Gut microbiota derived indole-3-acetic acid ameliorates precancerous inflammatory intestinal milieu to inhibit tumorigenesis through IL-35

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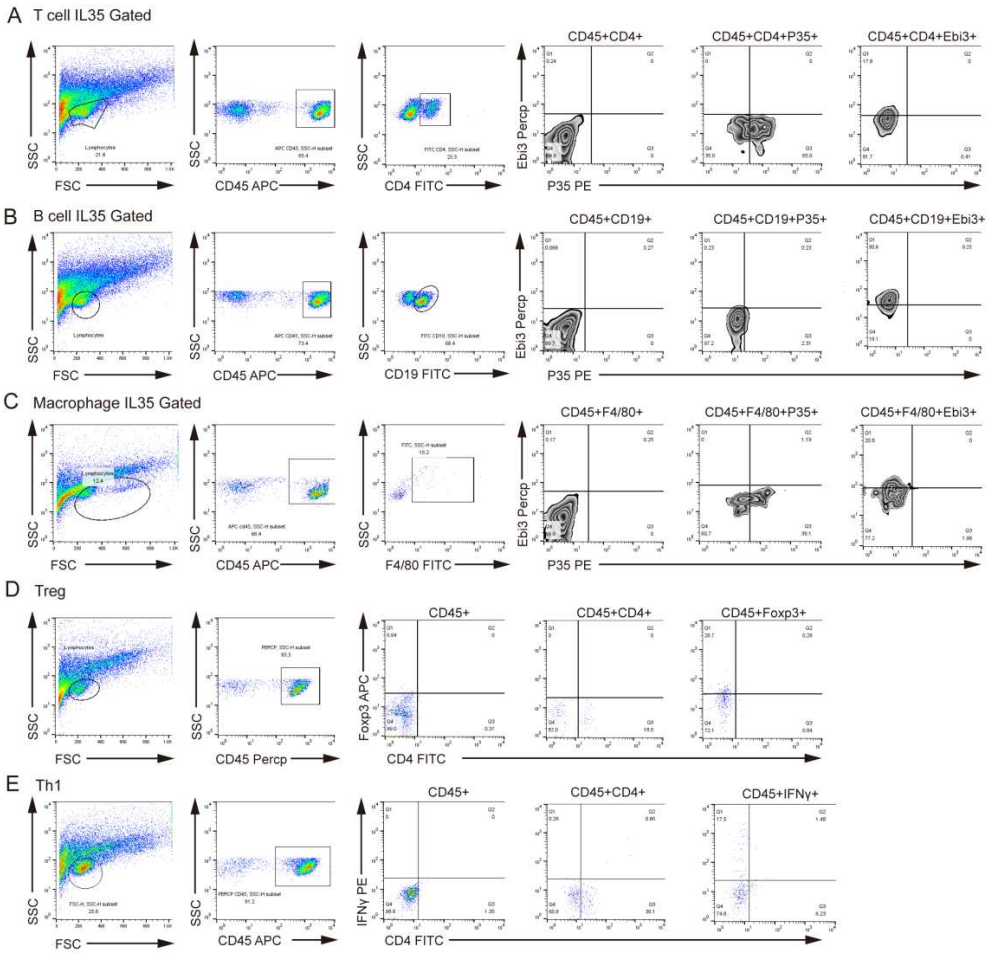


Figure S1. Gate strategies of flow cytometry used in this experiment.

（A） Gate strategy for IL-35+ T cells;
（B） Gate strategy for IL-35+ B cells;
（C） Gate strategy for IL-35+ macrophages;
（D） Gate strategy for Treg cells;
（E） Gate strategy for Th1 cells.

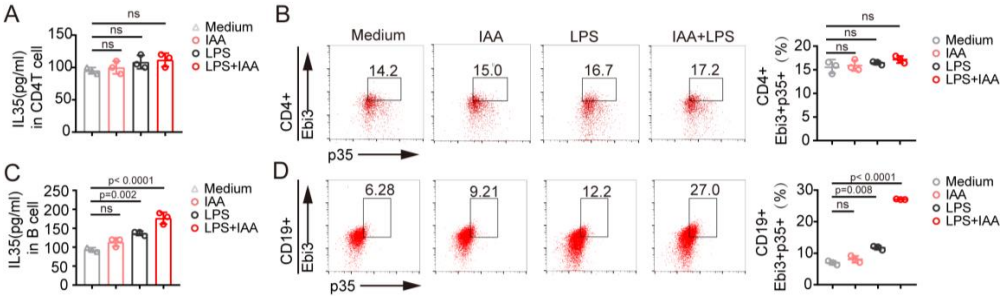


Figure S2. IAA induces IL-35^{+} B cells but not T cells in vitro.

(A) Concentration of IL-35 detected by ELISA in the supernatants of isolated CD4 T cells in indicated groups;

(B)Flow cytometry analyses of CD4^{+}Ebi3^{+}P35^{+} in isolated CD4 T after exposed to IAA (100 μM), LPS (100 ng/ml) and LPS (100 ng/ml) +IAA (100 μM) for 24 hours;

(C) Concentration of IL-35 detected by ELISA in the supernatants of isolated B cells in indicated groups;

(D)Flow cytometry analyses of CD19^{+}Ebi3^{+}P35^{+} in isolated B cells after exposed to IAA (100 μM), LPS (100 ng/ml) or LPS (100 ng/ml) +IAA (100 μM) for 24 hours.

Error bars indicate mean ± SD.

Statistic test: Unpaired Student's t test; Ns, no significance.

Data were a representative of three experiments.

IAA, indole-3- acidic acid; LPS, lipopolysaccharide.

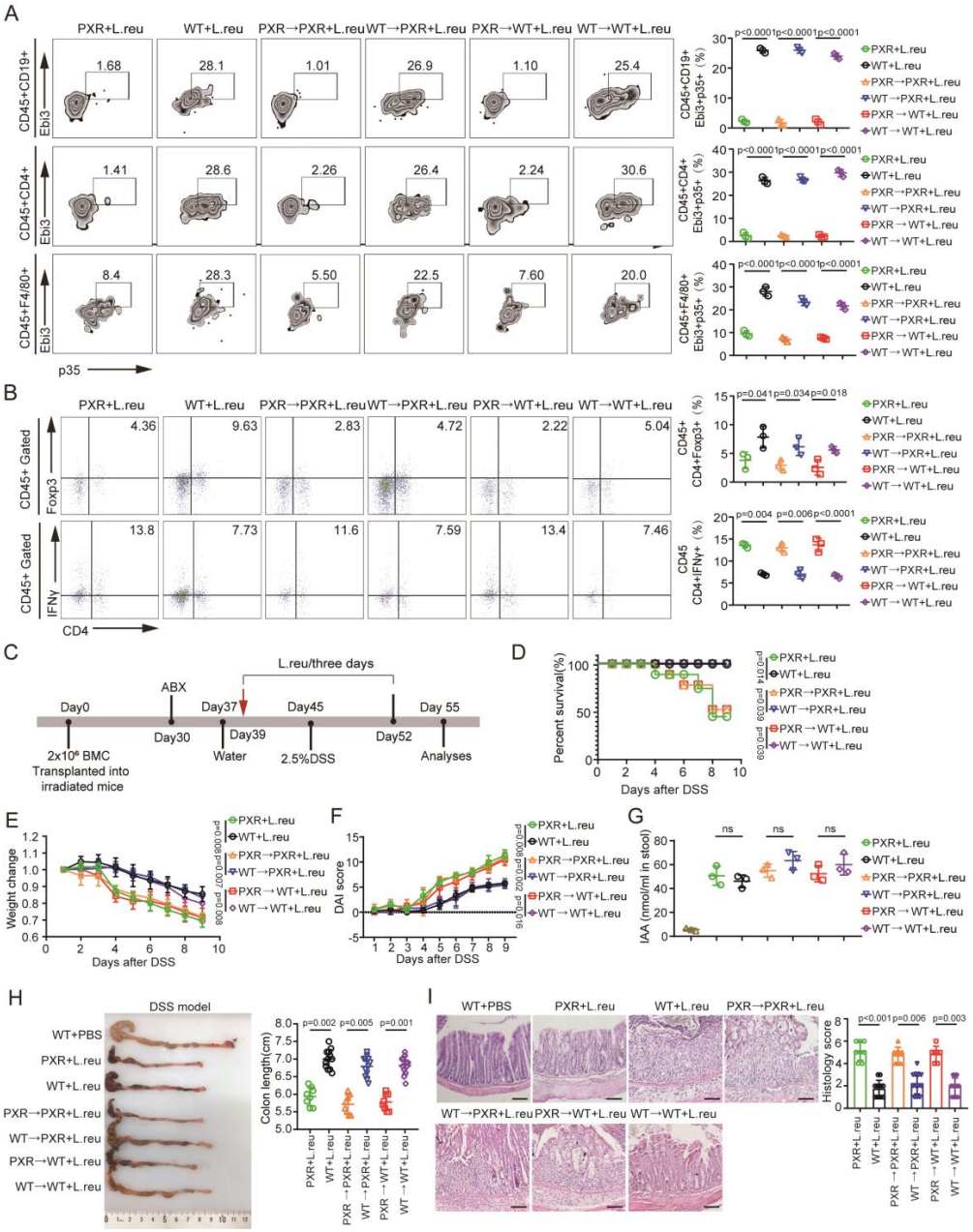
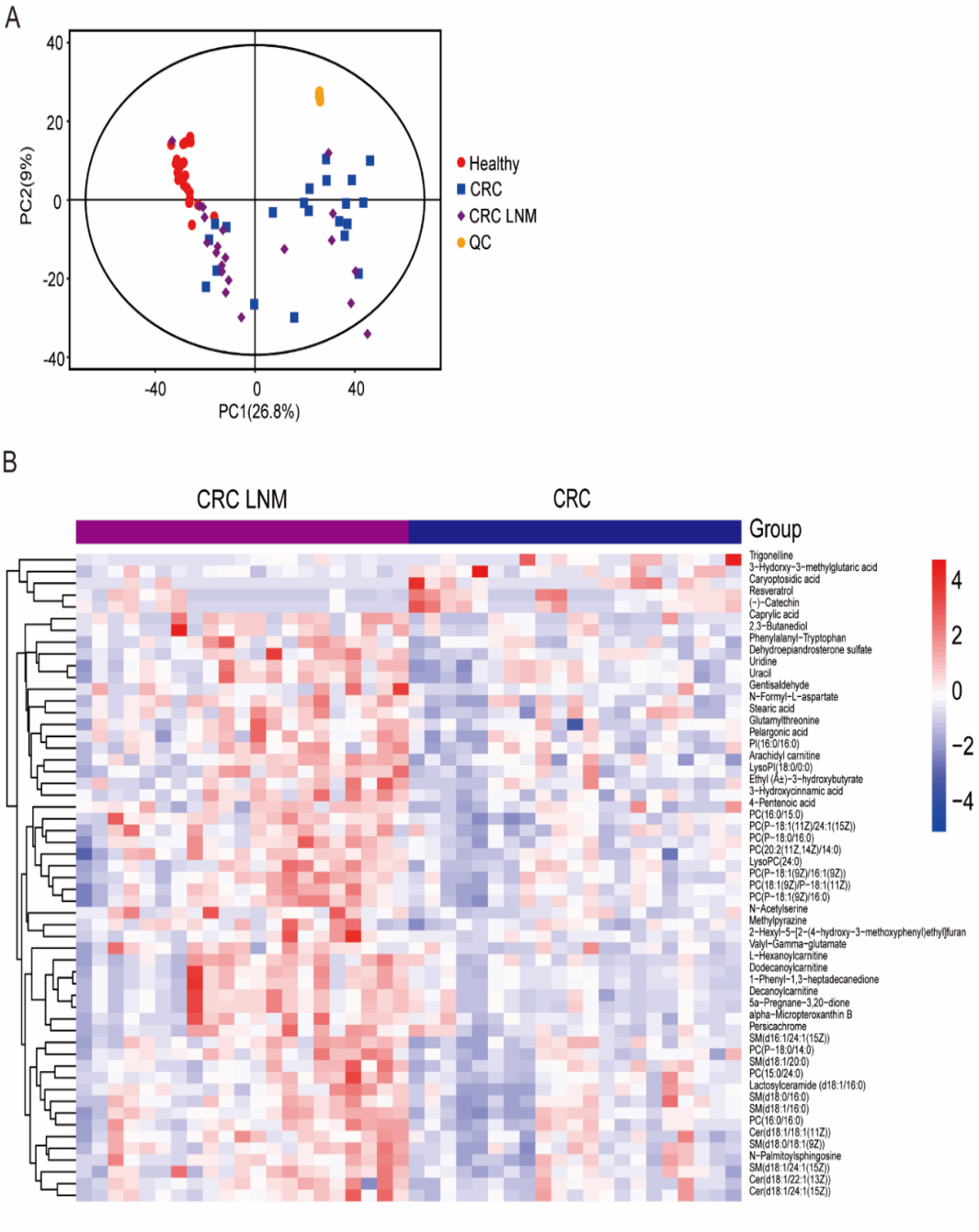


Figure S3. IAA-producing *L. Reu* mediated IL-35^{+} cells depend on PXR in Bone marrow cells.

(A) Flow cytometry analysis of CD4^{+}Ebi3^{+}P35^{+}, CD19^{+}Ebi3^{+}P35^{+} and F4/80^{+}Ebi3^{+}P35^{+} cells in the chimera mice treated with *L. Reu* gavage;

(B) Flow cytometry analysis of CD4^{+}Foxp3^{+} and CD4^{+}IFN γ $^{+}$ cells in the chimera mice treated with *L. Reu* gavage;

(C) Schematic diagram of the experimental design for the DSS-induced colitis model in the chimera mice; Mice received 8 Gy irradiation followed by transplantation of BMCs via tail vein injection. After 4 weeks, mice were treated with ABX and then with *L. reu* gavage (once every three days). After gavage, mice were exposed to 2.5% DSS;
(D)Survival rates in the chimera mice treated with *L. reu* (+L. reu); n=15 mice/group.
(E-F) Body weight changes (E) and disease activity index (DAI) (F) in the chimera mice treated with *L. Reu* gavage after DSS (PXR, n=8; WT, n=12; PXR→PXR, n=8; WT→PXR, n=12; PXR→WT, n=8; WT→WT, n=12);
(G) Concentration of IAA in the stool of the chimera mice treated with *L. Reu* gavage.
H) Representative colon images and statistical analysis of colon length from the indicated groups (PXR, n=8; WT, n=12; PXR→PXR, n=8; WT→PXR, n=12; PXR→WT, n=8; WT→WT, n=12);
(I) Representative H&E staining images and histological analysis of colon tissues from the indicated groups (PXR, n=8; WT, n=12; PXR→PXR, n=8; WT→PXR, n=12; PXR→WT, n=8; WT→WT, n=12).
Error bars indicate mean ± SD (A, B, E-H) or mean ± SEM (I);
Statistic test: Two-way ANOVA test (E-F); Unpaired Student's t test (A, B, G and H); Log-rank (Mantel-Cox) test (D); Ns, no significance.
Data were a representative of three experiments.
L. Reu, *Lactobacillus reuteri.*; DSS, dextran sulfate; PXR, pregnane X receptor; BMC, bone marrow cell.
Chimera mice: PXR→PXR, PXR KO BMCs were transplanted irradiated PXR KO mice; WT→PXR, WT BMCs were transplanted irradiated PXR KO mice; PXR→WT, PXR KO mouse BMCs were transplanted irradiated WT mice; WT→WT, WT mouse BMCs were transplanted irradiated WT mice.


A
PC2(9%)
PC1(26.8%)
Healthy
CRC
CRC LNM
QC
B
CRC LNM
CRC
Group
Trigoneliine
3-Hydoxy-3-methylglutaric acid
Caryoptosidic acid
Resveratrol
(-)-Catechin
Caprylic acid
2,3-Butanediol
Phenylalanyl-Tryptophan
Dehydroepiandrosterone sulfate
Uridine
Uracil
Gentisaldehyde
N-Formyl-L-aspartate
Stearic acid
Glutamylthreonine
Pelargonic acid
PI(16:0/16:0)
Arachidyl carnitine
LysoPI(18:0/0:0)
Ethyl (Az)-3-hydroxybutyrate
3-Hydroxycinnamic acid
4-Pentenoic acid
PC(16:0/15:0)
PC(P-18:1(11Z)/24:1(15Z))
PC(P-18:0/16:0)
PC(20:2(11Z,14Z)/14:0)
LysoPC(24:0)
PC(P-18:1(9Z)/16:1(9Z))
PC(18:1(9Z)/P-18:1(11Z))
PC(P-18:1(9Z)/16:0)
N-Acetylserine
Methylpyrazine
2-Hexyl-5-[2-(4-hydroxy-3-methoxyphenyl)ethyl]furan
Valyl-Gamma-glutamate
L-Hexanoylcarnitine
Dodecanoylcarnitine
1-Phenyl-1,3-heptadecanedione
Decanoylcarnitine
5a-Pregnane-3,20-dione
alpha-Micropteroxanthin B
Persicachrome
SM(d16:1/24:1(15Z))
PC(P-18:0/14:0)
SM(d18:1/20:0)
PC(15:0/24:0)
Lactosylceramide (d18:1/16:0)
SM(d18:0/16:0)
SM(d18:1/16:0)
PC(16:0/16:0)
Cer(d18:1/18:1(11Z))
SM(d18:0/18:1(9Z))
N-Palmitoylsphingosine
SM(d18:1/24:1(15Z))
Cer(d18:1/22:1(13Z))
Cer(d18:1/24:1(15Z))

C


D
CRC LNM
Healthy
Group
4
2
0
−2
−4

Figure S4. L-tryptophan metabolites in the sera of patients with colorectal carcinoma.

(A) PCA score scatter plot of all samples (including QC samples);

(B) Heatmap of differential metabolites between CRC LNM and CRC detected by LC-MS/MS;

(C) Heatmap of differential metabolites patients with CRC and healthy individual detected by LC-MS/MS;

(D) Heatmap of differential metabolites patients with CRC LNM and healthy individual detected by LC-MS/MS;

PCA, principal component analysis; QC, quality control; CRC, colorectal carcinoma; CRC LNM, colorectal carcinoma with lymph node metastatic; LC-MS/MS: liquid chromatography-tandem mass spectrometry;

Supplementary Table S1. Reagents used in this study.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies for flow cytometry analysis		
PerCP/Cy5.5 anti-mouse CD45	Thermo Fisher Scientific	Cat: 103132
APC anti-mouse CD45 antibody	Biolegend	Cat: 103112
Anti-mouse CD45 PE-Cy7	Invitrogen	Cat: 4323753
FITC anti-mouse/human CD11b antibody	Biolegend	Cat: 101206
FITC anti-mouse F4/80 antibody	Biolegend	Cat: 123108
FITC anti-mouse CD19 antibody	Biolegend	Cat: 152404
FITC anti-mouse CD4 antibody	Biolegend	Cat: 100406
APC anti- mouse Foxp3 antibody	Invitrogen	Cat: 2513557
PE anti-mouse IFN-γ antibody	Biolegend	Cat: 505808
PerCP anti-mouse Ebi3 Antibody	R&D systems	Cat: IC18341C
PE anti-mouse/human p35 antibody	R&D systems	Cat: IC2191P
PE anti-mouse IL-10	BioLegend	Cat: 505008
Antibodies for immunofluorescence		
DAPI Fluoromount-G	SouthernBiotech	Cat: 0100-20
Anti-mouse F4/80（C-7）antibody	Santa Cruz	Cat: #L3020
Anti-mouse CD4 antibody	Proteintech	Cat: 67786-1-Ig
Anti-mouse CD19 antibody	Proteintech	Cat: 27949-1-AP
Anti-mouse/human-Ebi3 Antibody	Proteintech	Cat: 12371-1-AP
Alexa Fluor 488 Goat Anti-Mouse IgG(H+L) Antibody	Proteintech	Cat: SA00003-1
Alexa Fluor 594 Goat Anti-Mouse IgG(H+L) Antibody	Proteintech	Cat: SA00013-3
Alexa Fluor 488 Goat Anti-Rabbit IgG(H+L) Antibody	Proteintech	Cat: SA00003-2
TRITC-Rabbit Anti-Goat IgG (H+L) Antibody	Proteintech	Cat: SA00007-4
TRITC-Goat Anti-Rat IgG (H+L) Antibody	Proteintech	Cat: SA00007-7
Reagents and Chemicals		

Dextran sulfate sodium salt (DSS)	MP Biomedicals	Cat: 160110
Mouse IAA ELISA KIT	Mlbio	Cat: Ml401842
3-Indoleacetic acid	MCE	Cat: HY-18569
Recombinant IL-35	PEPROTECH	Cat: 200-37
Monocolonal Anti-Ebi3(IL-35) blocking Antibody, clone V1.4C4.22	Sigma	Cat: 3144423
Lipopolysaccharides (LPS)	MCE	Cat: HY-D1056
FBS	Gibco	Cat:10099141
Collagenase IV	Sigma-Aldrich	Cat: C5138
Dnase I	Solarbio	Cat: D8071
Percoll	Solarbio	Cat: P8370
EDTA	Sigma-Aldrich	Cat: 798681
DMEM/F12	STEMCELL	Cat: 36254
Mouse IAA ELISA KIT	Mlbio	Cat: Ml401842
Cell stimulation cocktail	eBioscience	Cat: 00-4975-03
Permeabilization Buffer	eBioscience	Cat: 00-8333-56
Azoxymethane (AOM)	Sigma-Aldrich	Cat: A5486
Ampicillin	Sigma-Aldrich	Cat: BP021
Vancomycine	Sigma-Aldrich	Cat: V2002
Neomycin sulfate	Sigma-Aldrich	Cat: N6386
Metronidazole	Sigma-Aldrich	Cat: M3761
MRS broth	Basebio	Cat: BS1138

Supplementary Table S2. Patient information (all patients without treatment)**.**

Information of patient with colorectal carcinoma (1)

Number	Age	Tumor site	Type of pathology	Pathologic stage
Patient 1	Adult	Colon	Adenocarcinoma	T2N0Mx DukasA
Patient 2	Adult	Colon	Adenocarcinoma	T3N0Mx DukasB
Patient 3	Adult	Rectum	Adenocarcinoma	T2N0Mx DukasA
Patient 4	Adult	Rectum	Adenocarcinoma	T3N0Mx DukasB
Patient 5	Adult	Rectum	Adenocarcinoma	T2N0Mx DukasA
Patient 6	Adult	Rectum	Adenocarcinoma	T3N0Mx DukasB
Patient 7	Adult	Rectum	Adenocarcinoma	T3N0Mx DukasB
Patient 8	Adult	Colon	Adenocarcinoma	T4N0Mx DukasB
Patient 9	Adult	Colon	Adenocarcinoma	T3N0Mx DukasB
Patient 10	Adult	Rectum	Adenocarcinoma	T3N0Mx DukasB
Patient 11	Adult	Colon	Adenocarcinoma	T3N0Mx DukasB
Patient 12	Adult	Rectum	Adenocarcinoma	T1N0Mx DukasA
Patient 13	Adult	Colon	Adenocarcinoma	T1N0Mx DukasA
Patient 14	Adult	Rectum	Adenocarcinoma	T2N0Mx DukasA
Patient 15	Adult	Rectum	Adenocarcinoma	T3N0Mx DukasB
Patien 16	Adult	Colorectal	Adenocarcinoma	T3N0Mx DukasB
Patient 17	Adult	Rectum	Adenocarcinoma	T1N0Mx DukasA
Patient 18	Adult	Colon	Adenocarcinoma	T1N0Mx DukasA
Patient 19	Adult	Rectum	Adenocarcinoma	T2N0Mx DukasA
Patient 20	Adult	Colorectal	Adenocarcinoma	T2N0Mx DukasA
Patient 21	Adult	Colorectal	Adenocarcinoma	T2N1Mx DukasA

Information of patient with colorectal carcinoma and lymph node metastasis (2)

Number	Age	Tumor site	Type of pathology	Pathologic stage
Patient 1	Adult	Colorectal	Adenocarcinoma	T4N1Mx DukasC
Patient 2	Adult	Colon	Adenocarcinoma	T3N1Mx DukasC
Patient 3	Adult	Rectum	Adenocarcinoma	T3N2Mx DukasC
Patient 4	Adult	Colon	Adenocarcinoma	T4N1Mx DukasC
Patient 5	Adult	Colon	Adenocarcinoma	T4N1Mx DukasC
Patient 6	Adult	Colon	Adenocarcinoma	T4N2Mx DukasC
Patient 7	Adult	Rectum	Adenocarcinoma	T3N2Mx DukasC
Patient 8	Adult	Rectum	Adenocarcinoma	T3N1Mx DukasC
Patient 9	Adult	Rectum	Adenocarcinoma	T3N2Mx DukasC
Patient 10	Adult	Rectum	Adenocarcinoma	T3N1Mx DukasC
Patient 11	Adult	Rectum	Adenocarcinoma	T2N1Mx DukasC
Patient 12	Adult	Colorectal	Adenocarcinoma	T3N1Mx DukasC
Patient 13	Adult	Colorectal	Adenocarcinoma	T3N1Mx DukasC
Patient 14	Adult	Rectum	Adenocarcinoma	T3N1Mx DukasC
Patient 15	Adult	Rectum	Adenocarcinoma	T4N1Mx DukasC
Patient 16	Adult	Colon	Adenocarcinoma	T3N1Mx DukasC

Patient 17	Adult	Colorectal	Adenocarcinoma	T3N2Mx DukasC
Patient 18	Adult	Colon	Adenocarcinoma	T4N1Mx DukasC
Patient 19	Adult	Rectum	Adenocarcinoma	T3N1Mx DukasC
Patient 20	Adult	Rectum	Adenocarcinoma	T4N2Mx DukasC
Patient 21	Adult	Rectum	Adenocarcinoma	T3N1Mx DukasC
Patient 22	Adult	Rectum	Adenocarcinoma	T3N2Mx DukasC