PO Box 2345, Beijing 100023, China
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RAPID COMMUNICATION

Expression of $p21^{WAF1}$ is related to acetylation of histone H3 in total chromatin in human colorectal cancer

Ying-Xuan Chen, Jing-Yuan Fang, Rong Lu, De-Kai Qiu

Ying-Xuan Chen, Jing-Yuan Fang, Rong-Lu, De-Kai Qiu, Department of Gastroenterology, Renji Hospital, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine, Shanghai 200001, China
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Correspondence to: Jing-Yuan Fang, MD, PhD, Professor of Medicine, Shanghai Jiaotong University School of Medicine Renji Hospital, Shanghai Institute of Digestive Disease, 145 Shandong Zhong Lu, Shanghai 200001, China. jingyuanfang@yahoo.com
Telephone: +86-21-63200874 Fax: + 86-21-63266027
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Abstract

AIM: To explore the relationship between acetylation of histone in total chromatin and $p21^{WAF1}$ expression regulation in human colorectal carcinoma.

METHODS: We analyzed the expression of tumor suppressor gene $p21^{WAF1}$ mRNA by RT-PCR or real-time PCR in 33 samples of colorectal cancerous tissue, corresponding para-cancerous tissue and normal colorectal mucosa, and also examined the level of acetylated histone H3 in total chromatin using Western blotting.

RESULTS: The expression level of $p21^{WAF1}$ mRNA was significantly lower in colorectal cancerous tissue from 33 patients than in para-cancerous tissue and normal colorectal mucosa (2377.95 ± 865.80 *vs* 3216.58 ± 1149.42 and 3541.61 ± 1433.17 respectively, $P < 0.01$). In addition, when $p21^{WAF1}$ mRNA expression was undectectable or at very low level (50% less than that in adjacent tissue and normal colorectal mucosa) in all tissues, the level of acetylated histone H3 in colorectal cancerous tissue was significantly lower than that in corresponding para-cancerous tissue and normal colorectal mucosa in five of seven (71.43%) cases. The transcriptional level of $p21^{WAF1}$ in colorectal carcinoma might not be associated with its biological behaviors.

CONCLUSION: The down-regulation of $p21^{WAF1}$ transcription is involved in the tumorigenesis and development of colorectal carcinoma. The down-expression of $p21^{WAF1}$ mRNA in colorectal carcinoma might be associated with histone hypoacetylation in chromatin but not with biological behaviors.

Key words: Colorectal cancer; $p21^{WAF1}$; Histone acetylation

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INTRODUCTION

Cell cycle progression is regulated by interactions between cyclins and cyclin-dependent kinases (CDKs)[1]. $p21^{WAF1}$ is one of the CIP/KIP protein family members known to inhibit CDK activity. Increased $p21^{WAF1}$ expression may play an important role in the growth arrest of transformed cells[2]. Although stability of $p21^{WAF1}$ mRNA could be altered by different signals such as differentiation and various influencing factors, a recent study suggested that inactive chromatin induced by histone deacetylation may be a likely candidate mechanism for $p21^{WAF1}$ inactivation[3].

In recent years, some studies indicated that $p21^{WAF1}$ expression is regulated by gene-associated histone acetylation in tumors[3-5]. We have previously reported that the level of $p21^{WAF1}$ gene promoter-associated histone acetylation is decreased in human colon cancer cell line, SW1116[6]. However, these investigations were carried out *in vitro* experiments using certain cell lines, and very few data are available regarding $p21^{WAF1}$ expression and histone acetylation in excised human carcinoma tissues. Furthermore, whether acetylation of histone in total chromatin affects $p21^{WAF1}$ gene regulation in human colon cancer is unknown.

In this paper, we provide further evidence that the acetylation of histone in total chromatin is a mechanism of down-regulation of $p21^{WAF1}$ mRNA in patients with colorectal cancer.

MATERIALS AND METHODS

Patients and specimens

Thirty-three patients with colorectal carcinoma

Table 1 Primer sequence, PCR program and Genbank accession number

Primers	Sense (5′ -> 3′)	Antisense (5′ -> 3′)	Size and PCR condition	GenBank accession number
β-actin	GGC ATC GTG ATG GAC TCC G	GCT GGA AGG TGG ACA GCG A	94℃ 5 min; 92℃ 40 s; 58℃ 40 s; 72℃ 50 s, 30X	BC023204
$p21^{WAF1}$	CAG GGG ACA GCA GAG GAA GA	GGG CGG CCA GGG TAT GTA C	94℃ 5 min; 94℃ 1 min; 58℃ 1 min; 72℃ 1 min, 35X	NM_000389

Table 2 Sequences of primers and probes for real-time PCR

Gene	Primer (forward) (5′ -> 3′)	Primer (reverse) (5′ -> 3′)	Probe	GenBank No.
$p21^{WAF1}$	CTG GAG ACT CTC AGG GTC GAA	GGA TTA GGG CTT CCT CTT GGA	ACG GCG GCA GAC CAG CAT GA	NM_078467
β-actin	CTG GCA CCC AGC ACA ATG	GGA CAG CGA GGC CAG GAT	ATC ATT GCT CCT CCT GAG	BC016045

underwent resection at Shanghai Renji Hospital between May 2001 and July 2002. All patients gave their informed consent and none of them received radiation or chemotherapy before surgery. Fresh samples taken from the tumor and its corresponding adjacent tissue (obtained from areas less than 5 cm away from the margin of carcinoma) and normal colorectal mucosa (obtained from areas at least 10 cm away from the margin of carcinoma) were frozen immediately in liquid nitrogen, fixed in formalin solution and embedded in paraffin blocks for pathological diagnosis. Clinicopathological factors, tumor histology and disease stage were assigned according to the general rules for clinical and pathological studies on cancer of colon, rectum, anus and Dukes' stage[7]. Fifteen cases were identified as stage B, 11 cases as stage C, and 7 cases as stage D. There were 23 cases of tubular adenocarcinoma, 2 cases of mucinous adenocarcinoma and 8 cases of tubular-papillary adenocarcinoma. Twenty-one patients were males and 12 were females, and their mean age was 65.36 years (SD 13.00 years, range 39-89 years). The protocol was approved by the Ethics Committee of Shanghai Second Medical University and the research was carried out according to the Helsinki Declaration in 1975.

RT-PCR and real-time RT-PCR

Total RNA was extracted from clinical specimens using Trizol reagent (Gibco BRL, Gaithersburg, MD), following the manufacturer's recommendations. After DNAse I (Promega, UK) digest, the pellet was vacuum dried and resuspended in 20 μL sterile distilled water containing 1 U/μL RNasin (Promega, UK).

RT-PCR was performed as described previously[8]. The primer sequence and amplification profiles are shown in Table 1. The control consisted of an amplified 612 bp fragment of β-actin cDNA. The density of the bands in RT-PCR was quantitated by using a molecular dynamics phosphor-imager (Nucleo Tech Inc., San Mateo, CA), normalized in each lane to the amount of total RNA as determined by the density of β-actin band from RT-PCR[9].

The expression of the $p21^{WAF1}$ gene in 13 of 33 colorectal carcinoma specimens was detected using a real-time quantitative PCR system. Gene-specific TaqMan probes and PCR primers were designed using Primer Express software (PE Biosystems, Foster City, CA). The sequence for forward and reverse primers and the probe are shown in Table 2. Triplicate PCR was prepared for each cDNA sample. PCR consisted of 40 cycles of 95℃ denaturation at 95℃ for 15 s and annealing/extension at 60℃ for 1 min. Thermal cycling and fluorescent monitoring were performed using an ABI 7700 sequence analyzer (PE Biosystems). The point at which the PCR product was first detected above a fixed threshold, termed cycle threshold (Ct), was determined for each sample, and the average Ct of triplicate samples was calculated. The ratio of $p21^{WAF1}$ mRNA levels in colorectal carcinoma specimens relative to those in non-neoplastic mucosa was calculated.

Western blot analysis of acetylated histone H3 in total chromatin

Seven powdered colorectal cancerous tissue sections in which $p21^{WAF1}$ mRNA expression was undetectable or at very low levels (50% less than that in adjacent tissue and normal colorectal mucosa) (100 mg) and six such sections in which $p21^{WAF1}$ mRNA expression was comparable to that in non-neoplastic mucosa were suspended in 500 μL Radio-immunoprecipitation assay buffer (150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl pH 8.0, 0.2 mmol/L PMSF, 1 μg/μL aprotinin/ leupeptin) for 15 min with rotation at 4℃, then the soluble protein was collected by centrifugation.

Tissue extracts (200 μg) were boiled in loading buffer (125 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.005% bromphenol blue) for 5 min and then loaded onto a 15% SDS-polyacryamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS for 1 h at room temperature, incubated overnight at 4℃ with the first antibody (Upstate Biotechnology, Lake

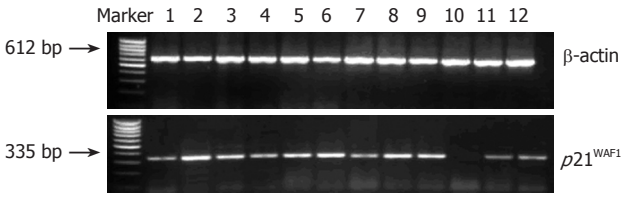

Figure 1 RT-PCR products of grouped samples of colorectal cancerous tissues (lanes 1, 4, 7, 10), corresponding para-cancerous tissue (lanes 2, 5, 8, 11) and normal colorectal mucosa (lanes 3, 6, 9, 12). β-actin amplified from the same cDNA served an internal control.

Table 3 Expression of $p21^{WAF1}$ mRNA in colorectal carcinoma tissue

	Cancerous tissues	Para-cancerous	Normal mucosa
$p21^{WAF1}$ mRNA	2377.95 ± 865.80[b]	3216.58 ± 1149.42	3541.61 ± 433.17

[b]$P < 0.01$: average density in cancerous tissue *vs* that in corresponding para-cancerous tissue and normal colorectal mucosa. The band density detected by RT-PCR was described in the "Material and Methods".

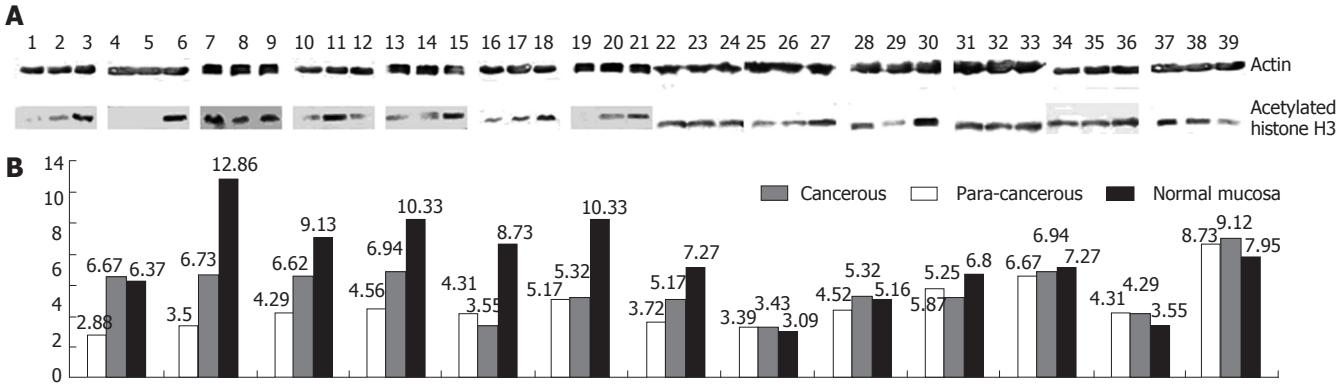

Figure 2 Level of acetylated core histone H3 (**A**) and mRNA $p21^{WAF1}$ mRNA (**B**) in colorectal carcinoma tissues. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37: tumor tissue; lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38: corresponding para-cancerous tissue; and normal colorectal mucosa lanes 3, 6, 9, 12, 15, 18, 21, 34, 27, 30, 33, 36, 39: normal colorectal mucosa.

Placid, NY) against acetylated histone H3, and incubated for 4 h at 4℃ with secondary antibodies against rabbit IgG-conjugated AP, and then exposed to Kodak BioMax film for 1 min. Antibody against β-actin (Sigma, St Louis, MO) was used as a control for protein concentration.

Statistical analysis

The level of tumor-related gene expression in cancerous and adjacent tissues and normal colorectal mucosa was compared by variance analysis or Student's *t*-test using SPSS 6.0. Variables associated with the expression of two tumor suppressor genes as well as the correlation to various clinicopathological variables were examined by the χ^2 method and correlation analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of $p21^{WAF1}$ mRNA was down-regulated in colorectal cancer tissue

As shown in Figure 1 and Table 3, the expression level of $p21^{WAF1}$ mRNA in colorectal cancerous tissue was significantly lower than that in adjacent tissue and normal colorectal mucosa ($P < 0.01$). However, there was no significant difference in the expression level of $p21^{WAF1}$ between the samples of adjacent tissue and normal mucosa ($P > 0.05$). Of the 33 tumor samples, 23 (69.70%) showed varying degrees of decrease in $p21^{WAF1}$ expression. In seven cases, $p21^{WAF1}$ mRNA expression was undetectable or at very low levels (50% less than that in adjacent tissue and normal colorectal mucosa). The data were consistent with the result from real-time RT-PCR (Figure 2).

Acetylation of histone H3 in total chromatin was related to $p21^{WAF1}$ expression

Futher study of the seven specimens with undetectable or very low level $p21^{WAF1}$ mRNA expression revealed that in five of seven (71.43%) cases (Figure 2), the level of acetylated histone H3 in cancerous tissue was significantly lower than that in corresponding para-cancerous tissue and normal colorectal mucosa. The level of acetylated core histone H3 in tumor specimen with $p21^{WAF1}$ mRNA expression was comparable to that in non-neoplastic mucosa specimen. However, it was lower than that in two of six (33.33%) non-neoplastic mucosa specimens (Figure 2).

Expression of $p21^{WAF1}$ might not be associated with clinicopathological characteristics

We examined the relationship between $p21^{WAF1}$ expression in cancer tissue and the clinicopathological characteristics of colorectal cancer patients. There was no significant correlation between $p21^{WAF1}$ expression and tumor size, extent of local tumor invasion, lymphatic invasion, Duke's stage or histological subtype (data not shown).

DISCUSSION

The initiation and progression of colorectal carcinoma involve unregulated epithelial cell proliferation associated with a series of accumulated genetic alterations[10]. The

molecular pathogenesis of human cancer is due to structural and/or functional alterations of specific genes whose normal function is to control cellular growth and differentiation. The development of colorectal cancer is associated with activation of oncogenes and/or inactivation of tumor suppressor genes.

Defects in the mechanisms controlling cell cycle are critical triggers in cell transformation and/or tumor progression. $p21^{WAF1}$ is a known inhibitor of cyclin-dependent kinases, which can block tumor progression through the cell cycle[11]. The discovery of the $p21^{WAF1}$ gene has illuminated the importance of down-regulation of cell cycle as an inhibitor of cyclin-cdk complexes. The $p21^{WAF1}$ gene causes G_1 arrest of the cell cycle, and inhibits growth of human tumor cells by blocking G_1/S transition through interference with cyclin-cdk function. In this study, we showed that $p21^{WAF1}$ mRNA expression was constantly and significantly suppressed in tumor tissues compared to that in the corresponding adjacent tissue and normal colorectal mucosa. We also found that $p21^{WAF1}$ expression was decreased in 69.70% of the tumor samples, which was higher than that in colorectal cancer detected by immunohistochemistry[12], and lower than that detected by Northern blot analysis[13]. The different results may be due to the geographical and racial factors. Our results suggest that tumor cell cycles are disturbed by reduction in $p21^{WAF1}$ mRNA expression, leading to uncontrolled cell proliferation in human colorectal carcinoma. That is to say, tumor cells may acquire more rapid growth activity and more malignant potential as $p21^{WAF1}$ expression decreases.

It was reported that two histone deacetylation inhibitors, trichostatin A and sodium butyrate, cause accumulation of acetylated histone H3 and H4[14]. This action most likely occurs in vivo because in rats fed with a high-fiber diet, high butyrate levels are correlated with histone hyperacetylation in colonic epithelial cells[15]. Moreover, accumulation of acetylated histones in total cellular chromatin and hyperacetylation of $p21^{WAF1}$ gene-associated histones H3 and H4 could activate the expression of $p21^{WAF1}$ mRNA and protein in SW1116 cells[16]. However, the in vivo status of histone acetylation in human colorectal carcinoma tissue is not well understood. In this study, we investigated colorectal cancerous tissues where $p21^{WAF1}$ mRNA expression was undetectable or at very low levels, and found that the level of acetylated histone H3 in total chromatin was significantly lower in cancerous tissue than in corresponding para-cancerous tissue and normal colorectal mucosa. Also, acetylated core histone H3 level in tumor specimens where $p21^{WAF1}$ mRNA expression was comparable to that in non-neoplastic mucosa specimens was lower than that in non-neoplastic mucosa specimens. These results suggest that the down-expression of $p21^{WAF1}$ mRNA in colorectal carcinoma might be associated with histone hypoacetylation in total chromatin. It is possible that nucleosome conformation was altered due to histone H3 hypoacetylation and that access of transcriptional regulatory proteins to chromatin might be reduced in colorectal cancer. Thus, we hypothesize that $p21^{WAF1}$ over-expression may be relevant to the possible therapeutic effects of anticancer drugs.

However, the data from this study indicate that none of the clinicopathological parameters correlates with $p21^{WAF1}$ expression, including different tumor size, tumor depth, lymph node metastasis, Dukes' classification and histological stage. These results suggest that the expression of $p21^{WAF1}$ detected by RT-PCR cannot be used per se as a predictive indicator for clinicopathological behaviors in colorectal carcinoma. To date, the potential correlation between tumor-related genes and biological behaviors remains controversial. For example, one recent report[17] demonstrated that allelic deletion on chromosome 18q is associated with poor survival of patients with stage Ⅱ colorectal cancer, while another study[18] showed that this phenomenon does not provide any prognostic information, suggesting that construction of composite genetic profiles of tumor tissue, with inclusion of several tumor markers, may be premature[14].

It is necessary to further investigate how histone modifications impact the neoplastic process, and to study the means for prevention and treatment of colorectal carcinoma, such as chromatin structure alteration and activation of specific tumor suppressor genes.

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