P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• COLORECTAL CANCER •

Expressing patterns of p16 and CDK4 correlated to prognosis in colorectal carcinoma

Po Zhao, Ying-Chuan Hu, Ian C. Talbot

Po Zhao, Department of Pathology, Chinese PLA General Hospital, Beijing 100853, Beijing, China and Academic Department of Pathology, St. Mark' s Hospital, Cancer Research UK, Colorectal Cancer Unit, Harrow, HA1 3UJ, London, UK
Ying-Chuan Hu, Department of Pathology, Huaxi Medical College, Sichuan University, Chengdu 610041, Sichuan Province, China
Ian C. Talbot, Academic Department of Pathology, St. Mark' s Hospital, Cancer Research UK, Colorectal Cancer Unit, Harrow, HA1 3UJ, London, UK
Correspondence to: Dr. Po Zhao, Department of Pathology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China. zhaopo@plagh.com.cn or Professor Ian C. Talbot, Academic Department of Pathology, St Mark' s Hospital, Cancer Research UK, Colorectal Cancer Unit, Watford Road, Harrow HA1 3UJ, London, UK. i.talbot@cancer.org.uk
Telephone: +86-10-66937954 **Fax:** +86-10-68181689
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Abstract

AIM: To describe the correlation between innunostaining patterns of p16 and CDK4 and prognosis in colorectal carcinoma.

METHODS: Paraffin sections of 74 cases of colorectal carcinoma were analysed immunohistochemically for expression of p16 and CDK4 proteins.

RESULTS: Most carcinomas showed stronger p16 and CDK4 immunostaining in the cytoplasm than the adenomas or the adjacent normal mucosa. Strong immunostaining of p16 was a predictor for better prognosis whereas strong cytoplasmic immunostaining of CDK4 was a predictor for poor prognosis. Both p16 and CDK4 immunostainings were correlated with histological grade or Dukes' stage.

CONCLUSION: These results support the experimental evidence that interaction of expression of p16 and CDK4 may play an important role in the Rb/p16 pathway, and the expression paterns of CDK4 and p16 may be imperative in the development of colorectal carcinoma, thus becoming a new prognostic marker in colorectal cancer.

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INTRODUCTION

p16 gene, an important tumor suppressor gene, regulates cell proliferation negatively through inhibition of the kinase activity of cyclin-dependent kinase 4/6 (CDK4/6), which promotes phosphorylation and therefore inactivation of a very important tumor suppressor product, the retinoblastoma (Rb) gene protein[1-4]. The Rb/p16 tumor-suppressor pathway is abrogated frequently in multiple types of human tumors, either through inactivation of Rb or p16 tumor-suppressor protein, or through overexpression of cyclin D1 or cyclin-dependent kinase 4 (CDK4) oncoproteins. But no deletion and only quite a low frequency of mutation on p16 gene have been found in colorectal cancer since this gene was identified in 1994[1,5]. CpG islands are areas rich in CpG dinucleotides, which are found within the promotors of about 60 % of human genes. These CpG islands normally lack DNA methylation, regardless of the expression status of the gene[6]. Methylation of promotor usually leads to irreversible inhibition of gene transcription[7]. It has become apparent that *de novo* methylation is an important alternate mechanism to code region mutation for inactivating tumor suppressor genes during neoplasia[8-11]. Herman *et al*.[9] previously reported that there was methylation of p16 gene in 40 % of primary colorectal cancers and 92 % of colorectal cancer cell lines, in which all the p16 genes were inactivited by Southern analysis. Gonzalez-Zulueta *et al*.[10] found the evidence of methylation of the 5' CpG island not only in cancer tissues but also in normal colonic tissues by the more sensitive PCR-based assay, in which methylation of p16 exon 1 was detected in 1 of 10 (10 %) colon carcinomas and methylation of p16 exon 2 was detected in 7 of 10 (70 %) colon cancers. It is interesting that in 50 % of the colon normal/tumor matched cases, p16 was methylated and not expressed in the normal tissues but unmethylated and highly expressed in the tumor tissues. It also suggested that methylation of exon 1, but not exon 2, of p16 was associated with transcriptional silencing although the CpG island in exon 2 of p16 underwent extensive *de novo* methylation in colorectal carcinoma. More recently, Ahuja *et al*.[12] found that 16 (34 %) of 47 colorectal cancers exhibited methylation in exon 1 of the p16 gene, which was associated with microsatellite instability. Transcriptional silencing of p16 gene could be due to either gene deletion or methylation in exon 1. In either event, there would be no expression product in the cells. Therefore, we used an immunohistochemical method to detect p16 protein level *in situ* and compared with its binding oncoprotein, CDK4, as a simple way to investigate their possible interaction in the development and prognosis of colorectal carcinoma. We also detected the mRNA levels of both p16 and CDK4 genes and checked the methylation status on the promoter region of p16 gene to confirm their expression results in immunocytochemistry.

MATERIALS AND METHODS

Colorectal carcinoma

Seventy-four cases of colorectal carcinoma were randomly and retrospectively selected from the files of the Academic Department of Pathology, St. Mark' s Hospital, London, UK and the Department of Pathology, the former West China University of Medical Sciences, China. Specimens obtained at surgery were routinely fixed in 10 % neutral formalin and embedded in paraffin. The clinical stage was determined according to the Dukes' stages A, B and C. The histological grade of tumors was also determined according to the WHO criteria as follows: grade I as well differentiated, grade II as moderately differentiated, and grade III as poorly differentiated. Follow-up data on 5-year survival rate in 32 cases were available for further analysis with variables.

Immunohistochemical staining

Immunohistochemical staining for p16 and CDK4 was performed according to the standard ABC method except that the pressure for cooking procedure was used for antigen retrieval pretreatment[13, 14]. Serial 4 μm thick sections were cut and dewaxed in xylene and rehydrated in a graded ethanol series. The sections were immersed in 3 % hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. and rinsed in running water. After that, sections were immersed in boiling 1 mM EDTA-NaOH (pH 8.0) buffer in a pressure cooker. The pressure cooker was then sealed and brought to full pressure. The heating time was 2 minutes which began only when full pressure was reached. At 2 minutes, the cooker was depressured and cooled under running water. The lid was then removed, and the hot buffer was flushed out with cold water from a running tap. The cooled sections were washed twice in PBS before immunohistochemical staining, then immersed in 0.05 % avidin for 30 minutes to block any possible endogenous biotin exposed to heating. Prior to immunohistochemical staining, the sections were first incubated with 10 % horse serum for monoclonal antibody and 10 % goat serum for polyclonal antibody respectively for 15 minutes to block non-specific binding. The primary monoclonal mouse antibody against human p16 protein (Pierce, USA) and polyclonal rabbit antibody against human CDK4 protein (Santa Cruz Biotechnology, USA) were diluted in 200 with 0.01 M PBS (pH 7.2), respectively. Then the sections were allowed to react by the standard ABC method using a VECTASTAIN Elite PK-6100 kit (Vector Labratories, Inc., USA), as directed by the manufacturer. A previously known positive pancreatic carcinoma was used as a positive control. The primary antibody was replaced by 0.01 M PBS or 10 % serum as a negative control. Normal colon mucosal tissue was used as a normal control.

Evaluation of score

The slides were examined and data regarding staining positivity were recorded before the clinical outcome was available. When p16 or CDK4 protein expression was scored, both the extent and intensity of immunopositivity were considered, according to Hao *et al*[15]. The intensity of positivity was scored as follows: 0 as negative, 1 as weak, 2 as moderate, 3 as strong as normal liver. The extent of positivity was scored as follows: 0 <5, 1>5-25 %, 2>25-50 %, 3>50-75 %, and 4>75 % of the tumor cells in the respective lesions. The final score was determined by multiplying the intensity of positivity and the extent of positivity scores, yielding a range from 0 to 12. Scores 9-12 were defined as strong staining pattern (++), 5-8 as weak staining pattern (+), and 0-4 as markedly reduced or negative expression (-).

RNA extraction and RT-PCR

Total RNA was isolated from the frozen tissues of 10 paired normal and tumor samples using the total RNA isolation system (Promega) and treated with RNase-free DNase I (GeneHunter). Equal amount (0.2 μg) of total RNA from each sample was added into the access RT-PCR system (Promega) which carried on both cDNA syntheses by reverse transcription and PCR in a same tube with a total volume of 25 μl reaction solution according to the manufacturer' s directions. The primer pairs used to amplify p16 and CDK4 genes were as follows. The sequence of p16 sense primer was 5' -CCCGCTTTCGTAGTTTTCAT-3' and that of antisense primer was 5' -TTATTTGAGCTTTGGTTCTG-3' [16] and that of CDK4 sense primer was 5' -ATGGCTGCCACTCGATAT GAACCC-3' and antisense primer, 5' -GTACCAGAGCGT AACCACCACAGG-3' [17]. In addition, primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene were used as an internal control to ensure the quality of the templet RNA and reagents in reactions. The sequence of sense primer was 5' -TGGTATCGTGGAAGGACTCATGAC-3' and antisense primer was 5' -ATGCCAGTGAGCTTCCCGTT CAGC-3' [16]. cDNA for each sample was synthesized at 48 ℃ for 45 min, followed by at 94 ℃ for 2 min to inactivate reverse transcriptase and was subjected to 40 cycles of amplification under the conditions described before[16,17] except for modified extension step during each cycle at 68 ℃ for 2 min according to the access RT-PCR system' s direction. Water controls, including primers and all reagents except for RNA in PCR reactions, were setup for potential DNA contamination.

Methylation-specific PCR

Samples from 24 cases of colorectal carcinoma with residual adenoma were selected for DNA extraction. DNA was extracted from these cases according to the conventional methods. The methylation status on the promoter region of p16 was assessed by methylation-specific PCR (MSP) as described previously[9] Genomic DNAs from the primary tumors were subjected to bisulfate modification using the CpGenome DNA modification kit (Intergen, New York, NY). Treatment of genomic DNA with sodium bisulfate converted unmethylated cytosines (but not methylated cytosines) to uracil, which was then converted to thymidine during the subsequent PCR step, yielding sequence differences between methylated DNA. PCR primers distinguishing these methylated and unmethylated DNA sequences were used. Primer sequences of p16 genes[9] for both the methylated and unmethylated forms were 5' -TTATTAGAGGGTGGGGCGGATCGC-3' /5' -GACCCCGAACCGCGACCGTAA-3' and 5' -TTATTAGAGGGTGGGGTGGATTGT-3' /5' -CAACCCCAAACCACAACCATAA-3' , with annealing temperature at 65 ℃ and at 60 ℃, and the expected PCR product sizes were 150 bp and 151 bp, respectively. For PCR amplification, 2 μl of bisulfate-modified DNA was added in a final volume of 25 μl PCR mixture containing 1×PCR buffer, $MgCl_2$, deoxynucleotide triphosphates, and primers (100 pmol each per reaction), and 1 unit of AmpiTaq Gold (Applied Biosystems, Branchburg, NJ). Amplification was performed at 95 ℃ for 12 min, 35 cycles at 95 ℃ for 1 min, the specific annealing temperature for both methylated and unmethylated at 65 ℃ and at 60 ℃ for 1 min, and at 72 ℃ for 1 min, followed by a final 7-min extension at 72 ℃. PCR products (15 μl) were loaded onto a 10 % nondenaturing polyacrylamide gel, stained with ethidium bromide after 2 hours of electrophoresis, visualized under UV illumination. MSP for all samples was repeated to confirm their methylation status.

Statistical analysis

Fisher' s exact test (two sided) and Pearson Chi square test for trends in proportions were used to assess the associations between Fhit expression and pathological indices. A $P<0.05$ was considered statistically significant.

RESULTS

The results are summarized in Tables 1-2.

The 5-year survival of the 32 patients according to the available data was significantly shorter in patients with Dukes' C tumors than in those with Dukes' A and B tumors ($P<0.01$).

p16 expression in colorectal carcinoma

In 61 patients in whom non-neoplastic mucosa was detected adjacent to the carcinoma, weak p16 expression was observed in the nuclei and moderate p16 expression only in the cytoplasm around nuclei. Most of the cytoplasm of goblet cells filled with

mucus was negative (Figures 1A, B). Of the 74 specimens examined, 73 (98.6 %) were p16-positive (Table 1). Expression of p16 was almost always observed in the cytoplasm but only sporadically in the nuclei. Of the p16 positive specimens, 53 showed a strong expression pattern and 20 a weak expression pattern (Figure 1C). Strong p16 expressions were detected in 15 (78.9 %) patients who survived more than 5 years, while weak p16 expressions were detected in 8 (61.5 %) patients who survived less than 5 years after operation (Table 1). The prognosis was significantly better in patients with tumors in strong expression pattern of p16 (P<0.001).

Table 1 Relationship between p16 expression and clinicopathological features

Clinicopathological features	No. of patients	p16 Immunostaining patterns		
		Strong	Weak	Negative
Dukes' stage				
A	23	16	7	0
B	22	16	6	0
C	29	21	7	1
Histological grade				
I	24	17	7	0
II	41	30	10	1
III	9	6	3	0
Total	74	53	20	1
5-year survival rate				
Alive	20	15	5	0
Dead	12	4	8	0
Total	32	19	13	0

CDK4 expression in colorectal carcinoma

In 49 of 61 non-neoplastic mucosaea adjacent to the carcinoma, CDK4 nuclear and cytoplasmic immunoreactivity was a little weaker than the p16 staining, but otherwise identical. The remaining 12 were negative. Expression of CDK4 was observed predominantly in the cytoplasm (Figure 1D). All the 74 specimens showed CDK4 expression (Table 2). Of the CDK-positive specimens, 33 showed a strong expressing pattern and 41 yielded a weak expressing pattern. Tumors with strong CDK4 expression were found in 4 (28.6 %) patients who had survived for more than 5 years after operation, whereas those with weak CDK4 expression were found only in 2 (11.1 %) patients who died within 5 years after operation. The prognosis was significantly poorers in patients with cancers in strong expression of CDK4 (P<0.001).

Table 2 Relationship between CDK4 expression and clinicopathological features

Clinicopathological features	No. of patients	CDK4 Immunostaining patterns		
		Strong	Weak	Negative
Dukes' stage				
A	23	9	14	0
B	22	10	12	0
C	29	14	15	0
Histological grade				
I	24	8	16	0
II	41	19	22	0
III	9	6	3	0
Total	74	33	41	0
5-year survival rate				
Alive	20	4	16	0
Dead	12	10	2	0
Total	32	14	18	0

Correlation between p16 and CDK4 expression

Of the 74 tumors with CDK4 expression (strong in 33, weak in 41), p16 expression was also found in all but one (strong in 53, weak in 20). There was a significantly reverse correlation between p16 and CDK4 expression patterns (P<0.001).

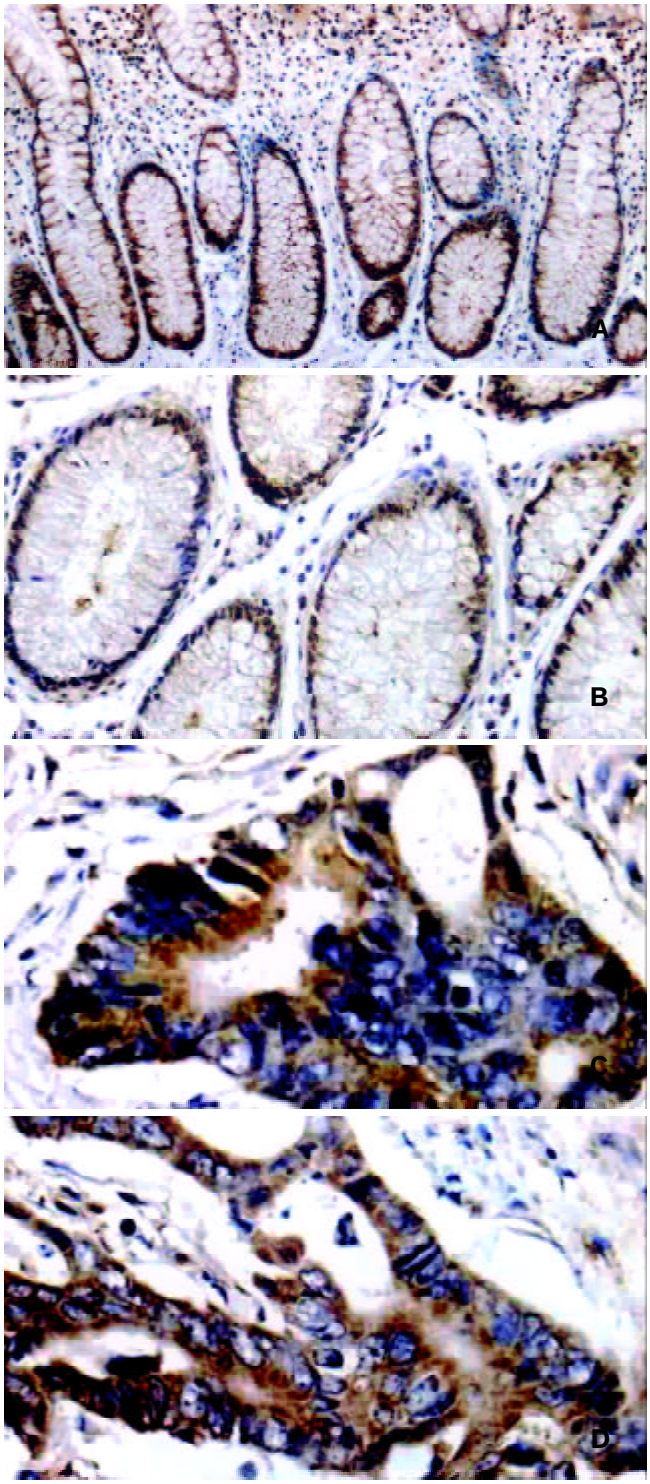

Figure 1 Expression patterns for p16 and CDK4 gene proteins. A, B: nuclear/cytoplasmic pattern of p16 protein in normal crypts (SP×100, 200), C: overexpression pattern of p16 in the cytoplasm of colon cancer cells (SP×400), D: overexpression pattern of CDK4 in the cytoplasm of colon cancer cells (SP×400).

Correlation between p16 or CDK4 expression and clinicopathological features

In our study, a significant correlation was found between p16 or CDK4 expression patterns and the tumor stage (P<0.001;

P<0.001) or the histological grade (*P*<0.001; *P*<0.001) (Tables 1, 2). The prognosis was significantly poorer in patients with weak p16 expression pattern (*P*<0.001), or with strong CDK4 expression pattern (*P*<0.001).

Results of RT-PCR and MSP

We investigated the mRNA expression of p16 and CDK4 genes in 10 frozen normal-tumor paired tissues. The results showed that the level of mRNA expressions of p16 (9/10) and CDK4 (10/10) of cancer tissues rose in accordence with that of proteins of p16 and CDK4 in immunostaining (Figures 2A, B). We also determined the frequency of promoter methylation of p16 gene by MSP-PCR, but the result showed no positively methylated signal in all the 24 cases of colorectal carcinoma with residual adenoma (Figure 2C).

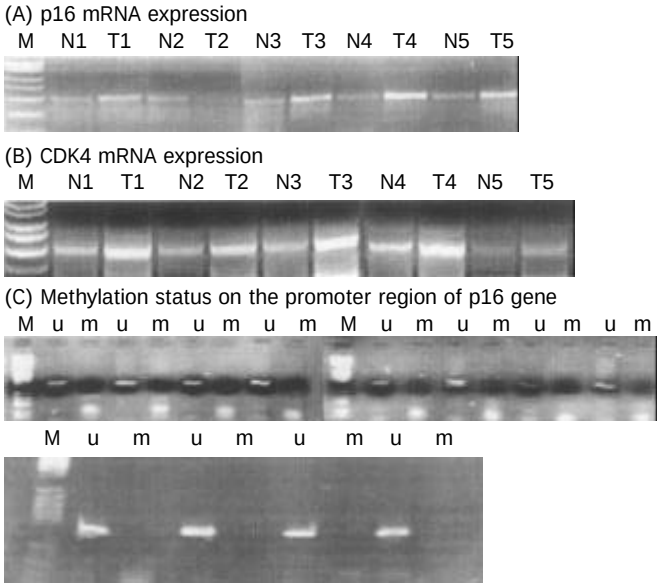

Figure 2 Results in p16 and CDK4 mRNA expressions as well as p16 gene methylation status on the promoter region. (A) and (B): mRNA levels of both p16 and CDK4 genes were elevated in colorectal carcinoma (T), compared with their matched normal (N) tissues. (C) There was no methylation detected on p16 promoter region in all 24 cases of cancer tissue with residue adenoma. u: unmethylated, m: methylated, M: molecular marker (PBR322 / Hae III).

DISCUSSION

Loss of expression of proteins such as p16 could occur as a consequence of either homozygous gene deletion or gene methylation. One aim of this study was to assess the extent to which both homozygous deletion and methylation on promoter region of p16 gene occurred with loss of gene expression in colorectal carcinoma. All carcinomas but one in this study showed the expression of p16 protein, confirmed not only by immunocytochemistry but also by RT-PCR and MSP, suggesting that loss of p16 gene expression is infrequent in primary colorectal carcinoma. The localization of p16 protein was similar to that of CDK4, almost always in the cytoplasm of adenocarcinoma cells. This indicates that the main site of interaction between p16 and CDK4 in adenocarcinoma cells is the cytoplasm. This contrasts with the localization of p16 protein in astrocytoma[18], in which nuclear expression is always more intense than any cytoplasmic expression. Based on these findings, our results indicate that CDK4 may contribute to phosphorylation of pRB, and to the loss of regulating function of p16 protein in nuclei, leading to the development of colorectal carcinoma. Induction of p16 overexpression would act as a brake at G1/S transition through pRB phosphorylation by CDK4 overexpression on the one hand, but predominant mislocation or only in cytoplasm might also imply the function of p16 in the nuclei, its ability to regulate transcriptions of other important genes related to proliferation and angiogenesis[19-21], was lost in colorectal carcinogenesis on the other hand.

The previous results[22-26] suggest that the deletion or mutational inactivity of both p16 and Rb proteins may be a rare event in cervical carcinogenesis. Moreover, overexpression of the p16 protein may be a useful diagnostic marker for cervical neoplastic lesions on routine laboratory screening. It was proposed that the expression of two viral oncogenes, E6 and E7, in epithelial stem cells be required to initiate and maintain cervical carcinogenesis and result in significant overexpression of the cellular p16 protein. Since this protein was not expressed in normal cervical squamous epithelia, screening for p16 over-expressing cells could specifically identify dysplastic lesions, and significantly reduced the inter-observer disagreement of the conventional cytological or histological tests. The similar results[28-30] have also been reported in prostate cancer, gastrointestinal stromal tumor and gastritis, in which overexpression of p16 in high grade prostate intraepithelial neoplasia (HGPIN) and cancer was correlated with, but independent of, pathological stage and was associated with early relapse in cancer patients treated with radical prostatectomy. Overexpression of p16 in HGPIN was also an independent predictor of disease relapse and increased the risk of recurrence 2.24-fold, providing the first evidence for a prognostic marker in HGPIN. The aberrant cytoplasmic expression as well as overexpression of p16 in colorectal carcinoma might be due to its binding to CDK4, CDK6 and some unknown proteins, thereby forming a larger volume of molecule which is uneasy to pass through the nuclear membrane. Nuclear expression of p16 would therefore imply extra unbound p16[5, 19], which might play other important roles in inhibiting transcription of genes related to tumor progression in the nuclei. For instance, VEGF for angiogenesis is besides or independent of binding CDK4/6[18-20]. It could be that CDK4 overexpression is an initial event, which is followed by a reactive overexpression of p16 in colorectal carcinoma, thus the oncoprotein function of CDK4 is inhibited to some extent by binding to p16. In our study, the histological grade, Dukes' stage and prognosis in colorectal carcinoma were closely related to p16 or CDK4 expression (*P*<0.001), respectively, suggesting that the expression of p16 or CDK4 protein may be a useful diagnostic and prognostic marker for colorectal neoplastic lesions. A more extended study is required to confirm the value of aberrant cytoplasmic expression or overexpression of p16 protein as a prognostic marker.

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