(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2015 (12.02.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/018308 A1

(51) **International Patent Classification**:
C12Q 1/68 (2006.01) *G01N 33/574* (2006.01)
C12Q 1/10 (2006.01)

(21) **International Application Number**:
PCT/CN2014/083664

(22) **International Filing Date**:
5 August 2014 (05.08.2014)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
PCT/CN2013/080872 6 August 2013 (06.08.2013) CN

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2015/018308 A1

(54) **Title**: BIOMARKERS FOR COLORECTAL CANCER

(57) **Abstract**: Biomarkers and methods for predicting the risk of a disease related to microbiota, in particular colorectal cancer (CRC), are provided.

BIOMARKERS FOR COLORECTAL CANCER

CROSS-REFERENCE TO RELATED APPLICATION

The present patent application claims priority to PCT Patent Application No. PCT/CN2013/080872, filed Aug. 6, 2013, which is incorporated herein by reference.

FIELD

The present invention relates to biomarkers and methods for predicting the risk of a disease related to microbiota, in particular colorectal cancer (CRC).

BACKGROUND

Colorectal cancer (CRC) is the third most common form of cancer and the second leading cause of cancer-related death in the Western world (Schetter AJ, Harris CRC (2011) Alterations of microRNAs contribute to colon carcinogenesis. Semin Oncol 38:734–742, incorporated herein by reference). A lot of people are diagnosed with CRC and many patients die of this disease each year worldwide. Although current strategies, including surgery, radiotherapy, and chemotherapy, have a significant clinical value for CRC, the relapses and metastases of cancers after surgery have hampered the success of those treatment modalities. Early diagnosis of CRC will help to not only prevent mortality, but also reduce the costs for surgical intervention.

Current tests of CRC, such as flexible sigmoidoscopy and colonoscopy, are invasive and patients may find the procedures and bowel preparation to be uncomfortable or unpleasant.

The development of CRC is a multifactorial process influenced by genetic, physiological, and environmental factors. Regarding environmental factors, the lifestyle, particularly dietary intake, may affect the risk of CRC developing. Western diet, rich in animal fat and poor in fiber, is generally associated with an increased risk of CRC. Thus, it has been hypothesized that the connection between the diet and CRC, may be the influence that the diet has on the colon microbiota and bacterial metabolism, making both relevant factors in the etiology of the disease (McGarr SE, Ridlon JM, Hylemon PB (2005). Diet, anaerobic bacterial metabolism, and colon cancer. J Clin Gastroenterol. 39:98–109; Hatakka K, Holma R, El-Nezami H, Suomalainen T, Kuisma M, Saxelin M, Poussa T, Mykkänen H, Korpela R (2008). The influence of Lactobacillus rhamnosus LC705 together with Propionibacterium freudenreichii ssp. shermanii JS on

potentially carcinogenic bacterial activity in human colon. Int J Food Microbiol. 128:406–410, incorporated herein by reference).

Interactions between the gut microbiota and the immune system have an important role in many diseases both within and outside the gut (Cho, I. & Blaser, M. J. The human microbiome: at the interface of health and disease. Nature Rev. Genet. 13, 260–270 (2012), incorporated herein by reference). Intestinal microbiota analysis of feces DNA has the potential to be used as a noninvasive test for finding specific biomarkers that may be used as a screening tool for early diagnosis of patients having CRC, thus leading to a longer survival and a better quality of life.

With the development of molecular biology and its application in microbial ecology and environmental microbiology, an emerging field of metagenomics (environmental genomics or ecogenomics), has been developed rapidly. Metagenomics, comprising extracting total community DNA, constructing genomic library, and analyzing library with similar strategies for functional genomics, provides a powerful tool to study the uncultured microorganisms in the complex environmental habitats. In recent years, metagenomics has been applied to many environmental samples, such as the oceans, soils, river, thermal vents, hot springs, and the human gastrointestinal tract, nasal passages, oral cavities, skin and urogenital tract, showing significant value in various areas including medicine, alternative energy, environmental remediation, biotechnology, agriculture and biodefense. For the study of CRC, the inventors performed analysis in the metagenomics field.

SUMMARY

Embodiments of the present disclosure seek to solve at least one of the problems existing in the prior art to at least some extent.

The present invention is based on the following findings by the inventors:

Assessment and characterization of gut microbiota has become a major research area in human disease, including colorectal cancer (CRC), one of the commonest causes of death among all types of cancers. To carry out analysis on gut microbial content in CRC patients, the inventors carried out a protocol for a Metagenome-Wide Association Study (MGWAS) (Qin, J. et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490, 55–60 (2012), incorporated herein by reference) based on deep shotgun sequencing of the gut microbial DNA from 128 Chinese individuals. The inventors identified and validated 140,455

CRC-associated gene markers. To exploit the potential ability of CRC classification by gut microbiota, the inventors developed a disease classifier system based on the 31 gene markers that are defined as an optimal gene set by a minimum redundancy - maximum relevance (mRMR) feature selection method. For intuitive evaluation of the risk of CRC disease based on these 31 gut microbial gene markers, the inventors calculated a healthy index. The inventors' data provide insight into the characteristics of the gut metagenome related to CRC risk, a paradigm for future studies of the pathophysiological role of the gut metagenome in other relevant disorders, and the potential usefulness for a gut-microbiota-based approach for assessment of individuals at risk of such disorders.

It is believed that gene markers of intestinal microbiota are valuable for increasing cancer detection at earlier stages due to the following. First, the markers of the present invention are more specific and sensitive as compared with conventional cancer markers. Second, analysis of stool promises accuracy, safety, affordability, and patient compliance. And samples of stool are transportable. As compared with colonoscopy requiring bowel preparation, polymerase chain reaction (PCR)-based assays are comfortable and noninvasive, so people will participate in a given screening program more easily. Third, the markers of the present invention may also serve as tools for therapy monitoring in cancer patients to detect the response to therapy.

BRIEF DISCRIPTION OF DRAWINGS

These and other aspects and advantages of the present disclosure will become apparent and more readily appreciated from the following descriptions taken in conjunction with the drawings, in which:

Fig.1 shows distribution of P-value association statistics of all microbial genes in this study. The association analysis of CRC p-value distribution identified a disproportionate over-representation of strongly associated markers at lower P-values, with the majority of genes following the expected P-value distribution under the null hypothesis. This suggests that the significant markers likely represent true rather than spurious associations.

Fig.2 shows minimum redundancy maximum relevance (mRMR) method to identify 31 gene markers that differentiate colorectal cancer cases from controls. Incremental search was performed using the mRMR method which generated a sequential number of subsets. For each subset, the error rate was estimated by a leave-one-out cross-validation

(LOOCV) of a linear discrimination classifier. The optimum subset with the lowest error rate contained 31 gene markers.

Fig.3 shows that discovering gut microbial gene markers associated with CRC. CRC index computed for CRC patients and control individuals from this study, shown along patients and control individuals from earlier studies on type 2 diabetes and inflammatory bowel disease. The box depicts the interquartile ranges between the first and third quartiles, and the line inside denotes the median. The calculated gut healthy index listed in Table 6 correlated well with the ratio of CRC patients in the population.CRC indices for CRC patient microbiomes are significantly different from the rest (***$P < 0.001$).

Fig.4 shows that ROC analysis of CRC index from 31 gene markers in Chinese cohort I shows excellent classification potential with an area under the curve of 0.9932.

Fig. 5 shows that the CRC index was calculated for an additional 19 Chinese CRC samples and 16 non-CRC samples in Example 2. The box depicts the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the line inside denotes the median, while the points represent the gut healthy index in each sample. The square represents the case group (CRC); the triangle represents the controls group (non-CRC); the triangle with * represents non-CRC individual that were diagnosed as CRC patient.

Fig.6 shows species involved in gut microbial dysbiosis during colorectal cancer. Differential relative abundance of two CRC-associated and one control-associated microbial species consistently identified using three different methods: MLG, mOTU and IMG database.

Fig.7 shows enrichment of *Solobacterium moore* and Peptostreptococcus stomati in CRC patient microbiomes.

Fig.8 shows the Receive-Operator-Curve of CRC specific species marker selection using random forest method and three different species annotation methods. (A) IMG species annotation using clean reads to IMG version 400. (B) mOTU species using published methods. (C) All significant genes clustered using MLG methods and the species annotation using IMG version 400.

Fig.9 shows stage specific abundance of three species that are enriched in stage II and later, using three species annotation methods: MLG, IMG and mOTU.

Fig.10 shows species involved in gut microbial dysbiosis during colorectal cancer. Relative abundances of one bacterial species enriched in control microbiomes and three enriched in

CRC-associated microbiomes, during different stages of CRC (three different species annotation methods were used).

Fig.11 shows correlation between quantification by the metagenomic approach versus quantitative polymerase chain reaction (qPCR) for two gene markers.

Fig. 12 shows evaluating CRC index from 2 genes in Chinese cohort II. (A) CRC index based on 2 gene markers separates CRC and control microbiomes. (B) ROC analysis reveals marginal potential for classification using CRC index, with an area under the curve of 0.73.

Fig.13 shows validating robust gene markers associated with CRC. qPCR abundance (in log10 scale, zero abundance plotted as -8) of three gene markers were measured in cohort II consisting 51 cases and 113 healthy controls. Two were randomly selected (m1704941: butyryl-CoA dehydrogenase from *F. nucleatum*, m482585: RNA-directed DNA polymerase from an unknown microbe) and one was targeted (m1696299: RNA polymerase subunit beta, *rpoB*, from *P. micra*). (A) CRC index based on the three genes clearly separates CRC microbiomes from controls. (B) CRC index classifies with an area under the receiver operating characteristic (ROC) curve of 0.84. (C) *P. micra* species specific *rpoB* gene shows relatively higher incidence and abundance starting in CRC stage II and III ($P = 2.15 \times 10^{-15}$) compared to control and stage I microbiomes.

DETAILED DESCRIPTION

Terms used herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

In one aspect, the present invention relates to a gene marker set for predicting the risk of colorectal cancer (CRC) in a subject, consisting of the genes as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO: 6.

In another aspect the present invention relates to use of the gene markers in the gene marker set of the present invention for predicting the risk of colorectal cancer (CRC) in a subject, via the steps of:

1) collecting a sample *j* from the subject and extracting DNA from the sample;

2) determining the abundance information of each of gene marker in the set of gene markers; and

3) calculating the index of sample *j* by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the abundance information of marker i in sample j, wherein i refers to each of the gene markers in said gene marker set;

N is a subset of all abnormal -enriched markers in selected biomarkers related to the disease,

$|N|$ is number (sizes) of the biomarkers in the subset, wherein $|N|$ is 3;

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer (CRC).

In another aspect, the present invention relates to use of the gene markers in the gene marker set of the present invention for preparation of a kit for predicting the risk of colorectal cancer (CRC) in a subject, via the steps of:

1) collecting a sample *j* from the subject and extracting DNA from the sample;

2) determining the abundance information of each of gene marker in the set of gene markers; and

3) calculating the index of sample *j* by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the abundance information of marker i in sample j, wherein i refers to each of the gene markers in said gene marker set;

N is a subset of all abnormal -enriched markers in selected biomarkers related to the disease,

$|N|$ is number (sizes) of the biomarkers in the subset, wherein $|N|$ is 3;

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer (CRC).

In another aspect, the present invention relates to a method of diagnosing whether a subject has colorectal cancer or is at the risk of developing colorectal cancer, comprising:

1) collecting a feces sample *j* from the subject and extracting DNA from the sample;

2) determining the abundance information of each of the marker as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO:6; and

3) calculating the index of sample *j* by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the abundance information of marker *i* in sample *j*, wherein *i* refers to each of the gene markers as set forth in said gene marker set;

N is a subset of all patient-enriched markers;

wherein the subset of CRC-enriched markers are the marker as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO:6;

$|N|$ is number (sizes) of the biomarker in the subset, wherein $|N|$ is 3,

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer.

In one embodiment, the abundance information is gene relative abundance of each of gene marker in the set of gene markers which is determined by means of sequencing method.

In another embodiment, the abundance information is qPCR abundance of each of gene marker in the set of gene markers which is determined by a qPCR method.

In another embodiment, the cutoff value is obtained by a Receiver Operator Characteristic (ROC) method, wherein the cutoff corresponds to when AUC (Area Under the Curve) reached at its maximum.

In one preferred embodiment, the cutoff value is determined as -14.39.

In another aspect, the present invention provides a kit for determining the gene marker set of the present invention, comprising one or more primers and probes as set forth in Table 15.

In another aspect, the present invention provides use of a marker as set forth in SEQ ID NO: 6 or *rpoB* gene encoding RNA polymerase subunit β as a gene marker for predicting the risk of colorectal cancer (CRC) in a subject, wherein the enrichment of said gene marker in a sample of

the subject relative to a control sample is indicative of the risk of colorectal cancer in the subject.

In another aspect, the present invention provides use of *Parvimonas micra* as a species marker for predicting the risk of colorectal cancer (CRC) in a subject, wherein the enrichment of said species marker in a sample of the subject relative to a control sample is indicative of the risk of colorectal cancer in the subject

The present invention is further exemplified in the following non-limiting Examples. Unless otherwise stated, parts and percentages are by weight and degrees are Celsius. As apparent to one of ordinary skill in the art, these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only, and the agents were all commercially available.

GENERAL METHOD

I. Methods for detecting biomarkers (Detect biomarkers by using MGWAS strategy)

To define CRC-associated metagenomic markers, the inventors carried out a MGWAS (metagenome-wide association study) strategy (Qin, J. et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490, 55–60 (2012), incorporated herein by reference). Using a sequence-based profiling method, the inventors quantified the gut microbiota in samples. On average, with the requirement that there should be $\geq 90\%$ identity, the inventors could uniquely map paired-end reads to the updated gene catalogue. To normalize the sequencing coverage, the inventors used relative abundance instead of the raw read count to quantify the gut microbial genes. However, unlike what is done in a GWAS subpopulation correction, the inventors applied this analysis to microbial abundance rather than to genotype. A Wilcoxon rank-sum test was done on the adjusted gene profile to identify differential metagenomic gene contents between the CRC patients and controls. The outcome of the analyses showed a substantial enrichment of a set of microbial genes that had very small *P* values, as compared with the expected distribution under the null hypothesis, suggesting that these genes were true CRC-associated gut microbial genes.

The inventors next controlled the false discovery rate (FDR) in the analysis, and defined CRC-associated gene markers from these genes corresponding to a FDR.

II. Methods for selecting 31 best markers from biomarkers (Maximum Relevance Minimum Redundancy (mRMR) feature selection framework)

To identify an optimal gene set, a minimum redundancy - maximum relevance (mRMR) (for

detailed information, see Peng, H., Long, F. & Ding, C. Feature selection based on mutual information: criteria of max-dependency, max-relevance, and min-redundancy. IEEE Trans Pattern Anal Mach Intell27, 1226-1238, doi:10.1109/TPAMI.2005.159 (2005), which is incorporated herein by reference) feature selection method was used to select from all the CRC-associated gene markers. The inventors used the "sideChannelAttack" package of the R software to perform the incremental search and found 128 sequential markers sets. For each sequential set, the inventors estimated the error rate by a leave-one-out cross-validation (LOOCV) of linear discrimination classifier. The optimal selection of marker sets was the one corresponding to the lowest error rate. In the present study, the inventors made the feature selection on a set of 140,455 CRC-associated gene markers. Since this was computationally prohibitive to perform mRMR using all genes, the inventors derived a statistically non-redundant gene set. Firstly, the inventors pre-grouped the 140,455 colorectal cancer associated genes that are highly correlated with each other (Kendall correlation > 0.9). Then the inventors chose the longest gene as representative gene for the group, since longer genes have a higher chance of being functionally annotated, and will attract more reads during the mapping procedure. This generated a non-redundant set of 15,836 significant genes. Subsequently, the inventors applied the mRMR feature selection method to the 15,836 significant genes and identified an optimal set of 31 gene biomarkers that are strongly associated with colorectal cancer for colorectal cancer classification, which were shown on Table 1. The gene id is from the published reference gene catalogue as Qin et al. 2012, *supra.*

Table 1. 31 optimal Gene markers' enrichment information

Gene id	Correlation coefficient with CRC	mRMR rank	Enrichment (1=Control, 0=CRC)	SEQ ID NO:
2361423	-0.558205377	1	0	1
2040133	-0.500237832	2	0	2
3246804	-0.454281109	3	0	3
3319526	0.441366585	4	1	4
3976414	0.431923463	5	1	5
1696299	-0.499397182	6	0	6
2211919	0.410506085	7	1	7
1804565	0.418663439	8	1	8
3173495	-0.55118428	9	0	9
482585	-0.454270958	10	0	10
181682	0.400814213	11	1	11
3531210	0.383705453	12	1	12
3611706	0.413879567	13	1	13
1704941	-0.468122499	14	0	14

4256106	0.42048024	15	1	15
4171064	0.43365554	16	1	16
2736705	-0.417069104	17	0	17
2206475	0.411512652	18	1	18
370640	0.399015232	19	1	19
1559769	0.427134509	20	1	20
3494506	0.382302723	21	1	21
1225574	-0.407066113	22	0	22
1694820	-0.442595115	23	0	23
4165909	0.410519669	24	1	24
3546943	-0.395361093	25	0	25
3319172	0.448526551	26	1	26
1699104	-0.467388978	27	0	27
3399273	0.388569946	28	1	28
3840474	0.383705453	29	1	29
4148945	0.407802676	30	1	30
2748108	-0.426515966	31	0	31

III. Gut healthy index (CRC index)

To exploit the potential ability of disease classification by gut microbiota, the inventors developed a disease classifier system based on the gene markers that the inventors defined. For intuitive evaluation of the risk of disease based on these gut microbial gene markers, the inventors calculated a gut healthy index (CRC index).

To evaluate the effect of the gut metagenome on CRC, the inventors defined and calculated the gut healthy index for each individual on the basis of the selected 31 gut metagenomic markers as described above. For each individual sample, the gut healthy index of sample j that denoted by I_j was calculated by the formula below:

$$I_j = \left[\frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|} - \frac{\sum_i \epsilon_M \log 10(A_{ij} + 10^{-20})}{|M|}\right]$$

A_{ij} is the relative abundance of marker i in sample j.

N is a subset of all patient-enriched markers in selected biomarkers related to the abnormal condition (namely, a subset of all CRC-enriched markers in these 31 selected gut metagenomic markers),

M is a subset of all control-enriched markers in selected biomarkers related to the abnormal condition (namely, a subset of all control-enriched markers in these 31 selected gut metagenomic markers),

$|N|$ and $|M|$ are number (sizes) of the biomarker respectively in these two sets,

IV.Receiver Operator Characteristic (ROC) analysis

The inventors applied the ROC analysis to assess the performance of the colorectal cancer classification based on metagenomic markers. Based on the 31 gut metagenomic markers selected above, the inventors calculated the CRC index for each sample. The inventors then used the "Daim" package in R software to draw the ROC curve.

V. Disease classifier system

After identifying biomarkers from MGWAS strategy, the inventors, in the principle of biomarkers used to classify should be strongest to the classification between disease and healthy with the least redundancy, ranked the biomarkers by a minimum redundancy - maximum relevance (mRMR) and found sequential markers sets (its size can be as large as biomarkers number). For each sequential set, the inventors estimated the error rate by a leave-one-out cross-validation (LOOCV) of a classifier. The optimal selection of marker sets was the one corresponding to the lowest error rate (In some embodiments, the inventors have selected 31 biomarkers).

Finally, for intuitive evaluation of the risk of disease based on these gut microbial gene markers, the inventors calculated a gut healthy index. Larger the healthy index, higher the risk of disease. Smaller the healthy index, more healthy the people. The inventors can build an optimal healthy index cutoff based on a large cohort. If the test sample healthy index is larger than the cutoff, then the person is in higher disease risk. And if the test sample healthy index is smaller than the cutoff then he is more healthy at low risk of disease. The optimal healthy index cutoff can be determined by a ROC method when AUC (Area Under the Curve) reached at its maximum.

Example 1. Identifying 31 biomarker from 128 Chinese individuals and use gut healthy index to evaluate their colorectal cancer risk

1.1 Sample collection and DNA extraction

Stool samples from 128 subjects (cohort I), including 74 colorectal cancer patients and 54 healthy controls (Table 2) were collected in the Prince of Wales Hospital, Hong Kong with

informed consent. To be eligible for inclusion in this study, individuals have to fit the following criteria for stool sample collection: 1) no taking of antibiotics or other medications with no particular diets (diabetics, vegetarians, etc) and with normal lifestyle (without extra stress) for a minimum of 3 months; 2) a minimum of 3 months after any medical intervention; 3) no history of colorectal surgery, any kind of cancer, or inflammatory or infectious diseases of the intestine. Subjects were asked to collect stool samples before colonoscopy examination in standardized containers at home and store samples in their home freezer immediately. Frozen samples were then delivered to the Prince of Wales Hospital in insulating polystyrene foam containers and stored at -80^{o}C immediately until use.

Stool samples were thawed on ice and DNA extraction was performed using the QiagenQIAamp DNA Stool Mini Kit according to manufacturer's instructions. Extracts were treated with DNase-free RNase to eliminate RNA contamination. DNA quantity was determined using NanoDrop spectrophotometer, Qubit Fluorometer (with the Quant-iTTMdsDNA BR Assay Kit) and gel electrophoresis.

Table 2 Baseline characteristics of colorectal cancer cases and controls in the cohort I. BMI: body mass index; eGFR: epidermal growth factor receptor; DM: diabetes mellitus type 2.

Parameter	Controls (n=54)	Cases (n=74)
Age	61.76	66.04
Sex (M:F)	33:21	48:26
BMI	23.47	23.9
eGFR	72.24	74.15
DM (%)	16 (29.6%)	29 (39.2%)
Enterotype (1:2:3)	26:22:6	37:31:6
Stage of disease (1:2:3:4)	n.a.	16:21:30:7
Location (proximal: distal)	n.a.	13:61

1.2 **DNA library construction and sequencing**

DNA library construction was performed following the manufacturer's instruction (Illumina HiSeq 2000 platform). The inventors used the same workflow as described previously to perform cluster generation, template hybridization, isothermal amplification, linearization, blocking and denaturation, and hybridization of the sequencing primers (Qin, J. et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490, 55–60 (2012), incorporated herein by reference).

The inventors constructed one paired-end (PE) library with insert size of 350bp for each sample, followed by a high-throughput sequencing to obtain around 30 million PE reads of length

2x100bp. High quality reads were extracted by filtering low quality reads with 'N' base, adapter contamination and human DNA contamination from the raw data, and by trimming low quality terminal bases of reads at the same time. 751 million metagenomic reads (high quality reads) were generated (5.86 million reads per individual on average, Table 3).

1.3 Reads mapping

The inventors mapped the high quality reads to the gene catalogue (Table 3) to a published reference gut gene catalogue established from European and Chinese adults (Qin, J. et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490, 55–60 (2012), incorporated herein by reference) (identity >= 90%), based on which the inventors derived the gene profiles using the same method of the published T2D paper in Qin et al. 2012, *supra*. From the reference gene catalogue as Qin et al. 2012, *supra*, the inventors derived a subset of 2,110,489 (2.1M) genes that appeared in at least 6 samples in all 128 samples.

Table 3. Summary of metagenomic data and mapping to reference gene catalogue. The fourth column reports P-value results from Wilcoxon rank-sum tests.

Parameter	Controls	Cases	P-value
Average raw reads	60162577	60496561	0.8082
After removing low quality reads	59423292 (98.77%)	59715967 (98.71%)	0.831
After removing human reads	59380535 ± 7378751	58112890 ± 10324458	0.419
Mapping rate	66.82%	66.27%	0.252

1.4 Analysis of factors influencing gut microbiota gene profiles

To ensure robust comparison of the gene content of 128 metagenomes, the inventors generated a set of 2,110,489 (2.1M) genes that were present in at least 6 subjects, and generated 128 gene abundance profiles using these 2.1 million genes. The inventors used the permutational multivariate analysis of variance (PERMANOVA) test to assess the effect of different characteristics, including age, BMI, eGFR, TCHO, LDL, HDL, TG, gender, DM, CRC status, smoking status and location, on gene profiles of 2.1M genes. The inventors performed the analysis using the method implemented in package "vegan" in R, and the permuted p-value was obtained by 10,000 times permutations. The inventors also corrected for multiple testing using "p.adjust" in R with Benjamini-Hochberg method to get the q-value for each gene.

When the inventors performed permutational multivariate analysis of variance (PERMANOVA) on 13 different covariates, only CRC status was significantly associated with

these gene profiles (q = 0.0028, **Table 4**), showing a stronger association than the second best determinant body mass index (q = 0.15). Thus the data suggest an altered gene composition in CRC patient microbiomes.

Table 4. PERMANOVA analysis using microbial gene profile. The analysis was conducted to test whether clinical parameters and colorectal cancer (CRC) status have significant impact on the gut microbiota with q<0.05. BMI: body mass index; DM: diabetes mellitus type 2; HDL: high density lipoprotein; TG: triglyceride; eGFR: epidermal growth factor receptor; TCHO: total cholesterol; LDL; low density lipoprotein.

Phenotype	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	q-value
CRC Status	1	0.679293	0.679293	1.95963	0.015314	0.0004	0.0028
BMI	1	0.484289	0.484289	1.39269	0.011019	0.033	0.154
DM Status	1	0.438359	0.438359	1.257642	0.009883	0.084	0.27272
Location	1	0.436417	0.436417	1.228172	0.016772	0.0974	0.27272
Age	1	0.397282	0.397282	1.138728	0.008957	0.1923	0.4487
HDL	1	0.38049	0.38049	1.083265	0.010509	0.271	0.542
TG	1	0.365191	0.365191	1.039593	0.010089	0.3517	0.564964
eGFR	1	0.358527	0.358527	1.023138	0.009471	0.38	0.564964
CRC Stage	1	0.357298	0.357298	1.002413	0.013731	0.441	0.564964
Smoker	1	0.347969	0.347969	0.999825	0.013511	0.4439	0.564964
TCHO	1	0.321989	0.321989	0.915216	0.008893	0.6539	0.762883
LDL	1	0.306483	0.306483	0.871306	0.00847	0.7564	0.814585
Gender	1	0.267738	0.267738	0.765162	0.006036	0.9528	0.9528

1.5 CRC-associated genes identified by MGWAS

1.5.1 Identification of colorectal cancer associated genes. The inventors performed a metagenome wide association study (MGWAS) to identify the genes contributing to the altered gene composition in CRC. To identify the association between the metagenomic profile and colorectal cancer, a two-tailed Wilcoxon rank-sum test was used in the 2.1M (2,110,489) gene profiles. The inventors got 140,455 gene markers, which were enriched in either case or control with $P<0.01$ (**Fig. 1**).

1.5.2 Estimating the false discovery rate (FDR). Instead of a sequential P-value rejection method, the inventors applied the "qvalue" method proposed in a previous study (J. D. Storey, R. Tibshirani, Statistical significance for genomewide studies. Proceedings of the National Academy of Sciences of the United States of America **100**, 9440 (Aug 5, 2003), incorporated herein by reference) to estimate the FDR. In the MGWAS, the statistical hypothesis tests were performed on

a large number of features of the 140,455 genes. The false discovery rate (FDR) was 11.03%.

1.6 Gut-microbiota-based CRC classification

The inventors proceeded to identify potential biomarkers for CRC from the genes associated with disease, using the minimum redundancy maximum relevance (mRMR) feature selection method. However, since the computational complexity of this method did not allow us to use all 140,455 genes from the MGWAS approach, the inventors had to reduce the number of candidate genes. First, the inventors selected a stricter set of 36,872 genes with higher statistical significance ($P < 0.001$; FDR=4.147%). Then the inventors identified groups of genes that were highly correlated with each other (Kendall's $\tau > 0.9$) and chose the longest gene in each group, to generate a statistically non-redundant set of 15,836 significant genes. Finally, the inventors used the mRMR method and identified an optimal set of 31 genes that were strongly associated with CRC status (Fig. 2, Table 5). The inventors computed a CRC index based on the relative abundance of these markers, which clearly separated the CRC patient microbiomes from the control microbiomes (Table 6), as well as from 490 fecal microbiomes from two previous studies on type 2 diabetes in Chinese individuals (Qin et al. 2012, *supra*) and inflammatory bowel disease in European individuals (J. Qin *et al.*, A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59 (Mar 4, 2010), incorporated herein by reference) (Fig. 3, median CRC-index for patients and controls in this study were 6.42 and -5.48, respectively; Wilcoxon rank-sum test, $q < 2.38 \times 10^{-10}$ for all five comparisons, see Table 7). Classification of the 74 CRC patient microbiomes against the 54 control microbiomes using the CRC index exhibited an area under the receiver operating characteristic (ROC) curve of 0.9932 (Fig. 4). At the cutoff -0.0575, true positive rate (TPR) was 1, and false positive rate (FPR) was 0.07407, indicating that the 31 gene markers could be used to accurately classify CRC individuals.

Table 6. 128 samples' calculated gut healthy index (CRC patients and non-CRC controls)

Sample ID	Type (Con_CRC:non- CRC controls; CRC:CRC patients)	CRC-index	Sample ID	Type (Con_CRC:non-CRC controls; CRC:CRC patients)	CRC-index
502A	Con_CRC	-7.505749695	A10A	CRC	13.26483131
512A	Con_CRC	-5.150023018	M2.PK002A	CRC	7.002094781
515A	Con_CRC	-4.919398163	M2.PK003A	CRC	5.108478224
516A	Con_CRC	-2.793151285	M2.PK018A	CRC	2.243592264
517A	Con_CRC	-8.078128133	M2.PK019A	CRC	-0.057498133

519A	Con_CRC	-7.556675412	M2.PK021A	CRC	7.878402029
530A	Con_CRC	-0.194519906	M2.PK022A	CRC	9.047909247
534A	Con_CRC	-5.251127609	M2.PK023A	CRC	5.428574192
536A	Con_CRC	-7.08635459	M2.PK024A	CRC	5.032760805
M2.PK504A	Con_CRC	-5.470747464	M2.PK026A	CRC	6.257085759
M2.PK514A	Con_CRC	-4.441183208	M2.PK027A	CRC	1.59430903
M2.PK520B	Con_CRC	-8.101427301	M2.PK029A	CRC	9.331138747
M2.PK522A	Con_CRC	0.269338093	M2.PK030A	CRC	4.728023967
M2.PK523A	Con_CRC	-6.980913756	M2.PK032A	CRC	6.055831256
M2.PK524A	Con_CRC	-9.027027667	M2.PK037A	CRC	4.227424374
M2.PK531B	Con_CRC	-5.483143199	M2.PK038A	CRC	2.669264211
M2.PK532A	Con_CRC	-5.96003222	M2.PK041A	CRC	4.558926807
M2.PK533A	Con_CRC	-7.718764145	M2.PK042A	CRC	3.47308125
M2.PK543A	Con_CRC	-9.844975269	M2.PK043A	CRC	5.347387703
M2.PK548A	Con_CRC	-4.062846751	M2.PK045A	CRC	8.09166979
M2.PK556A	Con_CRC	-4.15150788	M2.PK046A	CRC	9.235279951
M2.PK558A	Con_CRC	-9.712104855	M2.PK047A	CRC	8.45229555
M2.PK602A	Con_CRC	-7.380042553	M2.PK051A	CRC	6.602608047
M2.PK615A	Con_CRC	3.232971256	M2.PK052A	CRC	3.207800397
M2.PK617A	Con_CRC	-8.878473599	M2.PK055A	CRC	5.088317256
M2.PK619A	Con_CRC	-8.279540689	M2.PK056B	CRC	5.504229632
M2.PK630A	Con_CRC	-5.993197547	M2.PK059A	CRC	5.466091636
M2.PK644A	Con_CRC	1.230424198	M2.PK063A	CRC	3.758294225
M2.PK647A	Con_CRC	-7.181191393	M2.PK064A	CRC	3.763414393
M2.PK649A	Con_CRC	-1.576643721	M2.PK065A	CRC	6.486959786
M2.PK653A	Con_CRC	-4.246899704	M2.PK066A	CRC	1.199091901
M2.PK656A	Con_CRC	-5.80900221	M2.PK067A	CRC	9.938025463
M2.PK659A	Con_CRC	-7.805935646	M2.PK069B	CRC	-0.04402983
M2.PK663A	Con_CRC	-5.007057718	M2.PK083B	CRC	8.394697958
M2.PK699A	Con_CRC	-8.827532431	M2.PK084A	CRC	9.25322799
M2.PK701A	Con_CRC	-0.981728615	M2.PK085A	CRC	7.852591304
M2.PK705A	Con_CRC	-8.822384737	MSC103A	CRC	4.05476664
M2.PK708A	Con_CRC	-6.573782359	MSC119A	CRC	4.331580986
M2.PK710A	Con_CRC	-7.558945558	MSC120A	CRC	3.865826479
M2.PK712A	Con_CRC	-9.207916748	MSC1A	CRC	9.930238103
M2.PK723A	Con_CRC	-4.481542621	MSC45A	CRC	9.331894011
M2.PK725A	Con_CRC	-7.520375154	MSC4A	CRC	0.006971195
M2.PK729A	Con_CRC	-5.318926226	MSC54A	CRC	12.10968629
M2.PK730A	Con_CRC	-4.3710193	MSC5A	CRC	3.272778932
M2.PK732A	Con_CRC	-5.20132309	MSC63A	CRC	7.74197911
M2.PK750A	Con_CRC	-6.64771202	MSC6A	CRC	8.063701275
M2.PK751A	Con_CRC	-3.65391467	MSC76A	CRC	6.730976418
M2.PK797A	Con_CRC	-4.675123647	MSC78A	CRC	6.999247399
M2.PK801A	Con_CRC	-7.766321018	MSC79A	CRC	6.805539524
509A	Con_CRC	-2.479402638	MSC81A	CRC	8.465000094
A60A	Con_CRC	1.078322254	M118A	CRC	8.675933723
506A	Con_CRC	-4.246837899	M123A	CRC	8.627635602
A21A	Con_CRC	-4.440375851	M2.Pk.001A	CRC	7.78045553
A51A	Con_CRC	-2.809587066	M2.Pk.005A	CRC	4.534189338

			M2.Pk.009A	CRC	8.188718934
			M2.Pk.017A	CRC	6.225010462
			M84A	CRC	3.497922009
			M89A	CRC	0.394210537
			M2.Pk.007A	CRC	5.703428174
			M2.Pk.010A	CRC	7.231959163
			M122A	CRC	8.387516145
			M2.Pk.004A	CRC	4.246104721
			M2.Pk.008A	CRC	5.299578303
			M2.Pk.011A	CRC	6.354957821
			M2.Pk.015A	CRC	7.719629705
			M113A	CRC	7.528437656
			M116A	CRC	10.54991338
			M117A	CRC	0.072052278
			M2.Pk.006A	CRC	9.368358379
			M2.Pk.012A	CRC	1.112535148
			M2.Pk.014A	CRC	8.671786146
			M2.Pk.016A	CRC	8.898356611
			M115A	CRC	7.241420602
			M2.Pk.013A	CRC	7.331598086

Example 2. Validating the 31 biomarkers

The inventors validated the discriminatory power of the CRC classifier using another new independent study group, including 19 CRC patients and 16 non- CRC controls that were also collected in the Prince of Wales Hospital .

For each sample, DNA was extracted and a DNA library was constructed followed by high throughput sequencing as described in Example 1. The inventors calculated the gene abundance profile for these samples using the same method as described in Qin et al. 2012, *supra*. Then the gene relative abundance of each of the markers as set forth in SEQ ID NOs: 1-31 was determined. Then the index of each sample was calculated by the formula below:

$$I_j = \left[\frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|} - \frac{\sum_i \epsilon_M \log 10(A_{ij} + 10^{-20})}{|M|}\right]$$

A_{ij} is the relative abundance of marker i in sample j, wherein i refers to each of the gene markers as set forth in SEQ ID NOs 1-31;

N is a subset of all patient-enriched markers and M is a subset of all control-enriched markers; wherein the subset of CRC enriched markers and the subset of control-enriched markers are shown in Table 1;

$|N|$ and $|M|$ are number (sizes) of the biomarker respectively in these two subsets，wherein $|N|$ is 13 and $|M|$ is 18,

Table 8 shows the calculated index of each sample and Table 9 shows the relevant gene relative abundance of a representative sample V30.

In this assessment analysis, the top 19 samples with the highest gut healthy index were all CRC patients, and all of CRC patients were diagnosed as CRC individuals (Table 8 and Fig. 5) Only one of non-CRC controls (Fig. 5,the triangle with ＊) that were diagnosed as CRC patient. At the cutoff -0.0575, the error rate was 2.86%, validating that the 31 gene markers can accurately classify CRC individuals.

Table 8. 35 samples' calculated gut healthy index

Sample ID	Type (Con_CRC:non-CRC controls; CRC:CRC patients)	CRC-index	Sample ID	Type (Con_CRC:non- CRC controls; CRC:CRC patients)	CRC-index
V27	Con_CRC	0.269338056	V35	CRC	13.16483131
V19	Con_CRC	-0.981728643	V8	CRC	12.12968629
V26	Con_CRC	-2.793151257	V13	CRC	10.54991338
V10	Con_CRC	-4.371019	V7	CRC	9.958035463
V18	Con_CRC	-4.440375832	V17	CRC	9.2432279
V1	Con_CRC	-4.675123655	V2	CRC	9.235252955
V14	Con_CRC	-4.919398178	V15	CRC	8.465000028
V9	Con_CRC	-5.007057768	V25	CRC	8.188718932
V33	Con_CRC	-5.20132324	V20	CRC	7.852591353
V29	Con_CRC	-5.251127667	V3	CRC	7.74197955
V6	Con_CRC	-5.470747485	V24	CRC	7.528437632
V21	Con_CRC	-5.96003246	V16	CRC	6.225010478
V22	Con_CRC	-6.64771297	V30	CRC	6.055831257
V23	Con_CRC	-7.181191336	V31	CRC	5.088317266
V5	Con_CRC	-7.558945528	V28	CRC	3.865826489
V32	Con_CRC	-8.101427363	V4	CRC	3.758294237
			V11	CRC	2.669264236
			V34	CRC	2.243592293
			V12	CRC	1.199091982

Table 9. Gene relative abundance of Sample V30

Gene id	Enrichment (1=Control, 0=CRC)	SEQ ID NO:	Calculation of gene relative abundance
2361423	0	1	2.24903E-05

2040133	0	2	8.77418E-08
3246804	0	3	0
3319526	1	4	0
3976414	1	5	0
1696299	0	6	4.04178E-06
2211919	1	7	7.89676E-07
1804565	1	8	0
3173495	0	9	0.000020166
482585	0	10	0
181682	1	11	0
3531210	1	12	0
3611706	1	13	0
1704941	0	14	1.73798E-06
4256106	1	15	0
4171064	1	16	9.35913E-08
2736705	0	17	1.41059E-07
2206475	1	18	3.12301E-07
370640	1	19	0
1559769	1	20	0
3494506	1	21	0
1225574	0	22	0
1694820	0	23	4.57783E-07
4165909	1	24	0
3546943	0	25	0
3319172	1	26	0
1699104	0	27	4.74411E-06
3399273	1	28	6.0661E-08
3840474	1	29	0
4148945	1	30	3.00829E-07
2748108	0	31	8.14399E-08

Thus the inventors have identified and validated 31 markers set by a minimum redundancy - maximum relevance (mRMR) feature selection method based on 140,455 CRC-associated markers. And the inventors have built a gut healthy index to evaluate the risk of CRC disease based on these 31 gut microbial gene markers.

Example 3. Identifying species biomarker from the 128 Chinese individuals

Basing on the sequencing reads of the 128 microbiomes from cohort I in Example 1, the inventors examined the taxonomic differences between control and CRC-associated microbiomes to identify microbial taxa contributing to the dysbiosis. For this, the inventors used taxonomic profiles derived from three different methods, as supporting evidence from multiple methods would strengthen an association. First, the inventors mapped metagenomic reads to 4650 microbial genomes in the IMG database (version 400) and estimated the abundance of microbial species

included in that database (denoted IMG species). Second, the inventors estimated the abundance of species-level molecular operational taxonomic units (mOTUs) using universal phylogenetic marker genes. Third, the inventors organized the 140,455 genes identified by MGWAS into metagenomic linkage groups (MLGs) that represent clusters of genes originating from the same genome, annotated the MLGs at species level using IMG database whenever possible, grouped MLGs based on these species annotations, and then estimated the abundance of these species (denoted MLG species).

3.1 Species annotation of IMG genomes

For each IMG genome, using the NCBI taxonomy identifier provided by IMG, we identified the corresponding NCBI taxonomic classification at species and genus levels using NCBI taxonomy dump files. The genomes without corresponding NCBI species names were left with its original IMG names, most of which were unclassified.

3.2 Data profile construction

3.2.1 Gene profiles

The inventors mapped our high-quality reads to the gene catalogue to a published reference gut gene catalogue established from European and Chinese adults (identity >= 90%), based on which the inventors derived the gene profiles using the same method of the published T2D paper as Qin et al. 2012, *supra.*

3.2.2 mOTU profile

Clean reads (high quality reads in Example 1) were aligned to mOTU reference (total 79268 sequences) with default parameters (S. Sunagawa *et al.*, Metagenomic species profiling using universal phylogenetic marker genes. *Nature methods* **10**, 1196 (Dec, 2013) , incorporated herein by reference). 549 species level mOTUs were identified, including 307 annotated species and 242 mOTU linkage groups without representative genomes, which were putatively Firmicutes or Bacteroidetes.

3.2.3 IMG-species and IMG-genus profiles

Bacterial, archaeal and fungal sequences were extracted from IMG v400 reference database (V. M. Markowitz *et al.*, IMG: the Integrated Microbial Genomes database and comparative analysis system. *Nucleic acids research* **40**, D115 (Jan, 2012) , incorporated herein by reference) downloaded from http: //ftp.jgi-psf.org. 522,093 sequences were obtained in total, and SOAP reference index was constructed based on 7 equal size chunks of the original file. Clean reads were

aligned to reference using SOAP aligner (R. Li *et al.*, SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* **25**, 1966 (Aug 1, 2009) , incorporated herein by reference) version 2.22, with parameters "-m 4 -s 32 –r 2 -n 100 -x 600 -v 8 -c 0.9 -p 3". Then, SOAP coverage software was used to calculate read coverage of each genome, normalized with genome length, and further normalized to relative abundance for each individual sample. The profile was generated based on uniquely mapped reads only.

3.3 Identification of colorectal cancer associated MLG species

Based on the identified 140,455 colorectal cancer associated maker genes profile, the inventors constructed the colorectal cancer associated MLGs using the method described in the previous type 2 diabetes study as Qin et al. 2012, *supra*. All genes were aligned to the reference genomes of IMG database v400 to get genome level annotation. An MLG was assigned to a genome if > 50% constitutive genes were annotated to that genome, otherwise it was termed as unclassified. Total 87 MLGs with gene number over than 100 were selected as colorectal cancer associated MLGs. These MLGs were grouped based on the species annotation of these genomes to construct MLG species.

To estimate the relative abundance of an MLG species, the inventors estimated the average abundance of the genes of the MLG species, after removing the 5% lowest and 5% highest abundant genes. Relative abundance of IMG species was estimated by summing the abundance of IMG genomes belonging to that species.

These analysis identified 30 IMG species, 21 mOTUs and 86 MLG species that were significantly associated with CRC status (Wilcoxon rank-sum test, $q<0.05$; see Tables 10, 11). *Eubacterium ventriosum* was consistently enriched in the control microbiomes across all three methods (Wilcoxon rank-sum tests – IMG: q = 0.0414; mOTU: q = 0.012757; MLG: q = 5.446×10^{-4}), and *Eubacterium eligens* was enriched according to two methods (Wilcoxon rank-sum tests – IMG: q = 0.069; MLG: q = 0.00031). On the other hand, *Parvimonas micra* ($q<1.80 \times 10^{-5}$), *Peptostreptococcus stomatis* ($q<1.80 \times 10^{-5}$), *Solobacterium moorei* ($q<0.004331$) and *Fusobacterium nucleatum* ($q<0.004565$) were consistently enriched in CRC patient microbiomes across all three methods (**Fig. 6, Fig. 7**). *P. stomatis* has been associated with oral cancer, and *S. moorei* has been associated with bacteremia. Recent work with 16S rRNA sequencing has observed significant enrichment of *F. nucleatum* in CRC tumor samples, and this bacteria has been shown to possess adhesive, invasive and pro-inflammatory properties. Our

results confirmed this association in a new cohort with different genetic and cultural origins. However, a highly significant enrichment of *P. micra* – an obligate anaerobic bacterium that can cause oral infections like *F. nucleatum* – in CRC-associated microbiomes is a novel finding. *P. micra* is involved in the etiology of periodontis, and it produces a wide range of proteolytic enzymes and uses peptones and amino acids as energy source. It is known to produce hydrogen sulphide, which promotes tumor growth and proliferation of colon cancer cells. Further research is required to verify whether *P. micra* is involved in the pathogenesis of CRC, or its enrichment is a result of CRC associated changes in the colon and/or rectum. Nevertheless, it may represent opportunities for non-invasive diagnostic biomarkers for CRC.

3.4 species marker identification

In order to evaluate the predictive power of these taxonomic associations, the inventors used the random forest ensemble learning method (D. Knights, E. K. Costello, R. Knight, Supervised classification of human microbiota. *FEMS microbiology reviews* **35**, 343 (Mar, 2011), incorporated herein by reference) to identify key species marker in the species profiles from the three different methods.

3.4.1 MLG species marker identification

Based on the constructed 87 MLGs with gene numbers over than 100, the inventors performed the Wilcoxon rank-sum test to each MLG with Benjamini-Hochberg adjustment, and 86 MLGs were selected out as colorectal associated MLGs with $q < 0.05$. To identify MLG species makers, the inventors used "randomForest 4.5-36" package in R vision 2.10 based on the 86 colorectal cancer associated MLG species. Firstly, the inventors sorted all the 86 MLG species by the importance given by the "randomForest" method. MLG marker sets were constructed by creating incremental subsets of the top ranked MLG species, starting from 1 MLG species and ending at all 86 MLG species.

For each MLG makers set, the inventors calculated the false predication ratio in the 128 Chinese cohorts (cohort I). Finally, the MLG species sets with lowest false prediction ratio were selected out as MLG species makers. Furthermore, the inventors drew the ROC curve using the probability of illness based on the selected MLG species markers.

3.4.2 IMG species and mOTU species markers identification

Based on the IMG species and mOTU species profiles, the inventors identified the colorectal cancer associated IMG species and mOTU species with $q < 0.05$ (Wilcoxon rank-sum test with

6Benjamini-Hochberg adjustment). Subsequently, IMG species markers and mOTU species markers were selecting using the random forest approach as in MLG species markers selection.

This analysis revealed that 16 IMG species, 10 species-level mOTUs and 21 MLG species were highly predictive of CRC status (Tables 12, 13), with predictive power of 0.86, 0.90 and 0.94 in ROC analysis, respectively (Fig. 8). *Parvimonas micra* was identified as a key species from all three methods, and *Fusobacterium nucleatum* and *Solobacterium moorei* from two out of three methods, providing further statistical support for their association with CRC status.

3.5 MLG, IMG and mOTU species Stage enrichment analysis

Encouraged by the consistent species associations with CRC status and to take advantage of the records of disease stages of the CRC patients (**Table 2**), the inventors explored the species profiles for specific signatures identifying early stages of CRC. The inventors hypothesized that such an effort might even reveal stage-specific associations that are difficult to identify in a global analysis. To identified which species were enriched in the four colorectal cancer progress or health control, the inventors did Kruskal test for the MLG species with gene number over 100, and all IMG species and mOTU species with $q < 0.05$ (Wilcoxon rank-sum test with Benjamini-Hochberg adjustment) to get the species enrichment by the highest rank mean among four CRC stages and control. And the inventors also compared the significance between each two groups by pair-wise Wilcoxon Rank sum test.

In Chinesecohort I, several species showed significantly different abundances in different stages. Among these, the inventors did not identify any species enriched in stage I compared to all other stages and control samples. *Peptostreptococcus stomatis, Prevotella nigrescens* and *Clostridium symbiosum* were enriched in stage II or later compared to control samples, suggesting that they colonize the colon/rectum after the onset of CRC (**Fig.9**). However, *Fusobacterium nucleatum*, *Parvimonas micra*, and *Solobacterium moorei* were enriched in all four stages compared to controls and were most abundant in stage II (**Fig. 10**), suggesting that they may play a role in both CRC etiology and pathogenesis, and implying them as potential biomarkers for early CRC.

Example 4. Validation of markers by qPCR

The 31 gene biomarkers were derived using the admittedly expensive deep metagenome sequencing approach. Translating them into diagnostic biomarkers would require reliable

measurement by simple and less expensive methods such as quantitative PCR (TaqMan probe-based qPCR). Primers and probes were designed using Primer Express v3.0 (Applied Biosystems, Foster City, CA, USA). The qPCR was performed on an ABI7500 Real-Time PCR System using the TaqMan® Universal PCR Master Mixreagent (Applied Biosystems). Universal 16S rDNA was used as internal control and abundance of gene markers were expressed as relative levels to 16S rDNA.

To verify this, the inventors selected two case-enriched gene markers (m482585(SEQ ID NO: 10) and m1704941(SEQ ID NO: 14)) and measured their abundances by qPCR in a subset of 100 samples (55 cases and 45 controls). Quantification of each of the two genes by the two platforms (metagenomic sequencing and qPCR) showed strong correlations (Spearman r=0.93–0.95, **Fig. 11**), suggesting that the gene markers could also be reliably measured using qPCR.

Next, in order to validate the markers in previously unseen samples, the inventors measured the abundance of these two gene markers using qPCR in 164 fecal samples (51 cases and 113 controls) from an independent Chinese cohort (cohort II). Two case-enriched gene markers significantly associated with CRC status, at significance levels of q = 6.56×10^{-9} (m1704941, butyryl-CoA dehydrogenase from *F. nucleatum*), and q = 0.0011 (m482585, RNA-directed DNA polymerase from an unknown microbe). The gene from *F. nucleatum* was present only in 4 out of 113 control microbiomes, suggesting a potential for developing specific diagnostic tests for CRC using fecal samples. CRC index based on the combined qPCR abundance of the two case-enriched gene markers separated the CRC samples from control samples in cohort II (Wilcoxon rank-sum test, $P = 4.01 \times 10^{-7}$; Fig. 12A). However, the moderate classification potential (inferred from area under the ROC curve of 0.73; **Fig. 12B**) using only these two genes suggested that additional biomarkers could improve classification of CRC patient microbiomes.

Another gene from *P. micra* was the highly conserved *rpoB* gene (namely m1696299 (SEQ ID NO: 6), with identity of 99.78%) encoding RNA polymerase subunit β, often used as a phylogenetic marker (F. D. Ciccarelli *et al.*, Toward automatic reconstruction of a highly resolved tree of life. *Science* **311**, 1283 (Mar 3, 2006), incorporated herein by reference). Since the inventors repeatedly identified *P. micra* as a novel biomarker for CRC using several strategies including species-agnostic procedures, the inventors performed an additional qPCR experiment for this marker gene on Chinese cohort II as before and found a significant enrichment in CRC patient

microbiomes (Wilcoxon rank-sum test, P = $2.15x10^{-15}$). When the inventors combined this gene with the two qPCR validated genes, the CRC index from these three genes clearly separated case from control samples in Chinese cohort II (Wilcoxon rank-sum test, P =$5.76x10^{-13}$, Fig. 13A, Table 14) and showed good classification potential with an improved area under the ROC curve of 0.84 (best cutoff: -14.39, Fig. 13B). The CRC index of each sample was calculated by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the qPCR abundance of marker *i* in sample *j*, wherein *i* refers to each of the gene markers as set forth in said gene marker set;

N is a subset of all patient-enriched markers;

wherein the subset of CRC-enriched markers arethe marker as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO:6;

$|N|$ is number (sizes) of the biomarker in the subset, wherein $|N|$ is 3 ,

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer.

Abundance of *rpoB* from *P. micra* was significantly higher compared to control samples starting from stage II CRC (Fig. 13C, Table 14), agreeing with our results from species abundances, and providing further evidence that this gene could serve as a non-invasive biomarker for the identification of early stage CRC.

Table 15. Sequence Information for the primers and probes for the selected 3 gene markers

>1696299	Forward	AAGAATGGAGAGAGTTGTTAGAGAAAGAA
	Reverse	TTGTGATAATTGTGAAGAACCGAAGA
	Probe	AACTCAAGATCCAGACCTTGCTACGCCTCA
>1704941	Forward	TTGTAAGTGCTGGTAAAGGGATTG
	Reverse	CATTCCTACATAACGGTCAAGAGGTA
	Probe	AGCTTCTATTGGTTCTTCTCGTCCAGTGGC
>482585	Forward	AATGGGAATGGAGCGGATTC
	Reverse	CCTGCACCAGCTTATCGTCAA
	Probe	AAGCCTGCGGAACCACAGTTACCAGC

Table 5. The 31 gene markers identified by the mRMR feature selection method. Detailed information regarding their enrichment, occurrence in colorectal cancer cases and controls, statistical test of association, taxonomy and identity percentage are listed.

Marker gene ID	Wilcoxon Test P		Occurrence					Blastn to IMG v400		Blastp to KEGG v59
			Enrich	Control (n=54)		Case (n=74)				
	P-value	q-value		Count	Rate(%)	Count	Rate(%)	Identity	Taxonomy	Description
3546943	1.59E-06	1.90465E-06	Case	3	5.56	27	36.49	99.09	*Bacteroides* sp. 2_1_56FAA	zinc protease
1225574	1.47E-06	1.8957E-06	Case	0	0.00	13	17.57	88.88	*Clostridium hathewayi* DSM 13479	lactose/L-arabinose transport system substrate-binding protein
2736705	5.35E-07	8.4594E-07	Case	0	0.00	21	28.38	99.68	*Clostridium hathewayi* DSM 13479	NA
2748108	2.12E-07	4.38881E-07	Case	0	0.00	20	27.03	99.82	*Clostridium hathewayi* DSM 13479	RNA polymerase sigma-70 factor, ECF subfamily
2040133	7.46E-11	7.70506E-10	Case	7	12.96	44	59.46	99.4	*Clostridium symbiosum* WAL-14163	cobalt/nickel transport system permease protein
1694820	9.78E-08	2.52552E-07	Case	1	1.85	18	24.32	99.17	*Fusobacterium* sp. 7_1	V-type H+-transporting ATPase subunit K
1704941	1.16E-08	5.12764E-08	Case	1	1.85	21	28.38	99.13	*Fusobacterium nucleatum vincentii* ATCC 49256	butyryl-CoA dehydrogenase
482585	3.81E-09	2.36224E-08	Case	9	16.67	50	67.57	NA	NA	RNA-directed DNA polymerase
3246804	4.19E-08	1.44418E-07	Case	1	1.85	24	32.43	NA	NA	citrate-Mg2+:H+ or citrate-Ca2+:H+ symporter, CitMHS family
1696299	8.50E-10	6.58857E-09	Case	1	1.85	33	44.59	99.78	*Parvimonas micra* ATCC 33270	DNA-directed RNA polymerase subunit beta
1699104	1.00E-08	5.12764E-08	Case	1	1.85	31	41.89	98.08	*Parvimonas micra* ATCC 33270	glutamate decarboxylase
2361423	4.89E-13	1.51641E-11	Case	7	12.96	55	74.32	93.87	*Peptostreptococcus anaerobius* 653-L	transposase
3173495	1.14E-12	1.77065E-11	Case	4	7.41	44	59.46	93.98	*Peptostreptococcus anaerobius* 653-L	transposase
3494506	4.93E-06	5.27005E-06	Control	19	35.19	4	5.41	90.37	*Burkholderiales bacterium* 1_1_47	ribosomal small subunit pseudouridine synthase A
2211919	3.59E-08	1.3927E-07	Control	49	90.74	39	52.70	80.99	*Coprobacillus* sp. 8_2_54BFAA	NA
2206475	6.49E-07	9.58475E-07	Control	23	42.59	5	6.76	98.59	*Eubacterium ventriosum*	beta-glucosidase

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									ATCC 27560	
3976414	1.57E-07	3.48653E-07	Control	15	27.78	3	4.05	87.12	*Faecalibacterium* cf. *prausnitzii* KLE1255	adenosylcobinamide-phosphate synthase CobD
3319172	1.12E-07	2.666E-07	Control	19	35.19	2	2.70	84.22	*Faecalibacterium prausnitzii* A2-165	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase
3319526	7.04E-08	1.98403E-07	Control	21	38.89	7	9.46	90.01	*Faecalibacterium prausnitzii* L2-6	replicative DNA helicase
4171064	4.69E-08	1.45363E-07	Control	29	53.70	10	13.51	94.94	*Faecalibacterium prausnitzii* L2-6	cytidine deaminase
370640	4.06E-06	4.49308E-06	Control	12	22.22	0	0.00	99.4	*Bacteroides clarus* YIT 12056	NA
1804565	7.31E-07	9.85539E-07	Control	16	29.63	1	1.35	NA	NA	branched-chain amino acid transport system ATP-binding protein
3399273	4.88E-07	8.40846E-07	Control	41	75.93	23	31.08	NA	NA	two-component system, LytT family, response regulator
3531210	9.76E-06	9.75675E-06	Control	8	14.81	0	0.00	NA	NA	GDP-L-fucose synthase
3611706	1.67E-06	1.91677E-06	Control	13	24.07	0	0.00	NA	NA	anti-repressor protein
3840474	9.76E-06	9.75675E-06	Control	6	11.11	0	0.00	NA	NA	NA
4148945	5.46E-07	8.4594E-07	Control	23	42.59	8	10.81	NA	NA	NA
4165909	1.60E-06	1.90465E-06	Control	8	14.81	0	0.00	NA	NA	N-acetylmuramoyl-L-alanine amidase
4256106	3.69E-07	6.72327E-07	Control	21	38.89	4	5.41	NA	NA	integrase/recombinase XerD
181682	6.97E-07	9.82079E-07	Control	27	50.00	8	10.81	99.25	*Roseburia intestinalis* L1-82	NA
1559769	2.83E-07	5.48673E-07	Control	17	31.48	5	6.76	88.65	*Coprococcus catus* GD/7	polar amino acid transport system substrate-binding protein

Table 7. CRC index estimated in CRC, T2D and IBD patient and healthy cohorts.

Cohort/group	**Median CRC index**	**Comparison with CRC patients**	
		P-value	**q-value**
CRC patients	6.420958803	NA	NA
CRC controls	-5.476945331	1.96E-21	2.44E-21
T2D patients	-0.108110996	1.33E-27	2.21E-27

T2D controls	-1.471692382	6.21E-31	3.11E-30
IBD patients	-2.214296342	2.38E-10	2.38E-10
IBD controls	-4.724156396	7.56E-29	1.89E-28

Table 10. IMG and mOTU species associated with CRC with q-value < 0.05

30 IMG species					
	Control rank mean	**Case rank mean**	**Enrichment (1:Control;0:Case)**	**P-value**	**q-value**
Peptostreptococcus stomatis	37.25926	84.37838	0	1.29E-12	3.34E-09
Parvimonas micra	38.43519	83.52027	0	1.13E-11	1.46E-08
Parvimonas sp. oral taxon 393	39.81481	82.51351	0	1.28E-10	1.10E-07
Parvimonas sp. oral taxon 110	43.52778	79.80405	0	4.71E-08	3.04E-05
Gemella morbillorum	43.87037	79.55405	0	7.77E-08	4.01E-05
Burkholderia mallei	45.19444	78.58784	0	4.84E-07	0.000156
Fusobacterium sp. oral taxon 370	45.02778	78.70946	0	3.93E-07	0.000156
Fusobacterium nucleatum	45.09259	78.66216	0	4.33E-07	0.000156
Leptotrichia buccalis	45.60185	78.29054	0	7.30E-07	0.000209
Beggiatoa sp. PS	46.53704	77.60811	0	2.79E-06	0.000601
Prevotella intermedia	46.47222	77.65541	0	2.67E-06	0.000601
Streptococcus dysgalactiae	47.06481	77.22297	0	3.09E-06	0.000613
Streptococcus pseudoporcinus	47.5	76.90541	0	8.58E-06	0.001581
Paracoccus denitrificans	47.48148	76.91892	0	9.35E-06	0.001608
Solobacterium moorei	47.66667	76.78378	0	1.17E-05	0.001884
Streptococcus constellatus	48.2037	76.39189	0	2.20E-05	0.003153
Crenothrix polyspora	48.76852	75.97973	0	4.20E-05	0.005697
Filifactor alocis	49.06481	75.76351	0	5.84E-05	0.007533
Sulfurovum sp. SCGC AAA036-O23	52.12037	73.53378	0	6.60E-05	0.008105
Clostridium hathewayi	49.68519	75.31081	0	0.000115	0.013431

Lachnospiraceae bacterium 5_1_57FAA	50.10185	75.00676	0	0.000178	0.019084
Peptostreptococcus anaerobius	50.14815	74.97297	0	0.000186	0.019221
Streptococcus equi	50.58333	74.65541	0	0.00029	0.027747
Streptococcus anginosus	50.66667	74.59459	0	0.000316	0.029114
Leptotrichia hofstadii	50.99074	74.35811	0	0.000342	0.030424
Peptoniphilus indolicus	51.2963	74.13514	0	0.000581	0.048307
Eubacterium ventriosum	80.98148	52.47297	1	1.77E-05	0.00269
Adhaeribacter aquaticus	77.06481	55.33108	1	0.000271	0.026839
Eubacterium eligens	77.90741	54.71622	1	0.000482	0.041404
Haemophilus sputorum	77.66667	54.89189	1	0.000608	0.048977
21 mOTU species					
	Control rank mean	**Case rank mean**	**Enrichment (1:Control;0:Case)**	**P-value**	**q-value**
Parvimonas micra	46.2963	77.78378	0	4.11E-08	1.80E-05
Peptostreptococcus stomatis	46.25	77.81757	0	6.56E-08	1.80E-05
motu_linkage_group_731	50.42593	74.77027	0	1.08E-06	0.000198
Gemella morbillorum	47.93519	76.58784	0	1.57E-06	0.000215
Clostridium symbiosum	48.66667	76.05405	0	1.89E-05	0.00173
Solobacterium moorei	51.22222	74.18919	0	6.31E-05	0.004331
Fusobacterium nucleatum	54.62037	71.70946	0	9.15E-05	0.004565
unclassified *Fusobacterium*	54.22222	72	0	0.000176	0.00806
Clostridium ramosum	50.92593	74.40541	0	0.000289	0.012202
Clostridiales bacterium 1_7_47FAA	51.27778	74.14865	0	0.000365	0.013366
Bacteroides fragilis	51.09259	74.28378	0	0.00045	0.01371
motu_linkage_group_624	51.01852	74.33784	0	0.000448	0.01371
Clostridium bolteae	51.81481	73.75676	0	0.000952	0.026134
motu_linkage_group_407	81.13889	52.35811	1	6.00E-06	0.000659
motu_linkage_group_490	80.46296	52.85135	1	3.06E-05	0.002403

motu_linkage_group_316	79.61111	53.47297	1	8.17E-05	0.004487
motu_linkage_group_443	79.66667	53.43243	1	7.63E-05	0.004487
Eubacterium ventriosum	78.09259	54.58108	1	0.000325	0.012757
motu_linkage_group_510	77.84259	54.76351	1	0.000443	0.01371
motu_linkage_group_611	77.2963	55.16216	1	0.000606	0.017499
motu_linkage_group_190	75.16667	56.71622	1	0.001694	0.044273

Table 11. List of 86 MLG species formed after grouping MLGs with more than 100 genes using species annotation when available.

	Control rank mean	**Case rank mean**	**Enrichment (1:Control;0:Case)**	**P-value**	**q-value**
Parvimonas micra	38.40741	83.54054	0	3.16E-12	2.75E-10
Fusobacterium nucleatum	40.32407	82.14189	0	2.97E-11	1.29E-09
Solobacterium moorei	42.2037	80.77027	0	3.85E-09	1.12E-07
Clostridium symbiosum	46.31481	77.77027	0	1.64E-06	3.56E-05
CRC 2881	51.25926	74.16216	0	2.57E-06	4.46E-05
Clostridium hathewayi	46.77778	77.43243	0	3.92E-06	5.69E-05
CRC 6481	52.09259	73.55405	0	1.36E-05	0.000107
Clostridium clostridioforme	50.2037	74.93243	0	1.27E-05	0.000107
Clostridiales bacterium 1_7_47FAA	48.16667	76.41892	0	2.02E-05	0.000135
Clostridium sp. HGF2	48.27778	76.33784	0	2.36E-05	0.000147
CRC 2794	51.03704	74.32432	0	3.50E-05	0.000179
CRC 4136	50.99074	74.35811	0	5.22E-05	0.000233
Bacteroides fragilis	49.09259	75.74324	0	5.97E-05	0.000236
Lachnospiraceae bacterium 5_1_57FAA	49.96296	75.10811	0	7.37E-05	0.000273
Desulfovibrio sp. 6_1_46AFAA	53.33333	72.64865	0	0.000214	0.000546
Coprobacillus sp. 3_3_56FAA	50.53704	74.68919	0	0.000265	0.000623
Cloacibacillus evryensis	52.73148	73.08784	0	0.000359	0.000801
CRC 2867	52.31481	73.39189	0	0.000552	0.001162
Fusobacterium varium	54.57407	71.74324	0	0.000586	0.001186
Clostridium bolteae	51.39815	74.06081	0	0.000647	0.001223
Subdoligranulum sp. 4_3_54A2FAA	51.56481	73.93919	0	0.000758	0.001373

Clostridium citroniae	51.71296	73.83108	0	0.000861	0.001529
Lachnospiraceae bacterium 8_1_57FAA	51.88889	73.7027	0	0.001024	0.001782
Streptococcus equinus	54.52778	71.77703	0	0.001581	0.002457
CRC 4069	53.7963	72.31081	0	0.001632	0.00249
Lachnospiraceae bacterium 3_1_46FAA	52.53704	73.22973	0	0.00178	0.002612
Dorea formicigenerans	52.98148	72.90541	0	0.002703	0.003409
Synergistes sp. 3_1 syn1	54.37963	71.88514	0	0.003358	0.004002
Lachnospiraceae bacterium 3_1_57FAA_CT1	54.07407	72.10811	0	0.004478	0.005109
CRC 3579	54.05556	72.12162	0	0.005638	0.006289
Alistipes indistinctus	54.50926	71.79054	0	0.008262	0.008766
Con 10180	82.03704	51.7027	1	4.87E-06	6.05E-05
Coprococcus sp. ART55/1	80.85185	52.56757	1	8.22E-06	8.94E-05
Con 7958	75.27778	56.63514	1	1.36E-05	0.000107
butyrate-producing bacterium SS3/4	80.57407	52.77027	1	1.98E-05	0.000135
Haemophilus parainfluenzae	80.49074	52.83108	1	2.54E-05	0.000148
Con 154	80.35185	52.93243	1	3.30E-05	0.000179
Con 4595	77.21296	55.22297	1	4.17E-05	0.000202
Con 1617	76.12963	56.01351	1	5.61E-05	0.000233
Con 1979	79.94444	53.22973	1	5.62E-05	0.000233
Con 1371	78.46296	54.31081	1	7.54E-05	0.000273
Con 1529	75.05556	56.7973	1	9.25E-05	0.00031
Eubacterium eligens	79.53704	53.52703	1	9.03E-05	0.00031
Con 1987	79.42593	53.60811	1	0.000101	0.000324
Con 5770	79.39815	53.62838	1	0.000104	0.000324
Con 1197	75.42593	56.52703	1	0.000128	0.000383
Con 4699	78.78704	54.07432	1	0.000152	0.000441
Clostridium sp. L2-50	76.37963	55.83108	1	0.000167	0.000469
Con 2606	77.5	55.01351	1	0.000189	0.000514
Eubacterium ventriosum	78.62963	54.18919	1	0.000207	0.000545
Bacteroides clarus	75.55556	56.43243	1	0.000247	0.000597
Eubacterium biforme	74.68519	57.06757	1	0.000247	0.000597
Faecalibacterium prausnitzii	78.25926	54.45946	1	0.00034	0.000779
Con 563	72.7037	58.51351	1	0.000556	0.001162

Con 6037	77.5463	54.97973	1	0.000561	0.001162
Con 8757	77.17593	55.25	1	0.000634	0.001223
Ruminococcus obeum	77.53704	54.98649	1	0.000629	0.001223
Con 1513	76.59259	55.67568	1	0.000701	0.001298
Roseburia intestinalis	76.99074	55.38514	1	0.001079	0.001841
Ruminococcus torques	76.92593	55.43243	1	0.001186	0.001984
Con 4829	76.7963	55.52703	1	0.001335	0.002151
Con 569	73.41667	57.99324	1	0.001334	0.002151
Con 10559	76.59259	55.67568	1	0.001561	0.002457
Con 1604	71.92593	59.08108	1	0.001781	0.002612
Con 2494	74.35185	57.31081	1	0.001802	0.002612
Con 1867	76.38889	55.82432	1	0.001908	0.002722
Con 1241	76.27778	55.90541	1	0.002132	0.00294
Con 5752	73.65741	57.81757	1	0.002163	0.00294
Con 7367	76.23148	55.93919	1	0.002112	0.00294
Con 6128	76.22222	55.94595	1	0.002274	0.003043
Con 5615	76.07407	56.05405	1	0.002372	0.003104
Klebsiella pneumoniae	74.7037	57.05405	1	0.00239	0.003104
Con 4909	75.72222	56.31081	1	0.002685	0.003409
Con 356	75.94444	56.14865	1	0.002808	0.00349
Eubacterium rectale	75.90741	56.17568	1	0.002953	0.003619
Con 6068	75.74074	56.2973	1	0.003338	0.004002
Con 4295	74.98148	56.85135	1	0.004171	0.004904
Con 2703	74.55556	57.16216	1	0.00437	0.005069
Con 2503	74.14815	57.45946	1	0.004522	0.005109
Con 631	70.01852	60.47297	1	0.006178	0.006804
Con 561	70.5	60.12162	1	0.008137	0.00874
Con 8420	72.64815	58.55405	1	0.008068	0.00874
Con 425	73.19444	58.15541	1	0.008397	0.008802
Con 7993	73.74074	57.75676	1	0.009358	0.009692
Burkholderiales bacterium 1_1_47	72.37963	58.75	1	0.009707	0.009935
Con 600	69.53704	60.82432	1	0.026354	0.02666

Table 12. IMG and mOTU species makers. IMG and mOTU species markers identified using random forest method among species associated with CRC (**Table S9**). Spec
marker were listed by their importance reported by the method.

16 IMG species makers					
	Control rank mean	**Case rank mean**	**Enrichment (1:Control;0:Case)**	**P-value**	**q-value**
Peptostreptococcus stomatis	37.25926	84.37838	0	1.29E-12	3.34E-09
Parvimonas micra	38.43519	83.52027	0	1.13E-11	1.46E-08
Parvimonas sp. oral taxon 393	39.81481	82.51351	0	1.28E-10	1.10E-07
Parvimonas sp. oral taxon 110	43.52778	79.80405	0	4.71E-08	3.04E-05
Gemella morbillorum	43.87037	79.55405	0	7.77E-08	4.01E-05
Fusobacterium sp. oral taxon 370	45.02778	78.70946	0	3.93E-07	1.56E-04
Burkholderia mallei	45.19444	78.58784	0	4.84E-07	1.56E-04
Fusobacterium nucleatum	45.09259	78.66216	0	4.33E-07	1.56E-04
Leptotrichia buccalis	45.60185	78.29054	0	7.30E-07	2.09E-04
Prevotella intermedia	46.47222	77.65541	0	2.67E-06	6.01E-04
Beggiatoa sp. PS	46.53704	77.60811	0	2.79E-06	6.01E-04
Crenothrix polyspora	48.76852	75.97973	0	4.20E-05	5.70E-03
Clostridium hathewayi	49.68519	75.31081	0	1.15E-04	1.34E-02
Lachnospiraceae bacterium 5_1_57FAA	50.10185	75.00676	0	1.78E-04	1.91E-02
Eubacterium ventriosum	80.98148	52.47297	1	1.77E-05	2.69E-03
Haemophilus sputorum	77.66667	54.89189	1	6.08E-04	4.90E-02
10 mOTU species makers					
	Control rank mean	**Case rank mean**	**Enrichment (1:Control;0:Case)**	**P-value**	**q-value**
Peptostreptococcus stomatis	46.25	77.81757	0	6.56E-08	1.80E-05
Parvimonas micra	46.2963	77.78378	0	4.11E-08	1.80E-05
Gemella morbillorum	47.93519	76.58784	0	1.57E-06	0.000215
Solobacterium moorei	51.22222	74.18919	0	6.31E-05	0.004331
unclassified *Fusobacterium*	54.22222	72	0	0.000176	0.00806
Clostridiales bacterium 1_7_47FAA	51.27778	74.14865	0	0.000365	0.013366
motu_linkage_group_624	51.01852	74.33784	0	0.000448	0.01371

motu_linkage_group_407	81.13889	52.35811	1	6.00E-06	0.000659
motu_linkage_group_490	80.46296	52.85135	1	3.06E-05	0.002403
motu_linkage_group_316	79.61111	53.47297	1	8.17E-05	0.004487

Table 13. 21 MLG species markers identified using random forest method from 106 MLGs with gene number over 100.

21 MLG species makers					
	Control rank mean	**Case rank mean**	**Enrichment (1:Control;0:Case)**	**P-value**	**q-value**
Parvimonas micra	38.40741	83.54054	0	3.16E-12	2.75E-10
Fusobacterium nucleatum	40.32407	82.14189	0	2.97E-11	1.29E-09
Solobacterium moorei	42.2037	80.77027	0	3.85E-09	1.12E-07
CRC 2881	51.25926	74.16216	0	2.57E-06	4.46E-05
Clostridium hathewayi	46.77778	77.43243	0	3.92E-06	5.69E-05
CRC 6481	52.09259	73.55405	0	1.36E-05	0.000107
Clostridiales bacterium 1_7_47FAA	48.16667	76.41892	0	2.02E-05	0.000135
Clostridium sp. HGF2	48.27778	76.33784	0	2.36E-05	0.000147
CRC 4136	50.99074	74.35811	0	5.22E-05	0.000233
Bacteroides fragilis	49.09259	75.74324	0	5.97E-05	0.000236
Clostridium citroniae	51.71296	73.83108	0	0.000861	0.001529
Lachnospiraceae bacterium 8_1_57FAA	51.88889	73.7027	0	0.001024	0.001782
Dorea formicigenerans	52.98148	72.90541	0	0.002703	0.003409
Con 10180	82.03704	51.7027	1	4.87E-06	6.05E-05
Con 7958	75.27778	56.63514	1	1.36E-05	0.000107
butyrate-producing bacterium SS3/4	80.57407	52.77027	1	1.98E-05	0.000135
Haemophilus parainfluenzae	80.49074	52.83108	1	2.54E-05	0.000148
Con 154	80.35185	52.93243	1	3.30E-05	0.000179
Con 1979	79.94444	53.22973	1	5.62E-05	0.000233
Con 5770	79.39815	53.62838	1	0.000104	0.000324
Con 1513	76.59259	55.67568	1	0.000701	0.001298

Table 14 164 samples' qPCR abundance and calculated gut healthy index

sample name(CRC: cases; Con:controls)	482585 (SEQ ID NO: 10)	1704941 (SEQ ID NO: 14)	1696299 (SEQ ID NO: 6)	Stage	CRC mini index
CRC_1	0	0	0.006203293	2	-14.0691259
CRC_2	1.86E-05	0.087144293	0.002625577	2	-2.790341115
CRC_4	0	0.005819658	0	2	-14.07836751
CRC_5	0.37491878	0	0.001675491	2	-7.733973569
CRC_6	0.73039561	0	0	2	-13.37881395
CRC_7	0.235418565	7.05E-06	0.18349339	2	-2.172116584
CRC_8	0.429119094	0	0.018272274	2	-7.368543187
CRC_9	9.98E-06	0	0	3	-15.00028982
CRC_10	0	0	1.60E-06	2	-15.26529334
CRC_11	0	0	1.73E-07	3	-15.58731797
CRC_12	0.372006568	0	0.000316655	2	-7.976287681
CRC_13	0.721364334	0	0	2	-13.38061511
CRC_14	0	0	0.049138581	2	-13.7695258
CRC_15	0	0	0.009579061	2	-14.00622569
CRC_16	0	0	0.000802784	4	-14.36513376
CRC_17	0	0	0	2	-20
CRC_18	3.38E-07	8.53E-05	0.008910363	2	-4.19674629
CRC_19	0.000110781	5.55E-05	0.044982261	3	-3.186066818
CRC_20	0.000234301	2.89E-05	0.066693964	2	-3.115080495
CRC_21	0	0.006985843	0.063669666	3	-7.783949536
CRC_22	0.109450466	0	0	2	-13.65359413
CRC_23	0	0	0	3	-20
CRC_24	0.000152828	0	0	2	-14.60526569
CRC_25	0	9.72E-05	9.80E-06	3	-9.673702553
CRC_26	0.002291805	0.002622757	0.01946802	3	-2.310580833
CRC_27	9.35E-05	0.001461738	0.322093176	3	-2.452112443
CRC_28	0	0	1.61E-05	2	-14.93105804
CRC_29	0.000326642	7.85E-05	0	2	-9.197019439
CRC_30	0	0	0.003779209	2	-14.14086636
CRC_31	0.000675175	0.000711697	0.009892837	2	-2.774322553
CRC_32	0.008042167	0.000418046	0.011960736	2	-2.465214979
CRC_33	0.002654305	0.023680609	0.007125466	2	-2.116281012
CRC_34	0.00081495	0	0	1	-14.36295635
CRC_35	0.000571484	0	0.000169321	3	-9.0047617
CRC_36	0.000982742	0.0005857	0	1	-8.74662842
CRC_37	0.000180959	6.71E-05	0.012612517	2	-3.271631843
CRC_38	8.82E-06	5.37E-05	0	3	-9.774852376
CRC_39	0.003822017	0.002785496	0.000296681	4	-2.833505037
CRC_40	0.021036668	0.000248796	0.014980712	3	-2.368549066
CRC_41	0	0	0	1	-20
CRC_42	0	0	0	1	-20
CRC_43	0	0	0	3	-20
CRC_44	0	0	0	3	-20

CRC_45	0.000663002	0	0	3	-14.39282839
CRC_46	0	4.92E-06	0.013275868	4	-9.061657324
CRC_47	0	0	0.002163301	2	-14.22162768
CRC_48	0	0	2.18E-05	2	-14.88718117
CRC_49	0.00571136	0	9.22E-05	2	-8.759509848
CRC_50	0.0002221	0	9.01E-07	3	-9.89957555
CRC_51	0	0	0	3	-20
CRC_52	3.41E-06	0	0	4	-15.15574854
Con_1	2.78E-07	0	0	0	-15.51865173
Con_2	0	0	0	0	-20
Con_3	0	0	0	0	-20
Con_4	0	0	0	0	-20
Con_5	1.71E-06	0	0	0	-15.25566796
Con_6	0	0	0	0	-20
Con_7	0	0	0	0	-20
Con_8	2.34E-06	0	0.000211515	0	-9.76848099
Con_9	0	0	0	0	-20
Con_10	0	0	0	0	-20
Con_11	0	0	0	0	-20
Con_12	8.85E-06	0	0	0	-15.01768558
Con_13	0	0	0	0	-20
Con_14	0	0	0	0	-20
Con_15	0.006715916	0	0	0	-14.05763158
Con_16	0	0	0	0	-20
Con_17	0	0	0	0	-20
Con_18	0	0	0	0	-20
Con_19	0	0	1.49E-07	0	-15.60893791
Con_20	0	0	0	0	-20
Con_21	0.002499751	0	0	0	-14.20070108
Con_22	0	0	0	0	-20
Con_23	3.37E-05	0	0	0	-14.82412337
Con_24	0.00407976	0	0	0	-14.12978846
Con_25	0	0	2.11E-05	0	-14.89190585
Con_26	0.008105124	0	0	0	-14.03041345
Con_27	2.88E-06	0	0	0	-15.1802025
Con_28	4.91E-05	0	0	0	-14.7696395
Con_29	0	0	0	0	-20
Con_30	0	0	0	0	-20
Con_31	0	0	0	0	-20
Con_32	6.20E-05	0	0	0	-14.73586944
Con_33	0	0	0	0	-20
Con_34	0	0	0	0	-20
Con_35	0	0	0	0	-20
Con_36	0.001536752	0	0	0	-14.27113207
Con_37	0	0	0	0	-20
Con_38	0	0	0	0	-20
Con_39	0.000190886	0	0	0	-14.57307531
Con_40	0	0	0	0	-20

Con_41	1.68E-05	0	0	0	-14.92489691
Con_42	0	0	0	0	-20
Con_43	0	0	0	0	-20
Con_44	0.005333691	0	0	0	-14.09099072
Con_45	0.00045872	0	0	0	-14.44615077
Con_46	0	0	0	0	-20
Con_47	0	0	0	0	-20
Con_48	0.000121349	0	0	0	-14.6386546
Con_49	1.95E-06	0	0	0	-15.23665513
Con_50	0	0	0	0	-20
Con_51	0	0	0	0	-20
Con_52	0	0	0	0	-20
Con_53	0	0	0	0	-20
Con_54	0	0	1.03E-05	0	-14.99572093
Con_55	0	0	0	0	-20
Con_56	0	0	0	0	-20
Con_57	0	0	0	0	-20
Con_58	0	0	0	0	-20
Con_59	0	0	0	0	-20
Con_60	0	0	0	0	-20
Con_61	0	0	0	0	-20
Con_62	0	0	0	0	-20
Con_63	0	0	0	0	-20
Con_64	0	2.10E-05	0	0	-14.89259357
Con_65	0.00096125	0	0	0	-14.33905455
Con_66	0.000280561	0	0	0	-14.51732423
Con_67	0.004437614	0.000250648	0.00179637	0	-2.899796813
Con_68	0.000125259	0	0	0	-14.63406369
Con_69	0	0	0	0	-20
Con_70	0	0	0	0	-20
Con_71	0	0	0	0	-20
Con_72	0	0	0	0	-20
Con_73	0	0	0	0	-20
Con_74	0	0	0	0	-20
Con_75	0	0	0	0	-20
Con_76	1.56E-05	0	0.000315363	0	-9.436021554
Con_77	0.042785033	0	0	0	-13.78956938
Con_78	0.011668395	0	0	0	-13.97766296
Con_79	0	0	0	0	-20
Con_80	0	0	0	0	-20
Con_81	0	0	1.88E-06	0	-15.24194738
Con_82	2.23E-06	0	0	0	-15.21723171
Con_83	0.000446671	0	0	0	-14.45000408
Con_84	1.94E-05	0	0	0	-14.90406609
Con_85	0	0	0	0	-20
Con_86	0.000823554	1.02E-06	0.000177345	0	-4.2756296
Con_87	1.02E-05	0	0	0	-14.99713328
Con_88	0	0	0	0	-20

Con_89	9.38E-07	0	0	0	-15.34259905
Con_90	3.05E-06	0	0	0	-15.17190005
Con_91	0	0	0	0	-20
Con_92	0	0	0	0	-20
Con_93	0	0	0	0	-20
Con_94	0	0	0	0	-20
Con_95	4.75E-07	0	0	0	-15.44110213
Con_96	2.15E-06	0	0	0	-15.22252051
Con_97	0	0	0	0	-20
Con_98	0	0	0	0	-20
Con_99	2.93E-06	0	0	0	-15.17771079
Con_100	0.012223913	0	0	0	-13.97092992
Con_101	9.50E-06	0	0	0	-15.00742546
Con_102	0	0	0	0	-20
Con_103	0	0	0	0	-20
Con_104	8.39E-05	0	0	0	-14.69207935
Con_105	0	0	0	0	-20
Con_106	0	0.000689816	0	0	-14.38708891
Con_107	0	0	0	0	-20
Con_108	0	0	0	0	-20
Con_109	0	0	0	0	-20
Con_110	0.000307175	0	0	0	-14.50420471
Con_111	0.024307579	0	0	0	-13.87141943
Con_112	0	0	0	0	-20
Con_113	0	0	0	0	-20

Although explanatory embodiments have been shown and described, it would be appreciated by those skilled in the art that the above embodiments can not be construed to limit the present disclosure, and changes, alternatives, and modifications can be made in the embodiments without departing from spirit, principles and scope of the present disclosure.

WHAT IS CLAIMED IS:

1. A gene marker set for predicting the risk of colorectal cancer (CRC) in a subject, consisting of the genes as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO: 6.

2. Use of the gene markers in the gene marker set of claim 1 for predicting the risk of colorectal cancer (CRC) in a subject, via the steps of:

1) collecting a sample *j* from the subject and extracting DNA from the sample;

2) determining the abundance information of each of gene marker in the set of gene markers; and

3) calculating the index of sample *j* by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the abundance information of marker i in sample j, wherein i refers to each of the gene markers in said gene marker set;

N is a subset of all abnormal -enriched markers in selected biomarkers related to the disease,

$|N|$ is number (sizes) of the biomarkers in the subset, wherein $|N|$ is 3;

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer (CRC).

3. Use of the gene markers in the gene marker set of claim 1 for preparation of a kit for predicting the risk of colorectal cancer (CRC) in a subject, via the steps of:

1) collecting a sample *j* from the subject and extracting DNA from the sample;

2) determining the abundance information of each of gene marker in the set of gene markers; and

3) calculating the index of sample *j* by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the abundance information of marker i in sample j, wherein i refers to each of the gene markers in said gene marker set;

N is a subset of all abnormal -enriched markers in selected biomarkers related to the disease,

$|N|$ is number (sizes) of the biomarkers in the subset, wherein $|N|$ is 3;

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer (CRC).

4. The use of claim 2 or claim 3, wherein the abundance information is gene relative abundance of each of gene marker in the set of gene markers which is determined by means of sequencing method.

5. The use of claim 2 or claim 3, wherein the abundance information is qPCR abundance of each of gene marker in the set of gene markers which is determined by a qPCR method.

6. The use of any one of claims 2-5, wherein the cutoff value is obtained by a Receiver Operator Characteristic (ROC) method, wherein the cutoff corresponds to when AUC (Area Under the Curve) reached at its maximum.

7. The use of claim 6, wherein the cutoff value is determined as -14.39.

8. A method for diagnosing whether a subject has colorectal cancer or is at the risk of developing colorectal cancer, comprising:

1) collecting a feces sample *j* from the subject and extracting DNA from the sample;

2) determining the abundance information of each of the marker as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO:6; and

3) calculating the index of sample *j* by the formula below:

$$I_j = \frac{\sum_i \epsilon_N \log 10(A_{ij} + 10^{-20})}{|N|}$$

A_{ij} is the abundance information of marker i in sample j, wherein i refers to each of the gene markers as set forth in said gene marker set;

N is a subset of all patient-enriched markers;

wherein the subset of CRC-enriched markers are the marker as set forth in SEQ ID NOs: 10, SEQ ID NO: 14 and SEQ ID NO:6;

$|N|$ is number (sizes) of the biomarker in the subset, wherein $|N|$ is 3,

wherein an index greater than a cutoff indicates that the subject has or is at the risk of developing colorectal cancer.

9. The method of claim 8, wherein the abundance information is gene relative abundance of each of gene marker in the set of gene markers which is determined by means of sequencing method.

10. The method of claim 8, wherein the abundance information is qPCR abundance of each of gene marker in the set of gene markers which is determined by a qPCR method.

11. The method of claim 8, wherein the cutoff value is obtained by a Receiver Operator Characteristic (ROC) method, wherein the cutoff corresponds to when AUC (Area Under the Curve) reached at its maximum.

12. The method of claim 11, wherein the cutoff value is determined as -14.39.

13. A kit for determining the gene marker set of claim 1, comprising one or more primers and probes as set forth in Table 15.

14. Use of a marker as set forth in SEQ ID NO: 6 or *rpoB* gene encoding RNA polymerase subunit β as a gene marker for predicting the risk of colorectal cancer (CRC) in a subject, wherein

the enrichment of said gene marker in a sample of the subject relative to a control sample is indicative of the risk of colorectal cancer in the subject.

15. Use of *Parvimonas micra* as a species marker for predicting the risk of colorectal cancer (CRC) in a subject, wherein the enrichment of said species marker in a sample of the subject relative to a control sample is indicative of the risk of colorectal cancer in the subject.

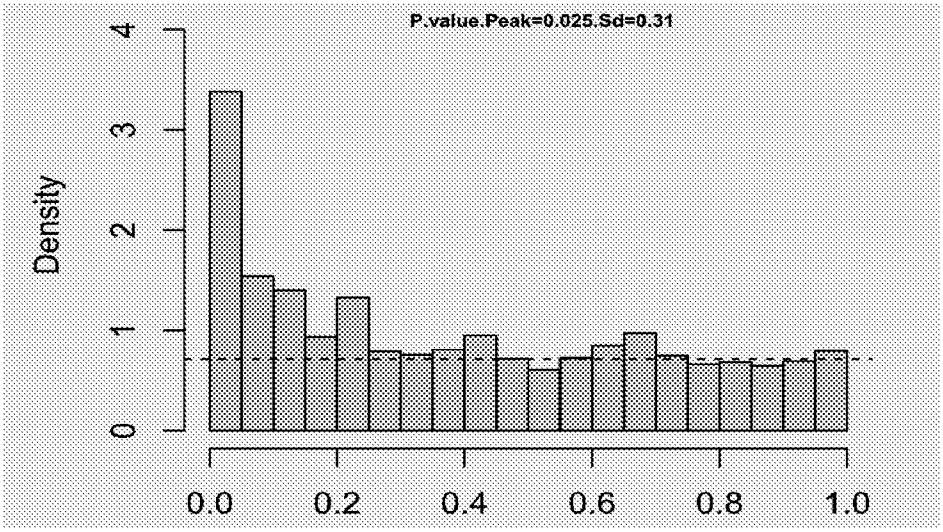

P.value.Peak=0.025.Sd=0.31
Density
0
1
2
3
4
0.0
0.2
0.4
0.6
0.8
1.0

Fig. 1

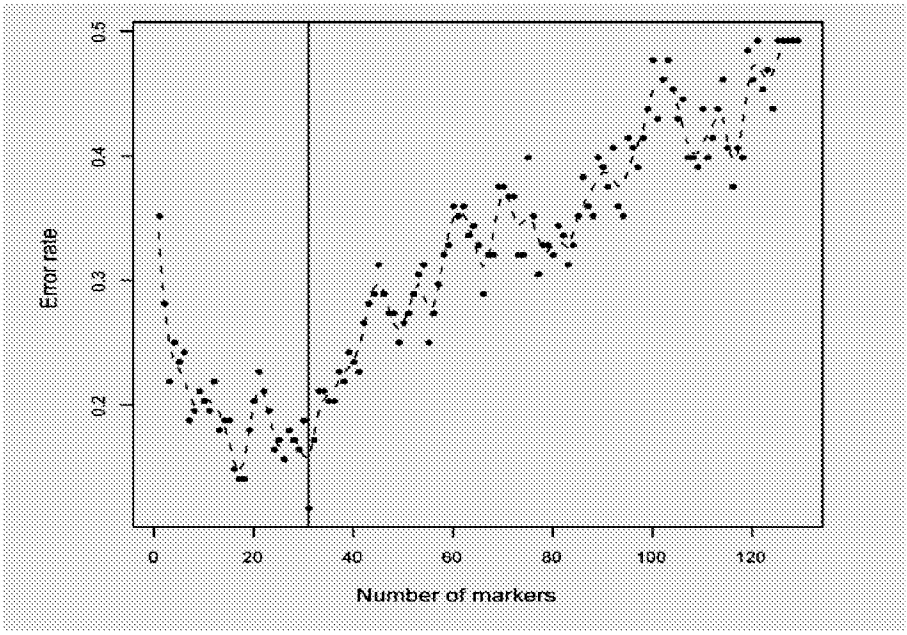

Error rate
0.2
0.3
0.4
0.5
0
20
40
60
80
100
120
Number of markers

Fig. 2

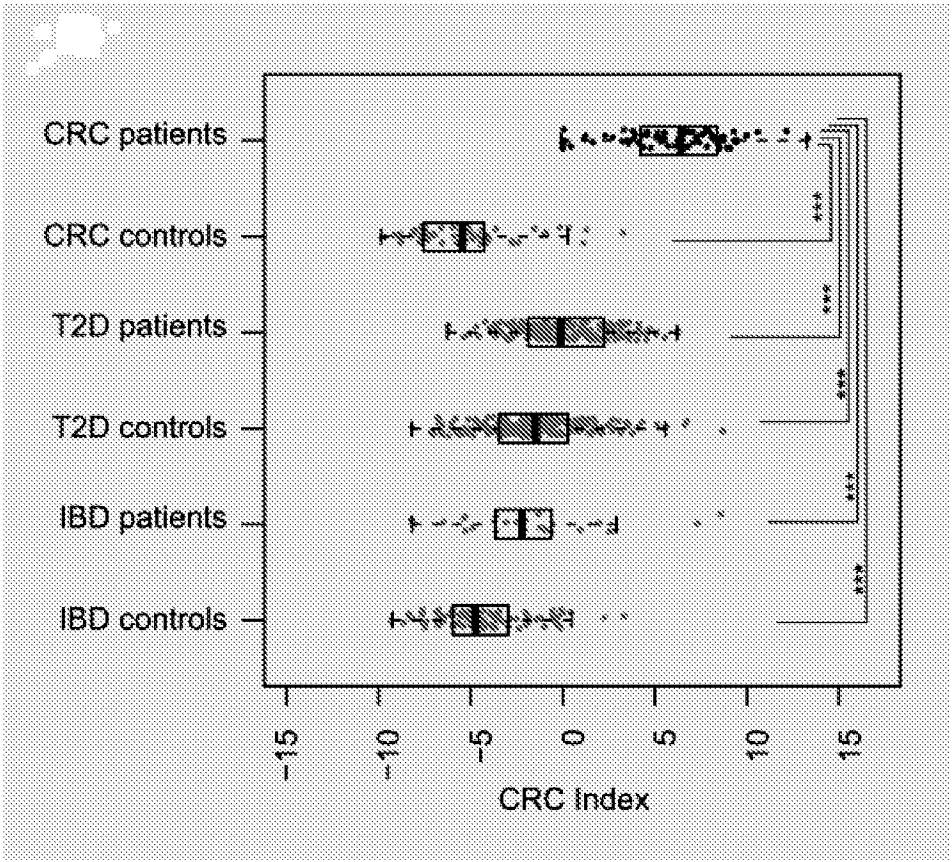

CRC patients
CRC controls
T2D patients
T2D controls
IBD patients
IBD controls
***
***
***
***
***
−15
−10
−5
0
5
10
15
CRC Index

Fig. 3

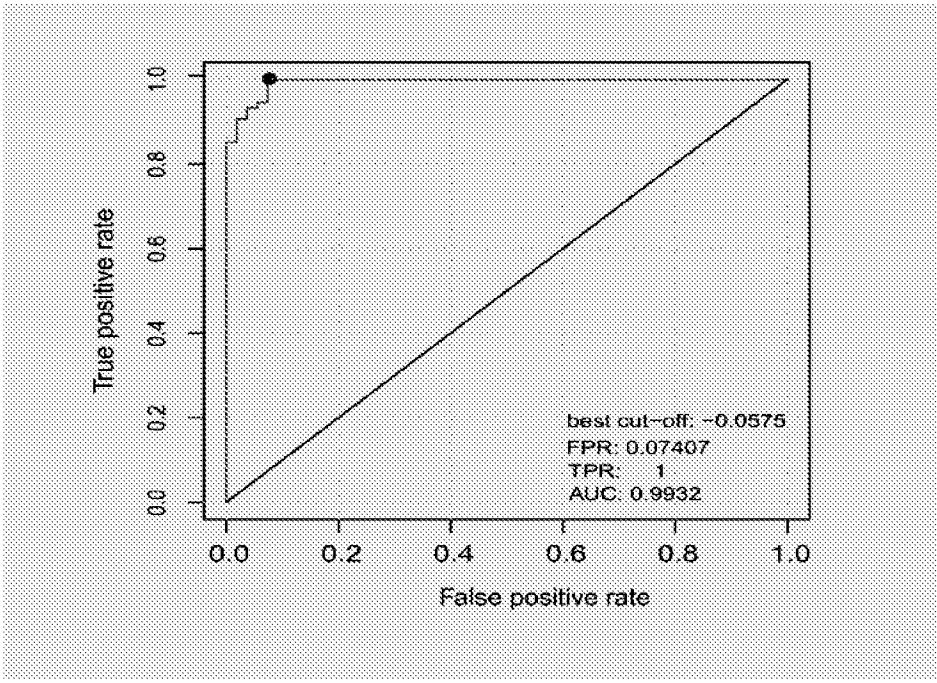

True positive rate
0.0
0.2
0.4
0.6
0.8
1.0
best cut−off: −0.0575
FPR: 0.07407
TPR: 1
AUC: 0.9932
0.0
0.2
0.4
0.6
0.8
1.0
False positive rate

Fig. 4

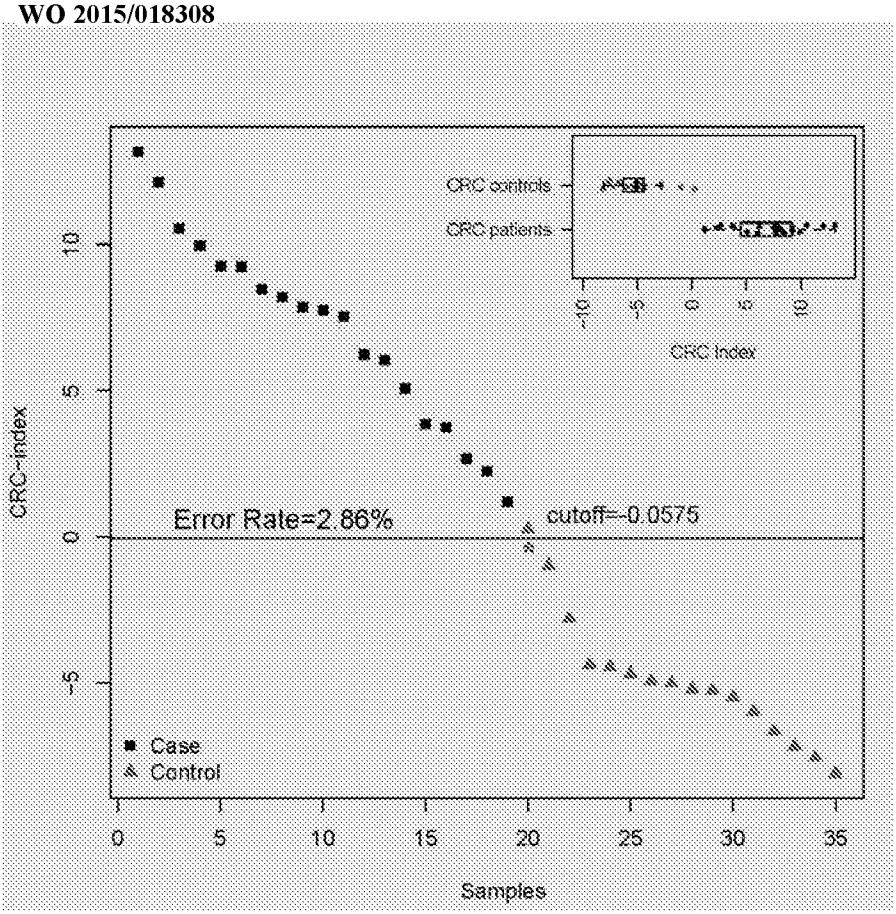

CRC controls
CRC patients
CRC index
CRC-index
Error Rate=2.86%
cutoff=-0.0575
Case
Control
Samples

Fig.5

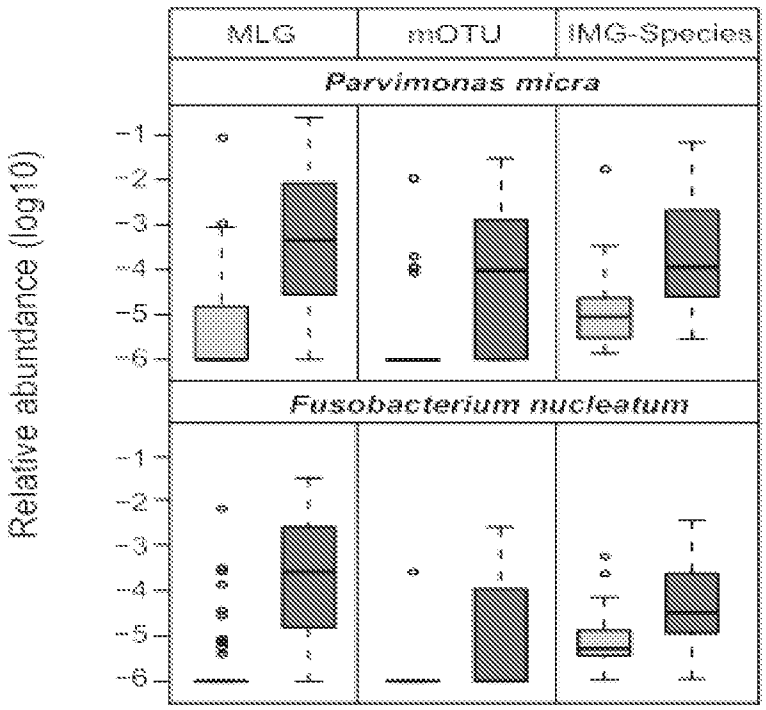

MLG
mOTU
IMG-Species
Parvimonas micra
Fusobacterium nucleatum
Relative abundance (log10)

Fig. 6

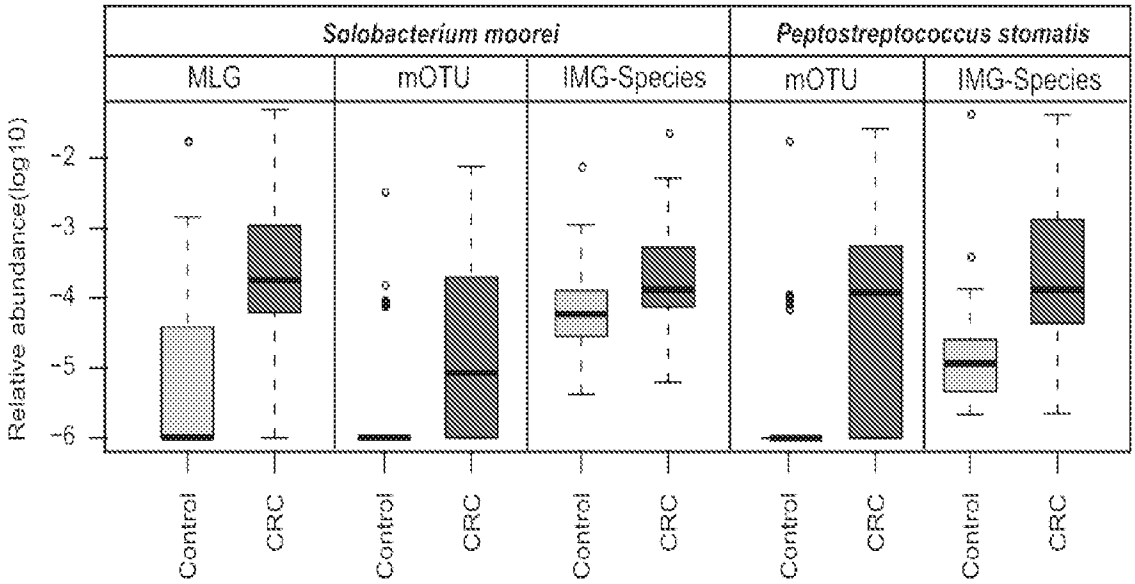

Solobacterium moorei
Peptostreptococcus stomatis
MLG
mOTU
IMG-Species
mOTU
IMG-Species
Relative abundance(log10)
-2
-3
-4
-5
-6
Control
CRC
Control
CRC
Control
CRC
Control
CRC
Control
CRC

Fig.7

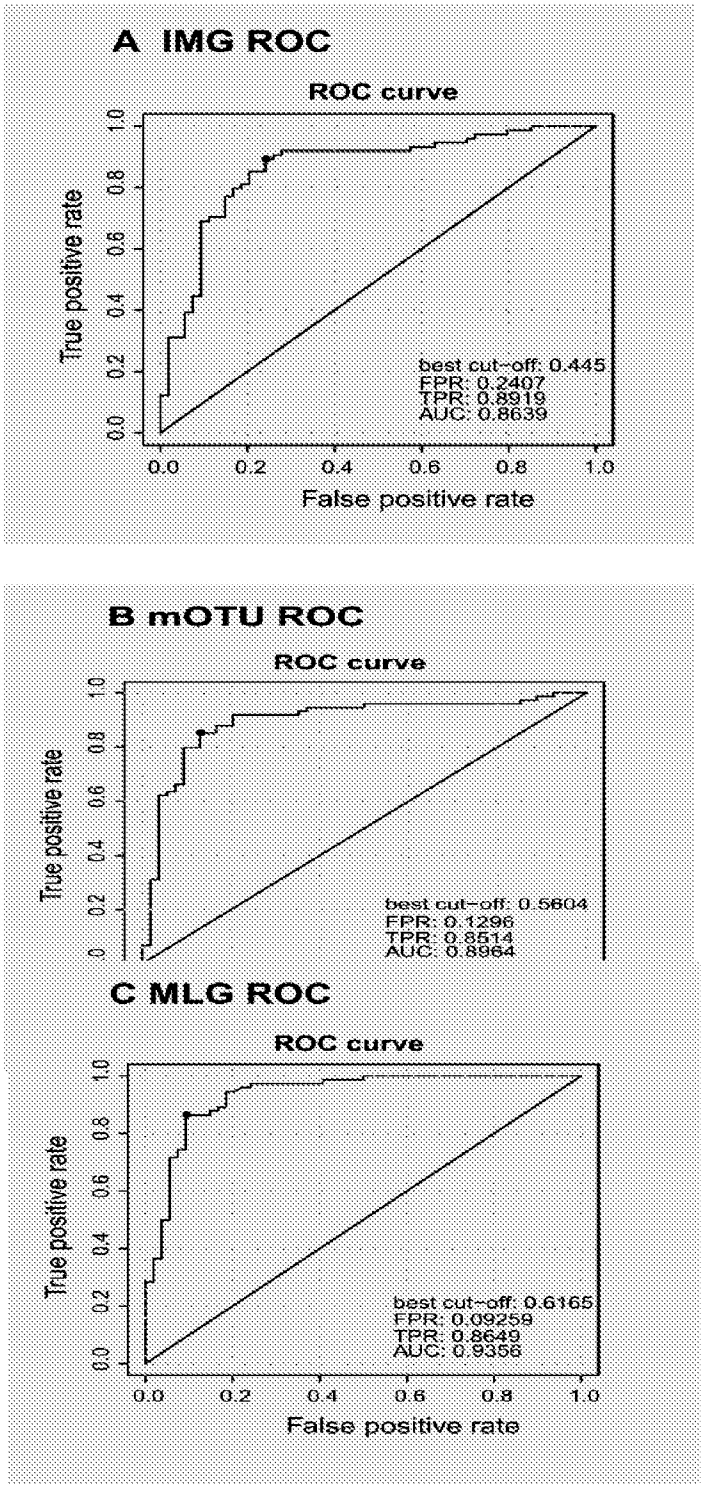

A IMG ROC
ROC curve
True positive rate
False positive rate
best cut-off: 0.445
FPR: 0.2407
TPR: 0.8919
AUC: 0.8639
B mOTU ROC
ROC curve
True positive rate
best cut-off: 0.5604
FPR: 0.1296
TPR: 0.8514
AUC: 0.8964
C MLG ROC
ROC curve
True positive rate
False positive rate
best cut-off: 0.6165
FPR: 0.09259
TPR: 0.8649
AUC: 0.9358

Fig. 8

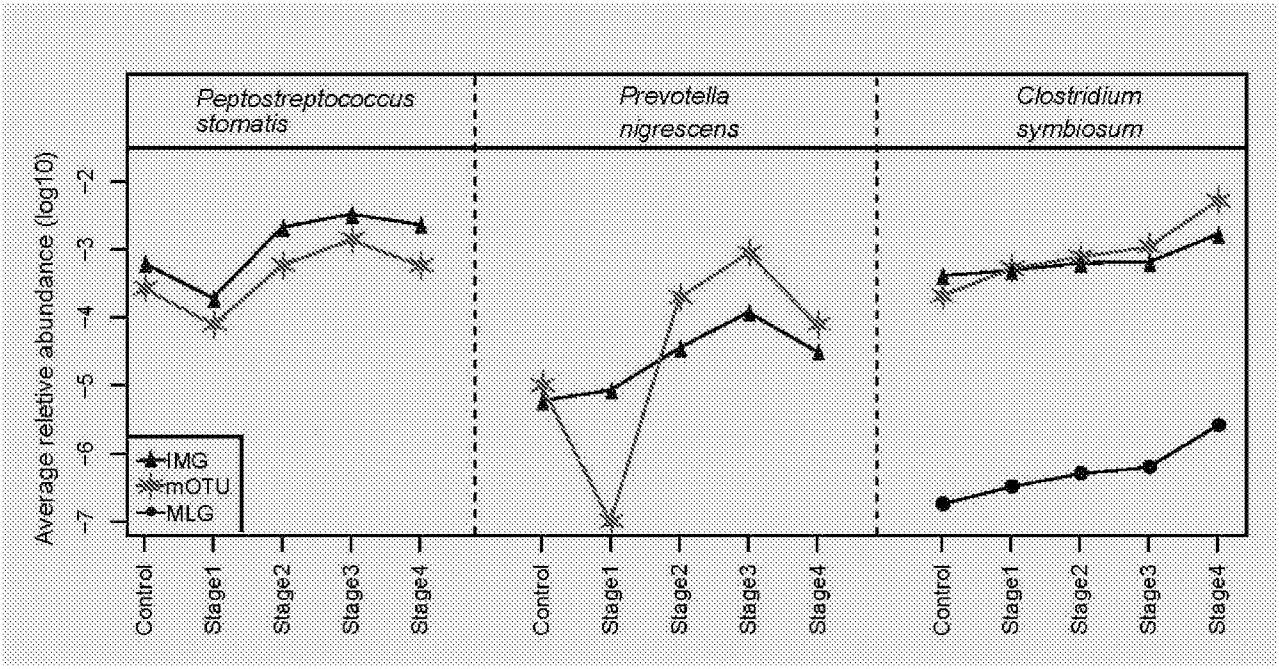

Peptostreptococcus stomatis
Prevotella nigrescens
Clostridium symbiosum
Average reletive abundance (log10)
-2
-3
-4
-5
-6
-7
IMG
mOTU
MLG
Control
Stage1
Stage2
Stage3
Stage4

Fig.9

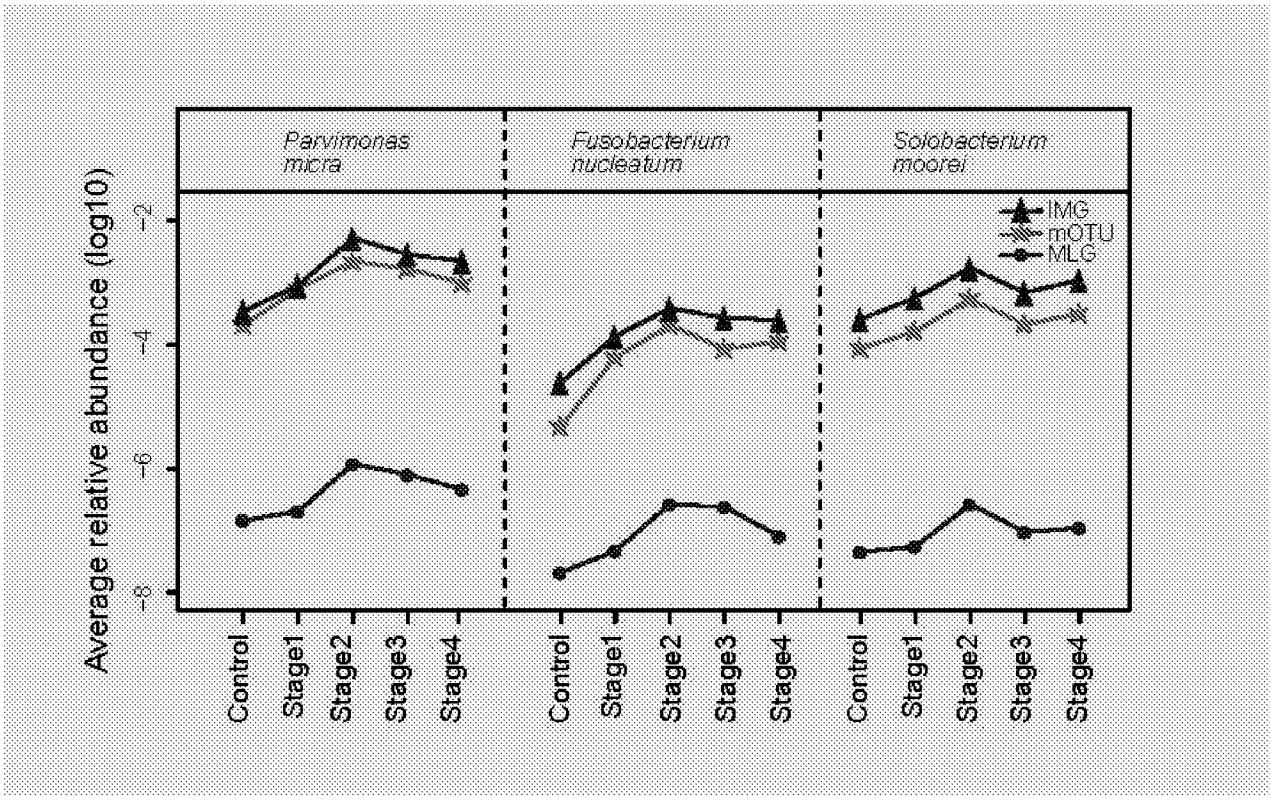
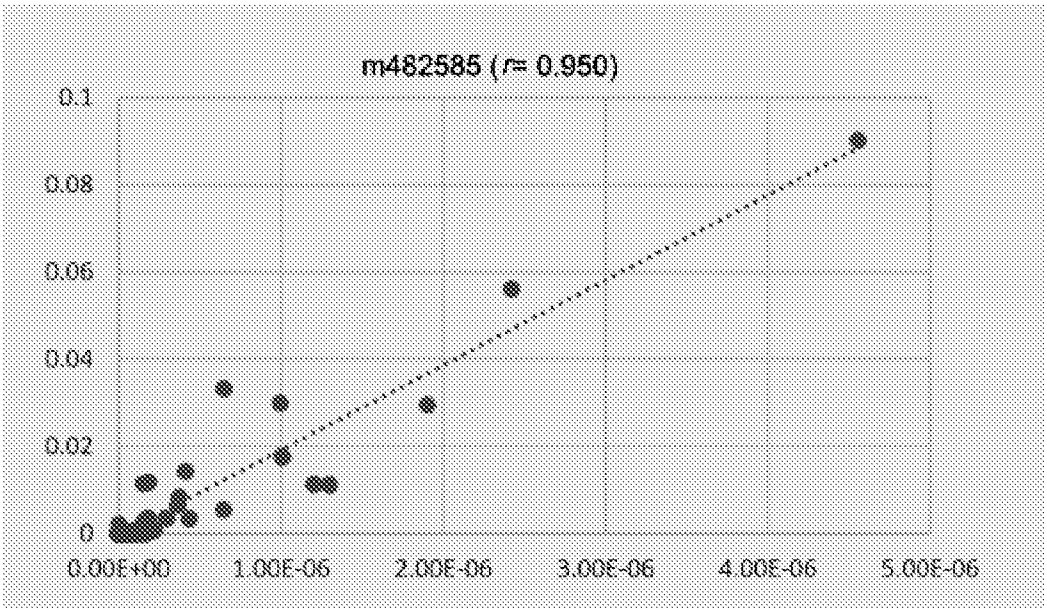

Parvimonas micra
Fusobacterium nucleatum
Solobacterium moorei
Average relative abundance (log10)
-2
-4
-6
-8
IMG
mOTU
MLG
Control
Stage1
Stage2
Stage3
Stage4

Fig.10

A


m482585 (r= 0.950)
0.1
0.08
0.06
0.04
0.02
0
0.00E+00
1.00E-06
2.00E-06
3.00E-06
4.00E-06
5.00E-06

B

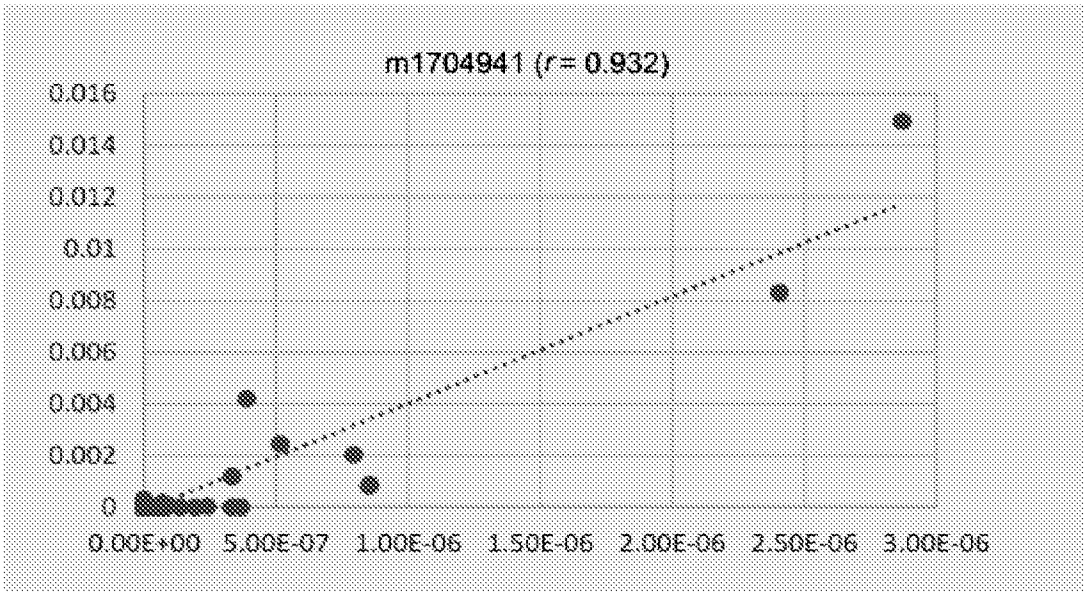

m1704941 (r= 0.932)
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0.00E+00
5.00E-07
1.00E-06
1.50E-06
2.00E-06
2.50E-06
3.00E-06

Fig.11

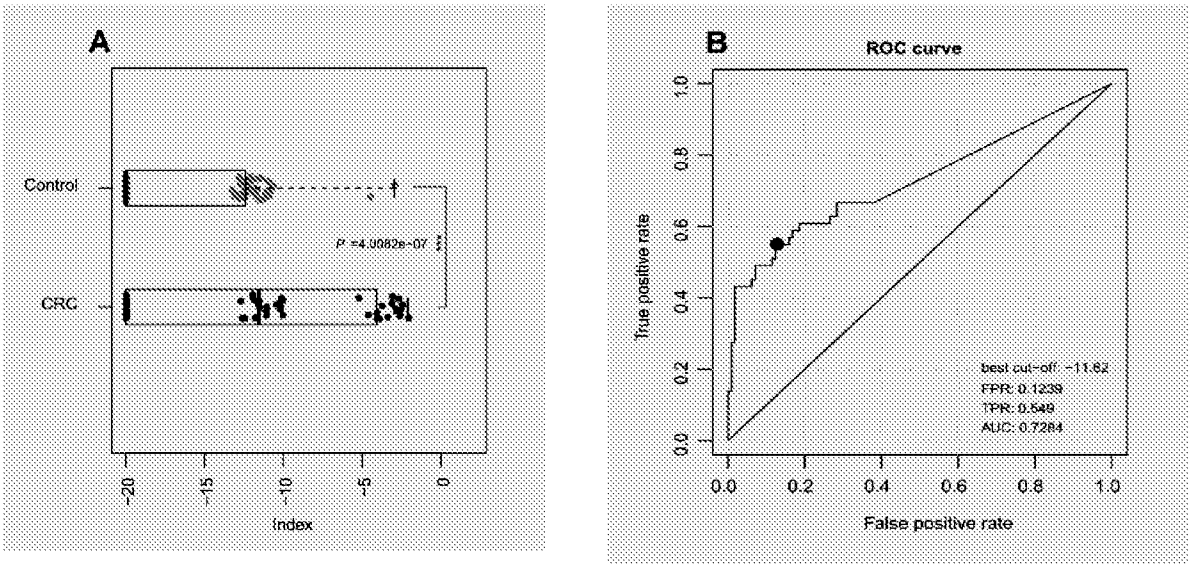

A
Control
CRC
P =4.0082e-07
-20
-15
-10
-5
0
Index
B
ROC curve
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
best cut-off: -11.62
FPR: 0.1239
TPR: 0.549
AUC: 0.7284

Fig.12

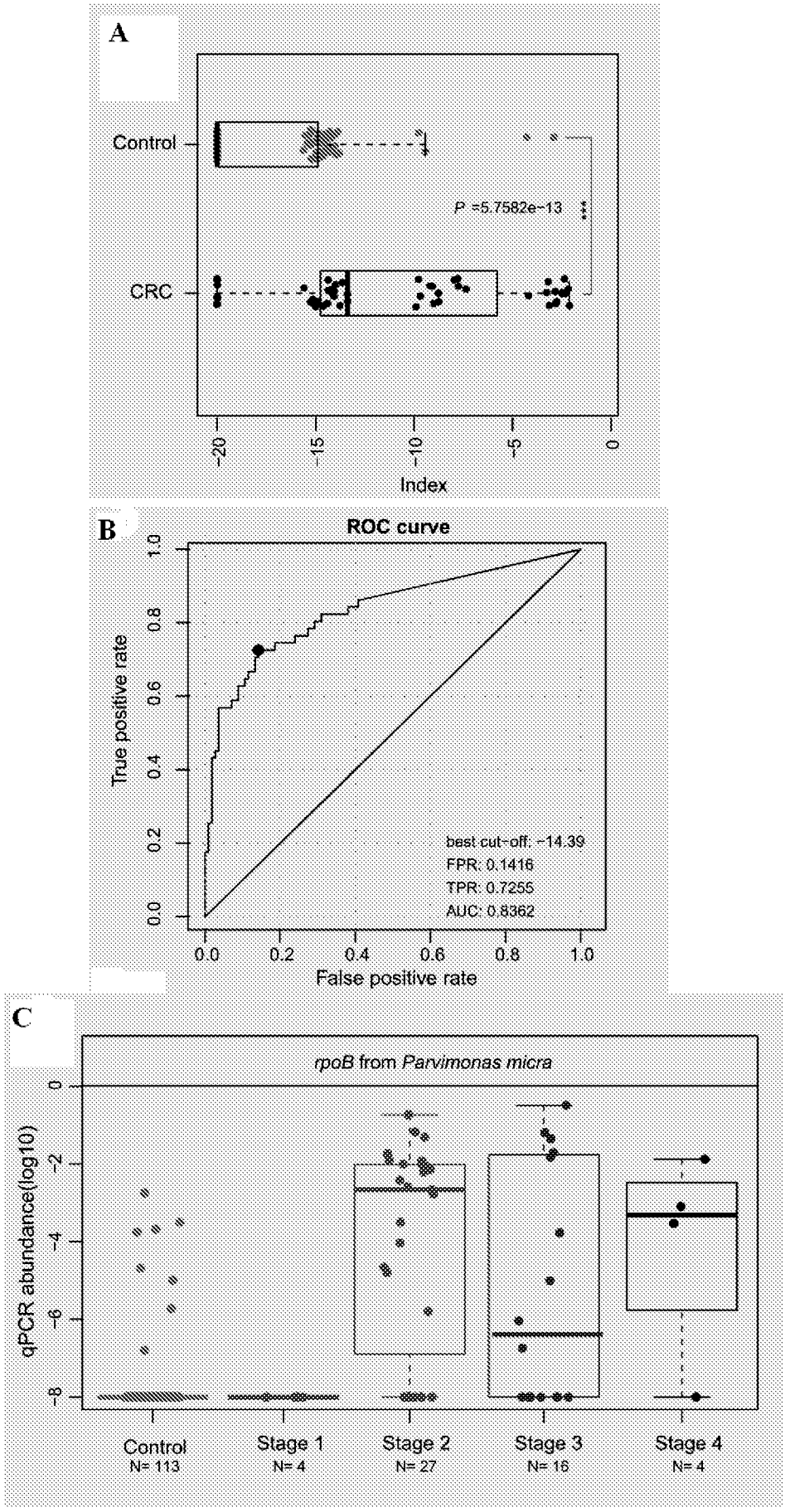

A
Control
CRC
P =5.7582e-13
-20
-15
-10
-5
0
Index
B
ROC curve
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
best cut-off: -14.39
FPR: 0.1416
TPR: 0.7255
AUC: 0.8362
C
rpoB from Parvimonas micra
qPCR abundance(log10)
0
-2
-4
-6
-8
Control
N= 113
Stage 1
N= 4
Stage 2
N= 27
Stage 3
N= 16
Stage 4
N= 4

Fig.13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2014/083664

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i; C12Q 1/10(2006.01)i; G01N 33/574(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12; A61; G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DWPI, CPRS, SIPOABS, CNKI:cancer, tumour, +marker?, gut, microbe, microbial?, intestine, risk, genemarker?, predic+, segmented, crc, microbion?, bacteria+, colon, microorganism?, microbiota, rectum, recta, colorectal; GenBank+embl+DDBJ:sequence search on SEQ ID Nos:10,14 and 6

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010096154 A2 (ONCONOME INCET AL.) 26 August 2010 (2010-08-26) see the whole document	1-12
A	CN 102936597 A (WENZHOU MEDICAL COLLEGE) 20 February 2013 (2013-02-20) see the whole document	1-12
A	CN 101988060 A (JIANGSU MINGMA BIOTECH CO LTD) 23 March 2011 (2011-03-23) see the whole document	1-12
A	WO 2013052480 A1 (UNIV TEXAS) 11 April 2013 (2013-04-11) see the whole document	1-12
A	Tingting Wang et al. ""Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers"" *The ISME Journal*, No. vol. 6, 18 August 2011 (2011-08-18), ISSN: pages 320-329, see the abstract and table 2	1-12

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2014	**24 November 2014**

Name and mailing address of the ISA/CN	Authorized officer
STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA(ISA/CN) **6,Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088 China**	**WANG,Qiyang**
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2014/083664

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Junjie Qin et al. "“Ametagenome-wide association study of gut microbiota in type 2 diabetes”" *NATURE*, No. Vol. 490, 04 October 2012 (2012-10-04), ISSN: pages 55-60, see the whole document	1-12

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2014/083664

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2010096154	A2	26 August 2010	EP	2398918	A2	28 December 2011
				WO	2010096154	A3	06 January 2011
				JP	2012517607	A	02 August 2012
				US	2012149022	A1	14 June 2012
				EP	2398918	A4	22 February 2012
CN	102936597	A	20 February 2013	CN	102936597	B	25 June 2014
CN	101988060	A	23 March 2011		Non	e	
WO	2013052480	A1	11 April 2013		Non	e	