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Diagnostic role of NPY methylation in patients with colorectal cancer

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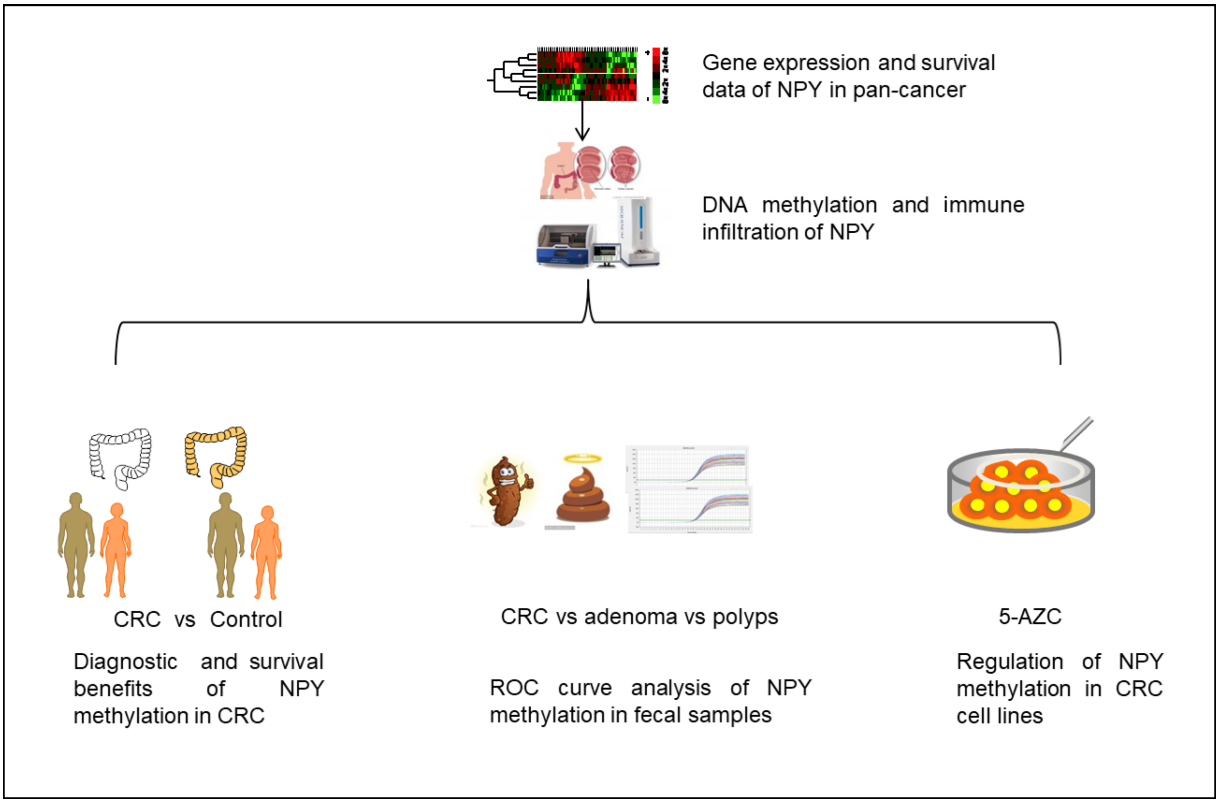
Graphical abstract



Diagnostic role of neuropeptide Y (NPY) methylation in colorectal cancer (CRC).

Public summary

■ The methylation level of europeptide Y (NPY) was increased in most carcinomas, including colorectal cancer (CRC).

■ Fecal NPY methylation appears to be associated with good diagnostic ability in patients with CRC.

■ In vivo experiments demonstrated that 5-AZC downregulated NPY methylation and restored its mRNA level.

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Abstract: *Objectives*: A growing number of studies have shown that methylation biomarkers play an important role in oncogenesis. This study aimed to explore the diagnostic role of neuropeptide Y (NPY) methylation in colorectal cancer (CRC). *Methods*: mRNA and protein expression, methylation, survival benefits, and immune cell infiltration were analyzed using bioinformatics tools across all tumors from The Cancer Genome Atlas. NPY methylation in CRC was further validated in CRC tissues, fecal samples, and cell lines. Analyses of NPY methylation were performed using Sequenome EpiTYPER and quantitative PCR. Retrieval of NPY expression in cell lines was tested using real-time PCR and western blotting. *Results*: Bioinformatic analysis showed that the methylation level of NPY increased in most carcinomas (P<0.05). Moreover, statistical correlations were observed between NPY transcriptional expression and $CD4^+$ T cells, macrophages, and dendritic cells in colon cancer (P<0.05). Similar results were obtained for $CD4^+$ T cells, neutrophils, and NPY in rectal cancer (P<0.05). Our results showed that NPY was hypermethylated in CRC tissues and fecal exfoliated cells (P<0.05). Fecal NPY methylation was observed in 82.5% sensitive for primary tumors, 46.3% for intestinal polyps (including adenomatous, serrated, and inflammatory polyps), and 23.4% of healthy controls. Overall, fecal NPY methylation was 76.6% specific. For cell lines, in vivo experiments demonstrated that 5-aza-2′-deoxycytidine downregulated the methylation of NPY and restored its mRNA level (P<0.05). *Conclusions*: This study indicates that NPY is hypermethylated in CRC, and that NPY methylation in fecal DNA is a potential noninvasive diagnostic biomarker for Chinese patients with CRC.

Keywords: colorectal cancer; neuropeptide Y; methylation; diagnostic; biomarker

CLC number: R735.3 **Document code:** A

Abbreviation

COAD: colon adenocarcinoma; READ: rectum adenocarcinoma; BRCA: Breast invasive carcinoma; ESCA: esophageal carcinoma; GBM: glioblastoma multiforme; KIRC: kidney renal clear cell carcinoma; LGG: brain lower grade glioma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; SCLC: small cell lung cancer; OV: ovarian serious cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PRAD: prostate adenocarcinoma; STAD: stomach adenocarcinoma; THCA: thyroid carcinoma; UCEC: uterine corpus endometrial carcinoma; CRC: colorectal cancer; TGCT: testicular germ cell tumors; UCS: uterine carcinosarcoma; LAML: acute myeloid leukemia; BLCA: bladder urothelial carcinoma; CHOL: cholangio carcinoma; UVM: uveal melanoma; SKCM: skin cutaneous melanoma.

1 Introduction

The incidence and mortality of colorectal cancer (CRC) in China have increased annually, and most patients have been diagnosed at a median or advanced stage [1].CRC develops in a multi-step process, from precancerous lesions to CRC. Early screening of the general at-risk population can significantly reduce the incidence of CRC by early prevention and intervention. Endoscopy, blood tests, and stool detection are common screening methods for CRC [2]. Methylated cell-free DNA has been developed as a biomarker in recent years, owing to its noninvasive detection and cost effectiveness[3].

Epigenetics is a promising mechanism that initiates and regulates genetic expression without affecting the genome sequence. DNA methylation, m6A modification, and histone acetylation are the classic means of epigenetic regulation [4]. Collectively, these findings illustrate the potential therapeutic benefits of epigenetic methylation regulation for cancer treatment. The methylation of cytosine at CpG dinucleotides is associated with transcriptional activation or repression[5]. Therefore, it is important to explore epigenetic functions to improve the diagnosis and treatment of cancer based on their biological significance.

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JUSTC, **2022**, 52(6): 2

Neuropeptide Y (NPY) is a sympathetic neurotransmitter composed of 36 amino acids and is mainly distributed in brain tissues (medullary brainstem and cerebral cortex). It is released by the peripheral sympathetic neurons under chronic stress and hypoxia. This peptide exhibits pleiotropic effects via Y1-Y5 receptors[6]. The physiological effects of NPY include ileal immobilization, decreased gastric and pancreatic secretion, and increased absorption of intestinal water and electrolytes[7]. An increased density of NPY neurons has been reported in the enteric nerve plexus of patients undergoing ileal surgery for Crohn's disease[8]. In the cardiovascular system, NPY is associated with the physiological processes of vasoconstriction, heart remodeling, and angiogenesis, leading to pathological processes such as hypertension, atherosclerosis, myocardial infarction, arrhythmia, and heart failure[9]. NPY has been used as an antidepressant and anxiolytic therapy[10]. In addition, increasing evidence has demonstrated that NPY is involved in breast cancer[7], liver cancer[11], prostate cancer[12], pancreatic cancer[13], bladder cancer[14], endometrial cancer[15], esophageal squamous cell carcinoma[16], head and neck tumors[17], venturia sarcoma, and neuroblastoma[18]. The NPY gene was found to be methylated in CRC[19–20] with potential predictive value[21,22]. However, there have been no reports on the use of fecal NPY detection for the early diagnosis of CRC in a Chinese cohort. In summary, this study aimed to explore the diagnostic role of NPY methylation in Chinese patients with CRC.

2 Materials and methods

2.1 Gene expression and survival analysis of NPY

NPY expression in tumor cell lines was analyzed using the Cancer Cell Line Encyclopedia database①. GEPIA2② was used to compare the differential expression and survival benefits in terms of overall survival (OS) and disease-free survival (DFS) between tumor and adjacent normal tissues for NPY across all tumors. The protein and methylation expression of NPY were analyzed using the clinical proteomic tumor analysis consortium and methylation part of UALCAN③, respectively.

2.2 Immune infiltration analysis of NPY

We used TIMER④ to conduct a comprehensive systematic analysis of immune infiltrates across all cancer types. The relationship between NPY and immune cell infiltration was estimated by deconvolution. The results are plotted as scatter plots. Pearson's correlation analysis was used to evaluate the relationship between NPY expression and immune cells.

2.3 Clinical colorectal cancer samples and cell lines

From January 2013 to December 2017, 42 tumors and 40 corresponding normal paraffin-embedded tissue samples were collected from the Sun Yat-sen University Cancer Center, Guangzhou, China. A total of 208 fecal exfoliated cell samples from newly diagnosed or healthy patients were obtained from the Department of Colorectal Surgery, Departments of Medical Oncology and Screening Center for Cancer Prevention of Sun Yat-sen University Cancer Center between September 2016 and December 2018. Patients with no detailed follow-up data were excluded from the study. Human cancer cell lines (HCT116 and SW480) were purchased from the cell bank of the Chinese Academy of Sciences in Shanghai. Incomplete Leibovitz L-15 was supplied by SW480 (KeyGen, Nanjing, China). HCT116 cells were cultured in McCoy's 5A medium (HyClone, Logan, UT, USA). All cells were incubated with 10% serum (Gibco, Waltham, MA, USA) at 37 °C and 5% CO_2.

2.4 Sequenom EpiTYPER analysis

The target genomic DNA was extracted and bisulfited using a Qiagen Nucleic Acid Isolation Kit (Hilden, Germany) and a Zymo Research Conversion Kit (Irvine, CA, USA). NPY methylation was evaluated quantitatively using the Agena Sequenom platform (San Diego, CA, USA). The forward primer for NPY was 5′-aggaagagagAAGTTTTGTCGCGATTCGTT-3′, and the reverse primer was 5′-cagtaatacgactcactatagggagaaggctCTCCCACCCCTAAACAAAC-3′. A total of 15 CpG sites were examined. The methylation results were analyzed and extracted using EpiTYPER (Agena).

2.5 Methylation analysis of NPY from fecal exfoliated cells

Serum was collected and centrifuged at 20,000 g within 5 h of sampling. The samples were stored at −80 °C until analysis. DNA was extracted using a stool nucleic acid kit (Qiagen). The entire DNA was bisulfite-converted using the EZ DNA Methylation Lightning Kit (Zymo) following the manufacturer's instructions. In total, fecal DNA was mixed with a primer/probe mix (BioRad, Hercules, CA, USA) in a volume of 20 μL. The reaction conditions were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 62 °C for 30 s, and finally 98 °C for 10 s. The results were analyzed by fluorescence quantitative PCR (ABI).

2.6 Demethylation of 5-aza-2′-deoxycytidine

The epigenetic function of NPY methylation was explored in HCT116 and SW480 cells. In brief, 1×10^5 cells were co-cultured with 5-aza-2′-deoxycytidine (5-AZC; 0 μmol/L vs. 5 μmol/L vs. 10 μmol/L) with fresh medium changed every 24 h for 3 d.

2.7 RNA extraction and quantitative real-time PCR

Total RNA from cell lines was extracted using TRIzol (Invitrogen, USA) and tested by CFX96 RT-PCR (Bio-Rad, USA). GAPDH was used as the internal reference. The NPY primers were proven to be effective: (sense 5′-TAGGTAACAAGCGACTGGGG-3' vs. antisense 5′-CAGCGCCGAGTAGTATCTGG-3'). All experiments were performed in triplicate.

2.8 Western blotting

The cells were collected and lysed in a mixture of RIPA buf-

① https://portals.broadinstitute.org/ccle/

② http://gepia2.cancer-pku.cn/#analysis

③ http://ualcan.path.uab.edu/analysis-prot.html

④ http://timer.cistrome.org/

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JUSTC, **2022**, 52(6): 2

fer and protease inhibitors (Beyotime, Shanghai, China). The protein concentration was determined using a BCA protein assay kit (Beyotime). The target protein was then separated and electroblotted onto a polyvinylidene fluoride ultrafiltration membrane (Merck, Burlington, MA, USA). The incubation concentrations of NPY and β-actin antibodies were 1∶200 (Abcam, Cambridge, UK) and 1∶1000 (Santa Cruz Biotechnology, Dallas, TX, USA), respectively. The results were compared with those of the naked eye using a Bio-Rad imaging system (Bio-Rad, USA).

2.9 Statistical analysis

SPSS software (version 26.0) was used for the statistical analysis. The Chi-square test was used to calculate the relationship between methylation biomarkers and clinicopathological characteristics. The *t*-test or one-way ANOVA were used to compare the differences in the overall average number of methylations between the groups. The diagnostic efficacy of the methylation biomarkers was evaluated using ROC curves, and the Kaplan-Meier method was used to analyze the relationship between NPY and patient survival prognosis. Statistical significance was set at $P<0.05$.

3 Results

3.1 Gene expression and survival data of NPY in pan-cancer

As depicted in Fig. 1a and 1b, the transcriptional level of NPY was explored in tumor cell lines and tissues. Most tumors showed decreased NPY expression, including CRC. Consistently, NPY protein expression was reduced in lung adenocarcinoma, ovarian cancer, uterine corpus endometrial carcinoma, and clear cell renal cell carcinoma (Fig. 1c, $P<0.05$). Pooling analysis of NPY transcriptional expression

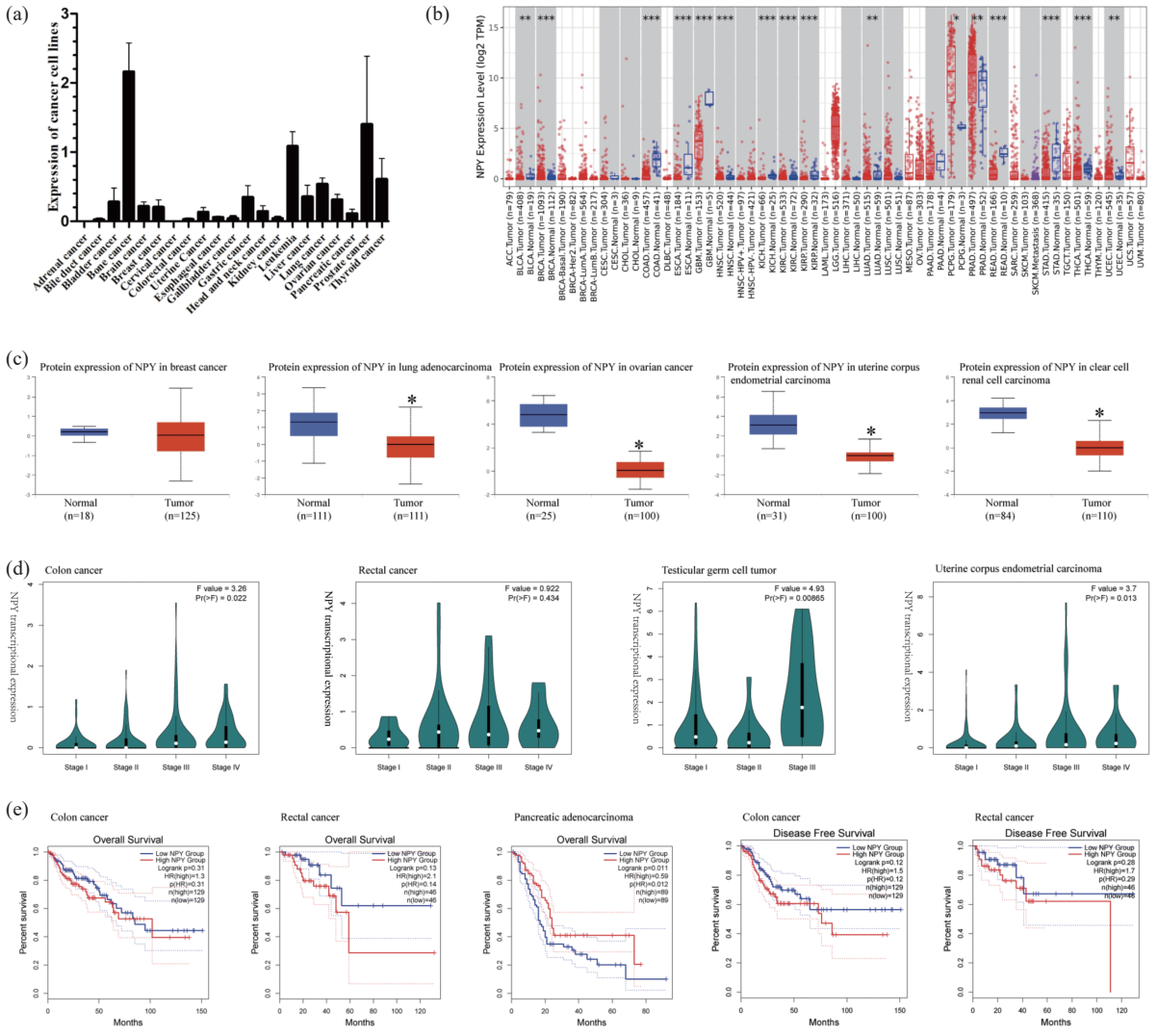

Fig. 1. (a) The expression status of NPY gene in different cancer cell lines through the database of Cancer Cell Line Encyclopedia. (b) The NPY expression was explored in tumor tissues from GEPIA (**$P<0.01$, ***$P<0.001$). (c) Based on the CPTAC dataset, the expression level of NPY total protein was analyzed between normal tissue and primary tissue of LUAD, BRCA, OV, KIRC, and UCEC (*$P<0.05$). (d) Based on the TCGA data, the expression level of NPY was analyzed by the pathological stages (stage I, stage II, stage III, and stage IV) of COAD, TGCT, and UCEC ($P<0.05$). (e) Correlation between NPY and overall survival / disease-free survival prognosis of cancers in TCGA.

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JUSTC, **2022**, 52(6): 2

and pathological stage revealed a significant correlation with colon cancer, testicular germ cell tumor, and uterine corpus endometrial carcinoma (Fig. 1d, P<0.05). Fig. 1e demonstrates that low NPY was linked to poor OS in patients with pancreatic adenocarcinoma (P<0.05). However, no significant correlation was found between NPY and DFS across all listed tumors (Fig. 1e, P>0.05).

3.2 DNA methylation and immune infiltration of NPY

Compared with normal tissues, the methylation level of NPY was increased in COAD, STAD, ESCA, LIHC, LUAD, LUSC, BRCA, HNSC, CHOL, PAAD, GBM, KIRP, PCPG, BLCA, KIRC, THYM, THCA, PRAD, and CESC (Fig. 2, P<0.05).

Statistically positive correlations between $CD4^+$ cells, macrophages, dendritic cells, and NPY transcription were observed in COAD (Fig. 3, P<0.05). Similar results were found for $CD4^+$ cells, neutrophils, and NPY in the READ group (Fig. 3, P<0.05).

3.3 Methylation role of NPY in colorectal cancer tissues

We investigated 42 CRC tissues to validate the epigenetic function of NPY methylation in specific tumors. Table 1 summarizes the clinicopathological characteristics of the patients. Compared to adjacent normal tissues, NPY was significantly hypermethylated in tumors (Fig. 4a, P<0.05). Moreover, all target CpG sites showed the same statistical difference (Fig. 4b, P<0.05). In addition, no obvious correlation was found between NPY methylation and sex, fecal immunochemical test results, tumor location, tumor differentiation, CEA, TNM stage, etc. (Table 1, all P>0.05). To assess the prognostic value of NPY methylation, patients were divided into two cohorts. The diagnostic sensitivity and specificity of NPY methylation for CRC were 78.5% and 87.5%, respectively (Fig. 4c). The area under the curve (AUC) was 0.83. The median follow-up was 878 d. According to the Kaplan-Meier analysis, the NPY methylation level was not an independent predictor of OS, but showed a correlative trend in patients with CRC (P=0.32, Fig. 4d).

3.4 ROC curve analysis of NPY methylation in fecal samples

Clinicopathological characteristics of the fecal samples are presented in Table 2. As depicted in Fig. 5 and Table 3, the sensitivity, specificity, and AUC values of the fecal NPY methylation test (Fig. 5a) for CRC diagnosis were 82.5%, 76.6%, and 0.79, respectively. The diagnostic sensitivity, specificity, and AUC values for adenoma+polyps were 46.3%, 76.6%, and 0.61, respectively. The diagnostic sensitivity, specificity, and AUC values for CRC+adenoma+polyps were 73.3%, 76.6%, and 0.74, respectively.

The sensitivity, specificity, and AUC values of the fecal

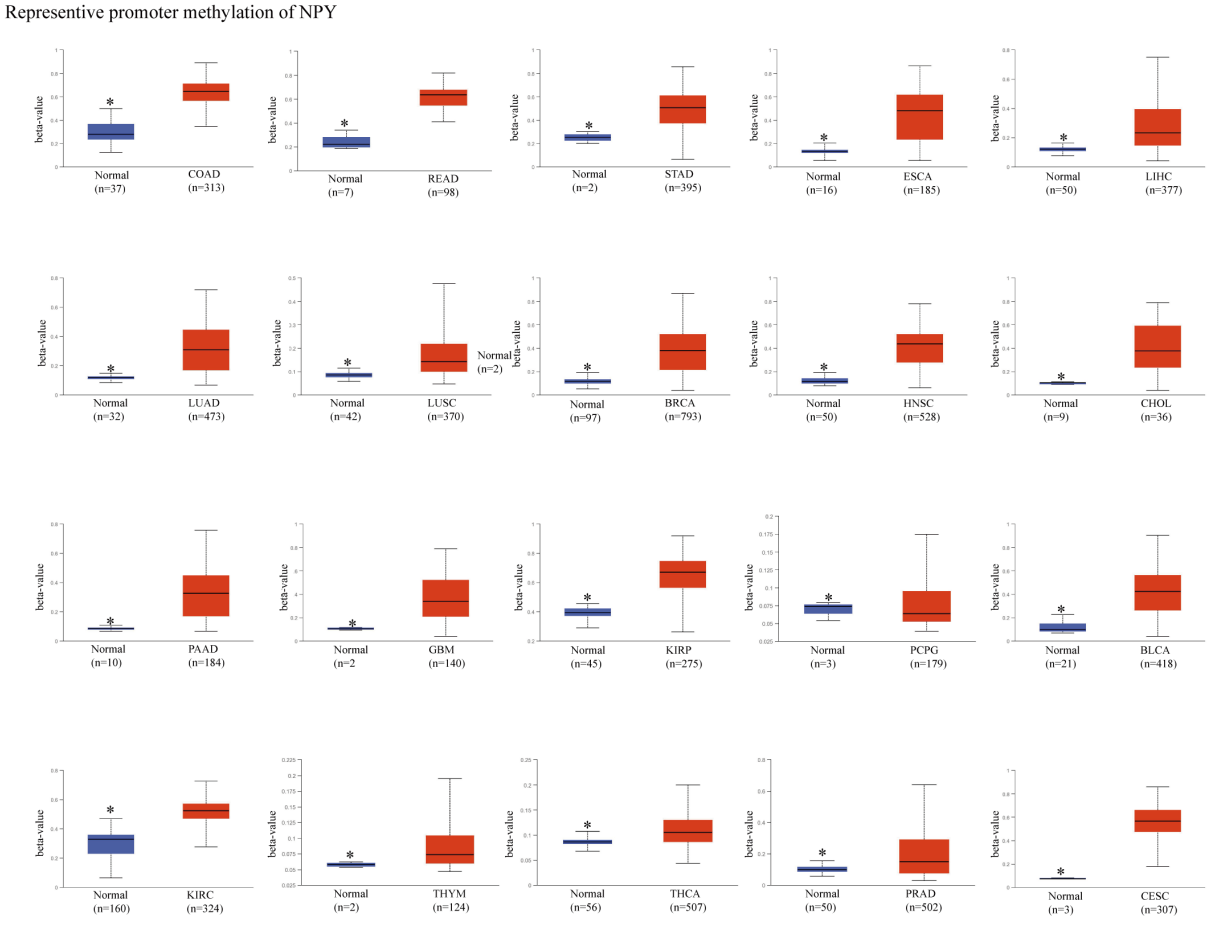

Fig. 2. Methylation feature of NPY in different tumors of TCGA (*P<0.05).

DOI: 10.52396/JUSTC-2022-0027
JUSTC, **2022**, 52(6): 2

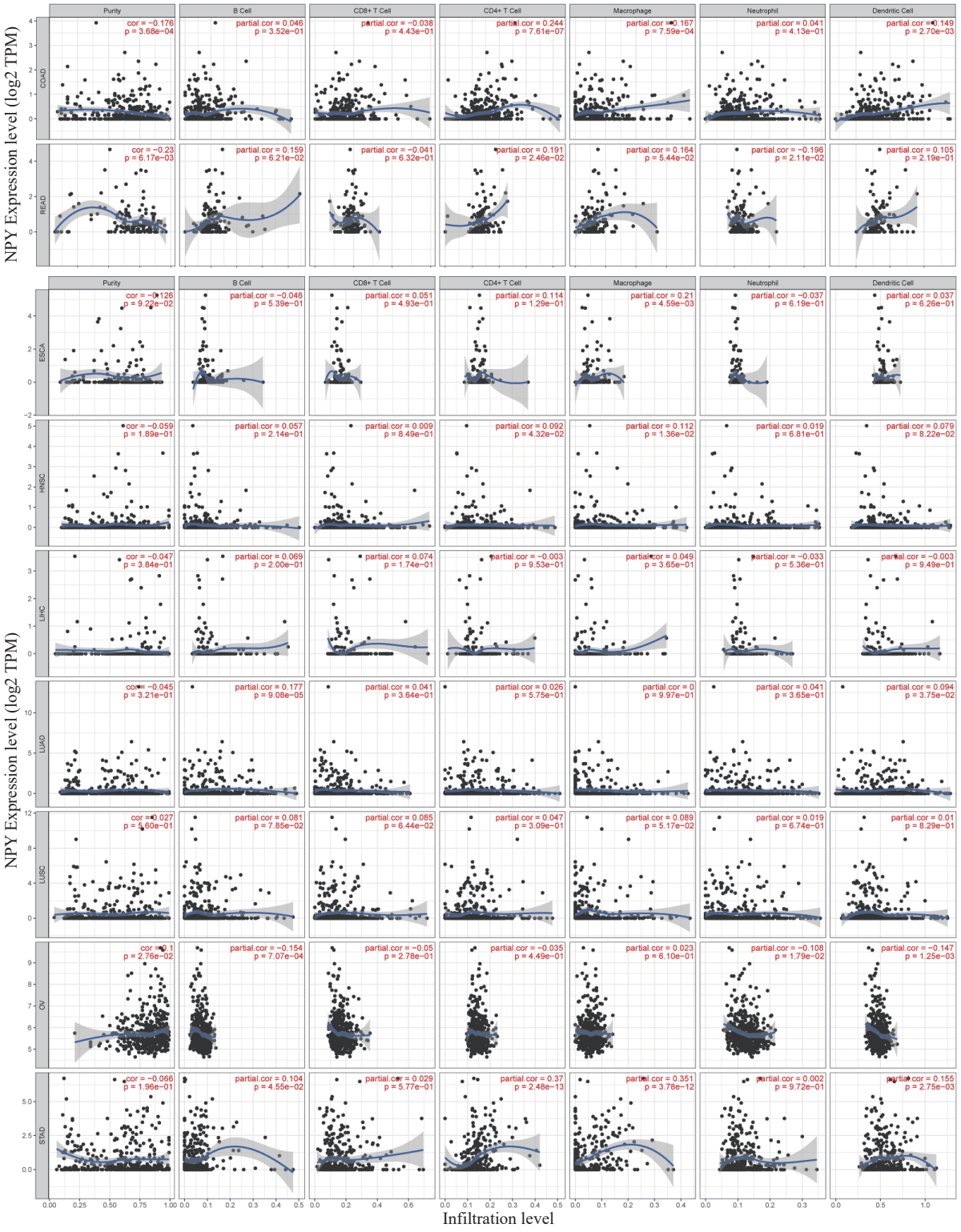

Fig. 3. Representive correlation analysis between NPY and immune infiltration cells.

NPY methylation and fecal immunochemical tests (Fig. 5b) for CRC diagnosis were 98.3%, 76.6%, and 0.87, respectively. Meanwhile, the diagnostic sensitivity, specificity, and AUC values for adenoma+polyps were 48.8%, 76.6%, and 0.62, respectively. Finally, the diagnostic values of sensitivity, specificity, and AUC for CRC+adenoma+polyps were 85.7%, 76.6%, and 0.81, respectively.

3.5 Regulation of NPY methylation in colorectal cancer cell lines

The function of NPY methylation was further explored in CRC cell lines (HCT116 and SW480). All cells were treated with various concentrations of 5-AZC for 72 h (0 μmol/L vs. 5 μmol/L vs. 10 μmol/L). NPY methylation gradually reduced after 3 d (Fig. 6a, P<0.05). Meanwhile, mRNA levels

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JUSTC, **2022**, 52(6): 2

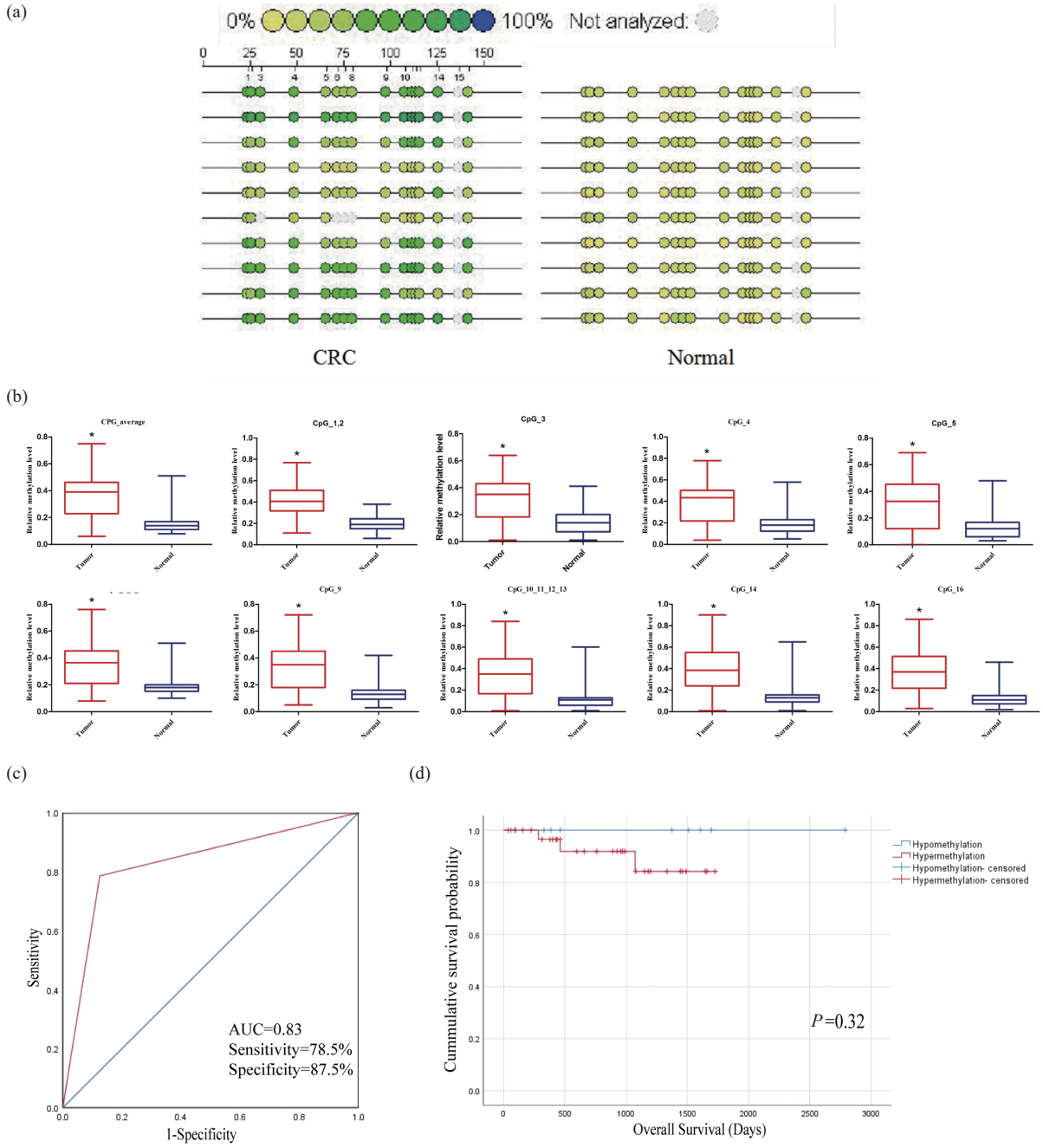

Fig. 4. Sequenom EpiTYPER system analysis of the average methylation ratio of NPY in CRC tissues. (a) The methylation level of NPY was significantly up-regulated in colorectal cancer compared to normal tissues (P<0.05). (b) All subgroup target CpG sites showed the same statistical differences (*P<0.0001). (c) ROC analysis of NPY methylation in CRC tissues: the AUC, sensitivity and specificity of NPY methylation were 0.83, 78.5%, 87.5%, respectively. (d) Kaplan-Meier survival analysis of NPY methylation for overall survival in colorectal cancer (P=0.32).

were increased (Fig. 6b, P<0.05). However, the protein expression was not consistently enhanced (Fig. 6d, P>0.05). Existing data suggest that NPY methylation may be due to the dysregulation of NPY transcriptional expression in CRC.

4 Discussion

Recently, methylation biomarkers have shown outstanding prospects in cancer diagnosis [3]. The present study found: (i) Bioinformatics analysis showed that NPY was dysregulated and linked to OS in some tumors. Compared with normal tissues, NPY is hypermethylated in carcinomas. (ii) A statistical correlation was found between NPY and $CD4^+$ T cells, macrophages, and dendritic cells in colon cancer. Similar results were found for $CD4^+$ T cells, neutrophils, and NPY in patients with rectal cancer. (iii) NPY is hypermethylated in CRC tissues. Fecal NPY methylation was sensitive in 82.5% of primary tumors, 46.3% of intestinal polyps (adenomatous, serrated, and inflammatory polyps), and 23.4% of healthy controls. Fecal NPY methylation was 76.6% specific. (iv) In vivo experiments demonstrated that 5-AZC downregulated NPY methylation and restored its mRNA level.

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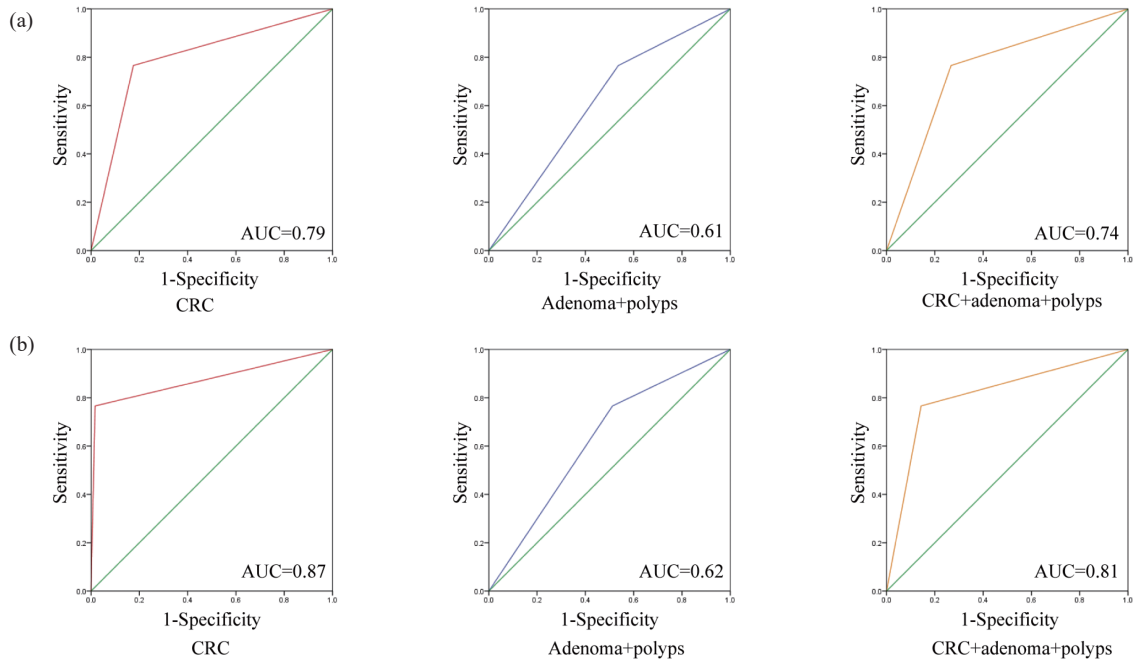

Fig. 5. ROC curve analysis of NPY methylation in fecal samples. (a) The sensitivity, specificity, and AUC value of fecal NPY methylation test for the diagnosis of colorectal cancer were 82.5%, 76.6%, and 0.79, respectively. (b) The sensitivity, specificity, and AUC value of fecal NPY methylation test and fecal immunochemical test for the diagnosis of colorectal cancer were 98.3%, 76.6%, and 0.87, respectively.

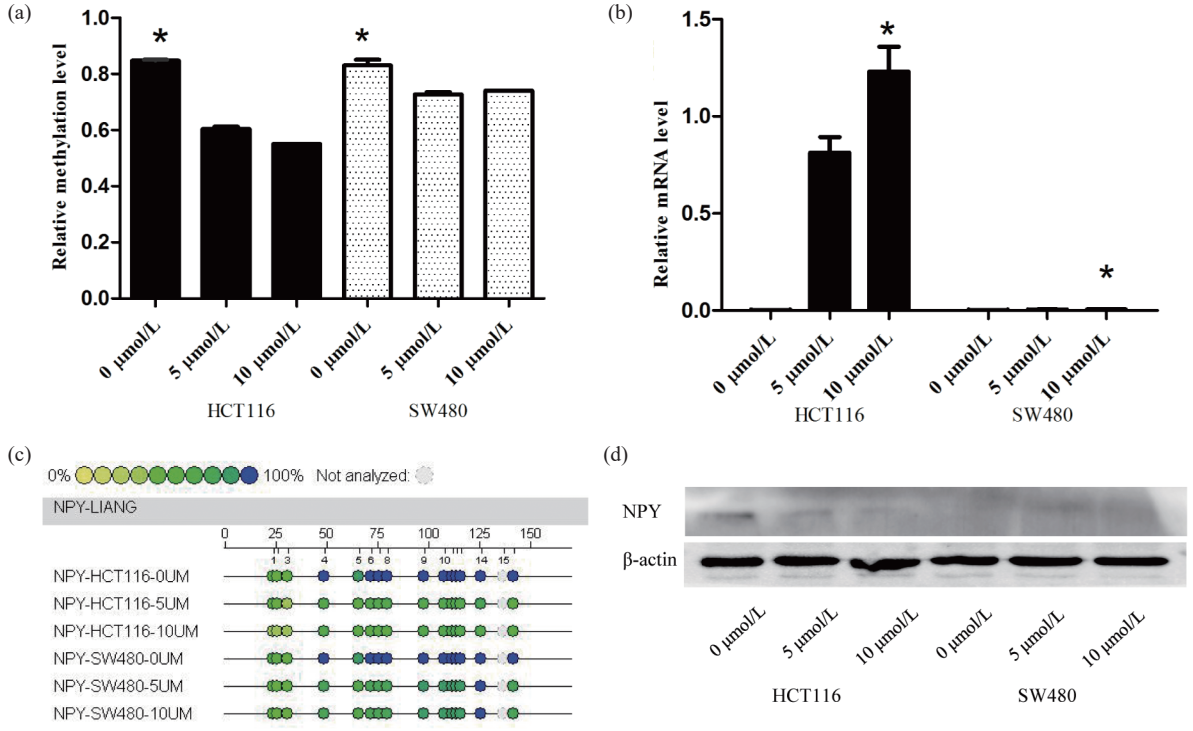

Fig. 6. (a, c) Sequenom EpiTYPER system analysis of the average methylation ratio of NPY in CRC cell lines treated with 5-Aza-2'-deoxycytidine (0 μmol/L vs 5 μmol/L vs 10 μmol/L) (**P*<0.05). (b) SYBR analysis of the relative mRNA level of NPY in CRC cell lines treated with 5-Aza-2'-deoxycytidine (0 μmol/L vs 5 μmol/L vs 10 μmol/L) (**P*<0.05). (d) Western blot analysis of the protein level of NPY in CRC cell lines treated with 5-Aza-2'-deoxycytidine (0 μmol/L vs 5 μmol/L vs 10 μmol/L) (*P*<0.05).

Previous studies have demonstrated NPY polypeptides and their receptors have some potential for tumor-targeted therapy; however, the molecular mechanism for their effects remain obscure. Increased NPY expression is associated with

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JUSTC, **2022**, 52(6): 2

Table 1. Correlations between the general clinicopathological characteristics and NPY methylation in patients with colorectal cancer.

Variants	NPY		*P*
	Positive	Negative	
Gender			
Male	49(79%)	13(21%)	0.3
Female	50(86.2%)	8(13.8%)	
Fecal immunochemical test			
Positive	97(83.6%)	19(16.4%)	0.28
Negative	2(50.0%)	2(50.0%)	
Tumor location			
Ascending	15(68.2%)	7(31.8%)	0.05
Transverse	5(62.5%)	3(37.5%)	
Descending	11(78.6%)	3(21.4%)	
Sigmoid	25(83.3%)	5(16.7%)	
Rectum	43(93.5%)	3(6.5%)	
Tumor differentiation			
High	3(75.0%)	1(25.0%)	0.87
Moderate	75(84.3%)	14(15.7%)	
Low	14(82.4%)	3(17.6%)	
NG	10(8.3%)		
TNM			
Carcinoma in situ+ Ⅰ + Ⅱ	55(83.3%)	11(16.7%)	0.79
Ⅲ+Ⅳ	44(81.5%)	10(18.5%)	
T			
1+2	29(82.9%)	6(17.1%)	0.94
3+4	70(82.4%)	15(17.6%)	
N			
Positive	42(82.4%)	9(17.6%)	0.97
Negative	57(82.6%)	12(17.4%)	
M			
Positive	7(77.8%)	2(22.2%)	0.69
Negative	92(82.9%)	19(17.1%)	
CEA(ng/ml)			
≥5	37(78.7%)	10(21.3%)	0.38
0−4.9	62(84.9%)	11(15.1%)	
CA199(U/ml)			
>37	17(73.9%)	6(26.1%)	0.23
0−37	81(84.4%)	15(15.6%)	
NG	1(0.8%)		

NG: not given

tumor cell growth, metastasis, angiogenesis, hypoxic tumor microenvironment, drug tolerance, and survival prognosis in neuroblastoma and Ewing's sarcomas [23]. The continuous activation of NPY/Y2R in neuroblastoma is associated with tumor cell growth through the p44/42-MAPK pathway, and apoptosis or drug resistance via the BCL-2 pathway [24,25]. Plasma NPY expression was elevated in 232 patients with Ewing's sarcoma, but there was no significant correlation with patient survival[26]. Spontaneous activation of NPY1R/5R receptors can lead to the death of Ewing's cells. The hypoxic microenvironment upregulates NPY and Y5R expression via Y2R[26]. Combined with IRF5, TNFRSF10C, and HOXA9, NPY is a liver cancer-specific hypermethylated tumor suppressor gene with diminished gene expression[11]. NPY antagonists reduce the proliferation and progression of prostate cancer cells by blocking the continuous phosphorylation of ERK1/2 and

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JUSTC, **2022**, 52(6): 2

Table 2. The general clinicopathological characteristics of fecal samples in patients with colorectal cancer.

Variants	CRC [n(%)]	Adenocarcinoma & Polyps [n(%)]	Normal [n(%)]	Variants	CRC[n(%)]
Total number	120	41	47	TNM	
Age	59*	52*	49*	Carcinoma in situ	10 (8.3%)
Gender				Ⅰ	21 (17.5%)
Male	62 (51.7%)	26 (63.4%)	21 (44.7%)	Ⅱ	35 (29.2%)
Female	58 (48.3%)	15 (36.6%)	26 (55.3%)	Ⅲ	38 (31.7%)
Fecal immunochemical test				Ⅳ	16 (13.3%)
Positive	116 (96.7%)	2 (4.9%)	0 (0.0%)	T	
Negative	4 (3.3%)	39 (95.1%)	47 (100.0%)	1+2	35 (29.2%)
Tumor location				3+4	85 (70.8%)
Ascending	22 (18.3%)			N	
Transverse	8 (6.7%)			Positive	51 (42.5%)
Descending	14 (11.7%)			Negative	69 (57.5%)
Sigmoid	30 (25.0%)			M	
Rectum	46 (38.3%)			Positive	9 (7.5%)
Tumor differentiation				Negative	111 (92.5%)
High	4 (3.3%)			CEA (ng/ml)	
Moderate	89 (74.2%)			≥5	47 (39.2%)
Low	17 (14.2%)			0−4.9	73 (60.8%)
NG	10 (8.3%)			CA199 (U/ml)	
				>37	23 (19.2%)
				0−37	96 (80.0%)
				NG	1 (0.8%)

CRC：colorectal cancer;*:median age; NG: not given

cAMP accumulation[27]. NPY is highly expressed in prostate intraepithelial neoplasia and prostate cancer, and is an independent risk biomarker for postoperative recurrence survival and tumor progression time[12]. In addition, scholars have reported that increasing NPY gradient concentrations promote breast cancer cell proliferation by regulating ERK1/2 phosphorylation, which could be reversed by NPY receptor antagonists[28]. Others have suggested that NPY is a differentially methylated gene in Chinese patients with breast cancer[7]. A case-control study reported that proline at NPY rs16139 presented a lower risk of pancreatic cancer than the leucine genotype[13]. Gene Expression Omnibus database analysis revealed that NPY was hypermethylated in bladder cancer[14], endometrial cancer[15] and oral squamous cell carcinoma[29]. In contrast, NPY can alleviate cancer pain caused by the spinal pain signaling pathway[30]. The expression of NPY affects the imbalance in food intake, not only leading to obesity but also to cancer-related cachexia. Being overweight or obese increases the risk of CRC, breast, and pancreatic cancer[31]. Studies have reported that cachexia is present in more than 60% of end-stage patients. Animal experiments have confirmed that tumor resection can upregulate NPY expression in mice[32]. It affects tumor growth and apoptosis by dysregulating the balance between energy metabolism and immune function[33]. NPY methylation showed a good ability to identify patients with a favorable responses to radiotherapy and chemotherapy (AUC=0.93) or survival benefits in esophageal squamous cell carcinoma. In addition, it was hypermethylated in patients with kidney cancer[34] and head and neck tumors[17], and was not significantly related to patient OS. Interestingly, NPY is related to intestinal inflammation by binding to Y1 and Y2 receptors on immune cells (T lymphocytes, macrophages, monocytes, and dendritic cells)[35]. Consistent with our findings, these findings illustrate the potential therapeutic benefits of combining NPY with immune cell analysis.

For CRC, some researchers have conducted a methylation test for 14,000 genes (27,578 CpG sites) and confirmed that NPY was hypermethylated in CRC[20]. The sensitivity and specificity of NPY combined with WIF-1 and PENK methylation detection in CRC were 87% and 80%, respectively[21]. Others found that NPY dysregulation predicted the effects of immunotherapy or chemotherapy in 82 patients with metastatic CRC[22]. Using digital PCR, methylated free DNA was found in 80% of patients with metastatic CRC and in 45% of patients with limited-stage CRC[36]. Plasma digital PCR detection of NPY methylation is useful for tumor treatment and follow-up monitoring[37]. In line with previous studies, we confirmed that NPY was hypermethylated in Chinese patients with CRC. Fecal NPY methylation was 82.5% sensitive for primary tumors, 46.3% for intestinal polyps (adenomatous, serrated, and inflammatory polyps), and 23.4% for healthy controls. Meanwhile, fecal NPY methylation was 76.6% specific. Previous studies have reported that NPY hy-

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JUSTC, **2022**, 52(6): 2

Table 3. Diagnostic role of fecal NPY methylation in patients with colorectal cancer.

Variables	CRC	Adenoma+polyps	CRC+Adenoma+polyps	Healthy control
NPY+	99	19	118	11
NPY−	21	22	43	36
Sensitivity	82.50%	46.30%	73.30%	/
Specificity	76.60%	/	/	/
NPY+/FIT+	118	20	138	11
NPY−&FIT−	2	21	23	36
Sensitivity	98.30%	48.80%	85.70%	/
Specificity	76.60%	/	/	/

CRC：colorectal cancer; FIT: Fecal immunochemical test

permethylation is associated with the invasion of CRC cells[38]. Furthermore, we found that NPY hypermethylation is related to the downregulation of gene transcription. However, we found that NPY demethylation drugs failed to significantly restore protein expression, which may be attributed to the following factors. First, the concentration of demethylation drugs needed to significantly upregulate protein expression was >10 μmol/L. Second, the expression of NPY protein may be affected by the demethylation of other genes. Third, NPY expression in CRC cell lines was extremely low. Finally, western blotting lacks necessary sensitivity. In contrast, no obvious correlation was found between NPY methylation and clinical parameters or OS benefits in our study, which was different from other heterogeneous tumors.

This study had some limitations. First, as a diagnostic marker, the sample size was insufficient, with only 208 stool samples used in this study. Second, this study did not explore the detailed mechanism of CRC, which needs further investigation. Third, fecal NPY methylation showed poor diagnostic specificity for CRC. The combined detection of multiple genes can improve this method to a certain extent. Therefore, it is necessary to explore new programs using other genes to improve their detection sensitivity and specificity. Finally, the failure rate of fecal NPY detection was high with an unsatisfactory burden. Methods of fecal DNA extraction to purify human-derived genes should be standardized, including nucleic acid extraction, PCR detection, and strict quality control.

5 Conclusions

This study indicated that NPY is hypermethylated in CRC and that NPY methylation in fecal DNA may be a potential noninvasive diagnostic biomarker for Chinese patients with CRC.

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Conflict of interest

The authors declared that they had no conflict of interest.

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