RESEARCH ARTICLE

P16ink4a Subcellular Expression Patterns in Colorectal Adenocarcinoma, Adenoma and Non-Neoplastic Tissue Samples

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Abstract

Background: Colorectal cancer (CRC) is one of the most common neoplasms with high mortality at advanced stages worldwide. Thus diagnosis of CRC at an early stage with sensitive molecular methods is a high priority. The aim of this study was to evaluate P16ink4a subcellular expression patterns in colorectal adenocarcinoma, adenoma and non-neoplastic tissue samples. **Methods:** A total of 137 colorectal formalin fixed paraffin-embedded tissue blocks from the pathology archives of Ali-Ebne-Abitaleb central hospital, Zahedan, Iran, were examined in three groups: adenocarcinoma (n= 63), adenoma (n= 38) and non-neoplastic (n= 36). The subcellular expression pattern was determined by immunocytochemistry. Data analysis was performed using Kruskal-Wallis and Fisher exact tests with the significance level set as $p<0.05$. **Results:** P16ink4a subcellular localization was observed in three different patterns, nuclear+cytoplasmic (73.33%), cytoplasmic (13.33%) and nuclear (13.33%). In most samples, nuclear+cytoplasmic was the predominant subcellular pattern. However, a significant difference in P16ink4a subcellular expression patterns was observed along the non-neoplastic, adenoma, adenocarcinoma sequence ($p<0.001$). An association with the histological tumor type was also noted ($p=0.021$). **Conclusion:** Considering variation in localization of P16ink4a under different pathological conditions, P16ink4a night be sensitive prognostic biomarker for benign colon lesions. Its use may improve strategies for screening, prognostic assessment and management of patients with CRC. Further studies are recommended in this field.

Keywords: Colorectal cancer- P16ink4a- immunohistochemistry- localization- aberrant expression

Asian Pac J Cancer Prev, **18 (11)**, 3049-3054

Introduction

Cancer is one of the main health problems in all countries. Colorectal cancer (CRC) is a malignant neoplasm in the alimentary canal with over one million new cases in each year that accompanied by more than 500,000 deaths annually (Jemal et al., 2011; Summers et al., 2013). In other words, CRC is known as the third most malignant disease and the fourth cancer-related death worldwide (Stewart and Wild, 2014). Although, surgical treatment is associated with satisfactory outcomes, however, due to late diagnosis in advanced metastatic stages, surgery alone is not enough and adjuvant chemotherapy and radiotherapy should be used for treatment of CRC patients (Melling et al., 2016). Nowadays, diagnostic and prognostic values of screening methods that are available for detection of colorectal cancers are very limited; due to discomfort for patients (colonoscopy), risks, costs and lack of sensitivity (fecal occult blood test) (Winawer et al., 2003; Collins et al., 2005).

Generally, genetics aberrant alterations occur in early tumor progression. Analysis and detecting specific biomarkers at molecular levels can predict the tumor's metastatic potential in benign conditions (Melling et al., 2016). Recently, various biomarkers are available for CRC detection. One of the most common is carcinoembryonic antigen (CEA). However, sensitivity and specificity of CEA for prognosis of CRC is limited (Bast Jr et al., 2001; Roessler et al., 2006; Ma et al., 2010). Use of novel biomarkers for precise diagnosis and prognosis of CRC in early stages seems to be necessary.

P16ink4a is a nucleoprotein that through binding to CDK4/6 and inhibition of cyclin D-CDK complex stops phosphorylation of protein retinoblastoma (pRB). Then, through interaction with other molecular pathways involved in cell cycle regulation can cause prevention of cell proliferation and arrest of the cell cycle in G1 to S phases. This protein acts as an antiproliferative factor in damage and aged cells and in this way, it plays a crucial role in pathogenesis and tumorgenesis of CRC (Lam et al., 2008; Qian-Qian, 2015). Results of previous studies on P16ink4a immunohistochemical (IHC) expression in various pathological conditions are very different and sometimes contradictory with each other (Romagosa et

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al., 2011). However, some of studies have shown that overexpression of P16^{ink4a} occurs in some neoplasms. High expression of P16^{ink4a} was used as a prognostic biomarker markedly in human papillomavirus (HPV) and cervical cancer (Gonzalez and Serrano, 2006; Mulvany et al., 2008; Buajeeb et al., 2009; Serup-Hansen et al., 2014). On the other hand, histopathological roles of P16^{ink4a} expression in CRCs have been studied by many researchers but there was no agreement between their reports (Schneider-Stock et al., 2003; Cui et al., 2004; Carneiro et al., 2006; Qian-Qian, 2015). The results of some studies suggested that P16^{ink4a} subcellular expression patterns in CRC occur in different pathological conditions (Zhao et al., 2006; Lam et al., 2008). It seems that finding out variations in expression patterns of cancer biomarkers in different clinical stages may improve strategies for early detection and management of patients with CRC (Zoratto et al., 2014; Heidari et al., 2017b).

Therefore, the aim of this study was to evaluate the subcellular expression patterns of P16^{ink4a} gene product different stages of CRC route comprising Adenocarcinoma, adenoma and non-neoplastic in colorectal human tissue samples.

Material and Methods

Study Design and Sample Selection

This case-control study was conducted on 137 cases of colorectal surgical samples archived as formalin fixed paraffin-embedded (FFPE) tissue blocks. All of the tissue blocks were retrospectively collected from pathology archives of Ali-Ebne-Abitaleb central hospital, in Zahedan, southeast of Iran, from 2010 to 2015. The samples were categorized based on their histopathological diagnosis into three groups; adenocarcinoma (n=63), adenomatous polyp (n=38) and non-neoplastic tissues (n=36). Inclusion criteria for sample selection were considered as: suitable FFPE tissue blocks with a complete clinicopathological data. Exclusion criteria of the study were: autolysis specimens, inadequate biopsies, metachronous CRC, having inflammatory diseases and other neoplasms of the gastrointestinal tract.

All colorectal tissues previously were re-examined by an expert pathologist to ensure the histopathological diagnosis before accomplishing immunohistochemistry (IHC) as described in our previous study (Heidari et al., 2017a).

This study was approved by the Ethics Committee of ZAUMS (No: IR.ZAUMS.REC.1394.0327).

P16^{ink4a} Immunohistochemistry

P16^{ink4a} expression was detected using IHC based on the manufacturer's instructions (Santa Cruz Biotechnology, Inc). The selected tissue blocks were cut into 3 µm thick sections using a fully automated microtome (Leica, RM2255, Germany) and mounted on HistoGrip (CEDARLANE, Canada) coated glass slides. The sections were dewaxed and rehydrated. Heat-induced antigen retrieval was conducted in sodium citrate buffer (PH=6, 10 mM) in an autoclave for 20 minutes and then placed the slides at room temperature to cooldown. Then, were incubated with P16 mouse monoclonal primary antibody (Santa Cruz, P16 C-7, SC-2053, USA) dilution 1:100 at 4◦ C overnight. Another stage of IHC procedure was described as precisely and with technical details in our previous study (Heidari et al., 2017a). The sections were counterstain with Papanicolau's hematoxylin and evaluations were done by two histologists were blinded to the pathological diagnosis.

The positive control for P16^{ink4a} detection was a ductal breast carcinoma sample. For negative control samples incubated with PBS instead of special antibodies.

P16^{ink4a} Immunocytochemical Evaluation

P16^{ink4a} scoring was conducted through the proportion of positive cells (extent) and intensity of immunoreactivity. The extent (score between 0-4) and intensity (score between 0-3) scores for each section were calculated by multiplying the extent by immunostaining intensity. Then, scores were categorized by semi-quantitatively: negative (0-4), weak (5-8) and strongly (9-12) immunoreactivity (Heidari et al., 2017a).

Only samples with IHC P16^{ink4a} positive expressions were selected for analysis of subcellular expression patterns and samples with negative expression were excluded from the study. For this purpose, the subcellular expression was determined based on the localization of the immunoreactivity of P16^{ink4a} positive cells and classified into three expression patterns: cytoplasmic+nuclear (C+N), cytoplasmic (C) and nuclear (N). All of observations and tissues examinations were done under a light microscope (Zeiss, Germany) with a 400X magnification.

Statistical Analysis

In order to evaluate the statistical differences of P16^{ink4a} subcellular expression status between different groups, Kruskal-Wallis test was used. To reveal a statistical association between P16^{ink4a} subcellular expression status and histopathological variables exact Fisher test was used. The statistical analysis was conducted by SPSS software under Windows edition 16.0 (SPSS Inc., Chicago, IL, USA). The significance level was set as $p<0.05$.

Results

A total of 137 tissue were enrolled in the study; 71 (51.80%) specimens were from male and 66 (48.20%) female patients. Also, the mean age of surveyed cases was 58.32±1.92, in range of 20-83 years. More clinicopathological details and their relationships with P16^{ink4a} expression patterns were presented in Table 2.

The results of this study showed that P16^{ink4a} protein was expressed with three different subcellular expression patterns in tissue samples (Figure 1). C+N, C and N expression patterns were 73.33%, 13.33% and 13.33%. According to this finding, the dominant expression pattern of P16^{ink4a} in all colorectal specimens was C+N. In addition, there was a significant difference between P16^{ink4a} subcellular expression patterns in non-neoplastic-adenomatous-adenocarcinoma sequence mucosa ($p<0.001$), (Table 1).

Regarding P16^{ink4a} subcellular expression pattern and

Table 1. P16ink4a Subcellular Expression Pattern in Colorectal Adenocarcinoma, Adenomatous and Non-Neoplastic Tissue Specimens

Tissue Samples	Number	Subcellular Expression Pattern of P16ink4a			p-value
		Cytoplasmic/Nuclear	Cytoplasmic	Nuclear	
Adenocarcinoma	16	11 (68.75%)	4 (25.00%)	1 (6.25%)	
Adenomatous	19	15 (78.94%)	1 (5.26%)	3 (15.80%)	
Non-neoplastic	25	18 (72.00%)	3 (12.00%)	4 (16.00%)	0.001
Total	60	44 (73.33%)	8 (13.33%)	8 (13.33%)	

Table 2. Relationship between P16ink4a Subcellular Expression Pattern and Histopathological Features in Colorectal Adenocarcinoma Tissue Specimens

Histopathological Characterized	Subcellular Expression Pattern of P16ink4a			p-value
	Cytoplasmic/Nuclear	Cytoplasmic	Nuclear	
Tumor Type (n)				
Mucinous adenocarcinoma	10	4	0	0.021
Non- Mucinous adenocarcinoma	1	0	1	
Histological Differentiate Grade (n)				
I	2	2	0	
II	3	0	0	0.526
III	6	2	1	
Location of Tumor (n)				
Cecum	1	1	0	
Descending colon	0	1	0	0.24
Sigmoid	8	1	0	
Anorectal	2	1	1	
Lymph Node Metastasis (n)				
Yes	3	0	0	0.432
No	8	4	1	
Distant Metastasis (n, %)				
Yes	8	3	1	0.834
No	3	1	0	

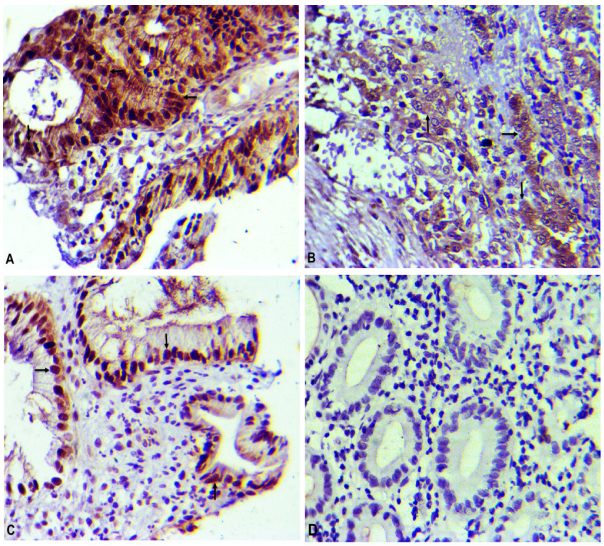

Figure 1. P16ink4a Different Subcellular Expression Patterns (A) cytoplasmic/nuclear; (B) predominantly cytoplasmic; (C) predominantly nuclear and (D) negative control. (IHC, Magnification 400X)

histopathological features in colorectal adenocarcinoma results showed that there was a significant relationship between P16ink4a subcellular expression pattern and histological tumor type (p=0.021). On the other hand, there was not any statistical association between P16ink4a expression pattern and tumor grade, the primary site of lesion, lymph node involvement and distant metastasis (p>0.05), (Table 2).

Discussion

Despite improvements and development of laboratory equipment and novel treatment approaches, CRC is still accompanied with high morbidity and mortality at worldwide. The main reason is a lack of effective diagnostic techniques in early stages of the malignancy. As, CRC in advanced metastatic stages almost is incurable or at least the five-year survival rate of patients is very low (Ma et al., 2010; Summers et al., 2013; Stewart and Wild, 2014) therefore, detecting methods at a molecular level in early stages can be useful for diagnosis and treatment of CRC.

Due to limited data regarding P16ink4a subcellular

localization patterns in colorectal adenoma-carcinoma sequence in comparison with normal samples, this study was carried out on colorectal adenocarcinoma, adenomatous and non-neoplastic tissue samples. The findings of the present study showed P16^{ink4a} protein in the examined samples was expressed with three different subcellular patterns including C+N, C and N. In most examined samples P16^{ink4a} notably was expressed with the C+N pattern. Zhao et al., (2003) stated that P16^{ink4a} expression was occurred sporadically weak in the nucleus of non-neoplastic mucosa adjacent to adenoma and carcinoma tissues, moderate to strong expression was found significantly higher in tumoral cells compared to the normal mucosa. They showed that this expression was found only in the cytoplasm of CRC cells. Another study about the aberrant cytological localization of P16^{ink4a} by Zhao et al., (2006) showed that; there was a significance difference between P16^{ink4a} subcellular expression patterns in non-neoplastic-dysplastic-malignant sequence mucosa. This result is in accordance with our findings between non-neoplastic-adenomatous polyp-adenocarcinoma. On the other hand, despite our results, they showed that two subcellular expression patterns (C+N and C) were predominantly observed in adenomatous and adenocarcinoma of CRC samples. However, in their study foci of weak expression was found in the nucleus of normal tissues adjacent to adenomatous and colorectal carcinoma. In contrast with the findings of our and Zhao studies regarding subcellular localization of P16^{ink4a}, Rao et al., (1997) in astrocytomas indicated that this biomarker expression was predominantly occurred in the nucleus of the malignant cells and claimed that subcellular expression of P16^{ink4a} was significantly higher in the nucleus than the cytoplasm. P16^{ink4a} known as a tumor suppressor and it was shown that its expression increased markedly in senescence and normal aging cells. Normally the main localization of P16^{ink4a} is in the nucleus (Lam et al., 2008; Qian-Qian, 2015). But, its expression in both of the nucleus and the cytoplasm can be an indicator of overexpression of P16^{ink4a}. Furthermore, it seems that the reason for variation in the subcellular expression pattern of P16^{ink4a} has been the impact of tumoral cells on normal cells adjacent to them. Another reason of different localization of P16^{ink4a} can be attributed to disturbance and interference with other participant molecules in the cell cycle. Some studies have claimed that subcellular cytoplasmic expression of P16^{ink4a} in colorectal tissues due to the formation of large molecular complexes (binding to CDK4) with other cell cycle regulators, consequently, P16^{ink4a} unable to passing from the nucleus membrane pores and its concentration in the cytoplasm rises (Zhao et al., 2006; Romagosa et al., 2011). Despite all this content, two forms of P16^{ink4a} protein in human tissues have been detected by 2D electrophoresis experiments with two subcellular expression patterns. One of them is predominantly located in the nucleolus and another form mainly is concentrated in the cytoplasm (Nilsson and Landberg, 2006). Probably, the difference among two forms of P16^{ink4a} can be determined by types of antibodies bind to them. According to aforementioned contents, it seems that an essential factor determining the subcellular localization of P16^{ink4a} in tissue are the type of its special antibody (Haller et al., 2010; Sawicka et al., 2013). More immunohistochemical researches in this field are needed. As high expression of P16^{ink4a} protein was occurred in two-third colorectal adenocarcinoma samples (Lam et al., 2006; Lam et al., 2008) it can be argued that variation in expression of this molecule might play a key role in CRC tumorigenesis. Moreover, based on the obtained results plausible variation in P16^{ink4a} subcellular localizations are an indicator of different roles of P16^{ink4a} in the human cells and can be changed in various pathological conditions. So that, Romagosa et al. claimed that cytoplasmic localization of P16^{ink4a} regarded as an alternative mechanism for modulating different pathways to regulating the cell cycle happened (Romagosa et al., 2011).

It is noteworthy that, the same of variation in subcellular expression of P16^{ink4a} in colorectal biopsies, interestingly, its IHC expression also is very different with the range from 17%-98% (Cui et al., 2004; Lam et al., 2006; Nikbakht Dastjerdi and Moeini, 2012; Qian-Qian, 2015). In our previous study, the rate of P16^{ink4a} positive expression in normal samples and colorectal adenocarcinoma was 96.50% versus 25.40%. Expression of this biomarker in non-neoplastic samples was significantly higher than adenomatous and adenocarcinoma tissue samples (Heidari et al., 2017a). On the other hand, Lam et al. in their study reported that the rate of P16^{ink4a} high expression in colorectal carcinoma was about 80% (Lam et al., 2008).

Experimental evidence revealed that overexpression, inactivation, and loss of P16^{ink4a} accompanied by poor clinical prognosis and low five-year survival rate in patients with malignancy (Zhao et al., 2006; Jemal et al., 2009; Chung et al., 2014; Li et al., 2015). Results of experimental and in vitro studies suggested that several factors could affect expression pattern of P16^{ink4a} and cell behavior in different pathological situations. Genetic changes (point mutations, deletion, and DNA damage) (Fordyce et al., 2010), epigenetic modifications such as hypermethylation and even oxidative stress (Quereda et al., 2007) can cause an alteration in molecular pathways of the cell cycle. In the next step, levels of gene products (proteins) that essential for the cell cycle controlling and cellular crucial functions are reduced. Then, normal cellular functions will be impaired and affect the cellular behavior and survival (Ding et al., 2003; Mokrowiecka et al., 2012; Fredericks et al., 2015). It was proposed that disruption in many molecular mechanisms controlling the cell cycle led to dysregulation of crucial cellular functions such as proliferation, growth, transformation and changed in programmed cell death patterns consequently, occurrence tumorgenesis in damage cells (Cesare et al., 2013).

In summary, previous studies showed that different P16^{ink4a} subcellular expression occurrence in different pathological conditions in various tissues (Zhao et al., 2006; Romagosa et al., 2011; Rao et al., 1997). Nonetheless, more cytoplasmic overexpression was reported in CRC and adenomatous versus non-neoplastic samples (Zhao et al., 2006). It seems that various factors

play roles in the subcellular expression of P16ink4a including antibodies diversity and IHC techniques limitations and even stage of the disease.

In conclusion, variation in subcellular expression patterns of P16ink4a in different pathological conditions of colorectal samples showed that P16ink4a can be used as a beneficial and sensitive prognostic biomarker in early stages of CRC. In addition, it may improve strategies for screening, prognosis and management of patients with CRC. Further studies are recommended for precisely determined mechanisms of P16ink4a aberrant localization.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Financial Disclosure

Zahedan University of Medical Sciences (ZAUMS) funded this study (grant no: 7548).

Funding/Support

This study was funded by ZAUMS.

Acknowledgments

This manuscript was a result of the MSc thesis of anatomical sciences (No: 130/K). The authors kindly appreciated vice chancellor of research deputy of Zahedan University of Medical Sciences (ZAUMS) for approving and financial support of the study. Our team also thanks from M. Jahantigh (clinical pathologist) for selection of the samples and confirmation diagnosis.

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