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Intratumoral polymorphism of peroxisome proliferator-activated receptor delta -87 T>C in colorectal cancer

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Peroxisome proliferator-activated receptor delta (PPARD) is a nuclear receptor transcription factor whose single nucleotide polymorphism (SNP), especially PPARD -87 T>C (rs2016520), may play an important role in regulation of PPARD expression. However its expression patterns as well as contribution to colorectal cancer (CRC) are still controversial. In this study, the presence of the intratumoral heterogeneity of PPARD -87 T>C (rs2016520) polymorphism and its influence in CRC were investigated. Tumor masses from primary CRC patients were collected during the tumorectomy, specimens from different sites of the same tumor mass were sampled and stored individually. The SNP of PPARD -87 T>C was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and the expression of PPARD in vivo was observed by immunohistochemistry. The correlation of PPARD -87 T>C intratumoral polymorphism and the clinicopathological parameters of patients was analyzed statistically. Tumor samples were collected from 106 CRC patients (70 males and 36 females) with an average age of 61.04±13.67 years. A total number of 808 samples (7.60±1.60 per patient) were mainly harvested at peripheral superficial (n=376), central superficial (n=163), invasive front (n=112) and mesenteric cancer foci (n=42) of tumor tissues as well as cancerous adjacent mucosa (n=104). PCR-RFLP analysis showed that T/T (n=460, 56.9%) and T/C (n=334, 41.3%) were the main genotypes of -87 T>C among these samples. Furthermore, intratumoral genotype of -87 T>C was homogeneous in 90 patients and heterogeneous in other 16 patients. The intratumoral heterogeneity was related to patient age (p=0.016), tumor location (p=0.011) and the grade of differentiation (p=0.022). For patients with intratumoral heterogeneity, immunochemistry showed that the expressions of PPARD were not influenced by T/T or T/C genotypes. Intratumoral heterogeneity of PPARD -87 T>C widely existed in CRC, and associated with patient age, tumor location and differentiation. However, the immunochemistry assay revealed that there is no significant link between heterogeneity and expression of PPARD.

Key words: intratumoral polymorphism, peroxisome, colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide that accounts for approximately 10% of cancer-related mortality [1, 2]. Such high prevalence of colorectal cancer is partially influenced by the increasingly aging population, unhealthy dietary habits (e.g., high fat and protein but low cellulose) and lifestyle (smoking, low physical exercise) [3, 4]. Several available treatments have been developed for the primary or metastatic colorectal cancer such as laparoscopic surgery, radiotherapy as well as palliative or other neo-adjuvant chemotherapies [5–7]. However, the cure rates or long-term survival of advanced or metastatic patients are still far from satisfactory [8].

There is a widely accepted consensus that tumor is a cell mass which originated from a single cell with off-tune mutation and constant proliferation [9–11]. However, with the development and application of new technologies such as high-throughput gene sequencing, the concept of tumor heterogeneity gradually captured the attentions of researchers [12–17]. Tumor heterogeneity can be divided into intertumor and intratumor heterogeneity. Unlike intertumor heterogeneity that comprises differences in synchronous primary tumors of the same type developing in the same patient, or between a primary tumor and its matched metastases, intratumor heterogeneity is described as the presence of various clones within one tumor, each with its own unique features on the level of morphology, inflammation, genetics, epigenetics, or transcriptomics [18, 19]. Although its origin is not fully understood, the existence of intratumor heteroge-

neity is well known both in primary CRC and corresponding metastases [20, 21]. Using multiple studies, such as comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays, researchers have revealed a more complex relationship between them [22].

Peroxisome proliferator-activated receptor (PPAR), a subfamily of nuclear receptor transcription factors,consists of three isoforms, i.e. alpha, beta/delta and gamma [23]. Among them, PPAR delta (PPARD) is highly expressed in keratinocytes, intestinal crypts and skeletal muscle [24, 25]. In the past few decades, the role of PPARD in cell proliferation and differentiation, metabolic diseases, inflammation, as well as tumorigenesis has been largely uncovered [26–28]. However, there is still a controversy regarding the promoting and inhibiting effect of PPARD in the carcinogenesis of colorectal cancer [26, 29–32].

PPARD gene consists of 10 introns and 11 exons and locates at 6p21.2-p21.1 chromosome. Single nucleotides polymorphism (SNP) on the coding region of genes usually results in altered expression at transcription level [33–35]. The SNP of PPARD embedded in the 87th nucleotide upstream of the start codon in the 5'-untranslated region of exon 4 (-87 T/C, also termed as +294 T>C (rs2016520)) has been widely studied and claimed to be associating with multiple physiological and pathological processes such as lipid metabolism and coronary artery disease [36–38]. Besides, our previous study revealed that PPARD -87 T>C with homozygotes for the C allele (C/C type) showed a tendency toward a higher risk for CRC compared to T/T homozygotes. Nevertheless, to the best of our knowledge, little is known about the distribution and proportion as well as the potential function of PPARD -87 T/C polymorphism in CRC. Thus, in this pilot study, the tumor tissues were collected from patients with CRC, specimens from the same tissue were sampled at different sites and their -87 T/C polymorphism were identified as homozygote type (T/T and C/C) or heterozygote type (T/C) individually. The inconsistent -87 T/C polymorphism among specimens resulting in the different expression of PPARD in the same tumor mass was examined.

Table 1. Clinico-pathological parameters of colorectal cancer patients (n=106).

Items		Case (n)	Average or Percent (%)
Sex	Male	70	66.0
	Female	36	34.0
Age (years)		106	61.04±13.67
Location	Ascending colon	18	17.0
	Transverse colon	4	3.8
	Descending colon	6	5.6
	Sigmoid colon	15	14.2
	Rectum	63	59.4
Types	Ulcerative	74	69.8
	Protuberant	29	27.4
	Others	3	2.8
T-staging	T1	0	0
	T2	6	5.7
	T3	8	7.5
	T4	92	86.8
N-staging	N0	55	51.9
	N1	23	21.7
	N2	28	26.4
M-staging	M0	96	90.6
	M1	10	9.4
Differentiation	High	8	7.5
	Middle	80	75.6
	Low	8	7.5
	Undifferentiation	10	9.4

Patients and methods

Patients and tissue samples. Participants were recruited from the patients with resectable primary CRC who underwent the tumorectomy at West China Hospital, Sichuan University (Chengdu, China) between April 2013 and September 2014, clinic pathological parameters of participants were recorded including patients' age, tumor type, location, the grade of differentiation and TNM stage (i.e. tumor size, nodal involvement, metastases), which was built by the American Joint Committee on Cancer (AJCC) as a robust system for classifying patients with cancer, defining prognosis and determining the best treatment approaches.

We excluded following below criteria: under 18 years of age familial polyposis, preoperative radio/chemotherapy and tumor size less than 2 cm. Resections of tumor tissues were performed by the same professional team of surgeons according to the guidelines of National Comprehensive Cancer Network (NCCN) [39]. Total of 106 CRC patients were enrolled (70 males and 36 females) and recorded in detail for the subsequent analysis, as demonstrated in Table 1.

Specimen collection and usage were granted approval by the ethics committee of West China Hospital, Sichuan University and the written informed consents were obtained from all participants. Resected CRC specimens were immediately washed by sterile saline to minimize the contaminations from feces and blood. Several specimens from each CRC tissue were harvested at the designated locations. Firstly, the resected bowl was slivered longitudinally and laid according to the anatomical position, as shown in Figure 1. Then, specimens from the peripheral superficial were sampled at the up (a1), left (a2), down (a3) and right (a4) margins of the tumor tissue; central superficial specimens were harvested at 1 cm from the tumor center (b1 and b2). Then the tumor mass was dissected and the deep specimens from the invasive front of the bowl wall (c) were collected. Mesenteric lymph nodes and metastasis nodes (d) were collected if positive. For liver metastasis, tissue was marked as e only if the tumor mass can be resected and verified. Besides, colorectal polyps were taken out partly and marked as f. Normal colorectal mucosal

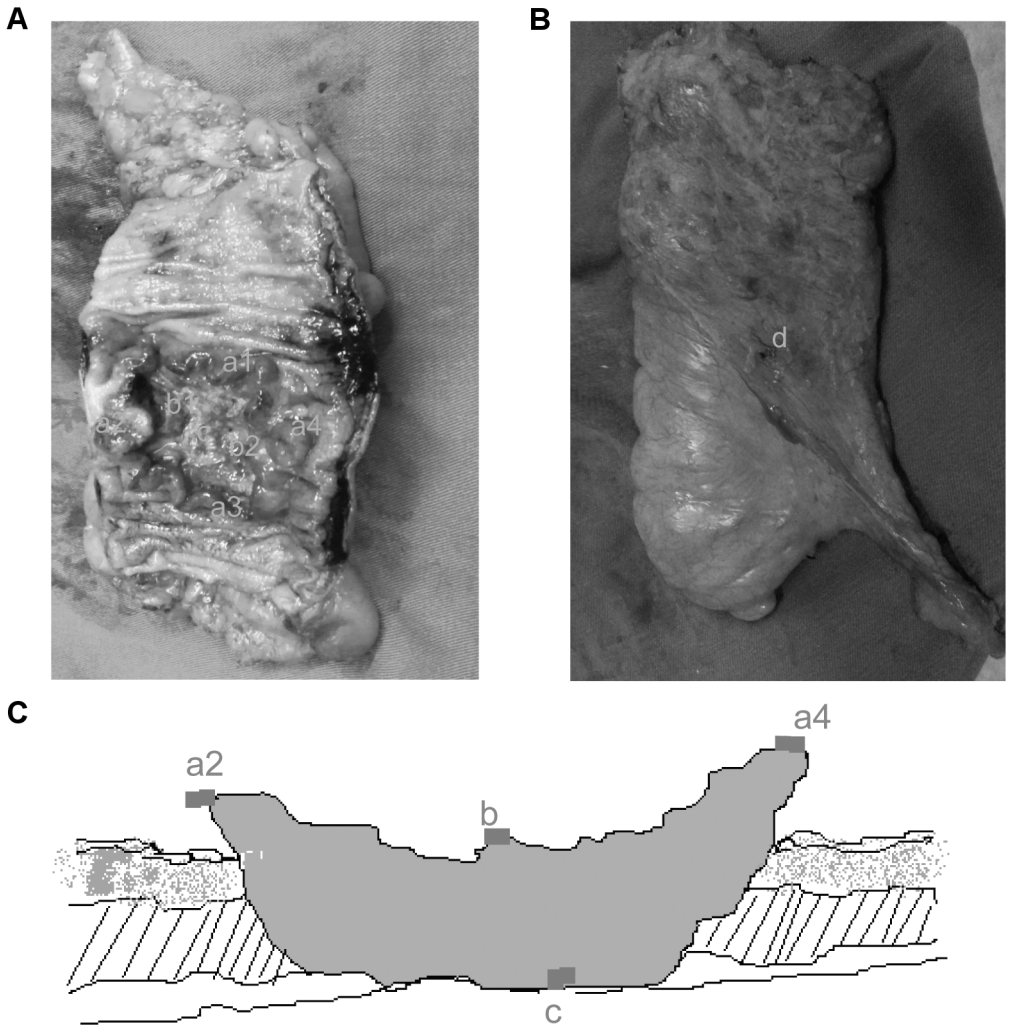

Figure 1. The designed sample sites in colorectal cancer tissue. A) Representative internal view of the resected tumor-carrying bowl after slivering longitudinally, the sample sites at the peripheral superficial (a), central superficial (b), deep invasive part (c) as well as normal colorectal mucosal tissue (n) are shown. B) Representative external view of colorectal cancer tissue, the sample site at mesenteric lymph nodes and metastasis nodes (d) is shown. C) Schematic representation of the transversely incised CRC tissue, the sample sites at the peripheral superficial (a), central superficial (b) as well as deep invasive part (c) are shown.

tissue (n) was taken from the site that was away from the tumor margin more than 5 cm. All specimens were harvested with the size less than 5 mm and then cut into two to three pieces, individually placed into 1.5 ml Eppendorf tube and stored in –80 °C refrigerator within one hour after resection. The same skilled pathologist operated all procedures.

DNA extraction. Tissue specimens were defrosted in an ice bath and then incubated with 400 µl of SNET buffer (1% SDS, 400 mM NaCl, 5 mM EDTA, 20 mM Tris, pH 8.0) and 5 µl of proteinase K (Merck, Billerica, MA) at 58 °C (water bath) overnight. Then, 200 µl of phenol were added into the reaction system followed by the centrifugation at 12 000 rpm for 10 min. The supernatant was harvested and transferred into a new tube containing phenol (200 µl) and chloroform-isoamyl alcohol mixture (24:1, 200 µl). Centrifugation was carried out again and the supernatant was washed by the mixture of chloroform and isoamyl alcohol (24:1, 200 µl). DNA contained in the upper aqueous phase was transferred into a new tube, precipitated by isopropanol, washed by 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

DNA amplification and restriction fragment length polymorphism analysis. DNA fragment containing the -87 T>C site of PPARD was amplified from the extracted genomic DNA using PCR reaction. A pair of specific primers (forward: 5'-TCTGGCATCGTCTGGGTCT-3'; reverse: 5'-ATAGGGCAGGTGACTTGTGA-3') was chemically synthesized by Sangon-Biotech (Shanghai, China) and then used to produce a 239 bp amplicons (Figure 2A). Mixtures (50 µl) containing 200 µM of dNTP, 1 µM of each primer,

3 mM of $MgCl_2$, 20 ng of extracted DNA and 2.5 U of Taq polymerase (Thermo Fisher Scientific, Waltham, MA) was prepared for PCR amplification. The reaction conditions consisted of a one-cycle initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72°C for 20 s, with a final extension at 72 °C for 10 min. Five microliters of PCR products were subjected to electrophoresis on 1.5% agarose gel with 0.5 μg/ml ethidium bromide and visualized under UV light irradiation (UVP GelDoc-It310, Upland, CA). Then, the PCR amplicons were purified with a bead-based purification kit (Mag-MK PCR products Purification Kit, Sangon-Biotech) and digested with FastDigest BslI restriction enzymes (Thermo Fisher Scientific). The restriction digest reaction system (10 μl) consisted of 0.2 μg PCR product, 0.5 μl FastDigest BslI and 1.5 μl 10×Green Buffer. Digests were performed at 37 °C for 5 min and then the enzymes were deactivated at 72 °C for 2 min. PCR amplicons would be cleaved to a long fragment (203 bp) and a short fragment (36 bp) if the nucleotide at -87 T>C is Cytosine (Figure 2A). The digested products were also analyzed by agarose gel electrophoresis.

Immunohistochemical assay. Protein expression of PPARD in primary CRC specimens was evaluated by immunohistochemistry as previously described [40]. In brief, the specimens were fixed by 4% formaldehyde, embedded in paraffin wax and sliced to 5 μm sections. The slices were then deparaffinized in xylene and rehydrated in alcohol with the decreased concentration. Antigen retrieval was performed by Tris-EDTA buffer (pH=8.0) at the sub-boiling temperature (about 95 °C) for 45 min. After naturally cooling to the room temperature, endogenous peroxidases were exhausted by the incubation with 3% H_2O_2 at 37 °C for 15 min. Then, the slices were blocked by 3% BSA in PBS for one hour and incubated with rabbit anti-PPARD antibody (1:1000, Abcam) at 4 °C overnight. Slices were then sequentially washed in PBST three times and incubated with HRP-labeled goat anti-rabbit antibody at 37 °C for 10 min. After visualizing by 3,3-diaminobenzidine (DAB), slices were counterstained with hematoxylin, dehydrated by alcohol and xylene and mounted permanently for examination.

Immunohistochemical evaluation was carried out by two researchers (blind to the clinical and pathological data of the patients) as previously described [41]. In particular, slices were scored semi-quantitatively for immunoreaction extension (score 0–3) as following: 0: 0–9% of immunoreactive cells; 1: 10–39% of immunoreactive cells; 2: 40–69% of immunoreactive cells; and 3: 70–100% of immunoreactive cells. Staining intensity was scored as follows: 0: negative (uncolored); 1: weak (yellowish); 2: intermediate (tan) and 3: strong (brown).The final score of immunoreactions was calculated as the product of staining extension and intension, which was only scored by 0, 1, 2, 3, 4, 6 and 9. The frequency of immunoreactive score in different genotypes (T/T, T/C and C/C) was recorded and compared using rank sum test (Kruskal-Wallis test and Mann-Whitney test).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis. TRIZOL reagent (Invitrogen, Grand Island, USA) was used for total RNA extraction. RNA was converted into cDNA using random hexamer primers by M-MLV reverse transcriptase (Invitrogen) following the construction of kit. Messenger RNA levels were estimated by qRT-PCR, carried out in the presence of SYBR green probe. The qRT-PCR primers sequences were as follows: PPARD-: 5'-ACAAGGCCTCAGGGTACCA-3', and PPARD-RTR: 5'-GCCGAAAGAAGCCCTTACAG-3'; GAPDH-RTF: 5'-TGCACCACCAACTGCTTAGC-3', and GAPDH-RTR: 5'-GGCATGGACTGTGGTCATGAG-3'.

Western blot analysis. Tissues were homogenized by sonication in PBS buffer (pH=7.4) with the presence of Halt protease inhibitor cocktail (Thermo Scientific, Mass, USA). Then the homogenate was centrifuged for 10 min with speed of 12 000 × g and temperature of 4 °C. After quantification and denaturation, protein samples were loaded into an electrophoresis gel and then blotted onto a nitrocellulose membrane (Amersham Ibérica®, Spain) with a semidry transfer system (Bio-Rad, CA, USA). The protein-blotted membrane was incubated with specific primary antibodies, including anti-GAPDH antibody (1:1000; Abcam), rabbit anti-PPARD antibody (1:1000, Abcam) and goat polyclonal to rabbit IgG.

Statistical analysis. Statistical analyses were carried out using SPSS16.0 software, the correlation between SNP of PPARD -87 T>C and clinicopathological parameters were analyzed by using Logistic Regression. Significance of difference was analyzed using Student's t test (for normal data) or Mann-Whitney test (for non-normal data) between two groups and one-way ANOVA (for normal data) or Kruskal-Wallis test (for non-normal data) between more than two groups. Two tailed $p<0.05$ was considered statistically different.

Results

SNP analysis of PPARD -87T/C. A total number of 822 tissue specimens were carefully taken and used to amplify the 239 bp fragment of PPARD by PCR with specific primers (Figure 2A). In RFLP analysis, the -87 T>C polymorphism of PPARD can be determined via BslI digestion. Specifically, PPARD -87 T>C with T/T type cannot be digested by BslI restriction enzymes, and thus presented single band with the length of 239 bp on the gel map (Figure 2B), while PPARD -87 T>C with T/C type was partially digested by BslI enzymes, presenting three bands with the length of 239 bp, 203 bp and 36 bp (Figure 2C). Besides, PPARD -87 T>C with C/C type only showed two bands with the length of 203 bp and 36 bp due to completely digestion by BslI enzymes (Figure 2D).

Among these tissue specimens, T/T-type was the most frequent genotype with 56.9% presence (n=460) in all specimens, while T/C-type accounted for 41.3% (n=334) and C/C-type only hold 1.7% (n=14) proportion, as shown in Table 2. Genotype distribution of -87 T>C polymorphism in

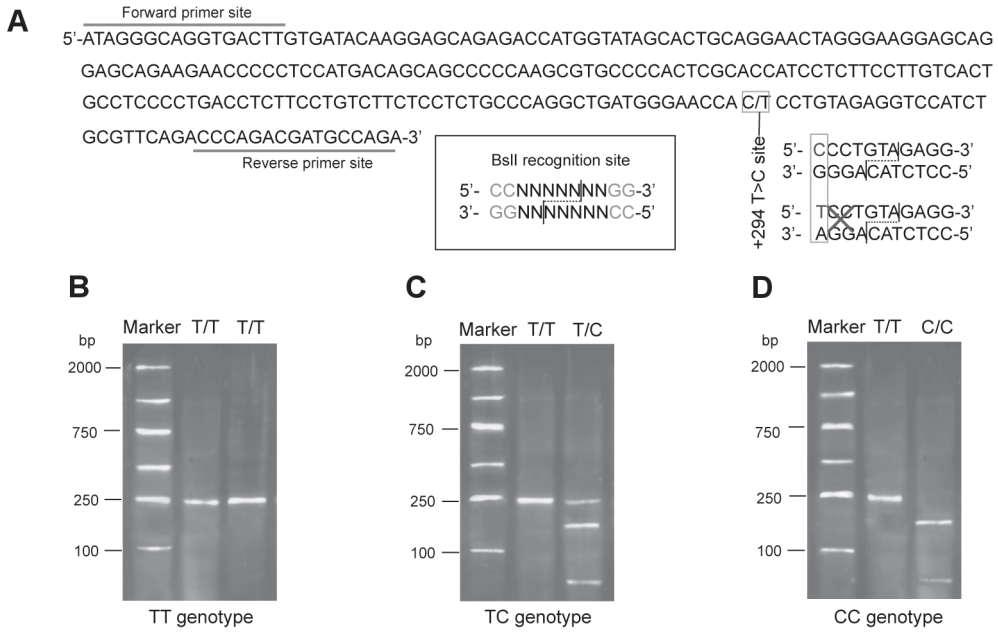

Figure 2. Single nucleotide polymorphism of PPARD -87 T>C. A) The expected sequence (239 bp) of PCR amplicons containing PPARD -87 T>C site. The specific f primers were designed according to the DNA base complementary matching principle. B) Schematic representation of the RFLP analysis for PPARD -87 T>C with T/T genotype. A single band with the length of 239 bp was presented on the gel map after the digestion with BslI enzymes. C) Schematic representation of the RFLP analysis for PPARD -87 T>C with T/C genotype. Three bands with the length of 239 bp, 203 bp and 36 bp were presented on the gel map after the digestion with BslI enzymes. D) Schematic representation of the RFLP analysis for PPARD -87 T>C with C/C genotype. Two bands with the length of 203 bp and 36 bp were presented on the gel map after the digestion with BslI enzymes. Notes: lane M: DL2000 DNA marker, Lane 1: PCR amplicons before BslI digestion, Lane 2: The products of FRLP.

the noncancerous colorectal mucosal tissue obeyed Hardy-Weinberg Equilibrium (HWE), while the overall distribution of -87 T>C genotype in all the 822 tissue samples failed to meet HWE. Moreover, majority of these patients (84.9%) only harbored one genotype of intratumoral PPARD -87 T>C, whereas evident intratumoral heterogeneity of PPARD -87 T>C was exhibited in other 16 patients (15.1%).

Logistic regression analysis showed the intratumoral heterogeneity was significantly associated with the age of patients (p=0.016), the tumor location (p=0.011) and the tumor differentiation (p=0.022) (Table 3 and 4). The average age of patients with intratumoral heterogeneity or intratumoral homogeneity was 65.44±9.26 or 60.36±14.21 years, respectively. The proportion of intratumoral heterogeneity in sigmoid colon cancer reached at 40% (6/15) which was higher than in any other location (Table 5). The rate of intratumoral heterogeneity in low-differentiated adenocarcinoma reached up to 50% (4/8) and higher than other differentiated degrees as well (Table 5).

Expression analysis of PPARD. A total of 87 specimens of tumor tissue with intratumoral heterogeneity (n=16) were subjected to the immunohistochemical analysis for the detection of the expression of PPARD protein. The results showed that the PPARD protein, both of T/T genotype (Figure 3A) and T/C genotype (Figure 3B), existed mainly in the CRC cells, while rarely expressed in the interstitial cells and normal mucosal cells. Besides, qRT-PCR and western blot were performed to evaluate mRNA and protein level of PPARD in these two genotype specimens. Likewise, there was no significant difference between these two groups (Figures 3C–D).

Table 2. Genotypic frequencies of the PPARD -87 T > C polymorphism in colorectal cancer tissue.

Specimens location	Genotype			Total
	TT	TC	CC	
Peripheral superficial	218	153	5	376
Central superficial	92	69	2	163
Deep invasive part	63	47	2	112
Mesenteric lymph nodes	21	18	3	42
Liver metastasis	2	0	0	2
Colorectal polyps	5	4	0	9
Noncancerous mucous	59	43	2	104
Total	460	334	14	808

The total immunoreactive activity was calculated as the product of immunoreactive intensity and extension. The frequency of immunoreactive score in different genotypes (T/T, T/C and C/C) was recorded in Table 6. Kruskal-Wallis test showed that the value of c^2=11.302 and p-value=0.004, which suggested that the differences of PPARD expression

Table 3. Dummy variables in unconditional logistic regression analysis.

Independent variables		Case (n)	Number of dummy variables			
			–1	–2	–3	–4
Tumor location	Transverse colon	4	1	0	0	0
	Descending colon	6	0	1	0	0
	Ascending colon	18	0	0	1	0
	Sigmoid colon	15	0	0	0	1
	Rectum	63	0	0	0	0
Differentiation	Undifferentiation	10	1	0	0	
	Low	8	0	1	0	
	Middle	80	0	0	1	
	High	8	0	0	0	
TNM stage	I	4	1	0	0	
	II	50	0	1	0	
	III	42	0	0	1	
	IV	10	0	0	0	
T-staging	2	6	1	0		
	3	10	0	1		
	4	90	0	0		
N-staging	0	55	1	0		
	1	23	0	1		
	2	28	0	0		
M-staging	0	96	1			
	1	10	0			
Types	Ulcerative	74	1	0		
	Protuberant	29	0	1		
	Others	3	0	0		
Sex	Male	70	1			
	Female	36	0			

among groups were of statistical significance. There were only 4 of the 87 specimens (4.6%) that showed C/C genotype, so the subsequent rank sum test (Mann-Whitney test) was only performed between T/T genotype and T/C genotype. However, the difference between these two groups was not statistically significant (p=0.854). These results indicated the expression of PPARD was not mediated by the genotype of -87 T/C.

Discussion

To this date, the CRC has developed into a predominant cancer globally and now ranks 3rd in the Western countries and 5th in Asia among malignant diseases [42, 43]. The pathogenesis of CRC is extremely complex and involves the collaboration of multiple factors including microsatellite instability, accumulating gene mutations, aberrant signal transduction pathways as well as the limited cell apoptosis and telomerase activation [1, 2, 44, 45]. In particular, a large number of signal pathways were aberrant throughout the whole processes of CRC including initiation, progression, and metastasis, represented by the Wnt signal pathway which loss of adenomatous polyposis coli (APC), a negative regulator of Wnt signal pathway and is considered as one of the hallmarks of CRC [46].

A compelling study has defined the PPARD as a downstream gene of the APC signaling pathway in CRC. The inactivation of APC caused evident up-regulation of PPARD expression, while APC overexpression significantly suppressed the level of PPARD [47]. PPARD is a member

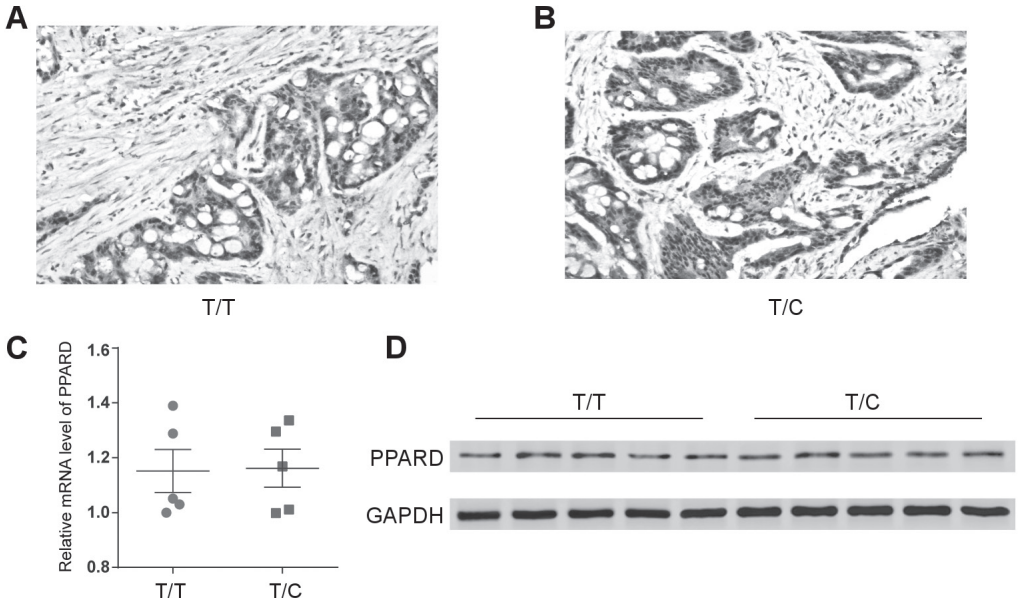

Figure 3. Expression of PPARD in the specimens with T/T or T/C genotype. In the same CRC tissue, the expressions of PPARD in different sites were detected by immunohistochemical staining (A-B). A) Expression of PPARD in specimen with T/T genotype; and B) expression of PPARD in specimen with T/C genotype. C) Quantitative RT-PCR was performed to determine mRNA level of PPARD. D) Western blot was conducted to confirm the protein level of PPARD.

Table 4. Unconditional logistic regression analysis estimating colorectal cancer associated with the PPARD +294 T>C genotype.

Independent variables	β±SE	Wald-value	p-value	OR	Lower 95% OR	Upper 95% OR
Sex (1)	−1.221±0.825	2.191	0.139	0.295	0.059	1.486
Age	0.103±0.042	5.854	0.016	1.108	1.02	1.204
Location		6.928	0.14			
Site (1)	2.325±1.641	2.008	0.156	10.226	0.41	254.772
Site (2)	−17.159±1.486E4	0	0.999	0	0	
Site (3)	1.175±1.166	1.016	0.314	3.239	0.329	31.844
Site (4)	2.740±1.076	6.482	0.011	15.488	1.879	127.674
T-staging		5.254	0.072			
T-staging (1)	3.876±2.179	3.164	0.075	48.229	0.674	3.45E+03
T-staging (2)	2.339±1.335	3.071	0.08	10.372	0.758	141.919
N-staging		1.277	0.528			
N-staging (1)	0.952±2.239	0.181	0.671	2.59	0.032	208.455
N-staging (2)	−0.865±1.461	0.35	0.554	0.421	0.024	7.381
M-staging (1)	−0.509±1.158	0.193	0.66	0.601	0.062	5.817
TNM-staging		0.841	0.657			
TNM-staging (1)	−22.093±1.895E4	0	0.999	0	0	.
TNM-staging (2)	−1.704±1.859	0.841	0.359	0.182	0.005	6.955
Tumor diameter	0.319±0.282	1.275	0.259	1.376	0.791	2.393
Positive lymph node	−0.009±0.156	0.003	0.954	0.991	0.729	1.347
Pathological types		0.219	0.896			
Type (1)	17.979±2.110E4	0	0.999	6.43E+07	0	.
Type (2)	17.498±2.110E4	0	0.999	3.98E+07	0	.
Differentiation degree		7.671	0.053			
Degree (1)	−0.160±1.714	0.009	0.926	0.852	0.03	24.532
Degree (2)	4.486±1.963	5.224	0.022	88.784	1.895	4.16E+03
Degree (3)	1.349±1.510	0.798	0.372	3.853	0.2	74.373
Constant term	−28.890±2.110E4	0	0.999	0		

Table 5. Frequency of intratumoral heterogeneity of the PPARD -87 T > C in colorectal cancer tissue at different site or differentiation grade.

Items	Heterogeneity		Total
	No	Yes	
Location			
Ascending colon	15 (83.3%)	3 (16.7%)	18 (17.0%)
Transverse colon	3(75%)	1 (25%)	4 (3.8%)
Descending colon	6 (100%)	0 (0%)	6 (5.7%)
Sigmoid colon	9 (60.0%)	6 (40.0%)	15 (14.2%)
Rectum	57 (90.5%)	6 (9.5%)	63 (59.3%)
Total	90 (84.9%)	16 (15.1%)	106
Differentiation			
High	7 (87.5%)	1 (12.5%)	8 (7.5%)
Middle	70 (87.5%)	10 (12.5%)	80 (75.5%)
Low	4 (50%)	4 (50%)	8 (7.5%)
Undifferentiation	9 (90%)	1 (10%)	10 (9.6%)
Total	90 (84.9%)	16 (15.1%)	106

Table 6. Immunoreactive activity scores of different genotypes of 87 specimens from 16 patients with intratumoral heterogeneity.

Score	Genotype			Total
	TT	TC	CC	
0	6	5	0	11
1	3	3	0	6
2	10	9	1	20
3	4	4	0	8
4	7	5	1	13
6	10	12	2	24
9	2	3	0	5
Total	42	41	4	87

of PPAR family that participate in cell proliferation and differentiation, as well as tumorigenesis of CRC; despite that whether it acts as tumor promoter or suppressor is still controversial [26, 29–32]. For the tumor-promoting role of PPARD, Takayama et al. [31] suggested that expression of PPARD was up-regulated in human and mouse colon cancer tissues; knockout of PPARD notably hampered the growth of the xenograft formed by CRC cells in nude-mice [48], yet the specific activator of PPARD (GW501516) positively promote the formation of intestinal tumors in rats [49]. Likewise, another nonignorable study also found that the depletion of PPARD did not significantly affect the polyp formation in the intestine and colon [50]. Evidences supporting the tumor suppressing role of PPARD in CRC were derived from the following studies: (1) Peter et al.

revealed that the number and volume of intestinal adenoma was largely increased in the mice with the knockout of PPAR beta/delta [32]; (2) the formation of intestinal tumor in $PPARD^{+/+}$ mice was attenuated compared to the $PPARD^{-/-}$ mice [30, 51]; (3) expression of PPARD was down-regulated in the intestine of rats with APC knockout [52]. In our previous studies, the proliferation of colon cancer cells with the silencing of PPARD was accelerated but the differentiation ability was weakened; additionally, the expression level of PPARD was positively related to the prognosis of colon cancer patients [53–55]. Here, we analyzed the polymorphism of PPARD -87 T/C (rs2016520) in CRC tissues and demonstrated the different existing probabilities of T/T, T/C, and C/C genotypes: T/T (56.9%) > T/C (41.3%) > C/C (1.7%). By the means of immunohistochemical staining, we revealed that expression of PPARD between T/T specimens and T/C specimens was not significantly different. However, in another unpublished study, we found out that the risk of carcinogenesis of normal colorectal tissues with the mutant C/C homozygote genotype was higher than that with the wild T/T homozygote genotype. Thus, whether the single nucleotides polymorphism of -87 T/C (rs2016520) affected the expression of PPARD and contributed to the role of PPARD in CRC still needs further investigation to illuminate.

Another intriguing result of our present study is the existence of non-negligible heterogeneity of the genotype of -87 T/C (rs2016520) in the same tumor mass. In the initiation and progression of malignancy that generates by a single cell, subclones with the distinct molecular biological characteristics often occurred upon the repeated proliferation and differentiation as well as the pressure of natural selection of microenvironment [9–11]. Besides, subclones may be formed at the initiation of malignancy and differentiated in the process of tumor growth [12, 14, 16]. These subclones differ in their histology, metabolism, growth rate, invasiveness and drug resistance [13, 15, 17]. Moreover, we found the intratumoral heterogeneity of -87 T/C (rs2016520) widely existed in CRC and closely correlated with tumor location and the degree of differentiation grade. Hence, although it failed to affect the expression of PPARD significantly, the role of single nucleotides polymorphism of -87 T/C (rs2016520) in the progression of CRC might not be ignorable.

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