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Predicting Adjuvant Chemotherapy Outcome by Simultaneous Analysis of Thymidylate Synthase Expression and p53 Nuclear Accumulation in Colorectal Cancer

Zihuan Yang[1,2*], Dandan Huang[2*], Lekun Fang[2], Xingzhi Feng[2], Huanliang Liu[1,2], Lei Wang[1,2], Jianping Wang[1,2#]

[1]Guangdong Institute of Gastroenterology, Guangzhou, China
[2]Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Diseases, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
Email: [#]wjp@mail.sysu.edu.cn

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Abstract

Studies have shown that the tumor suppressor gene p53 may regulate thymidylate synthase (TS) activity in colorectal cancer (CRC) cells, hence attributed to chemo-resistance to 5-flurouracil in CRC. In this study, a total of 299 primary CRC patients who underwent surgery alone or received an adjuvant 5-FU-based chemotherapy were retrospectively studied. TS expression and p53 nuclear accumulation on paraffin embedded primary tumor tissue arrays were immunohistochemically assessed, and their relationship to patient overall survival (OS) and disease free survival (DFS) were analyzed. No correlation was found between TS and p53 expression. p53 nuclear accumulation was significantly correlated with tumor location. In all, multivariate analysis shows that TNM stage is a good indicator of patient survival. TS or p53 is not an independent prognostic or predictive factor in the CRCs. In chemotherapy-treated group, simultaneous analysis of TS and p53 indicates patients in the p53−/TS− or p53+/TS+ group have significant better OS and DFS than the group p53−/TS+ or p53+/TS− ($P < 0.01$). Thus, our study suggests that simultaneous evaluation of both TS and p53 can help to predict the therapeutic effect of CRCs with 5-FU-based adjuvant chemotherapy.

[*]Authors contribute equally to this work.
[#]Corresponding author.

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Keywords

Colorectal Cancer, p53, TS, 5-FU, Chemotherapy

1. Introduction

Colorectal cancer ranks the third most common cancer globally. Over 1.2 million new cases and 608,700 deaths were estimated to have occurred in 2008 [1]. Surgery with postoperative adjuvant chemotherapy remains the standard treatment for high-risk stage II disease and advanced CRC. Addition of 5-fluorouracil (5-FU), which is the first-line chemotherapy drug, may improve three-year disease-free survival by approximately 25%, but there are still 30% of colorectal cancer patients will relapse [2].

Identifying molecular markers that can predict clinical outcomes would be of most importance to optimize individual therapy for CRC patients. The mechanism of cytotoxicity of 5-FU has been partially ascribed to the inhibition of the nucleotide synthetic enzyme TS, which consequently induces p53-dependent apoptosis [3]. TS and p53 are most studied markers that are considered to be of potential prognostic and predictive value in CRC. However, controversy remains about their role in colorectal cancer. Studies have indicated that high expression of TS contributes to a favorable prognosis and higher sensitivity to 5-FU [4] [5]. While other groups report opposite results or no correlation between TS levels and the prognosis of CRC [6]-[8]. A number of clinical studies have found that p53 over expression is correlated with patient survival and resistance to 5-FU [9] [10]. However, no clear evidence has proved the value of routine analysis of p53 status to predict CRC treatment response. It is still uncertain that whether p53 alteration will affect the clinical outcome of CRC [11] [12]. Interestingly, studies have demonstrated an interaction between TS and p53. Wild-type p53 can inhibit TS promoter activity [13], whereas, TS can repress p53 translation and result in the evasion of cell apoptosis [14].

In an effort to investigate the relationship of TS and p53, and their potential role as a prognostic or predictive factor, we analyze TS expression and p53 nuclear accumulation in colorectal cancer from 299 patients treated with surgery alone or with adjuvant 5-FU-based chemotherapy.

2. Materials and Methods

2.1. Patients

The patient cohort consisted of 299 patients, who underwent tumor resection between 2001 and 2006 at the First Affiliated Hospital of Sun Yat-sen University (SYSU). Among them, 75 (25.1%) patients received adjuvant 5-FU-based chemotherapy. Patients who received preoperative chemotherapy and/or radiotherapy were excluded from this study. Clinicopathologic data and follow-up data of enrolled patients were maintained by specialists. The study was approved by the Ethics Review Board of the Sixth Affiliated Hospital of SYSU. A written informed consent from each patient regarding tissue sampling had been obtained.

2.2. Tissue Microarrays Construction and Immunohistochemistry.

The paraffin-embedded tissue blocks and the corresponding histological H&E stained slides were overlaid for tissue TMA sampling. Duplicates of 1 mm diameter cylinder were punched from representative tumor areas of individual donor tissue block and re-embedded into a recipient paraffin block at a defined position using a tissue-arraying instrument (MiniCore, ALPHELYS, France).

Immunohistochemical staining was performed using the Polink-2 plus® Polymer HRP Detection System (GBI, WA, USA) according to the manufacturer's instructions. After deparaffinization in xylene and rehydration through a graded alcohol series, slides were transferred to sodium citrate buffer (Beijing Dingguo Changsheng Biotech Co. Ltd, #AR-0511, China) for 15 min in the microware and left at room temperature for 30 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 10 min at room temperature, then the slides were incubated with primary antibody to human TS (1:100, Proteintech #15047-1-AP, USA) or p53 (1:50, CST #2527, USA) overnight, respectively. The p53 (7F5) rabbit monoclonal antibody detects endogenous levels of both wild-type and mutant human p53 protein. Slides were washed three times with phosphate-buffered saline (PBS) and incubated with Polymer Helper (reagent 1, Polink-2 plus® supply) and Poly-HRP anti-Goat IgG (rea-

gent 2, Polink-2 plus® supply) for 30 min. Then the slides were stained with DAB and counterstained with hematoxylin. For negative control, isotype-matched antibodies were applied. Staining results were reviewed and scored independently by two pathologists (Y.B. and L.F).

For p53 nuclear staining, cells were assessed according to the proportion of nuclear positive cells. The staining pattern was graded from 0 to 4, with 0 being no staining; 1 when < 25% of the cell nuclei stained positive; 2 when 25% - 50% of cell nuclei stained positive; 3 when 50% - 75% cells displayed nuclear staining; and 4 when >75% cells displayed nuclear staining.

To evaluate TS expression level, each slide was assigned a score for intensity and staining positive pattern. The percentage of positive tumor cells as follows: 1 (up to 25% of positive cells), 2 (25% - 50% of positive cells), 3 (50% - 75% of positive cells) and 4 (>75% of positive cells). Intensity scores ranged from 0 - 3: 0, no staining; 1, weak; 2, moderate, 3, strong. Multiplication of the two scores resulted in a final score ranging from 0 to 12.

2.3. Statistical Analysis

Overall survival (OS) was calculated from the date of surgery to the date of death or the last follow-up time if follow-up was more than 5-years. For disease-free survival (DFS), an event was defined as the first clinical or pathologic evidence of local or distant recurrence.

Receiver Operation Characteristic (ROC) curve analysis was applied to determine the cut off point for tumor "high expression" by using the 0, 1-criterion. Under this condition, a score value of 8 was adopted as cut-off for stratification of TS expression into low (≤8, TS−) and high (>8, TS+); for p53 analysis, tumors were classified as p53-positive (p53+) if 50% or more cells demonstrated p53 nuclear accumulation (score > 2).

The relationship between TS, p53 and clinicopathologic features of CRC patients were analyzed by χ^2-test. The Kaplan-Meier method was used for the univariate survival analysis, and the differences between compared groups were assessed by the log-rank test. The Cox proportional hazards regression model was used to compare OS and DFS between marker categories and to obtain risk ratios. All statistical analyses were performed using SPSS software version 16 (Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant (bilateral).

3. Results

3.1. TS Expression, p53 Nuclear Accumulation and Patient Characteristics

IHC stainings were assessed using anti-p53 and anti-TS antibodies (**Figure 1(C)**). In the entire cohort of 299 patients (treated and untreated), 175 (58.5%) showed high TS expression (TS+) and 138 (46.2%) showed high p53 nuclear accumulation (p53+). The TS and p53 levels were correlated with the following parameters: sex and age of patients, tumor stage and location, and adjuvant chemotherapy. Positive p53 was seen more frequently in tumors located in the rectum compared to those in the colon (52.8% vs. 38.4%, $P = 0.013$). No other statistically significant associations were observed (**Table 1**).

3.2. Association between TS Expression, p53 Nuclear Accumulation and Prognosis

The expressions of TS and nuclear staining of p53, as well as other clinicopathologic factors were further examined by Cox regression analysis. Univariate analysis in all patients indicated a significant association between TNM stage and the survival of patients ($P < 0.001$ for both OS and DFS). Tumor located in rectum was associated with a significantly worse OS ($P = 0.004$) and DFS ($P = 0.004$). These were confirmed in a multivariate analysis, which revealed that TNM stage and tumor site were independent prognostic factors for CRC patients (**Table 2**). The significant interaction between tumor location and patient survival may attribute to higher percentage of tumors in rectum exhibited positive p53 nuclear staining. No significantly association between combined phenotypes of TS/p53 and DFS or OS.

3.3. Combined Effect of p53 and TS on the Survival of Chemotherapy-Treated Patients

In order to assess the relationship of TS and p53 level with treatment outcome, patients with 5-FU based chemotherapy were analyzed separately. In the chemotherapy-treated patients, 47 (72.3%) and 33 (44.0%) of 75

Table 1. Association of clinical annotations to TS and p53 level in all CRC patients.

Parameter	n	p53 nuclear accumulation			TS expression		
		Low	High	*P*	Low	High	P
All patient	299	161	138		124	175	
Sex				0.307			0.610
Male	166	94	72		71	95	
Female	136	69	67		53	80	
Age				0.429			0.610
≥59	166	86	80		53	80	
<59	133	75	58		71	95	
Tumor location				0.013			0.957
Colon	138	85	53		57	81	
Rectum	161	76	85		67	94	
TNM stage				0.064			0.317
1 - 2	178	88	90		78	100	
3 - 4	121	73	48		46	75	
Chemotherapy				0.484			0.401
No	224	118	106		96	128	
Yes	75	43	32		28	47	

Table 2. Univariate and multivariate analysis of molecular markers and clinicopathologic parameters in relation to OS and DFS in all patients.

Parameter	Value	Univariate				Multivariate			
		OS		DFS		OS		DFS	
		HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Sex	Male	0.94 (0.61, 1.44)	0.775	0.93 (0.61, 1.43)	0.749				
Age	<59	1.47 (0.94, 2.32)	0.201	1.26 (0.82, 1.96)	0.292				
Tumor location	Rectum	1.96 (1.24, 3.11)	0.004	1.94 (1.23, 3.05)	0.004	1.71 (1.07, 2.73)	0.025	1.88 (1.20, 2.96)	0.006
TNM stage	TNM1/2	0.36 (0.23, 0.56)	<0.001	0.37 (0.24, 0.57)	<0.001	0.36 (0.23, 0.57)	<0.001	0.38 (0.25, 0.59)	<0.001
Chemotherapy	Yes	1.07 (0.65, 1.78)	0.788	1.05 (0.64, 1.72)	0.855				
p53 expression	Low	0.96 (0.62, 1.49)	0.853	0.93 (0.61, 1.43)	0.739				
TS expression	Low	1.11 (0.71, 1.74)	0.638	1.14 (0.74, 1.76)	0.548				
p53/TS level	p53−/TS+	1.72 (0.91, 3.25)	0.098	1.61 (0.88, 2.95)	0.125				
	p53+/TS−	1.63 (0.81, 3.26)	0.170	1.42 (0.73, 2.77)	0.307				
	p53+/TS+	1.13 (0.56, 2.26)	0.741	1.10 (0.57, 2.13)	0.774				

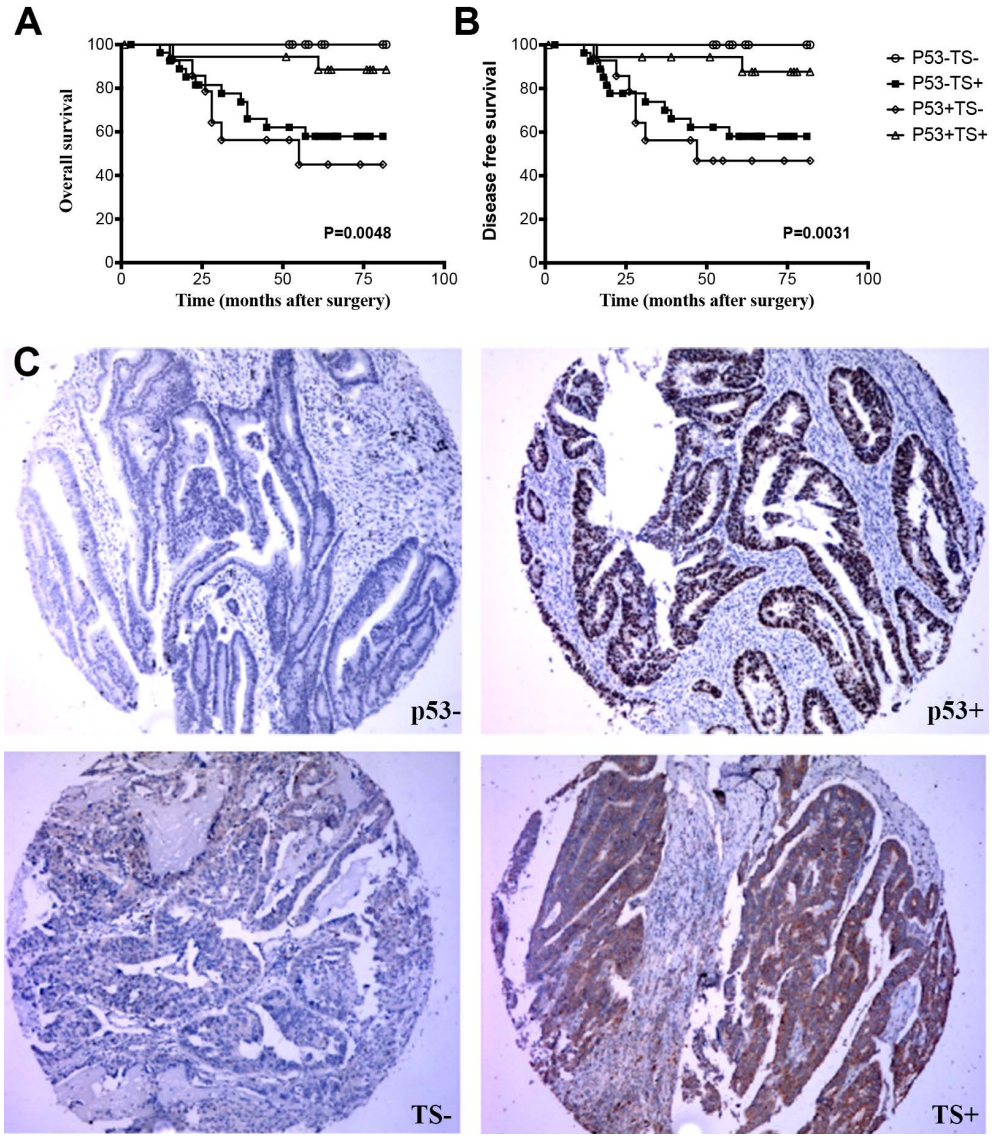

Figure 1. Kaplan-Meier OS (A) and DFS (B) curves of CRC patients received adjuvant chemotherapy caategorized according to different combinations of TS expression and p53 nuclear accumulation. (C) Representative immunohistochemical staining for p53 and TS in colorectal cancer samples.

tumor samples showed TS+ and p53+, respectively. No significant association was found between TS/p53 expression and patient characteristics, such as patient gender, age, TNM stage, and tumor location (**Table 3**).

According to the p53 and TS statues, patients were divided into the following four groups: p53 positive and TS high (p53+/TS+, n = 19), p53 negative and TS low (p53−/TS−, n = 14), p53 negative and TS high (p53−/TS+, n = 28), p53 positive and TS low (p53+/TS−, n = 14). There was no evidence of interaction between TS expression or p53 nuclear accumulation for either OS (P = 0.805 for p53, and P = 0.778 for TS) or DFS (P = 0.620 for p53, and P = 0.899 for TS). However, multivariate analysis revealed a significant association of combined phenotypes p53+/TS− with a worse OS (P = 0.022) and DFS (P = 0.025) (**Table 4**). Furthermore, p53−/TS+ group tended to have worse OS and DFS, although the difference did not reach statistical significance (P = 0.064 and 0.062, respectively). Survival curves among the 4 groups differed significantly (P = 0.0048 for OS, and P = 0.0031 for DFS, **Figure 1**). These findings indicate that CRC patients treated with 5-FU based chemotherapy could be stratified into better or worse outcome groups by TS expression when p53 nuclear staining was taken into account.

Table 3. Association of clinical annotations to TS and p53 level in chemotherapy-treated patients.

Parameter	n	p53 nuclear accumulation			TS expression		
		Low	High	P	Low	High	P
All patient	75	42	33		28	47	
Sex				0.954			0.301
Male	48	27	21		20	28	
Female	27	15	12		8	19	
Age				0.439			0.446
≥59	31	19	12		18	26	
<59	44	23	21		10	21	
Tumor location				0.424			0.571
Colon	38	23	15		13	25	
Rectum	37	19	18		15	22	
TNM stage				0.525			0.244
1 - 2	39	21	18		17	22	
3 - 4	36	22	14		11	25	

Table 4. Univariate and multivariate analysis of molecular markers and clinicopathologic parameters in relation to OS and DFS in chemotherapy-treated patients.

Parameter	Value	Univariate				Multivariate			
		OS		DFS		OS		DFS	
		HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Sex	Male	0.59 (0.25, 1.43)	0.243	0.53 (0.27, 1.26)	0.370				
Age	<59	1.43 (0.59, 3.43)	0.427	1.58 (0.67, 3.73)	0.124				
Tumor location	Colon	2.02 (0.81, 5.06)	0.135	2.08 (0.84, 5.18)	0.092				
TNM stage	TNM1/2	0.40 (0.16, 1.01)	0.054	0.43 (0.18, 1.05)	0.045	0.34 (0.13, 0.88)	0.026	0.36 (0.14, 0.91)	0.031
p53 nuclear accumulation	Low	0.89 (0.37, 2.19)	0.805	0.80 (0.33, 1.93)	0.499				
TS expression	Low	1.14 (0.45, 2.86)	0.778	1.06 (0.44, 2.56)	0.863				
p53/TS level	p53−/TS+	1.95 (0.75, 5.12)	0.056	4.41 (0.95, 20.48)	0.058	6.95 (0.90, 23.92)	0.064	4.36 (0.93, 20.53)	0.062
	p53+/TS−	2.15 (0.82, 5.60)	0.040	5.10 (1.00, 25.91)	0.050	12.12 (1.42, 33.23)	0.022	6.67 (1.27, 25.05)	0.025
	p53+/TS+	0.34 (0.12, 0.96)	0.680	0.87 (0.12, 6.22)	0.893				

4. Discussion

In the present study, we find that the immunohistochemical analysis of p53 and TS in colorectal cancer may be useful to predict the survival of patients who have received 5-FU based chemotherapy. TS or p53 alone is not an independent marker for the survival in CRC patients. However, the combination of the two markers acts as a good indicator for predicting 5-FU based chemotherapy outcome.

The tumor suppressor p53 is one of the most frequently mutated genes in human CRC [15] [16]. Compare to wild-type protein, the mutated p53 protein has a great stability, a significantly longer half-life and accumulated in tumor cells thus could be detected by IHC. It has been believed that 5-FU can activate p53-dependent apoptosis by incorporation of fluoronucleotides into RNA and DNA [3] [17]. Cells with p53 mutations are less sensitive to 5-FU than cells with wild-type 5-FU [18]. A study finds that an elevated expression of wild-type p53 is associated with better survival in CRC patients treated with adjuvant chemotherapy [19]. However, other studies have concluded that abnormal p53 has no effect on the chemotherapy treatment outcome [20] [21]. In our study, we find that p53 expression is associated with tumor location, but not associated with patient OS or DFS, no matter if the patient receives chemotherapy treatment. Further study of the cellular mechanisms of p53 in responses to 5-FU may provide insights into how this important gene functions. It is worth to notice that the sensitivity and specificity of the immunohistochemical assay may affect the correlation between molecular markers and clinical outcome. The antibody we use for p53 recognizes both wild-type and mutant forms of the p53 protein. Consequently, the present results might require further studies in which other mAbs or molecular analysis for different type of p53 detection is tested.

TS is a key enzyme in catalyzing the methylation of deoxythymidine to thymidylate, an essential precursor for DNA replication [22] [23]. Therefore, the first-line chemotherapy treatment of CRC is based upon the inhibition of TS level by 5-FU [24] [25]. However, controversy remains about the prognostic and predictive significance of the expression of TS [7] [26]-[29]. Our results suggest that TS is not an independent prognostic or predictive factor in CRC. *In vitro* studies have shown that TS is capable of binding to several cellular RNA species, including p53 mRNA, resulting in translational repression [30] [31]. Thus, in addition to the incomplete inhibition of TS through direct binding by 5-FU metabolite, the relevance of high TS and the resistance to 5-FU are considered to due to p53 suppression by TS, which consequently causes impaired cell cycle control and an inability of tumor cells to undergo apoptosis [32]. It is possible to hypothesize that the failure to predict patient survival of TS is possibly because of the regardless of p53 status. Paradiso *et al.* previously reported that the p53+/TS− tumors responded better to chemotherapy than other groups [21]. But we notice that their follow-up time is relatively short, and the patient number in p53+/TS− group is too small compared to other groups, which may result in a bias. Our analysis for the first time indicates that patients in the TS+/p53+ and TS−/p53− group have better OS and DFS than the other two groups. This can be explained by several ways: 1) When both TS and p53 level is low, 5-FU can effectively inhibit tumor DNA synthesis by direct binding to free TS; 2) When both TS and p53 is high, p53 high expression can rescue the drug resistance phenotype caused by the suppression of p53 by high TS.

Our results demonstrate that the analysis of TS and p53 together can be important in predicting chemotherapeutic efficacy in CRC patients to 5-FU based chemotherapy. Further studies on larger cohort are needed to verify the correlation between p53 and TS and clinical outcome in CRC patients treated with 5-FU based chemotherapy. If our observation is confirmed, TS and p53 status will need to be evaluated as a stratification criterion in future adjuvant treatment.

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