RAPID COMMUNICATION

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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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[1-4]. 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[1,5]. 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[6]. Methylation of promoter usually leads to irreversible inhibition of gene transcription[7]. It has become apparent that *de novo* methylation is an important alternate mechanism underlying coding region mutation which inactivates tumor suppressor genes during neoplasia[8-12]. Methylation 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[9,10]. 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[13], and p16 protein plays an important role in the regulation of glioma angiogenesis, suggesting a novel function of the p16 gene[14]. It was also reported that overexpression of p16 was observed in a majority of colorectal cancer[15-17] and a low p16 expression due to methylation[18] or not[15,19,20] 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[21], oral cavity[22,23], head and neck[24-26] and skin[27,28] 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)[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[15]. 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 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 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 Labratories, 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
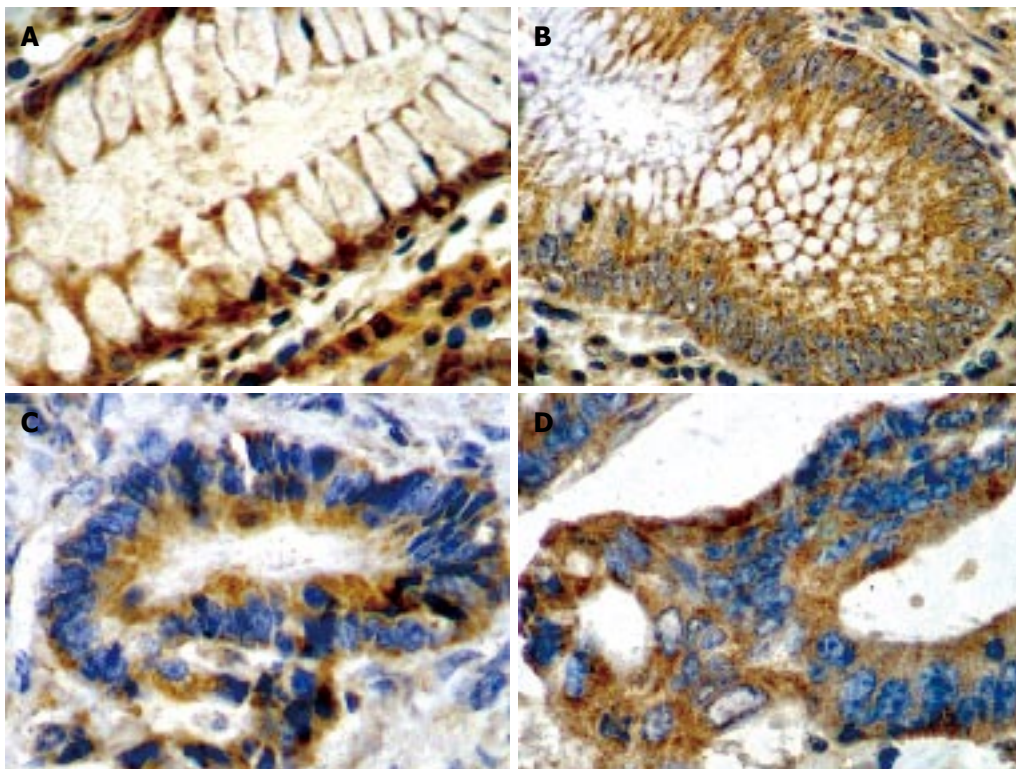
Figure 1 Subcellular expression of p16 and CDK4. **A**: Cytoplasmic/nuclear pattern of p16 in normal epithelia of colon mucosa (× 200); **B**: Cytoplasmic pattern of p16 in adenomatous epithelia of adenoma (× 200); **C**: Cytoplasmic pattern of p16 in colonic adenocarcinoma (× 400); **D**: Cytoplasmic pattern of CDK4 in colonic adenocarcinoma. Original magnifications (× 400).

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

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.

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 10 (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).

Table 3 Relationship between CDK4 subcellular expression pattern and clinicopathological features

| Clinicopathological feature | n | CDK4 subcellular pattern | |
		C/N	C
Dukes' stage			
A	22	4	18
B	22	4	18
C	30	7	23
Histological grade			
I	24	4	20
II	41	10	31
III	9	1	8
Total	74	15	59

C/N: cytoplasm/nucleus. There was no significant correlation between CDK4 subcellular expression pattern and the Dukes' stage ($P = 0.844$) or histological grade ($P = 0.584$) of the carcinoma.

Correlation between p16 or CDK4 subcellular expression and Dukes' stage or histological grade in carcinomas

There was no significant correlation between p16 or CDK4 subcellular expression pattern and the Dukes' stage ($P = 0.441$; $P = 0.844$) or the histological grade ($P = 0.865$; $P = 0.584$) of the carcinoma (Tables 2 and 3).

DISCUSSION

Research on expression of p16 has made much progress in gynecologic pathology in recent years[21,29-31]. p16 is found diffusely positive in most cervical carcinomas including squamous, glandular, and small cell type. In cervical glandular lesions, p16 is also useful to diagnose the lesions between adenocarcinoma *in situ*, which shows diffusely positive pattern and benign mimics, including tuboendometrial metaplasia and endometriosis, which usually shows p16-negative or focally positive pattern. Combined with other markers, p16 may be used to distinguish between a cervical adenocarcinoma (diffuse positivity) and an endometrioid-type endometrial adenocarcinoma (negative or focally positive). In some uterine serous carcinomas the staining is diffusely positive. In the vulva, p16 is positive in HPV-associated vulval intraepithelial neoplasia (VIN) but negative in VIN not associated with HPV. Similarly, HPV-associated invasive squamous carcinomas are p16-positive, whereas the more common non-HPV-associated neoplasms are largely negative or focally positive. In the uterus, p16 positivity is more common and widespread in leiomyosarcomas than in leiomyomas, and it may be a useful marker to diagnose problematic uterine smooth muscle neoplasms. Metastatic cervical adenocarcinomas in the ovary are usually diffusely p16-positive, and because these may closely mimic a primary ovarian endometrioid or mucinous adenocarcinoma, it may be a valuable diagnostic aid, although p16 expression in primary ovarian adenocarcinomas of these morphologic subtypes has not been widely investigated. Some ovarian serous carcinomas, similar to their uterine counterparts, are p16-positive. Darvishian *et al*[32] reported that the sensitivity and specificity of p16 immunoreactivity in the detection of anal intraepithelial neoplasia or squamous cell carcinoma

were 72% and 71%, respectively. The presence of p16 immunoreactivity is a good predictor of dysplasia in anal specimens. Nilsson *et al*[28] found that the expression of p16INK4a varied between the skin lesions including actinic keratosis, squamous cell carcinoma in situ and invasive squamous cell carcinoma, with weak and cytoplasmic p16INK4a expression and functional Rb in actinic keratosis. Strong nuclear and cytoplasmic p16INK4a expression was observed in all carcinomas in situ in parallel with a lack of Rb-phosphorylation but high proliferation, indicating 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[15-19]; 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

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[33] 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[15], 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.

REFERENCES

1 **Kamb A**, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH. A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994; **264**: 436-440

2 **Serrano M**, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993; **366**: 704-707

3 **Marx J**. New tumor suppressor may rival p53. *Science* 1994; **264**: 344-345

4 **Nobori T**, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994; **368**: 753-756

5 **Okamoto A**, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH. Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA* 1994; **91**: 11045-11049

6 **Bird AP**. CpG-rich islands and the function of DNA methylation. *Nature* 1986; **321**: 209-213

7 **Eden S**, Cedar H. Role of DNA methylation in the regulation of transcription. *Curr Opin Genet Dev* 1994; **4**: 255-259

8 **Sakai T**, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TP. Allele-specific hypermethylation of the retino-blastoma tumor-suppressor gene. *Am J Hum Genet* 1991; **48**: 880-888

9 **Herman JG**, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, Baylin SB. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995; **55**: 4525-4530

10 **Gonzalez-Zulueta M**, Bender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, Jones PA. Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res* 1995; **55**: 4531-4535

11 **Herman JG**, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci USA* 1994; **91**: 9700-9704

12 **Ahuja N**, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, Issa JP. Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* 1997; **57**: 3370-3374

13 **Emig R**, Magener A, Ehemann V, Meyer A, Stilgenbauer F, Volkmann M, Wallwiener D, Sinn HP. Aberrant cytoplasmic expression of the p16 protein in breast cancer is associated with accelerated tumour proliferation. *Br J Cancer* 1998; **78**: 1661-1668

14 **Harada H**, Nakagawa K, Iwata S, Saito M, Kumon Y, Sakaki S, Sato K, Hamada K. Restoration of wild-type p16 down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human gliomas. *Cancer Res* 1999; **59**: 3783-3789

15 **Zhao P**, Hu YC, Talbot IC. Expressing patterns of p16 and CDK4 correlated to prognosis in colorectal carcinoma. *World J Gastroenterol* 2003; **9**: 2202-2206

16 **Sanz-Casla MT**, Maestro ML, Vidaurreta M, Maestro C, Arroyo M, Cerdán J. p16 Gene methylation in colorectal tumors: correlation with clinicopathological features and prognostic value. *Dig Dis* 2005; **23**: 151-155

17 **Cui X**, Shirai Y, Wakai T, Yokoyama N, Hirano S, Hatakeyama K. Aberrant expression of pRb and p16 (INK4), alone or in combination, indicates poor outcome after resection in patients with colorectal carcinoma. *Hum Pathol* 2004; **35**: 1189-1195

18 **Kim BN**, Yamamoto H, Ikeda K, Damdinsuren B, Sugita Y, Ngan CY, Fujie Y, Ogawa M, Hata T, Ikeda M, Ohue M, Sekimoto M, Monden T, Matsuura N, Monden M. Methylation and expression of p16INK4 tumor suppressor gene in primary colorectal cancer tissues. *Int J Oncol* 2005; **26**: 1217-1226

19 **Tada T**, Watanabe T, Kazama S, Kanazawa T, Hata K, Komuro Y, Nagawa H. Reduced p16 expression correlates with lymphatic invasion in colorectal cancers. *Hepatogastroenterology* 2003; **50**: 1756-1760

20 **Hibi K**, Nakao A. Lymph node metastasis is infrequent in patients with highly-methylated colorectal cancer. *Anticancer Res* 2006; **26**: 55-58

21 **O'Neill CJ**, McCluggage WG. p16 expression in the female genital tract and its value in diagnosis. *Adv Anat Pathol* 2006; **13**: 8-15

22 **Kresty LA**, Mallery SR, Knobloch TJ, Song H, Lloyd M, Casto BC, Weghorst CM. Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. *Cancer Res* 2002; **62**: 5295-5300

23 **Fregonesi PA**, Teresa DB, Duarte RA, Neto CB, de Oliveira MR, Soares CP. p16(INK4A) immunohistochemical overexpression in premalignant and malignant oral lesions infected with human papillomavirus. *J Histochem Cytochem* 2003; **51**: 1291-1297

24 **Wang D**, Grecula JC, Gahbauer RA, Schuller DE, Jatana KR, Biancamano JD, Lang JC. p16 gene alterations in locally advanced squamous cell carcinoma of the head and neck. *Oncol Rep* 2006; **15**: 661-665

25 **Ai L**, Stephenson KK, Ling W, Zuo C, Mukunyadzi P, Suen JY, Hanna E, Fan CY. The p16 (CDKN2a/INK4a) tumor-suppressor gene in head and neck squamous cell carcinoma: a promoter methylation and protein expression study in 100 cases. *Mod Pathol* 2003; **16**: 944-950

26 **Namazie A**, Alavi S, Olopade OI, Pauletti G, Aghamohammadi N, Aghamohammadi M, Gornbein JA, Calcaterra TC, Slamon DJ, Wang MB, Srivatsan ES. Cyclin D1 amplification and p16(MTS1/CDK4I) deletion correlate with poor prognosis in head and neck tumors. *Laryngoscope* 2002; **112**: 472-481

27 **Kaabipour E**, Haupt HM, Stern JB, Kanetsky PA, Podolski VF, Martin AM. p16 expression in keratoacanthomas and squamous cell carcinomas of the skin: an immunohisto-chemical study. *Arch Pathol Lab Med* 2006; **130**: 69-73

28 **Nilsson K**, Svensson S, Landberg G. Retinoblastoma protein function and p16INK4a expression in actinic keratosis, squamous cell carcinoma in situ and invasive squamous cell carcinoma of the skin and links between p16INK4a expression and infiltrative behavior. *Mod Pathol* 2004; **17**: 1464-1474

29 **Sano T**, Masuda N, Oyama T, Nakajima T. Overexpression of p16 and p14ARF is associated with human papillomavirus infection in cervical squamous cell carcinoma and dysplasia. *Pathol Int* 2002; **52**: 375-383

30 **Shigemasa K**, Hu C, West CM, Clarke J, Parham GP, Parmley TH, Korourian S, Baker VV, O'Brien TJ. p16 overexpression: a potential early indicator of transformation in ovarian carcinoma. *J Soc Gynecol Investig* 1997; **4**: 95-102

31 **Raspollini MR**, Amunni G, Villanucci A, Baroni G, Taddei GL. P16INK4a overexpression is associated with poor clinical outcome in ovarian carcinoma. *J Chemother* 2004; **16**: 411-413

32 **Darvishian F**, Stier EA, Soslow RA, Lin O. Immunoreactivity of p16 in anal cytology specimens: histologic correlation. *Cancer* 2006; **108**: 66-71

33 **Armes JE**, Lourie R, de Silva M, Stamaratis G, Boyd A, Kumar B, Price G, Hyde S, Allen D, Grant P, Venter DJ. Abnormalities of the RB1 pathway in ovarian serous papillary carcinoma as determined by overexpression of the p16(INK4A) protein. *Int J Gynecol Pathol* 2005; **24**: 363-368