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Original Article

Molecular profiling analysis for colorectal cancer patients with Pi-Xu or Shi-Re syndrome

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ABSTRACT

Background: Traditional Chinese medicine (TCM) syndromes (*ZHENG* in Chinese) constitute the basis of understanding the disorders of patients and guiding the use of the Chinese herbs. Colorectal cancer is divided into various subtypes mainly according to the *ZHENG* identification.
Objective: We aimed to determine the molecular basis underlying *Pi-Xu* (spleen deficiency) and *Shi-Re* (dampness-heat) *ZHENG* that are commonly found in colorectal cancer patients.
Methods: About 80 colorectal cancer patients, including 47 *Pi-Xu ZHENG* and 33 *Shi-Re ZHENG* were enrolled. Blood and tissue samples of these patients were available for protein and mRNA expression. The protein expression was determined by Immunohistochemistry (IHC) staining and mRNA profiling was detected by expression microarray. Furthermore, mRNA fold change was evaluated by qRT-PCR.
Results: The colorectal cancer patients with *Shi-Re ZHENG* had a poor prognosis, compared with *Pi-Xu ZHENG* (95% CI: 0.05–0.33; $p < 0.0001$). Moreover, there was a significant difference in protein expression levels (especially for mutant TP53, PCNA, PD-L1 and Ki-67) among *Pi-Xu* and *Shi-Re ZHENG* ($p < 0.01$). Meanwhile, mRNA expression (especially for wild type TP53, KDM6A, PCNA, PD-L1, Ki-67, CCL-2, IL-1a and COX-2) was also remarkably different between *Pi-Xu* and *Shi-Re* groups ($p < 0.01$).
Conclusion: Our results suggest that *Shi-Re ZHENG* conditions may contribute to poor overall survival in patients with colorectal cancer. Compared with *Pi-Xu ZHENG*, high expression of mutant TP53, PCNA, PD-L1, Ki-67, CCL-2, IL-1a and COX-2 may serve as potential biomarkers for diagnosis and prognosis of colorectal cancer patients displayed *Shi-Re ZHENG*.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of death in cancer patients around the world.[1] However, mortality associated with a CRC diagnosis has declined progressively in the past decades, which may be attributed to cancer screening programs, improved surgical techniques and the availability of more-effective therapeutic strategies for early-stage and advanced-stage disease.[2] After curative treatment, however, 30% of patients with stages I–III and up to 65% of patients with stage IV CRC develop recurrent disease.[3] Therefore, identifying efficient prognostic factors is urgently needed to enable the stratification of patients with CRC into different prognostic subgroups and subsequently improve outcomes after cancer recurrence.

Traditional Chinese Medicine (TCM), a form of complementary medicine that emphasizes bringing the patient's body, mind,

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and spirit into harmony, has been widely used for treatment of tumor patients including CRC in China.[4] TCM rests squarely on *ZHENG* (syndrome differentiation) diagnosis, a process of analyzing and synthesizing data collected through four combined diagnostic methods: *WANG* (inspection), *WEN* (falling-rising tone, auscultation, and olfaction), *WEN* (falling tone, inquiry), and *QIE* (palpation).[5] *Pi-Xu* (spleen deficiency) and *Shi-Re* (dampness-heat) are two common *ZHENG* in CRC patients.[5] However, the impacts of these two patterns of *ZHENG* differentiation on colorectal cancer prognosis and their molecular basis are poorly understood.

The association between different *ZHENG* in the prognosis or biological characteristics of CRC has never been explored. Here, we for the first time showed that the colorectal cancer patients with *Shi-Re ZHENG* had a poor prognosis, compared with *Pi-Xu ZHENG*. Therefore, we demonstrated that there was a significant difference in protein and mRNA expression levels (especially for mutant TP53, PCNA, PD-L1, and Ki-67) among *Pi-Xu* and *Shi-Re ZHENG*, which could serve as potential biomarkers for diagnosis and prognosis of colorectal cancer patients displayed *Shi-Re ZHENG*.

2. Methods

2.1. Study subjects

This research protocol (No. 2018-0012) was approved by the local medical ethics committee of Hangzhou Normal University, based on the Declaration of Helsinki. A total of 80 CRC patients who underwent surgery were consecutively recruited from January 2012 to July 2017 in Hangzhou, Zhejiang, China. All subjects were genetically unrelated ethnic Han Chinese.

2.2. Diagnostic criteria

Diagnoses of all of the patients were confirmed by pathology. Trained interviewers used a uniform questionnaire to collect the TCM diagnostic information from the participants, namely, demographic factors such as age and gender, and known risk factors for CRC (including drinking, diet habit, individual disease history, marriage, and birth history). The standard criteria used for classification of CRC *ZHENG* were as described previously.[6]

Since many factors may affect the formation of TCM syndromes, more than one TCM syndrome was observed in the majority of patients. To ensure a uniform and standard CRC *ZHENG*, the most significant TCM syndromes functioned as units, which were worked out concurrently by two TCM clinical experts.

2.3. Inclusion criteria

Advanced colorectal cancer patients meet criterions of western medicine and TCM and the following characteristics were included in the study: (a) aged between 18 and 80 years, (b) Han Chinese ethnicity, (c) newly histopathologically diagnosed with primary CRC, (d) lack of previous malignant tumors in other organs, (e) had not had antitumor therapy before recruitment, including chemotherapy and radiotherapy, and (f) did not have severe heart failure, pulmonary insufficiency, or kidney disease.

2.4. Exclusion criteria

Patients with jejunum tumor, appendix tumor, colorectal adenoma, E. stromal tumor, large intestine malignant melanoma, and large intestine leiomyosarcoma and cases without pathological diagnosis and completed data were excluded. Patients who received pre-operative chemotherapy were excluded.

2.5. CRC sample preparation

CRC tissue samples were obtained from operation, and serum samples were purified through centrifugation of blood (3000 rpm, 10 min). Supernatant was collected and stored at −80°C until further analysis. Serum levels of embryonic antigen (CEA) and lactate dehydrogenase (LDH) were determined at the time of examination in our biochemical laboratory. Serum mRNA expression profiles were detected.

2.6. Antibodies

p53 antibody (DO-1): sc-126 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD-L1 (E1L3N®) (#13684) and Ki-67 (8D5) (#9449) antibody were obtained from Cell Signaling (Beverly, MA, USA). Anti-PCNA antibody (ab29) was obtained from Abcam.

2.7. Immunohistochemistry staining

The ChemMate EnVision Detection Kit (DAKO, Carpinteria, CA, USA) was used for immunohistochemistry according to company's recommended procedure. Briefly, after being deparaffinized and hydrated, the paraffin-embedded sections were placed in 0.01M sodium citratebuffer (pH 6.0), and subjected to pressure cooker treatment for 2 minutes at full pressure with a domestic pressure cooker. After cooling to room temperature, the slides were rinsed with Tris buffered saline (0.05 M Tris/0.15 M NaCl, pH 7.6). The endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide. The sections were incubated with the primary antibody overnight at 4°C. Then, the ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent was applied to the sections, followed by application of ChemMate DABt Chromogen included in the kit. The slides were lightly counterstained with hematoxylin. The area of positive cancer cells in each microscopic field was categorized as follows: 1+, 0% to 25%; 2+, 25% to 50%; 3+, 50% to 75% or 4+, 75% to 100%. The sum from 0% to 42% was assigned as "low expression." The sum from 43 to 80 was assigned as "high expression."

2.8. Gene expression profiling and data processing

Gene expression profiling was performed at the Genomic and Proteomics Core Facilities, German Cancer Research Center, Heidelberg, Germany.[7] A total of 200 ng RNA was analyzed by using HumanHT-12 Expression BeadChips (Illumina) according to the manufacturer's instructions. Samples were run in chronological order in which patients received tumor surgery. Paired samples from the same patient (SAT/VAT) were processed in the same batch for most samples. Raw data were transformed by using variance-stabilizing transformation and normalized with robust spline normalization. Possible batch effects were adjusted with ComBat.[8] All preprocessing steps were carried out in the statistical software R 3.1.0 (www.r-project.org) with the lumi and sva packages.[9,10]

2.9. qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Genomic DNA was removed using gDNA Eliminator spin columns (Qiagen). Total RNA was reverse-transcribed into cDNA using the SuperScript® III Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) analyses were performed using SYBR Green (Roche) on an ABI-7500 detector (Applied BioSystems). Relative gene expression levels were normalized to the internal control GAPDH.

Gene-specific primers were listed as follows: The primers for quantitative real time PCR (qRT-PCR) analysis were listed as follows:

KDM6A-F 5′-TGGAAACGTGCCTTACCTG-3′
KDM6A-R 5′-TGCCGAATGTGAACTCTGAC-3′
PD-L1-F 5′-TGTGACCAGCACACTGAGAA-3′
PD-L1-R 5′-AGTCCTTTCATTTGGAGGATGT-3′
CCL-2-F 5′-TGGGTTCACGATTCCATGGA-3′
CCL-2-R 5′-GGTTGTGGAGTGAGTGTTCAAGTC-3′
IL-1a-F 5′-GAAAGTGTGCATGGAGGAAACC-3′
IL-1a-R 5′-TGGGAATGGGACGCAGTT-3′
COX-2-F 5′-ATCAGAACCGCATTGCCTCT-3′
COX-2-R 5′-GCCAGCAATCTGTCTGGTGA-3′
GAPDH-F 5′-GCCTCAAGATCATCAGCAAT-3′
GAPDH-R 5′-TTCAGCTCAGGGATGACCTT-3′

2.10. Statistical analyses

All data are expressed as mean ± Std. Statistical differences were measured using an unpaired two-sided Student *t*-test or one-way ANOVA for multiple comparisons when appropriate. The two-tailed χ^2 test was used to analyze the association of protein expression with clinicopathological parameters. $p < 0.05$ was defined as statistically significant. All data analyses were conducted with GraphPad Prism Software Version 7 (GraphPad, San Diego, CA). Survival analyses were conducted using the Kaplan–Meier method and compared with a log-rank test.

3. Results

3.1. Characteristics of the Study Subjects

A total of 80 CRC patients were included in this study. Gender, age, and *ZHENG* distribution of subjects are shown in Table 1. However, there was no significant differences between gender, age, smoking and drinking status ($p > 0.05$).

3.2. ZHENG distribution and CRC patient prognosis

Pi-Xu and *Shi-Re* are two common *ZHENG* in CRC patients. *Pi-Xu* is the intrinsic weakness that means asthenia in origin. *Shi-Re ZHENG* refers to the excess in superficiality that represent the other side of CRC pathogenesis. Currently, the association between different *ZHENG* in the prognosis of CRC has never been explored. In this study, we evaluated whether different *ZHENG* types had an impact on CRC prognosis. As shown in Fig. 1, we found that the colorectal cancer patients with *Shi-Re ZHENG* had a poor overall survival, compared with *Pi-Xu ZHENG* (95% CI: 0.05–0.33; $p < 0.0001$).

Table 1
Characteristics of 80 Cases of Colorectal Cancer Patients

	Different syndromes		*p*-value[†]
	Pi-Xu Zheng ($n = 47$)	Shi-Re Zheng ($n = 33$)	
Gender			
Male	26 (55.32%)	17 (51.52%)	
Female	21 (44.68%)	16 (48.48%)	0.8212
Age			
<60	20 (42.55%)	15 (45.45%)	
≥60	27 (57.45%)	18 (54.55%)	0.8225
Smoking			
No	23 (48.94%)	14 (42.42%)	
Yes	24 (51.06%)	19 (57.58%)	0.6511
Drinking			
No	25 (53.19%)	13 (39.39%)	
Yes	22 (46.81%)	20 (47.62%)	0.2607
High fat diet			
No	21 (44.68%)	11 (33.33%)	
Yes	26 (55.32%)	22 (66.67%)	0.359
Family history			
No	46 (97.87%)	31 (93.94%)	
Yes	1 (2.13%)	2 (6.06%)	0.5658

[†] χ^2 test was used.

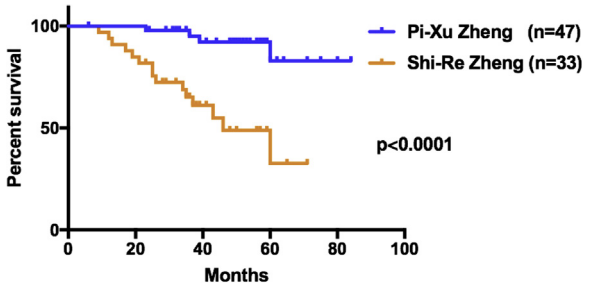

Fig. 1. The prognosis of CRC patients with *Pi-Xu* and *Shi-Re ZHENG* types.

Table 2
Correlation Between Clinic Pathological Background and Different Traditional Chinese Medicine Syndromes in 80 Cases of Colorectal Cancer Patients

		Different syndromes		*p*-value
		Pi-Xu Zheng ($n = 47$)	Shi-Re Zheng ($n = 33$)	
TP53 (mutant)	High	12 (25.53%)	26 (78.79%)	
	Low	35 (74.47%)	7 (21.21%)	<0.001**
PCNA	High	18 (38.30%)	22 (66.67%)	
	Low	29 (61.70%)	11(33.33%)	0.0125*
PD-L1	High	15 (31.91%)	25 (75.76%)	
	Low	32 (68.09%)	8 (24.24%)	0.0001**
Ki-67	High	15 (31.91%)	28 (75.76%)	
	Low	32 (68.09%)	5 (24.24%)	0.0001**

χ^2 test was used. *$p < 0.05$; **$p < 0.01$.

3.3. Analysis of protein expression profiles in Pi-Xu and Shi-Re groups of CRC patients

To determine the molecular expression of CRC patients with *Pi-Xu* and *Shi-Re ZHENG*, the protein expression of TP53, PCNA, PD-L1 and Ki-67 was detected by immunohistochemistry, as well as the p53 mutation status by direct DNA sequencing. The correlations between clinicopathological signatures of the patients with CRC and protein expression are shown in Table 2. Among these patients, mutant TP53, PCNA, PD-L1 and Ki-67 were highly expressed in CRC patients with *Shi-Re ZHENG*, compared with *Pi-Xu ZHENG* ($p < 0.01$) (Fig. 2), which indicated that these protein expressions were remarkably correlated with *Shi-Re ZHENG* in CRC.

3.4. Analysis of mRNA expression profiles in Pi-Xu and Shi-Re groups of CRC patients

Next, we used gene expression array to examine mRNA expression of the three blood samples of CRC from the *Pi-Xu* and *Shi*-Re groups. As shown in Fig. 3A, we found that there was a significant difference in mRNA expression among CRC from *Pi-Xu* and *Shi*-Re groups. The higher mRNA levels of wild type TP53 and KDM6A were found in *Pi-Xu* groups but not *Shi*-Re *ZHENG* types ($p < 0.01$). Meanwhile, the higher mRNA levels of PCNA, PD-L1, Ki-67, CCL-2, IL-1a and COX-2 were found in *Shi*-Re groups but not *Pi-Xu ZHENG* types ($p < 0.01$). We also examined the expression of KDM6A, PD-L1, CCL-2, IL-1a and COX-2 by qRT-PCR. Our results suggested that the *Shi-Re* group expressed the highest levels of PD-L1, CCL-2, IL-1a and COX-2 and the lowest levels of KDM6A ($p < 0.01$) (Fig. 3B–F). Therefore, some tumor-driving genes (PCNA, PD-L1 and Ki-67) and inflammatory cytokines (CCL-2, IL-1a and COX-2) were found higher

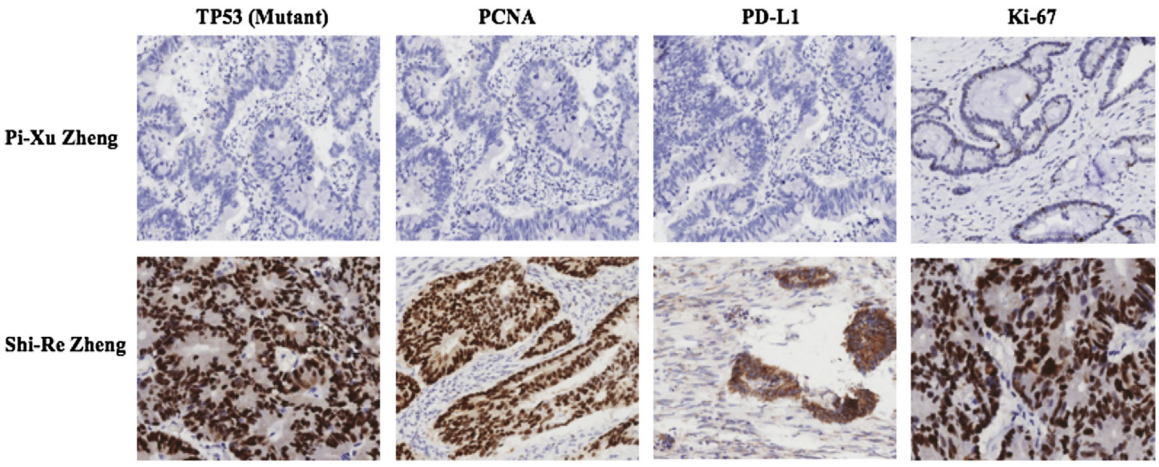

Fig. 2. Immunohistochemistry of TP53, PCNA, PD-L1 and Ki-67 in serial sections of CRC specimens with *Pi-Xu* and *Shi-Re ZHENG*.

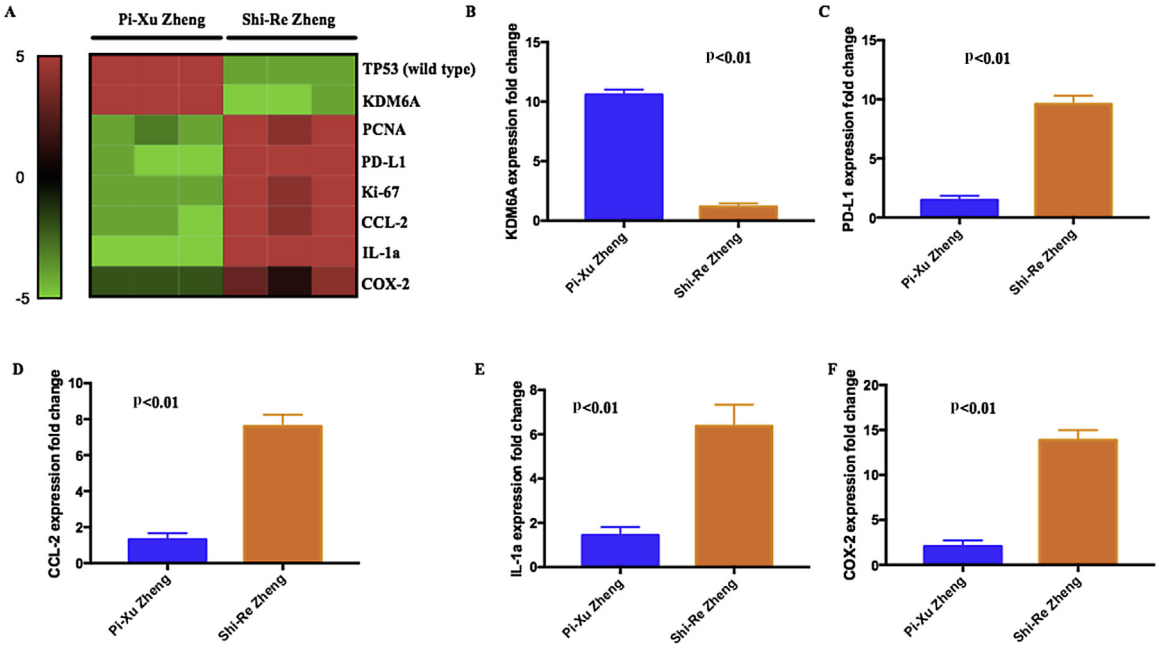

Fig. 3. The molecular basis of *Pi-Xu* and *Shi-Re ZHENG* in CRC. (A) mRNA expression profiles of blood samples of CRC from the *Pi-Xu* and *Shi-Re* groups are detected by gene expression array; (B) mRNA expression levels of KDM6A, PD-L1, CCL-2, IL-1a, and COX-2 are confirmed by qRT-PCR.

expression in *Shi-Re* groups but not *Pi-Xu ZHENG* types, which indicated that the mRNA expression profiles of *Shi-Re ZHENG* in CRC were significantly different from other *ZHENG* types and the CRC patients with *Shi-Re ZHENG* may have a poor prognosis.

4. Discussion

TCM is a popular complementary treatment which has been widely used to improve clinical symptoms and prolong the survival rate of colorectal cancer. *ZHENG*, also known as TCM syndrome, is an integral and essential part of TCM theory.[11] In TCM practice, the clinical diagnosis of *ZHENG* relies on the gathering of clinical information through inspection, auscultation, olfaction, inquiry, and palpation. TCM *ZHENG* is the basis of understanding the disorders of patients and the most essential principle that guides the prescription of Chinese herbal formulae.[12,13] However, there is still not a clear understanding of ZHENG-specific molecules and their effects on CRC.

Although great progresses have been made for CRC treatment in the past few years, the overall survival of CRC is still low due to cancer development. Various signaling pathways have been implicated during the CRC carcinogenesis and tumor progression. The tumor suppressor p53 is widely known for its potential to induce cell death or cell cycle arrest and could, thereby, prevent neoplastic progression.[14] X-linked lysine demethylase 6A (KDM6A, also known as UTX) is an epigenetic regulator that positively regulates gene expression programs associated with cell proliferation and invasion.[15] The programmed death-1 (PD-1), a coinhibitory receptor expressed on activated T cells and B cells, plays a critical role in tumor initiation and development. The cancer patients harboring PD-1 or PD ligand 1 (PD-L1) protein expression have often a poor prognosis and clinical outcome.[16] The proliferation-associated transcription factors (PCNA and Ki-67) and some inflammatory cytokines (CCL-2, IL-1a and COX-2) are also demonstrated to facilitate cancer cell proliferation and progression.[17,18] In recent years, there was a dramatic increase in the total number of publications reporting the concept of TCM ZHENG in cancer and their

molecular basis. However, the validity, prognosis, and the molecular basis of *ZHENG* is poorly understood. Therefore, a better understanding of TCM *ZHENG*, as well as its potential biological basis, will greatly contribute to the clinical diagnosis and the treatment of CRC patients.

Pi-Xu (spleen deficiency) and *Shi-Re* (dampness-heat) are two common *ZHENG* in CRC patients. In this research, we evaluated the impact of these two *ZHENG* types on CRC prognosis and found CRC patients with *Shi-Re ZHENG* had a poor overall survival, compared with *Pi-Xu ZHENG*. To determine the possible molecular mechanisms underlying different TCM *ZHENG*, we used Immunohistochemistry staining, gene expression array and qRT-PCR to examine protein and mRNA expression of CRC patients from the *Pi-Xu* and *Shi-Re* groups. Our results indicated that some genes or cytokines were specifically changed in CRC patients with *Shi-Re ZHENG* but not other *ZHENG* types, including KDM6A, mutant TP53, the proliferation-associated transcription factors (PCNA and Ki-67) and some inflammatory cytokines (CCL-2, IL-1a and COX-2). Our study will hopefully provide a promising approach for the diagnosis and prognosis of CRC patients with different *ZHENG* differentiation.

Data availability statement

All the data used to support the findings of this study are available from the corresponding author upon request.

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Conflicting interest

The authors declare no conflict of interest.

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