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Full Length Research Paper

Methylation of p14ARF and abnormal expression of p53 and mdm2 in colorectal cancer: Role and correlation

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Methylation of p14ARF in 42 samples of colorectal cancer and matched adjacent normal tissues was evaluated by methylation-specific PCR (MSP) method. Expressions of p53 and mdm2 were detected by using the semi-quantitative RT-PCR. Protein expressions of p14ARF, p53 and mdm2 in the two groups were assessed with immunohistochemical staining. Research data were analyzed with Student's t-test, chi-square test and Spearman rank correlation. p14ARF methylation was identified in 19 (45.2%) of 42 samples of colorectal cancer tissues and none of the 42 (0%) samples had normal adjacent tissues, indicating a statistically significant difference between the two groups ($p < 0.05$). Positive protein expression rates of p14ARF, p53 and mdm2 in 42 samples of colorectal cancer tissues were 16.7, 73.8 and 54.5%, respectively, while they were 95.2, 11.9 and 28.6% in normal adjacent tissues. A statistically significant difference was noted between the two groups ($p < 0.05$) regarding protein expression of three genes. Protein expressions of mdm2 and p53 were positively correlated ($r = 0.423$; $p < 0.05$) and protein expression of both was negatively correlated with p14ARF protein expression. p14ARF methylation or protein expressions of p53 plus mdm2 were not correlated with the age, sex or tumor size in patients with colorectal cancer, but were positively correlated with tumor differentiation and lymph node metastasis. p14ARF methylation is closely correlated with colorectal cancer and may be an early event in its occurrence; however, p14ARF methylation and abnormal expression of mdm2 plus p53 play a major role in the occurrence and development of colorectal cancer. As the p53-dependent pathway is one of the major regulation channels for growth of colorectal epithelial cells, simultaneous assessment of p14ARF methylation and abnormal expression of MDM2 plus p53 may work as a biological indicator for early diagnosis of colorectal cancer.

Key words: Colorectal cancer, p14ARF, methylation, p53, mdm2, p53 protein, mdm2 protein, immunohistochemistry.

INTRODUCTION

Tumor is regarded as a disease concerning cell cycle and it also holds true for colorectal cancer. Loss of cell cycle regulation leading to aberrant cell proliferation is a major factor in the occurrence of tumors. The cell cycle is regulated by several pathways involving various genes. Two major cell cycle pathways include the RB pathway (p16INK4a-RB) and the p53-dependent pathway (p14AR—>MDM2—>p53) in the occurrence of human tumors. p14ARF is the product of the variable reading frame of cyclin-dependent kinase 4 gene and it has been a hot spot in cancer research in recent years. In cell cycle regulation, p14ARF binds the oncoprotein Mdm2 to acelerate the degradation of Mdm2 and stabilize the level of p53 protein, thus resulting in cell arrest in G1 or G2 phase. In the present study, methylation of p14ARF and the abnormal expression of p53 and mdm2 were evaluated to investigate implications for occurrence and development of colorectal cancer, which the study would like to provide a theoretical basis for anti-tumor drug

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therapy and genetic intervention in clinical practice.

MATERIALS AND METHODS

Sample collection

Forty-two surgical samples of colorectal cancer were collected in the Second Affiliated Hospital, Nanchang University from 2008 - 2009, including 28 moderately differentiated samples and 14 lowly differentiated samples. A number of 15 samples involved lymph node metastasis, while the other 27 exhibited no metastasis and 42 addi-tional samples of normal adjacent tissues (distance from the tumor edge > 10 cm) were obtained. Patients enrolled ranged in age from 40 - 75 years old with a median age of 56.4 years old, including 25 males and 17 females. The patients did not undergo radiotherapy or chemotherapy prior to surgery and did not bear necrotic tissues. Paraffin-embedded sections of colorectal cancer (normal adjacent tissues included in each section) for the 42 samples of colorectal cancer were obtained from the Department of Pathology for immunochemistry. All patients were thoroughly informed about the study and gave written consent for the investigation in accordance with the ethical guidelines of the Local Ethical Committee.

Main reagents

The following reagents were used in the study: TRIZOL RNA kit(Promega), reverse transcription kit, 2×TaqPCR Master Mix(TaKaRa), DNA Marker DL600 (TaKaRa), protease K(TaKaRa), Wizard DNA Clean-up kit (Beijing Quanshijin Bio), mouse anti-human mdm2 monoclonal antibody (Shanghai Sagon Bio), rabbit anti-human p53 monoclonal antibody (Shanghai Sagon Bio), mouse anti-body p14ARF monoclonal antibody (Bejing Zhongshan Bio), SP kit (Bejing Zhongshan Bio) and DAB kit (Bejing Zhongshan Bio).

Methods

Methylation-specific PCR

Samples were digested with protease K and genomic DNA was extracted using the phenol-chloroform extraction method; (1) Purification and modification of DNA: DNA was purified using the Wizard clean-up system kit as per the manufacturer's instructions. (2) DNA modification using hydrosulfite: 2 µl DNA was collected and mixed with 50 µl sterile double distilled water and 3.3 µl NaOH (3 mol/L) for denaturalization for 15 min. 30 ml hydroquinone (10 mol/L) and 520 µl sodium bisulfate (3 mol/L) were added to mix uniformly and another 200 µl paraffin was added for sample blocking. Samples were wrapped in tinfoil and bathed in water at 55℃ for 16 h. (3) Methylation-specific PCR (MSP): Purified and modified DNA 2 µl was obtained by mixing 1 µl upstream primers and 1 µl downstream primers, 12.5 µl 2×Taq PCR Master Mix and 8.5 µL ribozyme-free water to establish the 25 µl reaction system for PCR amplification. Primers for methylation of p14ARF included 5′-GTGTTAAAGGGCGGCGTAGC-3′ and 5′-AAAACCCTCACTCGCG ACGA-3′. The amplification procedure consisted of 35 cycles of denaturation at 95℃ for 5 min, 94℃ for 45 s, 54℃ for 45 s, 72℃ for 1 min and an extension at 72℃ for 7 min. Detection of PCR amplification products: 10 µl amplification products each were selected to undergo agarose gel electrophoresis. The methylated p14ARF band was 122 bp and the unmethylated was 132 bp. Assessment of results: Sample unmethylation was considered when only unmethylated bands existed, while sample methylation was regarded when a methylated band was identified.

Semi-quantitative RT-PCR

Total RNA was extracted from samples of colorectal cancer and normal adjacent tissues using the Trizol RNA kit (A260/A280: 1.8 - 2.0). cDNA was synthesized using reverse transcriptase and Olingo(dT) as per instructions for the reverse transcriptase kit. Genome amplification (PCR): 2 µl products obtained from the above procedure was mixed in a uniform manner with 1 µl upstream primers and 1 µl downstream primers, 12.5 µl 2×TaqPCR Master-Mix and 8.5 µl ribozyme-free water to establish the 25 µl reaction system processed in the PCR device. Primers for p53 included the forward primer 5-CCCAAGCAATGGATGAT-3 and the reverse primer 5-TGACAGGAAGCCAAAGG-3. The amplification procedure consisted of 35 cycles of denaturation at 95℃ for 5 min, 94℃ for 45 s, 56℃ for 45 s, 72℃ for 1 min and an extension at 72℃ for 7 min. Primers for mdm2 included the forward primer 5-AGCTTCGGAACAAGAGACCCTGGTTAGACC and the reverse primer 5-ACTCTTTCACAGAGAAGCTTGGCACGCC. The ampli-fication procedure consisted of 35 cycles of denaturation at 95℃ for 5 min, 94℃ for 45 s, 58℃ for 45 s, 72℃ for 1 min and an extension at 72℃ for 7 min. Primers for the internal reference gene β-actin included the forward primer CCAGGCACCAGGGCGTGATGGTGGGCATGG and the reverse primer AGCAGCCGTGGCCATCTCTTGCTCGAAGTC. The ampli-fication procedure consisted of 35 cycles of denaturation at 95℃ for 5 min, 94℃ for 45 s, 56℃ for 45 s, 72℃ for 1 min and an extension at 72℃ for 7 min. 5 µl products obtained from the proce-dure mentioned above underwent electrophoresis on 2% agarose gel. Target bands were observed under the ultraviolet light and analyzed under the gel documentation system. The amplified band was 379bp for p53 and 227bp for mdm2. The band for internal reference gene β-actin was 581 bp. The density of the bands for PCR products was analyzed using the bandleader 3.0. The gene expression was expressed as (the density for the target gene / the density for β-actin) × 100%.

Immunohistochemistry

A detailed procedure was conducted according to the instructions of the immunohistochemistry kit, with PBS in replacement of the primary antibody as the negative control. Positive p14ARF and p53 proteins were mainly identified in the nucleus, while positive mdm2 protein was located in the nucleus and cytoplasm. According to the grading method by Sulzers, results were assessed as negative (-), if positive cells < 10%; positive (+), if positive cells are from 10 - 50%; strongly positive (++), if positive cells > 50%.

Statistical analysis

Research data were analyzed via the Student's t-test, the chi-square test and Spearman rank correlation using SPSS 13.0. A statistically significant difference was regarded if p was less than 0.05 ($\alpha = 0.05$).

RESULTS

p14ARF methylation

There was a statistically significant difference in p14ARF

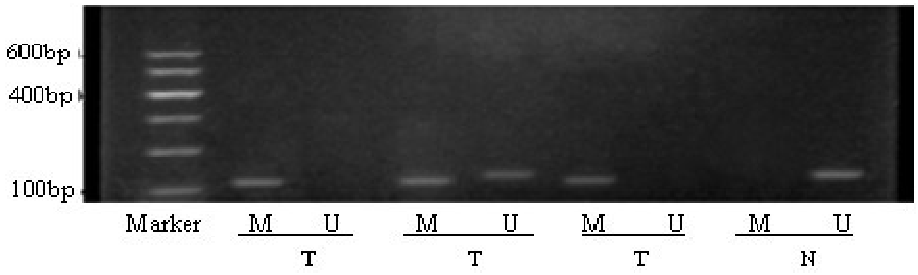

Figure 1. Electrophorogram for p14ARF methylation in colorectal cancer tissues and normal adjacent tissues. T: tumor tissue N: The adjacent normal tissues M: methylation U: Unmethylated).

Table 1. P14ARF methylation in colorectal cancer and in adjacent normal tissues.

Groups	n	p14ARF		χ^2	p
		Methylation	Unmethylation		
Cancer tissues	42	19	23	24.55	<0.05
Adjacent tissues	42	0	42		

Table 2. Relationship of p14ARF methylation and protein expression in colorectal cancer tissues.

p14ARF protein	n	p14ARF gene		χ^2	p
		Methylation	Unmethylation		
-	35	19	16	4.92	<0.05
+	7	0	7		
Total	42	19	23		

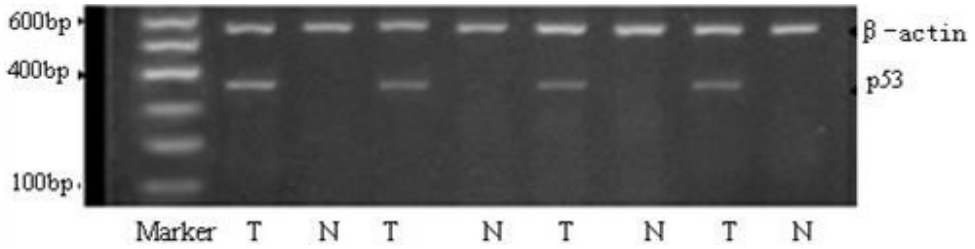

Figure 2. Electrophorogram for p53 mRNA expression in colorectal cancer tissues and normal adjacent tissues. T: tumor tissues N: Adjacent normal tissues.

methylation between colorectal cancer tissues and normal adjacent tissues. The p14ARF methylation occurred in about 45% samples of colorectal cancer tissues and in 54.3% samples with inactivated p14ARF. It was thus potentially a major approach for inactivation (Figure 1 and Tables 1 - 2).

mRNA expression of p53 and mdm2

Semi-quantitation result of p53 mRNA expression is shown in Figure 2. p53 mRNA was expressed in colorectal cancer tissues, but rarely in normal adjacent tissues. p53 mRNA expression was 0.862 ± 0.017 for

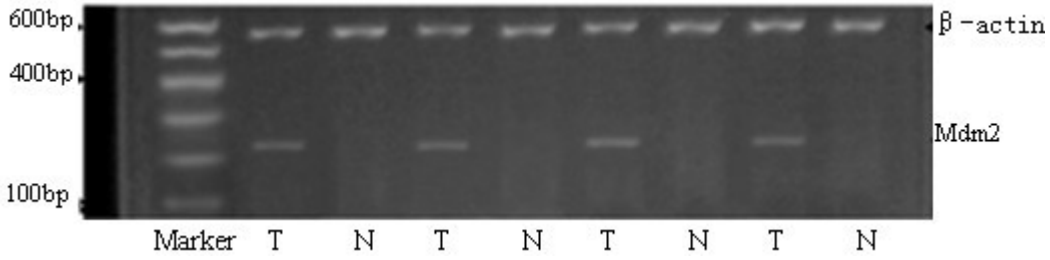

Figure 3. Electrophorogram for mdm2 mRNA expression in colorectal cancer tissues and normal adjacent tissues.
T: tumor tissues N: Adjacent normal tissues.

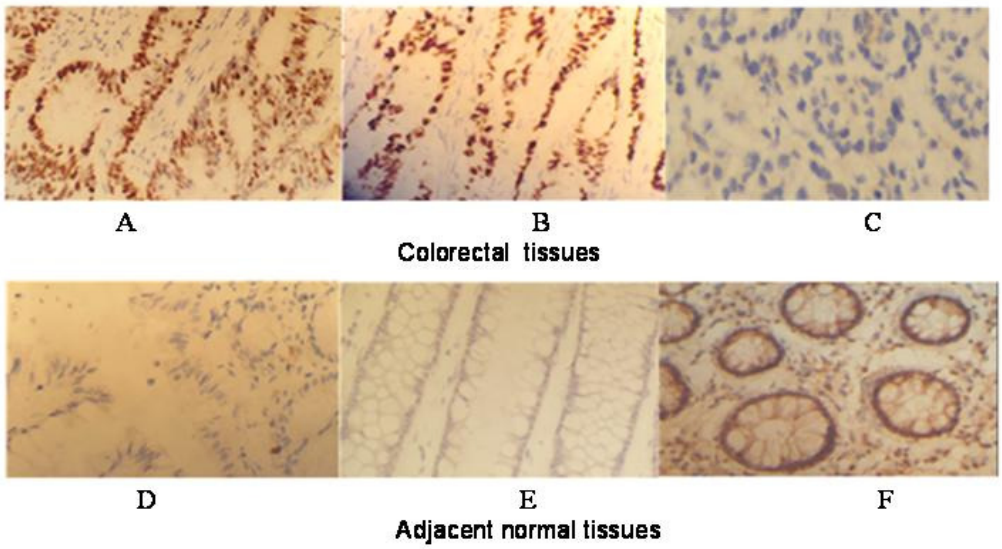

Figure 4. Immunohistochemistry for protein expression of mdm2, p53 and p14ARF in colorectal cancer tissues and normal adjacent tissues.
A: Postive expression of mdm2 protein in colorectal tissues.
B: Postive expression of p53 protein in colorectal tissues.
C: Negetive expression of p14ARF protein in colorectal tissues.
D: Negetive expression of mdm2 protein in the adjacent normal tissues.
E: Negetive expression of p53 protein in the adjacent normal tissues.
F: Postive expression of p14ARF protein in the adjacent normal tissues.

colorectal cancer tissues, significantly higher than 0.165 ± 0.013 for normal adjacent tissues ($p < 0.01$) .

Semi-quantitation result of mdm2 mRNA expression is shown in Figure 3. mdm2 mRNA was expressed in colorectal cancer tissues, but rarely in normal adjacent tissues. mdm2 mRNA expression was 0.675 ± 0.015 in colorectal cancer tissues, significantly higher than 00.276 ± 0.011 in normal adjacent tissues ($p < 0.01$).

Immunohistochemical results

There was a statistically significant difference in protein expression of p14ARF, p53 and mdm2 between colorectal cancer tissues and normal adjacent tissues. p14ARF protein expression was negatively correlated with mdm2 protein expression ($p < 0.05$), while p53 protein expression was positively correlated with mdm2 protein expression ($p < 0.05$). Methylation of p14ARF, and protein expression of p53 and mdm2 were not correlated with the age, sex and tumor size in patients with colorectal cancer, but were positively correlated with tumor differentiation and lymphnode metastasis. p14ARF methylation and p53 mutation occurred increasingly in lowly differentiated tumors (Figure 4, Table 3 - 6)

DISCUSSION

Colorectal cancer is one of the most common malignancies

Table 3. p14ARF, p53 and mdm2 proteins expression in colorectal cancer and adjacent normal tissues.

	N	p14ARF protein			p53 protein			mdm2 protein		
		+	-		+	-		+	-	
Cancer tissues	42	7	35	χ^2 52.6	31	11	χ^2 32.86	23	19	χ^2 6.05
Adjacent tissues	42	40	2	p < 0.01	5	37	p < 0.01	12	30	P < 0.05

Table 4. Relationship among p14ARF, p53 and mdm2 protein expression in colorectal cancer tissues.

		p14ARF protein			
Groups	n	-	+	r	p
Cancer tissues	42	p53 -	6	5	
		+	29	2	-0.765 <0.05
Cancer tissues	42	mdm2 -	14	5	
		+	21	2	-0.556 <0.05

Table 5. Relationship between expression of p53 and mdm2 protein in colorectal cancer tissues.

		mdm2 protein			
Groups	n	+	-	r	p
Cancer tissues	42	p53 +	20	11	
		-	3	8	0.423 <0.05

Table 6. Clinicopathological characteristics of p14ARF methylation with the expression of p53 and mdm2 protein in colorectal cancer.

		p14ARF methylation			p53 protein			mdm2 protein		
Features	**n**	**+**	**-**	**p**	**+**	**-**	**p**	**+**	**-**	**p**
Gender	25	11	14	>0.05	18	7	>0.05	14	11	>0.05
Female	17	8	9		13	4		9	8	
Age										
≤50	15	7	8	>0.05	13	2	>0.05	9	6	>0.05
>50	27	12	15		18	9		14	13	
Tumor size										
≤3cm	7	2	5	>0.05	5	2	>0.05	4	3	>0.05
>3cm	35	17	18		26	9		19	16	
Differentiation										
Moderately	26	8	20	<0.05	19	9	<0.05	14	14	<0.05
Poorly	14	11	3		12	2		9	5	
Lymphnode metastasis										
Without	30	9	21	<0.05	19	11	<0.05	18	12	<0.05
With	12	10	2		12	0		5	7	

of the digestive tract. As the standard of life improves and dietary habits change, colorectal cancer occurs in an increasing manner. Currently, it is mainly treated with surgery, radiology and chemotherapy and its early diagnosis and treatment are unfavorable. As the development of molecular biological techniques, the genetic therapy for colorectal cancer is increasingly desired. Tumor is regarded as a disease concerning cell cycle and

it also holds true for colorectal cancer. Loss of cell cycle regulation is an essential factor for tumor occurrence. Several studies reveal that p14ARF, p53 and mdm2 are major genes in cell cycle regulation. Aberrant expression or inactivation of them induces various human tumors. ARF is located at 9p21, a major inactivation site for human tumors. It encoded two proteins of p16INK4a and p14ARF through the open reading frame. p14ARF binds the proto-oncogene mdm2 to stabilize p53 and arrests the cell cycle in the G1 phase and in the G2/M transitional phase to induce apoptosis. mdm2 is a downstream gene for the pathway regulated by p53 and a major regulator for p53, participating in cell growth inhibition, apoptosis and cell cycle regulation. P53 located at 17q1311 has two types which includes; the wild type and the mutant type. The wild-type p53 is a major regulator for cell stress that monitors completeness of cell genome. The mutant p53, however, loses the cell monitoring function and tends to induce tumor cell growth. With a long half life and good stability, the mutant p53 can be detected in tumor tissues.

P53-dependent pathway (p14ARF—>MDM2—>p53)

Currently, several studies demonstrate that ARF weakens degradation of p53 moderated by mdm2 and forms the p53-dependent pathway with mdm2 and p53 in human cell growth cycle (Mikael et al., 2001; Vladimir et al., 2004). In the p53-dependent pathway, ARF protein is located in the nucleus, p53 shuttles between the nucleus and cytoplasm and promotes degradation of p53, and p53 inhibits mdm2 in return as an essential step (Lohrum et al., 2000; Tao and Levine, 1999). Two binding regions exist in mdm2, in which ARF and p53 binds respectively in a non-competitive manner.

At least, three molecular mechanisms are available for inhibiting transfer of p53 out of the nucleus moderated by mdm2: (1) ARF may bind mdm2 in the nucleus to inhibit transfer of mdm2 out of the nucleus and block the shuffle of mdm2 in order to stabilize p53 (Weber et al., 1999); (2) ARF may form a complex with mdm2 and p53 to inhibit transfer of mdm2 and p53 out of the nucleus (Bates et al., 1998); (3) ARF may bind mdm2 to inhibit the ubiquitin ligase activity of mdm2 and transfer of p53 out of the nucleus. mdm2 has E3 ubiquitin ligase activity and promotes ubiquitination of p53 when binding p53. The ubiquitinated p53 can be degraded and cleared by protease (Brooks and Gu, 2004). mdm2 has lower ubiquitin ligase activity when it binds to ARF, as compared to being alone.

The occurrence of colorectal cancer is possibly correlated with abnormal expression or inactivation of the three genes in the p53-dependent pathway. The present study found that methylation of p14ARF and gene and protein expression of mutant p53 and mdm2 in colorectal cancer were significantly correlated with colorectal cancer ($p < 0.05$), implying that the p53-dependent pathway may be a major regulation channel for growth of large intestine epithelial cells and its abnormity is a major molecular mechanism for colorectal cancer. The level of p14ARF methylation in colorectal cancer samples remains controversial. It was reported that methylation of p14ARF was detected in 14% patients (Lind et al., 2004). However, Anacleto et al. (2005) found the proportion of p14ARF methylation to be 38% and there was a positive correlation with microsatellite instability. Moreover, whether the methylation status of p14ARF was related to the onset, metastasis and infiltration of colorectal cancer still remains unclear. Therefore, it is necessary to explore the relationship of p14ARF promoter methylation status with the clinical and pathological characteristics of colorectal cancer. The present study also shows that p14ARF methylation occurred in about 45% samples of colorectal cancer tissues and was closely correlated with tumor differentiation and lymph node metastasis, though not being correlated with the age, sex and tumor size in patients with colorectal cancer, indicating that p14ARF methylation is closely correlated with colorectal cancer and is a major approach for inactivation of p14ARF. P14ARF methylation causes loss of normal p14ARF functions and abnormality of p14ARF induces its loss in nucleolus, so it cannot bind to mdm2 or inhibit the shuffle of mdm2 and ubiquitination of mdm2, blocking the normal cell cycle regulated by the p53-dependent pathway. Additionally, P14ARF methylation is not evidently correlated with the age, sex and tumor size in patients with colorectal cancer, indicating P14ARF methylation is an early event for colorectal cancer. The present study found that gene and protein expression of p53 and mdm2 were positively correlated with colorectal cancer ($p < 0.05$) and with a statistically significant difference as compared with that in normal adjacent tissues. P53 mutation and abnormal mdm2 expression may affect the occurrence and development of colorectal cancer synergistically. P53 and mdm2 composite has a negative feedback channel in the regulation mechanism for normal cell growth. Mdm2 is a transcription target gene for the wild-type p53. At the transcription level, p53 can induce an expression of mdm2 that binds p53 to generate an mdm2-p53 complex, leading to ubiquitination p53. Mdm2 also directly suppresses the transcriptional activity of p53. High-level mdm2 genetic products inactivate p53 and low-level p53, however, reduces mdm2 transcription which closes the negative p53-mdm2 feedback loop and brings p53 to a normal level. For colorectal cancer, abnormal expression of mdm2 results in over-degradation of the wild-type p53 and mutant p53 loses regulation of the mdm2 feedback, causing loss of cell cycle control.

Multiple p53-independent pathways

Currently, several p53-independent pathways are identified for cell cycle regulation, such as the Rb cell cycle

pathway. It has been found that p53-induced apoptosis was realized by a p53-independent pathway and that p53-independent ARF-responsive genes included the B-cell translocation gene family (Btgl, Btg2, Btg3 andTob1) (Kuo et al., 2003; Hemmati et al., 2002). The present study demonstrated those 7 p14ARF protein-positive samples and 4 samples with co-expression of p53 protein and mdm2 protein in 42 samples of colorectal cancer tissues, indicating that the other cell cycle regulation path-ways exist for occurrence of colorectal cancer, though they are still being investigated.

In conclusion, simultaneous assessment of p14ARF methylation and abnormal expression of MDM2 plus p53 may work as a biological indicator for early diagnosis of colorectal cancer, which may provide a theoretical basis for genetic intervention in clinical practice. Regarding outcomes from the present study, the following hypotheses are proposed: (1) Intervention of p14ARF methylation may be considered for colorectal cancer. Demethylation reagents may be administered to recover p14ARF demethylation and thus suppress p14ARF methylation for treatment of colorectal cancer; (2) Antisensedigonucleotides, RNA or Ribozyme may be applied to transfect normal genes in replacement of mutant genes, such as p53. Antisensedigonucleotide p53 may be transfected into vectors and transducted into colorectal cancer cells with defect p53 in order to attain apoptosis of tumor cells or recovery of normal cells; (3) Drugs may be administered to block abnormal genes in the p53-dependent pathway to maintain stability of genes for treatment purposes. To date, progress has been made for genetic research on occurrence of colorectal cancer, but clinical application still witnesses a series of problems including low efficacy of gene transduction and anti-tumor effects, targeting of gene transduction, safety of gene vectors, noninjurious gene expression monitoring, etc. Solutions to these problems will promote the genetic therapy as a key way to treat colorectal cancer.

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