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Immunohistochemical Expression of P16^{ink4a} in Colorectal Adenocarcinoma Compared to Adenomatous and Normal Tissue Samples: A Study on Southeast Iranian Samples

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Abstract

Background: Colorectal cancer (CRC) is one of the most common neoplasms of the digestive system with high morbidity and mortality. Treatment of CRC in advanced metastatic stages is problematic. Thus, early diagnosis in primary stages using sensitive molecular markers seems to be necessary.

Objectives: This study aimed at investigating the immunohistochemical expression of P16^{ink4a} in CRC compared to adenomatous and normal colorectal tissue samples of a population from the southeast of Iran.

Methods: This case-control study was conducted on 137 colorectal formalin fixed paraffin-embedded tissue blocks for P16^{ink4a} a protein using Immunohistochemistry (IHC). The tissue blocks were categorized in 3 groups, including adenocarcinoma (n = 63), adenomatous (n = 38), and normal (n = 36). All tissue blocks were collected from the pathology department of Ali-Ebne-Abitaleb central and referral Hospital, Zahedan, Iran from 2010 to 2015. The sections were evaluated using semi-quantitative scoring. The P16^{ink4a} expression was reported as negative and positive. Clinicopathological characteristics were also assessed. The data were analyzed by Kruskal-Wallis and Fisher exact tests. The significance level was considered as $P < 0.05$.

Results: The expression of P16^{ink4a} in adenocarcinoma, adenomatous, and normal colorectal tissues was 25.40%, 50.00%, and 69.50%, respectively. The P16^{ink4a} expression was significantly higher in non-neoplastic tissues compared to the adenomatous and colorectal tissues ($P < 0.001$). There was a significant association between P16^{ink4a} expression and differentiation grade ($P < 0.001$) and primary location of the tumor ($P = 0.010$).

Conclusions: Considering the significant expression of P16^{ink4a} in normal compared to adenomatous and cancerous samples, it seems that this biomarker could be used as a potential useful predictor for screening and diagnosis of CRC patients in early stages. Further researches should be conducted on this matter.

Keywords: Colorectal Cancer, Immunohistochemistry, P16^{ink4a}

1. Background

Colorectal cancer (CRC) is one of the most common malignancies of the alimentary canal with high morbidity and mortality. Colorectal cancer is the third most common cancer (almost 9.4% of all malignancies) and the fourth-leading cause of death due to cancer worldwide (1, 2). Annually, more than half a million people of the 1.4 million new cases diagnosed with CRC lose their lives (3, 4). Development of this neoplasm has its geographical and racial distribution. In countries such as North America, Australia, New Zealand, and Western Europe, the highest prevalence of CRC were reported. According to the statistics, its incidence is increasing in Asian countries and has become a major health problem globaly (5, 6). In Iran, reports have suggested that the mortality rate due to CRC was high. From 5000 (7 per 100,000 individuals) CRC new cases, 2263 case lose their lives, annually (7).

Furthermore, CRC is a multi-stage and multifactorial malignancy. Accumulation of genetic and epigenetic changes due to long-term exposure to various risk factors causes pathological changes in colorectal epithelium and results in polyp formation and then its progression to malignant changes (8).

Colorectal cancer treatment approaches include surgery (choice treatment), adjuvant chemotherapy, and radiotherapy (9). Despite the effectiveness of these treatments, advanced metastatic stages of CRC are difficult to cure and 5-year survival rate of the patients are considerably low (2). In order to diagnosis CRC in primary stages and for appropriate treatment according to the patient's condition, implication of sensitive and novel detecting

methods based on prognostic factors at the molecular level seems to be necessary.

$P16^{ink4a}$ is a nuclear protein that plays a crucial role in negative regulation of cell cycle, which leads to cell proliferation arrest. $P16^{ink4a}$, through the inhibition of cyclin-dependent kinase proteins (CDK4/6) and interaction with cyclin-D, could cause arrest of cell proliferation at phase G1 to S. Thus its expression increases markedly in senescence and normal aging cells (10).

Dysregulation of involved cell cycle mechanisms leads to uncontrolled cell proliferation and resistance to apoptosis in damaged cells, and finally tumorigenesis in tissues could occur (11). $P16^{ink4a}$, through the control of cell cycle mechanisms, prevents cell proliferation and averts tumorigenesis. Because of the important role of $P16^{ink4a}$, it is considered as a tumor suppressor protein (1, 10).

Immunohistochemical (IHC) evaluation of $P16^{ink4a}$ expression has been used as a diagnostic and prognostic tool in benign and malignant lesions of the skin, head, neck and genital tract (12-14). In the recent years, studies regarding $P16^{ink4a}$ IHC expression in CRC have shown that its upregulated expression in normal and senescence cells had occurred and was accompanied by high survival rate and good prognosis in the patients (15, 16). However, studies have stated that $P16^{ink4a}$ detection in colorectal tissue is related to worse (poor) prognosis and metastasis (17, 18). Results of several studies regarding expression rate of $P16^{ink4a}$ in colorectal normal, premalignant, and malignant tissues were very inconsistent, and expression rates of $P16^{ink4a}$ were reported at a range of 7% to 98% in CRCs (10, 19, 20). Understanding the expression pattern of biomarkers involved in normal control of cell cycle may contribute to improvement strategies for prevention, screening, diagnosis, treatment, and management of CRC patients (21).

Furthermore, due to controversies in findings of different previous studies regarding $P16^{ink4a}$ expression and its probable importance in early diagnosis of CRC prior to the development of malignancy and metastasis, it was decided to study $P16^{ink4a}$ expression in CRC samples compared to adenomatous and normal colorectal tissues at the molecular level.

2. Objectives

The aim of this study was to investigate the immunohistochemical expression of $P16^{ink4a}$ as a molecular biomarker in CRC compared to adenomatous and normal colorectal tissue samples.

3. Methods

3.1. Study Design and Sample Selection

This case-control study was carried out on 137 surgical resected specimens from colorectal region archived as formalin fixed paraffin-embedded tissue blocks. All archived tissue blocks were selected based on inclusion and exclusion criteria from pathology files of Ali-Ebne-Abitaleb referral hospital, Zahedan, Southeast of Iran, from year 2010 to 2015. The inclusion criterion for the specimens was suitable formalin fixed paraffin-embedded tissue along with complete clinicopathological data. The tissues with autolysis specimens, metachronous CRC, inadequate biopsy sample, inflammatory lesions, and other malignancies of gastrointestinal tract were excluded from the study. Accordingly, 22 samples were excluded and 137 tissue samples were enrolled in the present study.

The study samples, according to the histopathological diagnosis, were grouped as follows, colorectal adenocarcinoma (n = 63), polyp adenomatous (n = 38), and normal (n = 36) tissues.

All colorectal tissue specimens were re-examined by an expert pathologist for histopathological diagnostic confirmation prior performing IHC.

3.2. Ethical Considerations

This study was approved by the ethics committee of Zahedan University of Medical Sciences (No: IR.ZAUMS.REC.1394.327).

3.3. Immunohistochemistry Conventional Staining of $P16^{ink4a}$

The $P16^{ink4a}$ expression in colorectal tissue samples was detected using IHC, according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc, USA). Selected tissue blocks were cut to 3-μm thin sections using fully automated microtome (Leica, RM2255, Germany) and mounted on HistoGrip (CEDARLANE, Canada) coated glass slides. The sections were dewaxed in Xylene (Merk, Germany) and rehydrated with descending graded ethanol (Merk, Germany). Antigen retrieval by means of heat induction (20 minutes at 120°C) was conducted under pressure in sodium citrate buffer (10 mM solution, pH = 6) in an autoclave (Medical Prestige, Series 210003 Classic, England). Tissue sections were placed at room temperature to cooldown and eluted in phosphate buffered saline (PBS) (Santa Cruz Biotechnology, Inc, USA). The sections were incubated for 10 minutes in endogenous peroxidase block and rinsed with PBS, and serum block was added in a drop-wise manner for 5 minutes. The sections were then incubated with mouse monoclonal primary antibody P16 (Santa Cruz, P16 C-7, SC-2053, USA), with a dilution of 1:100

at 4°C overnight and placed in PBS. Biotinylated secondary antibody was added to the slides and were incubated at room temperature for 30 minutes and washed with PBS. The samples were incubated in Avidin D-HRP complex and eluted with PBS twice for 2 minutes. The sections were incubated with HRP substrate containing Diaminobenzidine (DAB) for 10 minutes and rinsed with distilled water. Ultimately, the sections were counterstained with Papanicolau's hematoxylin, rehydrated, and sealed with slides. The IHC scoring was conducted by 2 expert histologists that were blind regarding the pathological diagnosis. In case of discrepancy among their scores, to ensure the accuracy of assessments, a third histologist did further assesments.

The positive control samples for $P16^{ink4a}$ detection were periodontits. Negative control samples were incubated with PBS instead of special antibody.

3.4. $P16^{ink4a}$ IHC Scoring

$P16^{ink4a}$ evaluation was performed through determination of the extent (proportion of positive cells) and intensity of immunoreactivity positive cells. Percentage of positive cells was as follows, (0) less than 5% positive cells, (1) between 5% and 20%, (2) between 25% and 50%, (3) between 50% and 75%, and (4) more than 75% tumoral cells. Intensity score was defined as negative (0), weak (1), moderate (2), and strongly positive (3). The final score for each section was calculated by multiplying the extent by immunostaining intensity, yielding a range of 0 to 12. Scores were classified semi-quantitatively as follows, 0 to 4 as negative, 5 to 8 as weak, and 9 to 12 as strong immunostaining (22). Scoring of samples was done under a light microscope (Zeiss, Germany) with a 400X magnification.

3.5. Statistical Analysis

The data were represented as mean ± standard error of mean (SEM). To identify statistical differences between groups, the Kruskal-Wallis test was used. Also for analyzing the association between $P16^{ink4a}$ IHC expression status and clinicopathological parameters, Pearson's chi-square or Fisher's exact tests were conducted. All statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The significance level was considered as $P < 0.05$.

4. Results

4.1. Clinicopathological Data

The mean age of enrolled cases was 58.32 ± 1.92, in the range of 20 to 83 years. The majority of CRC cases were female (40; 63.50%), mucinous adenocarcinoma type (51; 81.00%), well differentiated (37; 58.70%) and distant metastasis (49; 77.80%) and located in the sigmoid region (38; 60.30%). More details on the history of patients are presented in Table 1.

Table 1. Clinicopathological Variables and Relationship with $P16^{ink4a}$ Expression Status in Colorectal Cancer Specimens[a]

Parameters	Value	P Value
Age, Mean ± SEM, y (range)	58.32 ± 1.92	0.909
Gender		0.458
Male	23 (36.50)	
Female	40 (63.50)	
Type		0.746
Mucinous adenocarcinoma	51 (81.00)	
Non- Mucinous adenocarcinoma	12 (19)	
Primary location of tumor		0.010
Cecum	2 (3.20)	
Ascending colon	5 (7.90)	
Transverse colon	8 (12.70)	
Descending colon	1 (1.60)	
Sigmoid	38 (6.30)	
Anorectal	8 (12.70)	
Histological differentiation grade		< 0.001
Well	37 (58.70)	
Moderate	15 (23.80)	
Poor	12 (19.00)	
Lymph node metastasis		0.540
Yes	15 (23.80)	
No	48 (76.20)	
Metastasis		0.874
Yes	49 (77.80)	
No	14 (22.20)	

[a]Values are expressed as N0.(%).

The results of statistical tests showed that there were significant associations between $P16^{ink4a}$ expression and primary site of tumor ($P = 0.010$) and histological differentiation grade ($P < 0.001$), whereas such association was not found between age ($P = 0.909$), gender ($P = 0.458$), type of tumor ($P = 0.746$), lymph node metastasis ($P = 0.540$), and distant metastasis ($P = 0.874$) (Table 1).

According to the findings, positive expressions of $P16^{ink4a}$ protein in CRC, adenomatous and normal colorectal tissues were 25.40%, 50%, and 69.50%, respectively. In addition, statistical analysis showed that $P16^{ink4a}$ positive expression in normal tissues was significantly higher than

adenomatous and CRC samples ($P < 0.001$) (Table 2) (Figure 1).

5. Discussion

The results of the present study showed that $P16^{ink4a}$ expression in normal colorectal tissues was significantly higher than adenomatous and CRC samples. A gradually decreasing trend of $P16^{ink4a}$ positivity was observed from CRC to adenomatous and finally to normal tissues.

Qian, in his study on $P16^{ink4a}$ protein expression in CRC samples and its clinical importance, showed that $P16^{ink4a}$ expression in normal samples was 73.33%, while in CRC samples, this was 23.33%, and the difference between the 2 groups was statistically significant (10). Several studies have also indicated that $P16^{ink4a}$ expression in normal colorectal tissues was higher than that of CRCs (17, 19, 23). Nikbakht Dastjerdi et al. in their study on the role of $P16^{ink4a}$ in tumor suppression, also stated that $P16^{ink4a}$ expression occurred in both normal and cancerous samples, yet its expression in cancerous tissues was significantly lower than adjacent normal ones (20). In accordance with these findings, other studies have also indicated that $P16^{ink4a}$ expression in normal colorectal tissues was higher than that of CRCs (17, 19, 23).

On the other hand, Zhao et al. showed that $P16^{ink4a}$ expression in colorectal carcinoma was higher than that of adenoma and adjacent normal tissues; $P16^{ink4a}$ was expressed in 98.6% of colorectal adenocarcinoma. They stated that $P16^{ink4a}$ expression in colorectal carcinoma was high while it was low or sometimes moderate in adjacent normal tissues. Considering the rate of $P16^{ink4a}$ expression in cancerous samples, they proposed that individuals with a high $P16^{ink4a}$ expression had a significantly better prognosis compared to individuals with low expression; high $P16^{ink4a}$ expression was found in 78.9% of patients with a survival rate of more than 5 years, while decreased survival rate in those with a low $P16^{ink4a}$ expression was seen (22). Lam et al. also found that $P16^{ink4a}$ expression in colorectal adenocarcinoma was significantly higher than colorectal normal tissues; expression was 80% in colorectal adenocarcinoma, yet in samples of normal colon, the $P16^{ink4a}$ was not expressed at all. These findings showed considerable expression of $P16^{ink4a}$ protein in cancerous samples compared to normal ones (24). The findings of the last 2 studies in terms of $P16^{ink4a}$ expression in normal and CRC tissues suggested significant conflict with the above-mentioned and the current study. Other studies about $P16^{ink4a}$ expression in cancerous cells, especially CRC, reported different levels of expression ranging from a low level of about 17% to a high level of more than 95% ((19, 20, 22, 25). This can be attributed to the different grades and level of differentiation in their specimens. It seems that higher $P16^{ink4a}$ expression is correlated with greater differentiation of the CRC cases.

The $P16^{ink4a}$ protein through negative controlling cell proliferation by inhibiting CDK4 kinase prevents cell proliferation and inhibits cell cycle in G1 to S phases (26). Considering the critical role of $P16^{ink4a}$ gene in a cell nucleus, its damage or any epigenetic modifications such as hypermethylation and genetic changes, such as point mutation or its absence, could have harmful effects on mechanisms controlling cell cycle and cause dysregulation in proliferation pattern of normal cells (27, 28). The $P16^{ink4a}$ protein is predominantly involved in cellular critical functions, particularly in cell cycle regulation, and development and progression of tumors. Therefore, damage or modifications of $P16^{ink4a}$ gene or gene product (protein) could cause modifications in cell behaviors, such as proliferation and apoptosis patterns. It seems that occurrence of these changes in molecular level could effect $P16^{ink4a}$ IHC expression pattern in adenomatous and adenocarcinoma samples compared to normal colorectal tissue samples.

The $P16^{ink4a}$ protein expression in other malignancies, such as breast cancer, cervical carcinoma, and gastrointestinal stromal tumors, revealed that the $P16^{ink4a}$ biomarker is closely associated with patients' prognosis, occurrence, drug resistance, response to treatment, trend disease, and even tumorigenesis (29-31).

Regarding the clinicopathological features, the current study revealed that there was no significant relationship between $P16^{ink4a}$ expression and age, gender, type of tumor, lymph node involvement, and distant metastasis. On the other hand, there was a significant association between $P16^{ink4a}$ expression and cell differentiation degree and primary tumor site so that higher level of $P16^{ink4a}$ expression was noted in tissues with poor differentiation grade compared to moderate and well differentiation grade. The $P16^{ink4a}$ expression was also significantly higher in left colorectal tumors than right ones.

Lam et al., regarding histopathological variables, showed that $P16^{ink4a}$ expression in moderately to well-differentiated cells was higher than poorly differentiated cells and left colorectal tumors had a higher level of $P16^{ink4a}$ expression than right ones. They also reported a significant relationship between $P16^{ink4a}$ expression and lymph node involvement, metastasis, and patient's gender (24). Researchers have found that it is possible to diagnose left, right, and colorectal tumors using clinical and molecular methods; after molecular research and determination of the rate of expression of biomarkers, they stated that at older ages and in females, right colorectal tumors occur more than left tumors, and more importantly they found that right-sided tumors response significantly to 5-flurouracil-based chemotherapy (32-

Table 2. P16^{ink4a} Expression Status in Colorectal Adenocarcinoma, Adenomatous, and Normal Tissues

Biomarker	Tissue Sections	Negative	Positive		Mean ± SEM
			Weak	Strong	
P16	Colorectal adenocarcinoma	47 (74.60)	10 (15.90)	6 (9.50)	2.90 ± 0.429
	Adenomatous	19 (50.00)	15 (39.50)	4 (10.50)	4.37 ± 0.623
	Normal	11 (30.60)	11 (30.60)	14 (38.90)	6.77 ± 0.648

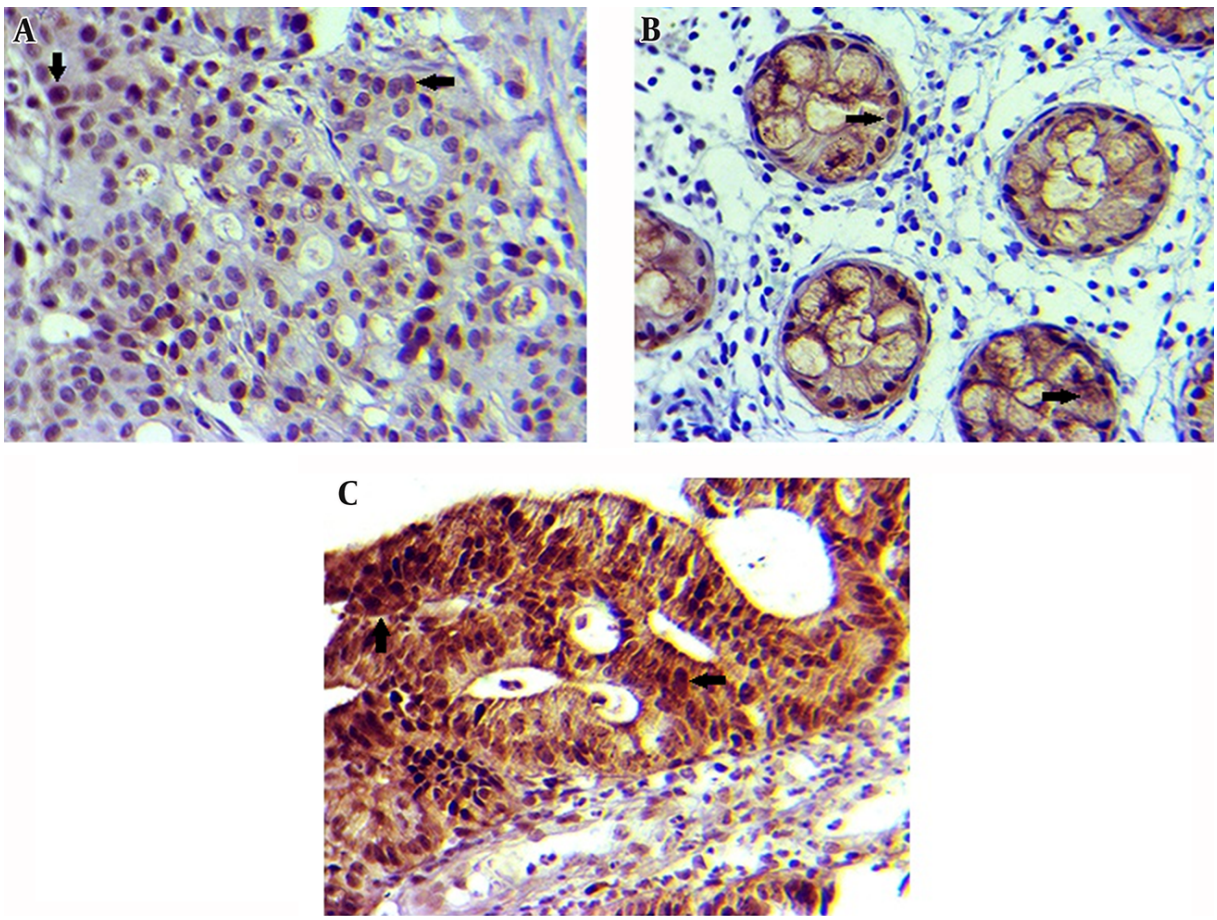

Figure 1. Expression of P16^{ink4a} in Adenocarcinoma (A), Adenomatous (B) and Normal (C) Human Colorectal Specimens (IHC, 400X Magnification)

34). It was proposed that right and left tumors of colon show different behaviors. It seems that the difference in bacterial flora and intestine passing time in right-sided and left-sided colon through exposure to normal colon epithelium (mucosa) with potential carcinogen factors may effect the pattern of P16^{ink4a} protein expression in neoplastic and normal cells.

Qian's study about P16^{ink4a} expression and its relationship with clinicopathological features showed that P16^{ink4a} expression in poorly-differentiated tissues was significantly higher than moderately to well-differentiated tissues (10), and this finding was in accordance with the current results. On the other hand, Carneiro et al. could not show any significant association between differentiation degree and tumor histological type and P16^{ink4a} expression (19).

King-Yin Lam et al. showed that although the positive P16^{ink4a} expression was higher in distal colorectal region,

there was no significant correlation between $P16^{ink4a}$ expression and clinicopathological features such as age, gender, lymph node involvement and metastasis, and even primary tumor site (35).

Various studies have shown different and controversial results regarding the relationship between $P16^{ink4a}$ expression and clinicopathological features; nevertheless, it seems that $P16^{ink4a}$ expression is influenced by various factors, the most important being insufficient sample size, restrictions in immunohistochemistry technique, and population heterogeneity and genetic differences. Despite this content, considering the effects of these variables on the rate and pattern of $P16^{ink4a}$ expression in patients with CRC, one can obtain useful information about the natural history of this disease, prognosis, survival rate, and response to treatment, and this information could be used for therapeutic purposes. The main weak point of the study was the low sample size (one of the limitations of this study). The strong points of this study was that the evaluation of P16 IHC expression as a cancer biomarker and its relationship with clinicopathological parameters of colorectal cancer patients are novel issues and there is limited data in this regard, especially in Iran. Further studies with larger sample sizes in different subgroups of CRC are needed.

In conclusion, the findings of the current study revealed that the IHC expression of $P16^{ink4a}$ in normal samples was significantly higher than CRC and adenomatous tissue samples. Considering differences in IHC expression of $P16^{ink4a}$ protein in normal cells compared to neoplastic cells, it seems that $P16^{ink4a}$ protein could be used as a potential useful independent biomarker in screening and diagnosis of early stages of neoplasms, prognosis, target therapy, and even evaluation of response to treatment in CRC cases. Further researches are needed to clarifying the importance of $P16^{ink4a}$ expression and its role in tumorigenesis route.

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Footnotes

Authors' Contribution: Zahra Heidari and Hamidreza Mahmoudzadeh-Sagheb co-designed the study, supervised all the experiments, and analyzed the results. Enam Alhagh Charkhat Gorgich participated in the experimental techniques, collecting and selection of samples, analyzed the results, and drafted the manuscript. All authors read, modified, and approved the final version of the manuscript.

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