9%) (Supplementary Table 3). Over 70,000 SNVs and InDels were identified by comparison with the current reference hg19 (http://genome.ucsc.edu) in each pool.

Gene functional categories of the significant SNVs (IBS-pool or DD-pool vs. HC-pool, $P_{cmh} \leq 0.05$) were annotated which shows in Fig. 2A. The IBS-significant SNVs mainly clustered in transferase, cell adhesion molecules, and voltage-gated ion channel activity, while DD-significant SNVs clustered in phospholipase, chaperone, and DNA-binding activity. There are 11 SNVs were overlapped between the IBS-significant SNVs and DD-significant SNVs, there functions were annotated as calcium ion binding and extracellular matrix structural constituents.

Considering the association between SNVs and diseases (OR ≥ 1.5 or ≤ 0.5) and the reference minor allele frequency in the 1000 Genomes Project of

Fig. 1. Flow-chart of the study methodology. HC: healthy controls; IBS: diarrhea predominant irritable bowel syndrome patients; DD: depressive disorder patients; WES: whole-exome sequencing; SNV: single nucleotide variations; InDel: insertion or deletion; SIFT: sorting intolerant from tolerant; LRT: likelihood ratio test; p_{cmh}: significance in comparison of the patient group with healthy controls, Cochran–Mantel–Haenszel test.

Asian population ($0.01 \leq$ MAF ≤ 0.5), seven candidate SNVs for IBS-D and six candidate SNVs for the DD were identified (Fig. 2B and C; Table 2). *FAM129A* rs28927681, *SYT8* rs3741231, *SSPO* rs12536873, and *COL6A1* rs13051496 were found in association with both IBS (IBS-pool *vs.* HC-pool, *P* < 0.05 separately) and DD (DD-pool *vs.* HC-pool, *P* < 0.05 separately). *SLC7A6OS* rs8063446, *RECQL4* rs4251691 and *ANKRD11* rs113527563 were uniquely identified in the IBS-pool (IBS-pool *vs.* HC-pool, *P* < 0.05 separately). *EDN3* rs11570255 and *COMT* rs6267 were uniquely identified in the DD-pool (DD-pool *vs.* HC-pool, *P* < 0.05 separately). The data shows in Table 3.

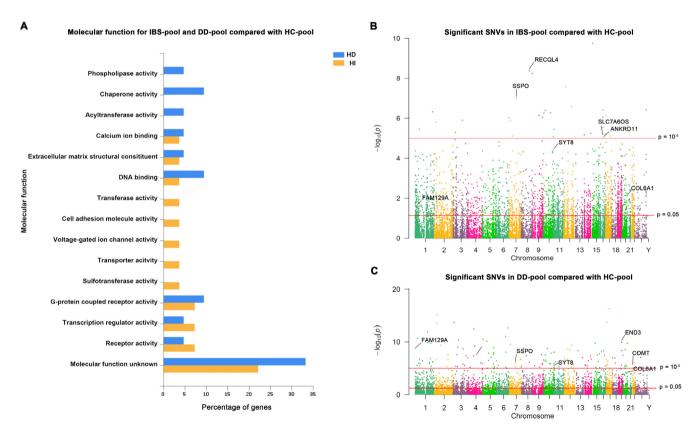*Validation of candidate SNVs through SNaPshot*

Frequencies for the SNVs are presented in supplementary table 5 and those in HC group were closed to 1000 Genomes Asian frequency. Therefore, we compared the patients with HC in allele model (Table 4) and different genotyping models (Table 5) to analyze the association for candidate SNVs with IBS-D or DD.

SYT8 rs3741231 was associated with both IBS-D and DD risks (AM: G *vs.* C; OR$_{IBS}$ = 3.287, P_{IBS} = 0.001; OR$_{DD}$ = 2.193, P_{DD} = 0.034). In genotype analysis, variation in *SYT8* rs3741231 significantly increased in the IBS group compared with the HC group

Table 1
Clinical characteristics of the study.

	Pooled-WES			SNaPshot		
	HC-pool (n = 35)	IBS-pool (n = 35)	DD-pool (n = 35)	HC (n = 144)	IBS (n = 120)	DD (n = 150)
Sex (male %)	77.14%	82.85%	74.28%	54.00%	67.00%	55.10%
Age (years old)	32.42 ± 10.45	34.84 ± 10.23	36.40 ± 10.33	41.61 ± 13.13	38.69 ± 9.89	39.59 ± 13.38
BMI	22.86 ± 3.91	23.69 ± 3.72	21.75 ± 3.15	24.10 ± 4.35	22.64 ± 3.72	23.41 ± 4.46
Rectal distension						
Initial sensory (mmHg)	12 (10, 14)	8 (6, 12) [ac]	11 (8, 12)	11 (7.5, 12.5)	8 (8, 12) [a]	8 (4, 10) [b]
Initial defecation(mmHg)	20 (18, 28)	16 (13.5, 18.5) [ac]	19 (17, 21.5)	19 (14, 20)	16 (14, 20) [ac]	12 (11, 12.5) [b]
Defecation urgency (mmHg)	28 (26, 38)	24 (20, 26) [ac]	28 (23.5, 32)	28 (21.5, 32)	24 (20, 28) [ac]	28 (22, 35)
HAD/HAMD[n]	5.29 ± 4.59	10.27 ± 4.81	32.43 ± 3.09	5.85 ± 6.68	9.68 ± 6.89	24.47 ± 5.19
IBS-SSS						
Abdominal pain				0 (0, 0)	50 (30, 81.5) [a c]	3.5 (0, 12.25)
Abdominal bloating				0 (0, 0)	30 (0, 36.25)[a]	12.5 (0, 26.25)
Dissatisfaction with bowel habits				20 (5, 50)	30 (0, 30) [a]	33 (20, 66) [b]
Life disturbance				3 (0, 20)	70 (40, 70) [a]	41.5 (27.25, 74.5) [b]

Note: HC: Healthy volunteers; IBS: Irritable bowel syndrome; DD: depressive disorders; HAD/HAMD: Hospital Anxiety and Depression Scale/ Hamilton Depression Scale; [n]: Because all the depressive patients had a HAD score over 11, thus we present the HAMD socres in DD-pool and DD; IBS-SSS: IBS Symptom Severity Scores; [a]: IBS *vs.* HC, $P < 0.05$; [b]: DD *vs.* HC, $P < 0.05$; [c]: IBS *vs.* DD, $P < 0.05$. Data conform to normal distribution presented as mean ± standard deviation was performed by *t*-test, otherwise, data presented as median (Q25, Q75) were performed by Mann–Whitney *U* test.

Fig. 2. A. Gene ontology (GO) molecular function of filtered SNVS for IBS-pool and DD-pool. HD: DD-pool compared with HC-pool; HI: IBS-pool compared with HC-pool. B. Manhattan plot for SNVs in the IBS-pool compared with the HC-pool. C. Manhattan plot for SNVs in the DD-pool compared with the HC-pool. *P* value indicates the significance according to Cochran–Mantel–Haenszel test.

(DM: GG + GC *vs.* CC, OR_{IBS} = 3.29, P_{IBS} = 0.019; RM: GG *vs.* GC + CC, OR_{IBS} = 3.29, P_{IBS} = 0.019; HoM: GG *vs.* CC, OR_{IBS} = 3.29, P_{IBS} = 0.019).

T allele of *SSPO* rs12536873 increased the risk for IBS-D (AM: T *vs.* G; OR_{IBS} = 1.867, P_{IBS} = 0.003). As for the genotype analysis, frequency of mutant genotype significantly increased in the IBS group compared with the HC group (DM: TT + GT *vs.* GG, OR_{IBS} = 2.07, P_{IBS} = 0.004; HoM: TT *vs.* GG, OR_{IBS} = 8.13, P_{IBS} = 0.03; HeM: GT *vs.* GG, OR_{IBS} = 1.92, P_{IBS} = 0.012). In the genotype analysis, variation of *SSPO* rs12536873 increased the risk of DD as well (RM: TT + GT *vs.* GG, OR_{DD} = 6.75, P_{DD} = 0.046; HeM: TT *vs.* GG, OR_{DD} = 6.72, P_{DD} = 0.042).

T allele of *COL6A1* rs13051496 was associated with IBS-D risk (AM: T *vs.* C, OR_{IBS} = 2.067, P_{IBS} = 0.016). The frequency of genotype TT + CT significantly increased in IBS-D patients (DM: TT + CT *vs.* CC, OR_{IBS} = 2.08, P_{IBS} = 0.026). No association was found in *COL6A1* rs13051496 and DD. No other significant association was found in the validation group of other SNVs.

We compared the clinical symptoms, such as IBS-SSS scores and HAD scores, among different genotypes of candidate SNVs. Subjects with *COL6A1* rs13051496 TT genotype presented significantly higher scores of abdominal pain, bloating, and dissatisfaction with bowel habits compared with the CC carrier which show in Fig. 3

Table 2
Identified candidate SNVs in IBS-pool or DD-pool through pooled-WES.

Group	Gene	avsnp150	Position	Ref	Alt	AA Change
HI; HD	COL6A1	rs13051496	chr21:46003595 (GRCh38.p12)	C	T	COL6A1: c. C2669T:p.Ser890Leu
HI; HD	FAM129A	rs28927681	chr1:184795938 (GRCh38.p12)	A	G	FAM129A: c.T1826C:p.Leu609Pro
HI; HD	SSPO	rs12536873	chr7:149820889 (GRCh38.p12)	G	T	SSPO: c.G12321T:p.Gln4107His
HI; HD	SYT8	rs3741231	chr11:1836521 (GRCh38.p12)	C	G	SYT8: c.C658G:p.Pro220Ala
HI	ANKRD11	rs113527563	chr16:89281630 (GRCh38.p12)	G	C	ANKRD11: c.C4912G:p.Pro1638Ala
HI	RECQL4	rs4251691	chr8:144512433 (GRCh38.p12)	C	T	RECQL4: c. G3014A:p.Arg1005Gln
HI	SLC7A6OS	rs8063446	chr16:68310460 (GRCh38.p12)	A	C	SLC7A6OS: c.T346G:p.Ser116Ala
HD	COMT	rs6267	chr22:19962740 (GRCh38.p12)	G	T	COMT: c.G64T:p.Ala22Ser
HD	EDN3	rs11570255	chr20:59300861 (GRCh38.p12)	G	A	EDN3: c.G49A:p.Ala17Thr

Note: avsnp150: SNPs reference database; Ref: reference allele; Alt: alteration allele; HI: IBS-pool compared with HC-pool; HD: DD-pool compared with HC-pool; c.: Variant effect on cDNA; p.: Variant effect on protein.

Table 3
Association of candidate SNVs with IBS-D or DD patients through pooled-WES.

SNVs	OR_{IBS}	P_{IBS}	OR_{DD}	P_{DD}
COL6A1 rs13051496	5.62	0.0001176	4.32	0.004224
FAM129A rs28927681	1.36	0.0236554	2.59	9.03×10^{-07}
SSPO rs12536873	2.83	0.000493	3.98	2.32×10^{-07}
SYT8 rs3741231	1.67	3.6×10^{-05}	2.04	2.42×10^{-05}
ANKRD11 rs113527563	0.33	6.027×10^{-06}	/	/
RECQL4 rs4251691	2.58	5.533×10^{-09}	/	/
SLC7A6OS rs8063446	2.41	7.977×10^{-06}	/	/
COMT rs6267	/	/	1.56	9.59×10^{-06}
EDN3 rs11570255	/	/	3.77	1.85×10^{-10}

Note: SNVs: single nucleotide variations; OR_{IBS}: odd ratio of IBS-pool compared with HC-pool; P_{IBS}: significance of IBS-pool compared with HC-pool, FDR corrected; OR_{DD}: odd ratio of DD-pool compared with HC-pool; P_{DD}: significance of DD-pool compared with HC-pool, FDR corrected; /: $0.5 < OR < 1.5$ and $P_{cmh} > 0.05$.

Table 4
Association of candidate SNVs with IBS-D or DD patients in allele model through SNaPshot validation.

SNVs	OR_{IBS}	P_{IBS}		OR_{DD}	P_{DD}	
ANKRD11 rs113527563	1.072	0.652	C	0.84	0.417	C
COL6A1 rs13051496	2.067	0.016*	C	1.109	0.756	C
COMT rs6267	1.254	0.543	C	0.84	0.519	C
EDN3 rs11570255	1.226	0.731	C	0.483	0.477	F
FAM129A rs28927681	0.894	0.714	C	1.028	0.924	C
RECQL4 rs4251691	1.130	0.503	C	1.119	0.534	C
SLC7A6OS rs8063446	1.457	0.207	C	0.631	0.182	C
SSPO rs12536873	1.867	0.003*	C	1.264	0.290	C
SYT8 rs3741231	3.287	0.001*	C	2.193	0.034*	C

Note: SNVs: single nucleotide variations; OR_{IBS}: odd ratio of IBS cohort compared with HC cohort; P_{IBS}: significance of IBS cohort compared with HC cohort, *: $P < 0.05$; OR_{DD}: odd ratio of DD cohort compared with HC cohort; P_{DD}: significance of DD cohort compared with HC cohort, *: $P < 0.05$; C: Chi-square Test; F: Fisher Exact Test; If there is a theoretical value that is smaller than 5, use fisher exact test, otherwise use chi-square test.

Table 5
Associations of candidate SNVs with IBS-D and DD in genotype model through SNaPshot validation.
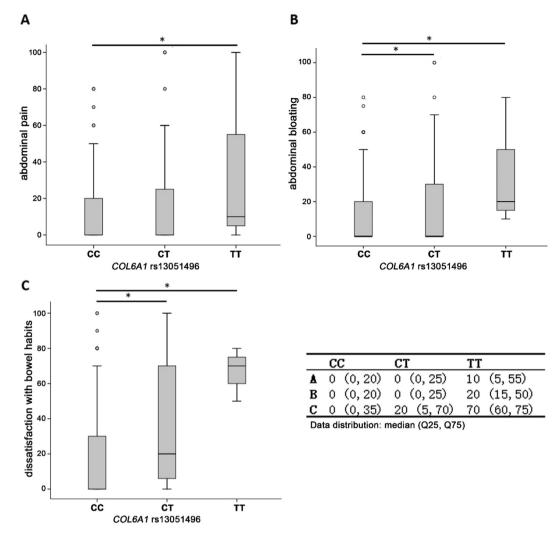
SNVs	DM				RM				HoM				HeM			
	OR_{IBS}	P_{IBS}	OR_{DD}	P_{DD}	OR_{IBS}	P_{IBS}	OR_{DD}	P_{DD}	OR_{IBS}	P_{IBS}	OR_{DD}	P_{DD}	OR_{IBS}	P_{IBS}	OR_{DD}	P_{DD}
ANKRD11 rs113527563	1.23	0.408 C	0.95	0.81 C	0.78	0.643 C	0.48	0.133 C	0.88	0.836 C	1.07	0.156 C	1.33	0.301 C	0.49	0.813 C
COL6A1 rs13051496	2.08	0.026 C	1.08	0.832 C	3.61	0.375 F	1.64	1 F	4.04	0.369 F	1.04	1 F	1.95	0.051 C	1.64	0.914 C
COMT rs6267	0.61	0.535 C	0	0.615 C	1.22	1 F	0	0.488 F	1.21	1 F	0	0.484 F	0	0.543 C	0	0.722 C
EDN3 rs11570255	1.23	0.728 C	0.48	0.474 F	0	1 C	0	1 C	0	1 C	0.48	1 C	1.23	0.728 C	0	0.474 F
FAM129A rs28927681	0.88	0.699 C	0.86	0.638 C	0	1 C	0	0.13 F	0.72	0.135 F	0.88	0.699 C	0	0.325 C		
RECQL4 rs4251691	1.44	0.149 C	1.29	0.307 C	0.72	0.42 C	0.91	0.807 C	0.94	0.88 C	1.35	0.862 C	1.58	0.083 C	1.07	0.251 C
SLC7A6OS rs8063446	1.46	0.23 C	0.61	0.166 C	0	1 F	0	1 C	0	1 F	0.61	1 C	1.42	0.275 C	0	0.166 C
SSPO rs12536873	2.07	0.004 C	1.13	0.64 C	6.22	0.073 F	6.75	0.046 F	8.13	0.03 F	0.99	0.042 C	1.92	0.012 C	6.72	0.962 C
SYT8 rs3741231	3.29	0.019 C	2.19	0.134 C	3.29	0.019 C	2.19	0.134 C	3.29	0.019 C	0	0.134 C	0	1 C	2.19	1 C

Note: SNVs: single nucleotide variations; DM: Dominant model; RM: Recessive model; HoM: Hmozygote model; HeM: Heterozygote; P_{IBS}: significance of IBS cohort compared with HC cohort; P_{DD}: significance of DD cohort compared with HC cohort; C: Chisquare Test; F: Fisher Exact Test; If there is a theoretical value that is smaller than 5, use fisher exact test, otherwise use chisquare test.

($P_{pain} = 0.034$, $P_{bloating} = 0.034$ and $P_{dissatisfaction} = 0.004$). CT carriers presented a significantly higher bloating and dissatisfaction score compared with CC carriers as well ($P_{bloating} = 0.021$ and $P_{dissatisfaction} = 0.004$). No association was found in other SNVs and clinical symptoms.

Discussion

IBS and DD are polygenetic diseases with significant family aggregation. Genetic studies on IBS [13,32,55] or DD [14] have been carried out separately, while few studies have analyzed the

Fig. 3. A. Abdominal pain scores in different genotype of *COL6A1 rs13051496*. B. Abdominal bloating scores in different genotype of *COL6A1 rs13051496*. C. Dissatisfaction with bowel habits scores in different genotype of *COL6A1 rs13051496*. (N_{CC} = 211, N_{CT} = 44, N_{TT} = 5; Box plot depicts median and IQR and error bars depict minimum to maximum values, single plot defines the outlier; *: $P < 0.05$; Mann–Whitney *U* test).

genetic risk factors for IBS and DD simultaneously. Here we analyze the genetic susceptibilities of patients with IBS-D or DD synchronously in Chinese participants, using an economical and effective tool of pooled-WES to distinguish variants associated with adaptive divergence in population genomics. After pooled-WES filtering and cohort validating, *SYT8* rs3741231 and *SSPO* rs12536873 were found to be correlated with both IBS-D and DD. *COL6A1* rs13051496 increased IBS-D risk and was associated with abdominal bloating and dissatisfaction with bowel habits.

G allele of *SYT8* rs3741231 was associated with both IBS-D and DD. Synaptic vesicle exocytosis-related genes (Syt8) were found to be involved in the initial desensitization steps of a number of G-protein coupled receptors which encode a member of the synaptotagmin protein family [33]. Synaptotagmins are important membrane proteins in neurotransmission and hormone secretion, both of them involve in regulating exocytosis. *SYT8* polymorphisms are found in patients with attention-deficit/hyperactivity disorder or MDD, and experimental studies have demonstrated that methylphenidate modulates synaptic vesicle trafficking [17,34]. Kanda and colleagues found *SYT8* variations might be a novel marker for peritoneal metastasis of gastric cancer [35,36] according to the results of a recurrence pattern-specific transcriptome analysis.

In humans, the *SSPO* gene encodes the SCP-spondin protein, which associates with commissural axon growth. Neurons cross the white commissure and ascend into higher brain centers where they exert mostly subservient regulatory functions of lower gastrointestinal pain, such as the visceral hypersensitivity in IBS-D patients [37]. A recent whole-exome sequencing studies in European patients with Parkinson's disease have identified potential risk variants of *SSPO* gene and being variated in Chinese population [49]. In this study, we found *SSPO* rs12536873 associated with both IBS-D and DD. T allele of *SSPO* rs12536873 was found to be associated with an increased risk of IBS-D. Meanwhile, the frequency of the TT genotype significantly increased in DD patients. There perhaps have some evidence in another study, the researchers found that methylation-based epigenetic changes in *SSPO* have been reported to be positively correlated with HAD depression scores in IBS patients [38].

COL6A1 encodes the collagen type VI alpha-1 chain, which is an extracellular matrix structural protein. It is a very important in intestinal structure composition and neurogenesis. In the intestine, it has been shown that epithelial cells are a major site of collagen VI production. *COL6A1* knockdown in human intestinal epithelial cells stimulates cell spreading, adhesion, and migration, most likely via increased expression and deposition of fibronectin which is associated with intestinal inflammation [39]. Fibronectin has been shown to be increased in the intestinal mucosa of IBS patients, and might be a new biomarker for IBS [40,41]. The extra-

cellular matrix protein COL6A1 has been found to be upregulated in rectal cancer tissue [42]. Our data showed *COL6A1* rs13051496 significantly increased IBS-D risk. Moreover, we found the T variant carrier corresponded with higher abdominal pain, bloating, and dissatisfaction with bowel habits scores. An *in vitro* study [43] shows that COL6A1 expression increased 1.57-fold in reactive spinal cord neuron cells after IL-1β treatment, which suggests collagen is critical in the early phase of neuronal repair. *COL6A1* is primarily be involved in the development of Parkinson [44] and Alzheimer disease [45]. *COL6A1* deficient leads to spontaneous apoptosis and defective autophagy in neural cells [46–48]. This suggest *COL6A1* might influences intestinal neurogenesis and intestinal motility.

There are some limitations for this study. Firstly, the most significant associations in the pooled-WES were not replicated in the validation cohort. For example, *RECQL4* rs4251691 significantly associated with IBS-D in pooled-WES in our data. It also had been reported that 12 out of 14 patients with *RECQL4* variations in Rothmund-Thomson syndrome exhibiting a severe diarrheal symptoms [50]. But no association was found for *RECQL4* rs4251691 and IBS-D in validation cohort. What's more, variants of *COMT* or *END3*, which had been reported in IBS or DD previously [55], were not validate in our cohort. One possible reason is the limited number of subjects. Some of SNVs are incapable compared and come up with positive result in the limited samples in this study according to its allele frequency. Another reason, the method of pooled-seq amplify the homogeneity in limited subjects, it might increase the false positive rate at the same time [52]. The identified variations in validation cohort in this study are poorly reported in IBS or DD patients before. Racial difference may have some effects because the majority of publications on IBS genetics are Caucasians, it also been found in our previous meta-analysis of IBS polymorphisms [55]. Phenotypes or variety caused by detection methods may also have an impact on this.

Finally, for the purpose of further analyzing the genetic characteristics of IBS and DD comorbidities, we detected susceptible SNVs in 22 IBS-D and DD comorbidity patients (unshown data). Owing to the limited number of comorbidity patients, no evidence of comorbidity risk was found in candidate SNVs. Hence, further validations in larger cohorts, especially in comorbidity patients, are required. Moreover, most of the SNVs function significance identified were unclear. Thus, transcriptional, translational, and protein functional analysis of SNVs are necessary as well.

Conclusion

Some unique variants were found to be associated with both IBS-D and DD, which have been identified herein as variations in *SYT8* rs3741231 and *SSPO* rs12536873. The gene of these variants are important in neurotransmission and neurogenesis. Moreover, a new risk of *COL6A1* rs13051496 for IBS was identified and positively correlated with abdominal bloating and dissatisfaction with bowel habits. Our findings provide important hints for understanding the genetic basis of the comorbidity between IBS-D and DD.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.01.016.

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