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Original Article

Changes of TCR repertoire diversity in colorectal cancer after Erbitux (cetuximab) in combination with chemotherapy

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Abstract: We have previous found a positive correlation between post-therapy TCR repertoire normalization and remission of colorectal cancer (CRC) patients following fluorouracil, leucovorin, and irinotecan (FOLFIRI) plus bevacizumab or Rh-endostatin therapy. To further define the TCR repertoire diversity changes following treatment in CRC patients, and confirm its potential prognostic value, the present study extended the sample size of follow-up and used an alternative therapy regime to investigate changes of TCR repertoires following Erbitux plus FOLFIRI therapy. Inclusion and exclusion criteria have been established to screen out 26 patients to receive Erbitux plus FOLFIRI therapy. Efficacy and toxicity assessment have been made for them after 3 months' treatment as well as the TCR repertoire diversity has been determined. A CDR3 complex scoring system was used to quantify the diversity of TCR repertoire. The results showing that the diversity of $CD4^+$ T cells in PR group was significantly higher than that of SD and PD groups, and the difference was enlargement after treatment. The diversity of $CD8^+$ T cells in PR group has no difference before and after treatment, but significant decrease in SD and PD group after treatment. In conclusion, analysis the diversity of T cell repertoire has an important prognosis value for CRC patients.

Keywords: Colorectal cancer (CRC), T cell receptor (TCR), TCR repertoire, complementarity determining region 3 (CDR3), Erbitux

Introduction

Colorectal cancer (CRC) is the third most common malignant tumors in the world which presents a serious threat to public health [1]. Approximately 75% of CRC patients are diagnosed as advanced metastasis disease and associated with bad curative effect and poor prognosis. Current imaging methods, such as computed tomography (CT) or magnetic resonance imaging (MRI), only can be used to measure the tumor's size, unable to provide information about patients' physical rehabilitation, especially the recovery of immune systemic function.

The emergence and progression of colorectal cancer was associated with a variety of factors, in which the function and distribution of tumor infiltrating T lymphocytes and circulating tumor antigen-specific T cells was crucial on account of T cells is immune effector cells involved in immune-mediated tumor surveillance [2]. The prognostic role of tumor infiltrating T cells have been reported not only in CRC patients [3], but also in other solid tumors such as breast cancer [4] and head and neck carcinomas [5]. Monitoring T cells' immune function before and after treatment could help to provide objective data to illustrate whether the treatment is effective, which have an important reference value for evaluation of treatment efficacy and judgment on recurrence, metastasis and prognosis.

T cells are functionally active cell population *in vivo* mediating cellular immune response and playing an important role in humoral immune response. T cell recognizes antigens via its surface receptors (TCR), 95% of T cells' TCR are composed of α and β chains. Complementarity determining region 3 (CDR3) is a critical region

in TCR both chains responsible for specifically recognize and bind antigen peptide. Each T cell has its own unique CDR3 sequence. According to the homology of CDR3 variable region (V) gene sequence, TCR Vβ genes were divided into about 24 families. Testing each Vβ gene family' CDR3 spectratype could therefore reflect the clonal expansion of T cells [6].

Healthy individual without antigen stimulation can be detected polyclonal and Gaussian distribution of CDR3 spectratype in almost each TCR gene families, indicating a highly diverse TCR repertoire against countless antigens in the environment [7]. While continuous tumor antigen stimulation may elicit clonal expansion of T cells result in monoclonal or oligoclonal expansion of certain TCR gene families and skewed distribution of CDR3 spectratype, which indicating a restricted T cell repertoire with compromised immune functions [8]. Therefore, detecting the diversity of TCR repertoire could reflect the cloned distribution and function of T cells [9].

We have previous first reported that the post-therapy TCR repertoire normalization was positive correlation with remission of metastatic CRC patients treated with FOLFIRI plus bevacizumab [10] or Rh-endostatin [11] therapy. This study is attempted to further illuminate the TCR repertoire diversity in CRC patients treated with another therapy regime. Findings in this study are consistent with our previous results, which suggest that the change of TCR repertoire diversity was associated with overall physical condition of CRC patients, patients undergoing remission have a broader diversity of TCR repertoire than patients showing progression, which further suggesting that monitoring of TCR repertoire diversity may have potential value for treatment efficacy evaluation and prognosis.

Materials and methods

Patient eligibility

From September 2011 to July 2013, CRC patients from Foshan First People's Hospital Cancer Center who fulfilled the following inclusion criteria and did not have any obvious exclusion criteria were enrolled in this study. Inclusion criteria consisted mainly of histologically confirmed metastatic colorectal adenocarcinoma, age ≥ 18 y, widespread metastatic disease that could not be resected for curative purposes, immunohistochemical evidence of EGFR expressed in tumor, have written informed consent form, ready to receive Erbitux plus FOLFIRI treatment, ECOG scores ≤ 2, and both mental and physical status good enough to permit 6-months participation. Exclusion criteria were: i) previous exposure to anti-EGFR therapy, chemotherapy or other therapy that was terminated ≤ 6 months before the start of present study. ii) have a history of infectious diseases, autoimmunity disease, transplant and other tumor etc. immune-related diseases within the last year. iii) known grade 3 or 4 allergic reaction to any of the treatment components. iv) had a medical or psychological condition that would prevent them from completing the study or properly signing the informed consent form.

Healthy controls

Four age-matched healthy donors (each patient was matched with a healthy donor of age difference no more than 2 years, two females aged 56 and 61 years, and two males aged 51 and 68 years, with no clinical or laboratory evidence of connective tumor disease or immunological disorders) were used as controls.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and approved by the institutional ethics commission of the Foshan First People's Hospital. All patients and healthy donors provided written informed consent for their participation.

Treatment plan

Patients were scheduled to receive weekly Erbitux and biweekly FOLFIRI. Erbitux was administered intravenously on day 1 of 400 mg per square meter of body-surface area (mg/m^2) in an initial 120-minute infusion, followed by weekly 60-minute infusions of Erbitux at a dose of 250 mg/m^2. FOLFIRI consisted of irinotecan infusion at a dose of 180 mg/m^2 30- to 90-minute, and infusion leucovorin at a dose of 200 mg/m^2 over 2 hr, immediately followed by fluorouracil in a bolus of 400 mg/m^2 on day 1, and then continuous infusion fluorouracil for 46 hours of 2400 mg/m^2. FOLFIRI was given once biweekly after the Erbitux infusion on day 1 of

each period. Treatment was continued until disease progression, unacceptable toxic effects, or withdrawal of consent occurred. Protocol-specified treatment modifications were performed according to predefined guidelines based on toxic effects related to chemotherapy or Erbitux.

Efficacy and toxicity assessment

Radiologic evaluation according to Response Evaluation Criteria in Solid Tumors (RECIST) consisted of computed tomography (CT) or magnetic resonance imaging (MRI) was performed at least every 12 weeks during the follow up. The original radiologic results were reviewed for RECIST and changes in tumor size and density by three radiologists who were blinded to the investigator's evaluation. Using the smallest sum longest diameter (LD) as the reference, criteria to assess complete responses (CRs) and partial responses (PRs) were based on those reported. A partial response (PR) was defined as a > 30% reduction in the sum of the LD of the target lesions, progressive disease (PD) was defined as the appearance of one or more new lesions or > 20% increase in the sum LD of the target lesions, stable disease (SD) was defined as a < 30% reduction or a < 20% increase in the sum LD of the measurable tumor lesions. Toxicity and adverse events (the severity of which were assessed according to the National Cancer Institute Common Toxicity Criteria Version 3.0.) were recorded continuously.

Isolation of mononuclear cells

Fresh blood samples were obtained from 26 patients with advance CRC and 4 healthy volunteers. This protocol approved by the ethics committee of Southern Medical University. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood. The isolation procedure was performed as previously described [12].

Magnetic bead separation of $CD4^+$ and $CD8^+$ T cells

The PBMCs were incubated with anti-CD4 and anti-CD8 monoclonal antibodies coupled with magnetic beads (Miltenyi Biotec, Germany). The cells were then loaded onto MidiMACS columns (M Miltenyi Biotec, Germany) and T cells were positively selected according to the manufacturer's instructions. The purity of the separated $CD4^+$ and $CD8^+$ T cells were > 95% by fluorescence-activated cell sorting (FACS) analysis (data not shown).

Amplification of TCR Vβ family CDR3 gene by RT-PCR

Total RNA from 2×10^6 $CD4^+$ and $CD8^+$ T cells after separation was extracted with a RNA extraction kit (Omega Biotech Company, Doraville, GA, USA), and quantified by spectrophotometry. One microgram of RNA was transcribed to first strand cDNA with a cDNA synthesis kit (MBI, Fermentas, Hanover, MD, USA), according to the manufacturer's instructions. Total RNA was incubated at 42°C for 1 h with 250 pM oligo-(dT) primer, 200 U Moloney murine leukemia virus (M-MuLV) reverse transcriptase and 250 μM of each dNTP in a total volume of 20 μl. PCR amplifications of BV-specific cDNA were carried out in a volume of 50 μl, containing 2 μl of the 5' BV primer, 2 μl of the 3' BC primer, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, 200 mM of each dNTP, and 1 μl of cDNA. The primers used for the TCR BV gene family-specific amplification were designed using a previous study [10]. Following an initial denaturing step at 94°C for 3 min, the PCR was carried out with 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

CDR3 spectratype analysis of $CD4^+$ and $CD8^+$ T cell

GeneScan analysis of TCR CDR3 spectratyping was performed as previously described [10]. In brief, the PCR products were electrophoresed on a pre-warmed 6% acrylamide sequencing gel and run for 2 h on a 50 lane Applied Biosystems model 373A DNA sequencer (Applied Biosystems, USA). The data were automatic collected and analyzed by GeneScan software version 672.

Spectratype complexity scoring

A CDR3 spectratypes complexity scoring system was used to assess the diversity of the TCR repertoire. Spectratype complexity scoring was performed as previously described [11]. Specifically, complexity scoring within a given fami-

Table 1. Baseline demographic and clinical characteristics of CRC patients (n = 26)

Characteristics	n (%)
Gender	
Male	15 (57.7)
Female	11 (42.3)
ECOG performance status- no. (%)	
0	3 (11.5)
1	23 (88.5)
Tumor Markers values- no. (%)	
CEA > 5 ng/ml	22 (84.6)
CA199 > 37 u/ml	18 (69.2)
Type of cancer- no. (%)	
Colon	13 (50.0)
Rectum	13 (50.0)
Number of metastatic sites- no. (%)	
1	6 (23.1)
> 1	20 (76.9)
Sites of metastasis	
Liver	14 (53.8)
Lung	11 (42.3)
Lymph nodes	11 (42.3)
Others	13 (50.0)
KRAS status	
Wild-type	26 (100)
Mutation	0 (0)

Table 2. Response to treatment (n = 26)

Response	n (%)
Completed response	0 (0.0)
Partial response	4 (15.4)
Stable disease	13 (50)
Disease progression	9 (34.6)

ly was determined by counting the number of peaks. Each family was given a score from 0 to 8 based on the number of peaks. The peak whose area accounted for 10% of the total peak area was included. A score of 0 was assigned to an absent family. A score of 1 was given to a family when only a single dominant peak was observed. A score of 2 was given to a family that showed biclonal peaks, and so on. Finally, a score of 8 denoted the normal spectratype of approximately eight peaks showing a complex, diverse, and polyclonal profile. The overall spectratype complexity score per sample was calculated as the summation of score of all families. A higher CDR3 score denoted a more diverse TCR repertoire.

Table 3. Toxicity

Toxicity	NCI-CTC grade			
	1	2	3	4
Hematological			9	
Anemia	4	1	10	
Leukopenia	1	3	3	
Neutropenia	1	3		
Thrombocytopenia	1	4	2	
Non-hematological				
Nausea/vomiting	4	7	1	
Mucositis	1	3	1	
Diarrhea	3	1		
Proteinuria	3	1		
Hematuria	3			

Statistical methods

Paired samples t-test were used to determine if there have differences in CDR3 complex scores between patients before treatment and three months after treatment, and also used to examine differences between CRC patients and age-matched healthy controls. Differences among patients with PR, SD and PD group post-treatment were analyzed by an one-way analysis of variance (ANOVA) and least significant difference (LSD) multiple comparison tests. *P*-values < 0.05 were considered statistically significant. All of the continuous variables are given as mean ± standard deviation. Statistical analyses were performed using the SPSS software package, version 16.0 for Windows (SPSS, Chicago, IL, USA).

Results

Efficacy

Twenty-six CRC patients age ranged from 50-69 (average 57) years screened for eligibility to participate in the present study (Supplementary Table 1). The baseline demographic and clinical characteristics of CRC patients are shown in **Table 1**. Of the 26 patients, 4 patients achieved PR, 13 patients showed SD, and 9 patients had PD. The overall response rate (ORR) was 15.4%. The treatment efficacy of the patients is shown in **Table 2**.

Adverse reactions

All patients received at least one cycle of chemotherapy and cetuximab were assessed for

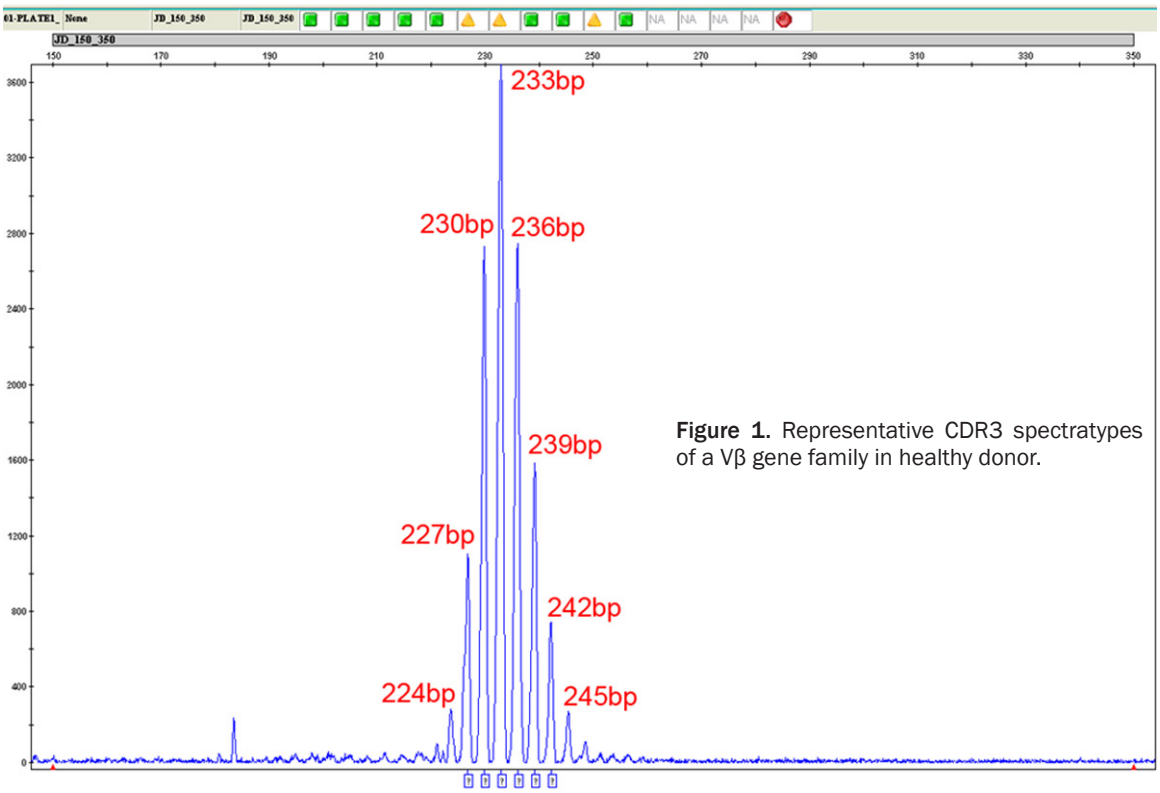

Figure 1. Representative CDR3 spectratypes of a Vβ gene family in healthy donor.

adverse events. Sixteen (61.5%) patients were received dose modifications or interruptions. The incidence of adverse events and selective non-hematological toxicity was summarized in **Table 3**. As for the hematological toxicity, the incidence of leukopenia and neutropenia were more happen. The most common grade 3 and 4 non-hematologic toxicities was nausea and vomiting. One patient with liver and lung metastasis was removed from the study because of severe nausea and vomiting. There were no other adverse events such as hypertension, bowel perforation, thromboembolic disease and treatment-related death.

CDR3 spectratypes in CRC patients

Polyclonal CDR3 spectratype with roughly or more than 8 peaks in a Gaussian distribution has been observed in overwhelming majority of TCR BV gene families in healthy controls' $CD4^+$ and $CD8^+$ T cells. Only one to six BV gene families showed skewed distribution of oligoclonal CDR3 pattern (the representative CDR3 spectratypes of a Vβ gene family in healthy donor has been shown in **Figure 1**). However, different degree of more restricted CDR3 profile in CRC patients has been detected, skewed distribution with several peaks fewer than eight or even a single peak of CDR3 spectratypes has been found in most of BV gene families in $CD4^+$ and $CD8^+$ T cells of CRC patients, as well as several families almost no peaks and being absent. Analysis of the pre-treatment CDR3 spectratype of CRC patients indicated a preferential usage of TCR BV7 and BV12 gene families within the $CD4^+$ T cell subset and BV3, BV5 and BV21 families within the $CD8^+$ T cell subset.

When compared the CDR3 spectratypes of each BV gene family in patients before treatment and three months after treatment, certain families showed oligoclonal expansion before treatment were restored to normal Gaussian distributions or to more skewed distributions post-treatment, several families absent before treatment was changed to oligoclonal pattern after treatment, and some other families showed monoclonal that was changed to oligoclonal or polyclonal pattern with Gaussian distribution after treatment. The representative result showing the $CD4^+$ and $CD8^+$ T cells' CDR3 spectratype of all 24 TCR BV gene families for CRC patients before and after treatment is shown in **Figure 2**.

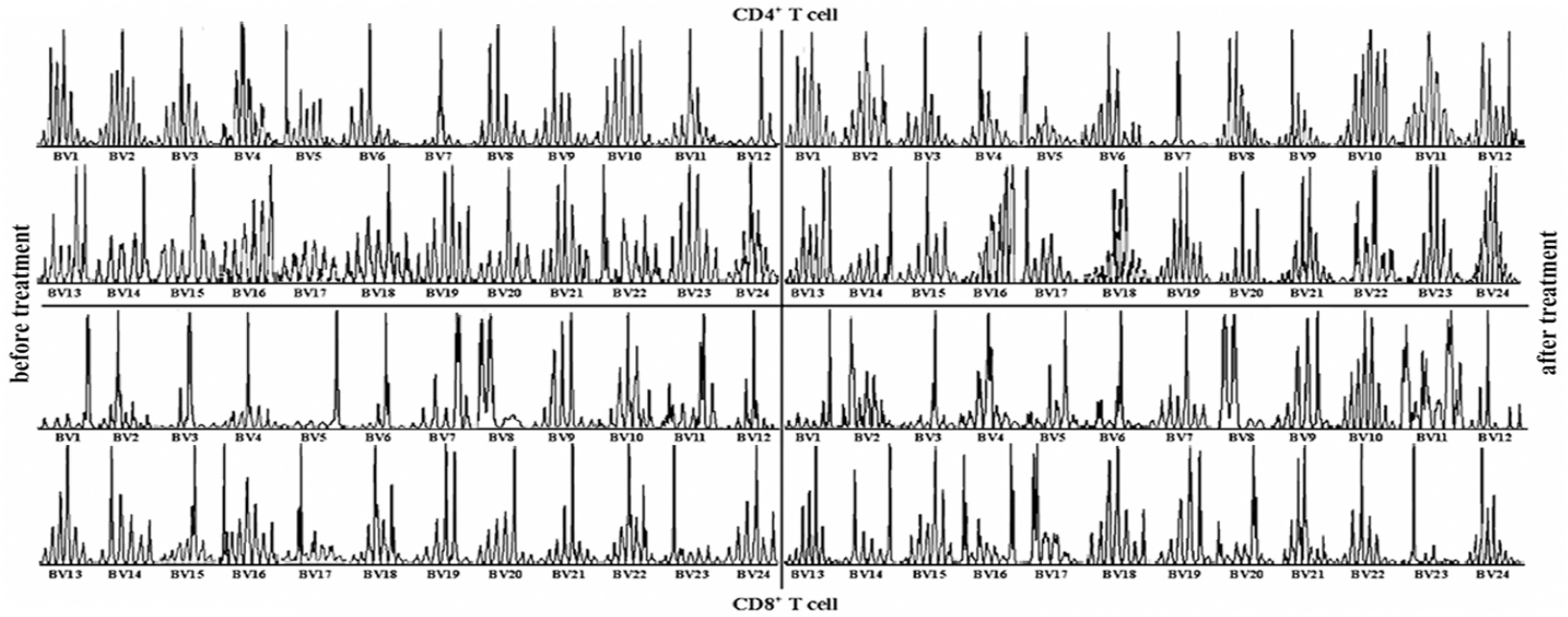

Figure 2. CDR3 spectratypes of a representative patient with partial remission before treatment and after treatment.

Table 4. CDR3 scores of patients before and 3 months after treatment

Group	Patient				Age-matched Healthy controls		Patient/healthy controls				*P*-values (pre *vs.* post)	
	Pre-treatment		Post-treatment				Pre-treatment		Post-treatment			
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
PR (n = 4)	152.2 ± 1.5	134.0 ± 3.2	148.0 ± 1.4	128.8 ± 3.1	166.0 ± 0.0	168.0 ± 0.0	0.92 ± 0.01	0.80 ± 0.02	0.89 ± 0.01	0.77 ± 0.02	.000	.095
SD (n = 13)	144.9 ± 4.6	123.9 ± 5.8	134.0 ± 8.8	116.9 ± 6.2	163.4 ± 1.5	163.3 ± 3.3	0.89 ± 0.02	0.76 ± 0.03	0.82 ± 0.05	0.72 ± 0.03	.000	.000
PD (n = 9)	145.8 ± 2.5	124.0 ± 7.1	126.3 ± 5.6	114.3 ± 9.5	163.1 ± 1.5	162.0 ± 3.7	0.89 ± 0.01	0.77 ± 0.03	0.77 ± 0.03	0.71 ± 0.05	.000	.000
P-values	.007	.019	.000	.010	.005	.016	.037	.103	.000	.055	/	/

CDR3 complexity scores of CRC patients

To obtain a uniform standard to quantify the diversity of TCR repertoire, A CDR3 scoring system was introduced. To control for the influence of age on T cell repertoire diversity, each patient was matched with a healthy control with age difference no more than 2 years. As can been seen from **Table 4**, the CDR3 scores of both $CD4^+$ and $CD8^+$ T cells in CRC patients were significantly lower than those of age matched healthy controls. And the scores of $CD4^+$ T cells were significantly higher than those of $CD8^+$ T cells in CRC patients both before treatment and after treatment.

The CDR3 scores of $CD4^+$ and $CD8^+$ T cells in CRC patients in PR group was significantly higher than that in SD and PD group. However, when the patients' CDR3 scores were calibrated by using the age matched healthy controls data (patients' CDR3 score/healthy controls' CDR3 score), there were only scores of $CD4^+$ T cells not $CD8^+$ T cells in PR group significantly higher than the other two groups. When compare the calibration data of each group before and after treatment, it can been seen that the scores of $CD4^+$ T cells pre-treatment was significantly higher than that of post-treatment in all three groups. The scores of $CD8^+$ T cells pre-treatment was also significantly higher than that of post-treatment in both SD and PD group, but have no difference in PR group.

Discussion

T cells-mediated cellular immune response plays an important role in anti-tumor immune response. The prognosis of CRC patients is directly associated with the functional disturbance of the specific cellular immune response, which is reflected by the presence of T cells both locally and systemically. The independent prognostic value of intra-tumor T cells has been reported [13, 14], low T-cells infiltration has been demonstrated associated with poor cancer-specific survival [15, 16]. However, the prognostic value of circulating T cells has never been explored in spite of clonal expansion of tumor antigen-specific T cells has also been found in peripheral blood of patients with CRCs and other solid tumors [17, 18].

When encounter tumor-expressed antigens, primed T cells will activation and clonal expansion, which result in a restricted T cell repertoire. We have previously characterized the T cell repertoire changes of metastatic CRC patients following FOLFIRI plus bevacizumab treatment [10] as well as advanced colorectal adenocarcinomas patients following FOLFIRI plus Rh-endostatin therapy [11], and found the restricted degree of TCR repertoire changed dynamically in accordance with patient conditions during treatment. To further define the TCR repertoire diversity changes following different course of CRC, and confirm its potential prognostic value, we extended the sample size of follow-up in the present study and used alternative therapy regime to investigate changes in TCR repertoires following cetuximab plus FOLFIRI therapy.

Cetuximab (also known as Erbitux®) is one of the most well-known monoclonal antibody drugs for targeted cancer therapy. Cetuximab acts by bind to the extracellular domain of the epidermal growth factor receptor (EGFR), where they prevent ligand-induced EGFR dimerization. EGFR is overexpressed in many solid tumors, which involved in signaling pathways affecting tumor cell growth, proliferation, differentiation and programmed death. It has been reported that over-expression of EGFR and the presence of mutant KRAS was associated with poor prognosis in CRC patients for lack of response to EGFR inhibitors [19, 20]. So it has been stating that all CRC patients who are candidates for EGFR antibody therapy should tested for KRAS mutations, and persons with KRAS mutation should not receive EGFR antibody as part of their treatment [21]. However, even among patients with wild-type KRAS, the response rate to EGFR monoclonal antibodies has been reported less than 20% [22]. The combination of cetuximab and chemotherapy drugs showed improved response rates and an increased median time to disease progression compared with chemotherapy alone [23]. In this study, we evaluated the efficacy and safety of cetuximab plus FOLFIRI for carry wild-type KRAS patients with irinotecan and oxaliplatin-refractory advanced colorectal cancer. The therapy regime has been shown to be effective that achieving an objective response rate of 15.4% and overall disease control rate of 65.4%, both of which are higher than our previous treatment effect-from FOLFIRI plus Rh-endostatin therapy or FOLFIRI therapy alone [11].

It is not surprising that CDR3 scores post-treatment were lower than pre-treatment, which suggest that the restricted T cells repertoire maybe result from not only tumor-associated antigen primed but also side effects of chemotherapy drug. The two main subsets of T cells are $CD4^+$ T cells and $CD8^+$ T cells, the former accounted for approximately 65% of total T cells. The role of $CD4^+$ Th in anti-tumor immune response has been largely considered to providing regulatory signals required for priming of CD8 CTL, which can serve as the dominant effector cells mediated killing of malignant cells. It has been found that $CD4^+$ T cells play a far broader role in the orchestration of host immune response [24]. Maybe this is why the TCR repertoire diversity of $CD4^+$ T cells was significantly higher than that of $CD8^+$ T cells in CRC patients. A more diversity rather than restrict $CD4^+$ T cell repertoire may protect tumor patients against pathogens infection. The diversity of $CD4^+$ T cells in PR group was significantly higher than that of SD and PD groups, and the difference was enlargement after treatment. These results are consistent with our previous findings. While the corrected data based on healthy controls have not showing any difference of the diversity of $CD8^+$ T cells among the three groups, even though the data in PR group was obvious higher than that of SD and PD groups. Therefore, we presumed that the diversity of $CD4^+$ T cell repertoire reflected the overall defense of the body, which might have more important prognostic value. A higher diversity of $CD4^+$ T cell repertoire is likely brought about a better prognosis.

Conclusions

We analyzed the changes of T cell repertoire diversity before and after cetuximab plus FOLFIRI therapy for advanced CRC patients. The results showing that the diversity of $CD4^+$ T cells in PR group was significantly higher than that of SD and PD groups, and the difference was enlargement after treatment. The diversity of $CD8^+$ T cells in PR group has no difference before and after treatment, but significant decrease in SD and PD group after treatment. These results suggest that the TCR repertoire diversity were highly correlated with the treatment efficacy of Erbitux in combination with FOLFIRI and analysis the diversity of T cell repertoire have an important prognosis value for CRC patients.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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Supplementary Table 1. Patient characteristics and CDR3 score

Case No.	Sex	Age	Diagnose of cancer			Tumor markers				ECOG score		Imaging Evaluation after treatment	CDR3 score				Age-match healthy's CDR3 score	
			Type of cancer	KRAS status	Sites of metastasis	Before-treatment		After-treatment		Base-line	After treatment		$CD4^+$ T cells		$CD8^+$ T cells			
						CEA (ng/ml)	CA199 (u/ml)	CEA (ng/ml)	CA199 (u/ml)				Base-line	After treatment	Base-line	After treatment	$CD4^+$	$CD8^+$
1	male	69	Rectal	wild-type	Lung; lymph nodes	5.3	23.9	6.2	27.1	1	2	SD	136	127	113	109	160	158
2	male	50	Colon	wild-type	Liver; others	2.9	> 240.0	1.9	208.0	1	1	SD	151	147	128	119	166	168
3	female	53	Rectal	wild-type	Liver; lymph nodes	37.5	155.8	11.7	88.9	0	1	PD	149	131	127	115	166	168
4	male	56	Colon	wild-type	Liver; lymph nodes	9.3	88.6	16.2	186.6	1	1	PD	143	131	137	132	164	165
5	male	61	Colon	wild-type	Liver	120.7	> 240.0	148.7	> 240.0	1	1	PD	145	119	123	110	162	159
6	female	57	Rectal	wild-type	Lung; lymph nodes	30.7	> 240.0	36.0	> 240	1	1	SD	147	136	123	116	164	165
7	male	58	Colon	wild-type	Lung; liver; others	7.8	24.1	13.6	26.7	1	1	SD	143	136	130	124	164	165
8	male	50	Rectal	wild-type	Lung; liver	28.9	59.7	30.2	40.5	1	1	PR	151	147	133	127	166	168
9	female	59	Rectal	wild-type	Liver; lymph nodes	207.3	11.2	117.3	10.5	1	1	SD	145	141	123	110	162	159
10	male	51	Rectal	wild-type	Lung; others	3.0	8.1	2.5	9.0	0	0	PR	151	147	130	129	166	168
11	male	62	Colon	wild-type	Liver	62.8	44.1	42.7	45.8	1	1	SD	145	130	127	121	162	159
12	male	55	Rectal	wild-type	Lung	5.7	37.9	4.6	44.2	1	1	PD	146	131	125	113	164	165
13	female	52	Colon	wild-type	Liver; others	9.1	25.9	7.0	24.1	1	1	PR	153	148	137	126	166	168
14	female	63	Colon	wild-type	Lung; liver; others	0.9	8.7	1.1	10.9	1	2	PD	148	121	113	96	162	159
15	male	57	Rectal	wild-type	Lung	3.5	48.7	4.3	51.4	1	1	SD	152	149	120	117	164	165
16	female	61	Rectal	wild-type	Lymph nodes; others	5.9	49.8	4.7	39.1	1	1	SD	143	125	118	111	162	159
17	male	61	Colon	wild-type	Lymph nodes; others	73.8	63.7	66.1	58.9	1	1	PD	145	133	121	118	162	159
18	female	60	Rectal	wild-type	Lung; liver; others	75.2	65.3	81.8	70.2	1	2	PD	145	127	120	116	162	159
19	male	56	Colon	wild-type	Lymph nodes; others	14.5	149.8	11.9	127.8	1	1	SD	145	134	129	123	164	165
20	female	59	Rectal	wild-type	Liver; others	5.3	> 240.0	4.5	89.3	1	1	SD	137	119	118	109	164	165
21	female	51	Colon	wild-type	Lymph nodes; others	14.8	> 240.0	3.5	81.3	0	0	PR	154	150	136	133	166	168
22	male	55	Rectal	wild-type	Lymph nodes; others	61.2	34.1	207.3	7.5	1	1	SD	147	138	121	112	164	165
23	male	58	Colon	wild-type	Lung; liver	58.1	> 240.0	86.9	> 240.0	1	1	PD	149	119	131	119	164	165
24	female	58	Colon	wild-type	Liver	9.1	> 240.0	4.7	76.2	1	1	SD	146	135	128	123	164	165
25	male	60	Rectal	wild-type	Lung	47.1	> 240.0	74.2	> 240.0	1	1	PD	142	125	119	110	162	159
26	female	57	Colon	wild-type	Lymph nodes; others	6.3	5.9	6.7	9.1	1	1	SD	147	125	133	126	164	165