Immunohistochemical detection of $p16^{INK4a}$ proteins expression in paraffin embedded sections of colorectal cancer tissues.

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Summary:

Background: Uncontrolled tumor cell proliferation is a reality in tumor cells, and the progression from a normal cell into a transformed cell probably includes genetic events affecting checkpoints in the cell cycle machinery**.**

Materials and methods: This study investigated the immunoexpression of $p16^{INK4a}$ in the paraffin sections from 43-cancers of colorectal tissue (CRC),26-hyperplastic polyps and adenomas(CRHPA) and 35-normal tissues (CRN),using immunohistochemical assay. We correlated the expression patterns with tumor histopathological type, site of the tumor, distance metastasis according to the TNM system.

Results: In colorectal cancer (CRC) patients' $p16^{INK4a}$,immunoexpression was detected in 32/43, there was significant association for $p16^{INK4a}$, which showed high reaction with monoclonal antibodies in the distal colon and rectal area with P value < 0.001. There was no significant correlation between tissue invasion stage and $p16^{INK4a}$. There was no significant difference in the immunoexpression of $p16^{INK4a}$ in relation to the two groups of patients with the stage of the disease.

Conclusion: $p16^{INK4a}$, expression have high level in CRHPA than in CRN tissue. Also, their expression in CRC is more than in CRN tissue. In addition, $p16^{INK4a}$ immunoexpression in CRC was significantly more than in CRHPA.

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Introduction:

Development of colorectal cancer (CRC) is a complex and multistep process in which several gene defects coordinate with each other in genotypic and phenotypic outcome (1).Mutations in many tumor suppressor and proto-oncogenes in the development of sporadic and hereditary colorectal cancers are well established (2). Uncontrolled tumor cell proliferation is a reality in tumor cells, and the progression from a normal cell into a transformed cell probably includes genetic events affecting checkpoints in the cell cycle machinery (3). The mammalian cell division cycle are cooperatively regulated by several classes of cyclin-dependent kinases (CDKs) whose activities are in turn constrained by CDK inhibitors (CKIs). CKIs that govern these events have been assigned to one of two families based on their structures and CDK targets. The first class includes the INK4 proteins (inhibitors of CDK4), so named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6. The proteins $p16^{INK4a}$, $p15I^{NK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ bind only to CDK4 and CDK6 but not to other CDKs or to D-type cyclins. The INK4 proteins can be differentiated with more broadly acting inhibitors whose actions affect the activities of cyclin D-, E-, and A-dependent kinases.

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The latter class includes p21Cip1, p27Kip1, and p57Kip2, all of which contain characteristic motifs within their amino-terminal moieties that enable them to bind both to cyclin and CDK subunits (4). The major substrate of D-type cyclin-CDK4/6 complexes is the retinoblastoma protein (pRb) (5). The first molecular event involving the actions of a CDK required for G1 progression is the phosphorylation of a substrate pRb by D-type cyclin-CDK4/6 complexes (6). Boonstra, in 2003(7), analyzed the expression of pRb, cyclin D1 and p27Kip1 in CRC and observed abnormalities in the protein expression in various fractions of the tumors and he reported that almost all cancers are associated with some defect in the pRb pathway. Unregulated phosphorylation of pRb by CDK4/6 due to either cyclin D1 overexpression or loss of functional p16INK4a could lead to uncontrolled cellular proliferation (8). Richard et al., (9) studied small and large tumor cell clusters at the invasive margin in CRCs patients and applied immunohistochemistry to explain the proliferative activity and the expression of G1/S regulatory proteins in those tumors. They found that the decrease in proliferation was correlated with the $p16^{INK4a}$ protein up-regulation (10). The $p16^{INK4a}$ methylation that caused gene silencing and loss of $p16^{INK4a}$ tumor suppressor function in colorectal tumors was associated with proximal location in the gut. This, reflect the difference in the loss of $p16^{INK4a}$ protein expression by promoter methylation between left- and right-sided primary colorectal carcinomas (11).

Materials and methods:

This study was carried out on 43 patients with colorectal carcinomas (CRC) (22 men and 21 women with an average age of 54 years and a range of 21 to 82 years). Clinical information was collected through direct interview with the patient, and by seeking his /her hospital record (Alkadhemia teaching hospital), as well as previous medical history. In addition, 26 paraffin blocks of colorectal hyperplastic polyp and adenoma (CRHPA) lesions were taken from archive of pathology laboratory of the above hospitals (14 male and 12 female patients) with an average age of 33.1 years (range between 19 to 60 years). Two specialized consultant histopathologist examined the H & E sections with heamatoxylin and eosin (H & E) for histological typing, stage grouping, and grading of the colorectal carcinoma. Normal colorectal tissue samples were taken as control.

Immunohistochemical detection:This was done according to Satoshi et al.,(2003) (12),using immunohistochemistry detection kit from DakoCytomation LSAB2 System- Horse Reddish Peroxidase (HRP) code KO673 (DakoCytomation, USA)and Anti-Human p16^{INK4a} Protein Code No./ K5336 ,Clone E6H4 as the monoclonal antibody.

Evaluation of the immunostaining:

We count the stained cells with the assistance of experienced histopathologist in order to avoid non-tumorous areas in the sections and the following slides examined:

1) Controls: The evaluations involve two types of control specimens

a) Positive tissue control: For each set of test conditions is included in each staining run. The positive tissue controls give weak positive staining so that we can detect slight changes in the primary antibody sensitivity.

b) Nonspecific negative control: To determine the signal specificity, we used the negative control antibody instead of the primary antibody with each patient specimen to evaluate the nonspecific staining and allow better interpretation of specific staining at the antigen site. The incubation period for the negative control antibody is the same as that of the primary antibody. It contains an antibody that exhibits no any specific reactivity with human tissues or normal/non-immune serum in the matrix/solution. The negative control antibody should be of the same subclass and animal species as the primary antibody and diluted to the equivalent immunoglobulin or protein concentration as the diluted primary antibody also using the same diluents. Therefore, as a negative control containing monoclonal mouse IgG2a anti Aspergillus niger glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissues. It used in the same dilution of the mouse antihuman antibody.

2) Scoring: reactivity was evaluated by counting the number of positively stained cells in 100 cells per HP field in 10 HP field.

A) For p16^{INK4a} the intensity of positivity scored as (Zhao et al., 2003):

i) Less than 5 % positive cell as negative (cutoff value)

ii) 5-25 % positive cell as (+)

iii) 26-50 % positive cell as (++)

iv) 51-75 % positive cell as (+++)

v) More than 75 % positive cell as (++++)

Statistical analysis:

Descriptive statistics were expressed as number and percentage. Chi-square ($\chi 2$) test of significance was adopted for the comparison and calculation of association in qualitative data**.** Mann-Whitney Test used to evaluate the comparison between (Colorectal Normal Tissue (CRN) and Colorectal Hyperplastic Polyp and Adenoma (CRHPA) and between Colorectal Hyperplastic Polyp and Adenoma (CRHPA) and Colorectal Cancer (CRC) and Colorectal Normal Tissue (CRN) and Colorectal Cancer (CRC)).

Results

In colorectal cancer (CRC) patients' p16INK4a immunoexpression was detected in 32/43 patient (table1). Patients with (CRC) were grouped according to the site of the tumor into proximal, distal and rectal. There was a significant statistical association between tumor site and detection of p16^{INK4a}, which showed high reaction with the monoclonal antibody in the distal colon and rectal area with P value < 0.001 (table2). Differences in the immunoexpression of p16^{INK4a} in relation to the histopathological types of tissue specimen was studied. There was no significant relationship between the histopathology of CRC and the marker's expression, (table3). In colorectal hyperplastic polyp and adenoma specimens (CRHPA), p16^{INK4a}, expression was detected in 12/26 patients (table1). The expression of this marker in relation to the site of the tumor had been studied and we found that, there was a significant high immunoexpression in distal colon and low expression in proximal colon with P value < 0.001 (table2). For the determination of the expressions in relation to the histopathological type(table 4), we found that the expression, were significant in tubular and villous adenoma with P value < 0.001. In colorectal normal specimens p16^{INK4a}, expression was detected below the used cutoff value and regarded as negative. Mann-Whitney test was used to detect the degree of significance in expression of this marker in each two groups (CRN and CRHPA, CRN and CRC, or CRC and CRHPA). Accordingly p16^{INK4a} immunoexpression has high level in CRHPA than in CRN tissue (table 5) with p value <0.001. Moreover, expression in CRC was more than in CRN tissue by means of p value <0.001 .Yet, p16^{INK4a} has more immunoexpression in CRC than in CRHPA with less degree of significance (p value = 0.011) (table 5).

Table 1: Immunexpression of p16 ^{INK4a} marker in patients groups (Colorectal cancer, CRC, and hyperplastic polyp and adenoma,CRHPA)

CRC (n=43)		CRHPA (n=26)	
Positive	Negative	Positive	Negative
(32) 74.4	(11) 25.6	(12) 46.2	(14) 53.8

Table 2: The effect of tumor site on the expression of p ^{16INK4a}in patients groups (colorectal cancer, CRC, and hyperplastic polyp and adenoma, CRHPA) According to Chi-square Test

	CRC		CRHPA	
Tumor Site	Positive (n)%	Negative (n)%	Positive (n)%	Negative (n)%
Proximal	(8) 44.4	(10) 55.6	(0) 0	(6) 100
Distal	(14) 93.3	(1) 6.7	(8)66.7	(4) 33.3
Rectal	(10) 100	(0) 0	(4) 50	(4) 50
P value	*P*=0.00006		*P*=0.27	

Table3: Distribution of the expression of p ^{16INK4a}in patients with colorectal cancer, CRC according to histopathological type According to. Chi-square Test.

Histopathological type	CRC	
	Positive (n)%	Negative (n)%
Adenocarcinoma	24 (72.7)	(9) 27.3
Mucinous carcinoma	(6) 75	(2) 25
Signet ring cell	(2) 100	(0) 0
P value	*P* = 0.691	

Table4: Distribution of the expression of p ^{16INK4a}in patients with hyperplastic polyp and adenoma (CRHPA) according to histopathological type. Chi-square Test.

Histopathological type	CRHPA	
	Positive (n)%	Negative (n)%
Hypertrophic	(2) 15.4	(11) 84.6
Tubular	(8) 72.7	(3) 27.3
Villous	(2) 100	(0) 0
P value	*P* = 0.0055	

Table 5: The Mean Ranks of the Immunoexpression of p16^{INK4a}, mRNA Expression in the Colorectal Normal Tissue (CRN) and Colorectal Hyperplastic Polyp and Adenoma (CRHPA) and between Colorectal Hyperplastic Polyp and Adenoma (CRHPA) and Colorectal Cancer (CRC) According to Mann-Whitney Test.

CR group	No.	Mean ranks	P value
CRN	35	27.13	<0.001
CRC	43	49.57	
CRHPA	26	27.15	0.011
CRC	43	39.74	
CRN	35	19.36	<0.001
CRHPA	26	46.67	

Discussion

Results demonstrated that cell cycle regulatory protein (p16INK4a) is disturbed during colorectal cancer progression. And that, p16INK4a immunoexpression was found in human colorectal tissue including, colorectal normal (CRN), colorectal hyperplasia and adenoma (CRHPA), and colorectal cancer (CRC). We showed that 14.3 % of the normal colorectal tissue had no immunoexpression and the other slides have < 5-percentage immunoexpression and regarded as negative. This finding goes with other study done by (9) that revealed all normal samples were essentially p16^{INK4a} negative, with only few scattered positive cells along the crypt axis with no specificity. In the CRHPA group 46.2% of patients gave positive immunoexpression for p16^{INK4a}. However, if we exclude the hyperplastic polyp (which are similar in immunoexpression to normal tissue) from the calculation and include the tubular and villous adenoma cases only this study find the percent of immunoexpression rises to 76.9% positive, most of them with low immunoexpression score (5-25%). When we compared this result with the CRC, this study found that 74.4% have positive reaction after IHC staining, most of them with high immunoexpression score. In other studies, Nadir et al., in 1999(13), found that the most common alteration involved an increase in the p16INK4a protein immunoexpression in 92% of the adenomas and 91% of the adenocarcinoma. Using Mann-Whitney U test, there was significance high immunoexpression of p16^{INK4a} in CRC than in CRN and CRHPA, in addition to high immunoexpression in CRHPA than in CRN specimens. This can be discussed if we know that p16^{INK4a} is a tumor-suppressor protein and increased in relation to increase of proliferation rate in attempt to overcome this increasing cell cycle rate in colorectal tissue, and presence of p16^{INK4a} genetic alterations was associated with shorter survival in CRC patients (14, 15). In addition, The CDKN2/ p16^{INK4a} gene product has been found to them nonfunctional in a high percentage of cell lines (75%) and various malignancies (16).Richard et al. in 2000(9), suppose that decrease in the proliferation rate was correlated with a p16^{INK4a} up-regulation and CRCs lacking p16^{INK4a} immunoexpression or tumors with other aberrations in the p16^{INK4a} /cyclin D1/pRb pathway had a less pronounced decrease in proliferation. An interesting point found that p16^{INK4a} significantly have high immunoexpression in distal colon and rectal area than in proximal colon in both tumor type CRHPA and CRC with P = 0.027 and 0.000004 respectively. In conclusions: p16^{INK4a}, expression have high level in CRHPA than in CRN tissue with p value <0.001. Also, their expression in CRC is more than in CRN tissue with a p value <0.001 In addition, p16INK4a immunoexpression in CRC was significantly more than in CRHPA with p value = 0.01.

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