Aberrant cytological localization of p16 and CDK4 in colorectal epithelia in the normal adenoma carcinoma sequence

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Abstract

AIM: To study the correlation between the patterns of subcellular expression of p16 and CDK4 in colorectal epithelia in the normal-adenoma-carcinoma sequence.

METHODS: Paraffin sections of 43 cases of normal colorectal epithelia and corresponding adenomas as well as carcinomas were analysed immunocytochemically for subcellular expression of p16 and CDK4 proteins.

RESULTS: Most carcinomas showed more cytoplasmic overexpression for p16 and CDK4 than the adenomas from which they arised or the adjacent normal mucosa. Most normal or non-neoplastic epithelia showed more p16 and CDK4 expression in the nucleus than their adjacent adenomas and carcinomas. There was a significant difference between the subcellular expression pattern of p16 and CDK4 in normal-adenoma-carcinoma sequence epithelia (P < 0.001). Neither p16 nor CDK4 subcellular patterns correlated with histological grade or Dukes' stage.

CONCLUSION: Interaction of expression of p16 and CDK4 plays an important role in the Rb/p16 pathway. Overexpression of p16 and CDK4 in the cytoplasm, as well as loss expression of p16 in the nucleus might be important in the evolution of colorectal carcinoma from adenoma and, of adenoma from normal epithelia.

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Key words: Colorectal neoplasm; p16; CDK4; Immunocytochemistry

INTRODUCTION

p16, as a cyclin-dependent kinase-4 inhibitor is expressed in a limited range of normal tissues and tumors. The Rb/p16 tumor-suppressor pathway is frequently abrogated in many types of human tumors, either through inactivation of Rb or p16 tumor-suppressor proteins, or through alteration or overexpression of cyclin D1 or cyclin-dependent kinase 4/6 (CDK4/6) oncogenes. However, no deletion and only quite a low frequency of mutation of p16 gene have been found in colorectal cancer and adenoma-carcinoma-sequence since this gene was identified in 1994. CpG islands are areas rich in CpG dinucleotides, which are found within the promoters of about 60% of human genes. These CpG islands normally lack DNA methylation, regardless of the expression status of the gene. Methylation of promoter usually leads to irreversible inhibition of gene transcription. It has become apparent that de novo methylation is an important alternate mechanism underlying coding region mutation which inactivates tumor suppressor genes during neoplasia. CpG islands of the 5' CpG island in p16 gene was previously reported not only in colorectal cancer but also in normal colonic tissue by the more sensitive PCR-based assay, in which all p16 genes were inactivated. Furthermore, it has been found that the aberrant cytoplasmic expression of the p16 protein without gene alteration is associated with accelerated tumor proliferation in breast cancer, and p16 protein plays an important role in the regulation of glioma angiogenesis, suggesting a novel function of the p16 gene. It was also reported that overexpression of p16 was observed in a majority of colorectal cancer and a low p16 expression due to methylation or not may contribute to tumor enlargement, expansion and metastasis and even prognosis of colorectal cancer. Immunohistochemistry with p16 antibody has been used as a diagnostic adjunct in premalignant and malignant lesions of gynecologic pathology, oral cavity, head and neck, and skin in recent years. Diffuse positivity but not focal one with p16 staining in the cervix can be regarded as a surrogate marker of the presence of...
high-risk human papillomavirus (HPV)\textsuperscript{[29-31]}. Most high-grade cervical intraepithelial neoplasia (CIN) and some cases of low-grade CIN, are usually associated with high-risk HPV. In cervical squamous lesions, the expression of p16 is found almost diffusely positive. And also p16 expression may be helpful to identify small focal high-grade CIN lesions, to distinguish CIN involving immature metaplastic squamous epithelium from immature metaplastic squamous epithelium not involved by CIN and to distinguish high-grade CIN from benign mimics. p16INK4A stain is also found to be a valuable ancillary test in making the diagnosis of squamous dysplasia. Combined with the conventional hematoxylin and eosin stain, the p16INK4A staining may help to identify truly dysplastic foci in tissues which is particularly challenging, because of poor orientation or small biopsies, tissues that show severe inflammation or ulceration or keratinizing dysplasia. Other series indicated that p16 overexpression within malignant epithelium in the female genital tract should not be interpreted as synonymous with HPV-induced carcinogenesis. Data also indicated that p16 overexpression could occur in non-HPV-related cancers of the gynecological tract and that p16 overexpression suggested generic functional RB1 pathway abnormalities in cancer evolution. These preliminary results are promising; however, there is no corresponding report on colorectal carcinogenesis from normal-adenoma-carcinoma sequence. Therefore additional studies are needed to identify whether the p16INK4A as well as its binding protein, CDK4 has the similar prognostic value in predicting lesions that are likely to progress in colorectal neoplasm. We used the p16INK4A and CDK4 antibodies to stain reactive and dysplastic lesions in this series of colorectal normal-adenoma-carcinoma sequence and to investigate the possible role of p16 or CDK expression in the evolution of colorectal neoplasia.

**MATERIALS AND METHODS**

**Samples**

Forty-three cases of colorectal carcinoma with residual adenoma, in which adjacent normal mucosa was available in 42 of the cases, were randomly and retrospectively selected from the pathology files of St. Mark’s Hospital, London and 31 cases of colorectal carcinoma were from the Department of Pathology, Chinese People’s Liberation Army (PLA) General Hospital. Specimens obtained at surgery were routinely fixed in 10% neutral formalin and embedded in paraffin. The clinical stage was classified according to Dukes: 22 in Dukes’ A, 22 in Dukes’ B and 30 in Dukes’ C, respectively. The histological grade of the tumors was determined according to WHO criteria as follows: 24 in grade I, well differentiated; 41 in grade II, moderately differentiated; and 9 in grade III, poorly differentiated.

**Immunocytochemical analysis**

Immunocytochemical staining for p16 and CDK4 was performed with a standard ABC method except that the pressure cooking procedure was used for antigen retrieval pretreatment\textsuperscript{[18]}. Serial sections were cut 4 µm thick and dewaxed in xylene and rehydrated in a graded ethanol series. The sections were immersed in 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity and rinsed in running water. Sections were then immersed in boiling 1 mmol/L 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 min, beginning only when full pressure was reached. At 2 min the cooker was depressurized 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 min to block any possible endogenous biotin exposed by heating. Prior to application of antibodies, the sections were incubated with 10% horse serum, for monoclonal antibodies and 10% goat serum, for polyclonal antibodies, respectively for 15 min to block non-specific binding. The primary monoclonal mouse antibody against human p16 protein (Pierce, USA) was produced, using a full-length recombinant bacterially produced GST-p16INK4 fusion protein as the immunogen, and could only be reactive to human tissue. The polyclonal rabbit antibody against human CDK4 protein (Santa Cruz Biotechnology, USA), was raised against the epitope corresponding to amino acids 282-303 mapping at the carboxy terminus of mouse CDK4 and is specific for CDK4 with no cross-reactivity with other cyclin dependent kinases in mouse, rat and human tissue. The antibodies were diluted 1:200 with 0.01 mol/L PBS (pH 7.2). After exposure to primary antibody the sections were allowed to react with the standard ABC method with VECTASTAIN Elite PK-6100 kit (Vector Laboratories, Inc. USA) as directed by the manufacturer. A previously known positive glioma was used as a positive control. The primary antibody was replaced by 0.01 mol/L PBS as a negative control. Normal colon mucosa tissue was used as a normal control.

**Interpretation of p16 and CDK4 immunocytochemical staining**

Immunostained sections were evaluated according to the localization of staining of positive tumor cells as follows: tumors were classified as cytoplasmic/nuclear (C/N) and cytoplasmic (C) pattern, respectively, when immunoreactivity was present in a large proportion of epithelial cells. When immunoreactivity was not visible, the result was classified as negative.

**Statistical analysis**

The correlation between variables was determined using the nonparametric test with SPSS10.0. A value of \( P \) less than 0.05 was accepted as statistically significant. The results are summarized in Table 1, Table 2, Table 3.

**RESULTS**

**p16 subcellular expression in normal-adenoma-carcinoma sequence**

Of the 42 normal epithelia examined, cytoplasmic/ nuclear expression pattern was found in 40 (95.2%) and
cytoplasmic only in 2 (4.8%) (Figure 1A). Of the 43 adenomas, 32 (74.4%) showed cytoplasmic/nuclear and 11 (25.6%) cytoplasmic only for p16 (Figure 1B). Of the 74 carcinomas arising from the adenomas, 27 (36.5%) showed cytoplasmic/nuclear and 46 (62.1%) cytoplasmic expressing pattern for p16 (Figure 1C). Only in one additional advanced carcinoma was the poorly differentiated signet cells negative. There was a significant difference between the patterns of p16 expression in any two positive types of epithelia of the normal-adenoma-carcinoma sequence ($P < 0.001$) (Table 1). In non-neoplastic mucosa adjacent to carcinoma, p16 expression was present weakly in the nucleus and moderately only in the cytoplasm around the nucleus. Most of the cytoplasm of goblet cells filled with mucus was negative. Expression for p16 was almost always observed strongly in the cytoplasms and sporadically weakly in the nuclei of cancer cells.

**CDK4 subcellular expression in normal adenoma-carcinoma sequence**

In non-neoplastic mucosa adjacent to carcinoma, CDK4 nuclear and cytoplasmic expression was slightly weaker than the p16 expression, but was otherwise identical. Of the 42 normal epithelia examined, CDK4 expression was cytoplasmic/nuclear in 29 (69.0%), cytoplasmic in 9 (21.4%), and negative in 4 (9.5%). Of the 43 adenomas, CDK4 expression was cytoplasmic/nuclear in 15 (23.3%) and cytoplasmic positive in 33 (76.7%). In 74 carcinomas, CDK4 staining was cytoplasmic/nuclear in 15 (20.3%) and cytoplasmic alone in 59 (79.7%) and like p16, almost always observed strongly in the cytoplasms (Figure 1D). There was a significant difference between the subcellular patterns of CDK4 expression in any two positive types of epithelia in the normal-adenoma-carcinoma sequence ($P < 0.001$) (Table 1).

![Figure 1](https://www.wjgnet.com)

**Table 1** Relationship between the subcellular expression pattern of p16 or CDK4 and the histology of epithelia of normal-adenoma-carcinoma sequence

| Histology  | Subcellular expression pattern | Total | C/N | C |
|------------|--------------------------------|-------|-----|---|
| p16        |                                |       |     |   |
| Normal     |                                | 42    | 40  | 2 |
| Adenoma    |                                | 43    | 32  | 11|
| Carcinoma  |                                | 73    | 27  | 46|
| $P < 0.001$|                                |       |     |   |
| CDK4       |                                |       |     |   |
| Normal     |                                | 38    | 29  | 9 |
| Adenoma    |                                | 43    | 10  | 33|
| Carcinoma  |                                | 74    | 15  | 59|
| $P < 0.001$|                                |       |     |   |

C/N: Cytoplasm/nucleus.

**Table 2** Relationship between p16 subcellular expression pattern and clinicopathological features

| Clinicopathological feature | $n$ | p16 subcellular pattern |
|-----------------------------|-----|-------------------------|
|                            |     | C/N | C    |
| Dukes' stage               |     |     |     |
| A                          | 22  | 8   | 14  |
| B                          | 22  | 6   | 16  |
| C                          | 29  | 13  | 16  |
| Histological grade         |     |     |     |
| I                           | 24  | 8   | 16  |
| II                          | 40  | 16  | 24  |
| III                         | 9   | 3   | 6   |
| Total                       | 73  | 27  | 46  |

| CDK4                        |     |     |     |
|-----------------------------|-----|-----|-----|
| Normal                      | 38  | 29  | 9   |
| Adenoma                     | 43  | 10  | 33  |
| Carcinoma                   | 74  | 15  | 59  |

C/N: Cytoplasm/nucleus. There was no significant correlation between p16 subcellular expression pattern and the Dukes' stage ($P = 0.441$) or histological grade ($P = 0.865$) of the carcinoma.
anal intraepithelial neoplasia or squamous cell carcinoma positive.
carcinomas, similar to their uterine counterparts, are p16-
metastatic cervical adenocarcinomas in the ovary
diagnose problematic uterine smooth muscle neoplasms.

In the vulva, p16 is positive in HPV-associated vulval
tuboendometrial metaplasia and endometriosis, which
distinguish between a cervical adenocarcinoma (diffuse
in situ), which shows
basal and the transformation from normal mucosa to
expression was observed strongly in the cytoplasms and sporadically
expression was observed in all carcinomas in situ in parallel
with a lack of  Rb-phosphorylation but high proliferation,
distinguishing a nonfunctional Rb. More interestingly, despite
this disability of  p16INK4a to inhibit proliferation there is
an upregulation of  cytoplasmic p16INK4a in infiltrative
cells compared to tumor cells towards the tumor center,
suggesting a potentially proliferation independent
function for p16INK4a in infiltrative behavior. It has been
reported that p16 overexpression is a potential early
indicator of  transformation[30] and associated with poor
clinical outcome in ovarian carcinoma[31]. In addition, it
was also reported that intensity of  p16 expression may
play an important role in clinicopathological features and
prognosis of  colorectal adenocarcinoma[16-18]. However,
there has been no subcellular localization analysis on
colorectal epithelia of  adenoma-carcinoma sequence
compared with their normal counterpart.

In the current study, we aimed to clarify if  p16
overexpression, with its binding protein, CDK4
overexpression has any role in colorectal neoplasia. Our
result showed that p16 overexpression was almost always
observed strongly in the cytoplasms and sporadically
weakly in the nuclei of  colorectal cancer cells and most
carcinomas showed more cytoplasmic overexpression for
p16 and CDK4 than the adenomas from which they were
arising or the adjacent normal mucosa. Most normal or
non-neoplastic epithelia showed more p16 and CDK4
expression in the cell nucleus than their adjacent adenomas
and carcinomas. There was a significant difference
between the subcellular expression pattern of  p16 and
CDK4 in normal-adenoma-carcinoma sequence epithelia
(P < 0.001). The findings suggest that CDK4 may be
activated as one of  the initial events, which is followed
by activation of  the p16 gene, possibly as a feedback
mechanism, with the effect of  preventing G1/S transition
through pRB phosphorylation by CDK4 overexpression.
In normal colorectal epithelia there was much mucin in the
cytoplasm. In the tumorigenesis, from normal epithelia
to adenoma, and then to carcinoma, the mucin loss was
observed gradually with the increase of  p16 and CDK4
expression in the cytoplasm of  epithelia. It is further
suggested that cytoplasmic overexpressions for p16 and
CDK4 may be involved in the mechanism for the loss of
mucin and the transformation from normal mucosa to
adenoma, and from adenoma to carcinoma. Cytoplasmic
localization of  p16 might be due to its binding with
CDK4, forming a larger molecule, difficult to pass through
the nuclear membrane. Loss of  expression of  p16 in the
nucleus might suggest that there is a loss of  function
of  p16 besides binding with CDK4, which negatively
regulates the transcription of  some important genes in
tumorigenesis. p16 overexpression is known to occur in
neoplastic cells as an indirect phenomenon of  an aberrant
neoplastic cells as an indirect phenomenon of  an aberrant
RB1 functional pathway, as is well known in carcinomas of the lower genital tract, in which high-risk types of HPV have caused inactivation of RB1. Some data also indicate that p16 overexpression may occur in non-HPV-related cancers of the gynecological tract and possibly indicate generic functional RB1 pathway abnormalities in cancer evolution in which p16 overexpression may not be caused by mutational silencing of the RB1 gene directly in the majority of cases, but rather the retinoblastoma pathway is rendered dysfunctional by some other mechanism. Unlike our previous report in intensity analysis, there were no differences in p16 or CDK4 subcellular expression between Dukes' stages and histological grades in this research, indirectly suggesting that the subcellular expression of the two proteins may be significant in the evolution, but not in the progression, of colorectal carcinomas. Extensive research is needed to further clarify the mechanism.

In summary, our results support experimental evidence that interaction of expression of p16 and CDK4 plays an important role in the Rb/p16 pathway, and cytoplasmic overexpression of CDK4 and p16 as well as loss expression of p16 in nucleus might be important in the evolution of colorectal carcinoma from adenoma and, of adenoma from normal epithelia. Thus cytoplasmic overexpression of CDK4 and p16 may be a potentially early marker of transformation in colorectal carcinoma.

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