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Expression of CDKN2A (p16/Ink4a) among Colorectal Cancer Patients: A cohort study

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Abstract

Colorectal cancer (CRC) exhibits significant rates of morbidity and mortality all over the world. Purpose: The present study aims to determine the expression level of CDKN2A (p16/Ink4a), a tumor suppressor protein with prognostic significance, in a moderate size cohort of colorectal cancer (CRC) patients in Baghdad, Iraq. Patients and methods: The cohort study included 43 CRC patients, all admitted to hospitals for elective CRC surgery between October 2012 and March 2016. Thirty-five resection margins taken from the same patients and proven to be free from malignancy were used as controls (CRN). Immunohistochemistry was use to estimate p16/Ink4a expression levels of both malignant and normal tissues. Possible point mutations in p16 genes isolated from the CRN and CRC sections were determined using PCR followed by DNA sequencing. Results: A negligible expression of p16/Ink4a was observed in the 35 CRN sections. Conversely, 75 % (32 / 43) of the CRC sections showed positive p16/Ink4a expression. Statistical analyses inferred a significantly high level of p16/Ink4a expression in the CRC sections ($P < 0.001$) compared to CRN sections from the same patients. Conclusion: detection of point mutation in the p16 gene via DNA sequencing of the CRN and CRC sections of the cohort study indicated no strong correlation between point mutations and a low level of p16 expression.

Key words: Point mutations, p16 gene, Tumor suppressor protein.

INTRODUCTION

Cancer is one of leading deaths causes in Iraq due to multiple environmental factors and special conditions that have developed over the last three decades.(Al-Shammari, 2016) These cancers are very aggressive and chemoresistant.(Al-Shammari et al., 2014; Al-Shammari et al., 2015b) It is particularly important to examine the gene expression and proteins of these tumors in order to understand their biology and determine the best therapeutic approach.(Al-Shammari et al., 2015a) In Iraq, colorectal tumors occur in males nearly three times as often as in females (2.75 times as often) and, and 80% of colorectal cancer patients are over 50. Colorectal cancer is fairly rare in patients under 30.(Sabeha Moosa, 2009) In colorectal cancers, CDKN2A has significant prognostic value and can be used to design patient-specific therapies.(Maeda et al., 2003) CDKN2A (the HUGO-approved official gene symbol for p16/ink4a) is an inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, and it functions as a tumor suppressor.(Collado et al., 2007) CDKN2A (p16) expression is up-regulated in cellular senescence, and it increases markedly with aging in a wide variety of human tissues.(Collado et al., 2007) CDKN2A is activated to prevent cancer and can promote aging by inducting cell growth arrest and senescence.(Liu and Sharpless, 2009) Loss of CDKN2A function due to deletion, point mutation, and/or promoter methylation may cause uncontrolled cell proliferation, leading to neoplastic transformation. However, the role of p16 in colorectal cancer is not yet established. The data from previous studies on the role of p16 in CRC carcinogenesis varies widely.(Trzeciak et al., 2001; Shima et al., 2011) The present study aims to determine the expression level of CDKN2A (p16/Ink4a), a tumor suppressor protein. We explored the protein expression in colorectal adenocarcinoma patients from Iraq, in the cancer tissue, adjoining normal tissue. The relationship of p16 expression with point mutations parameters was studied for the first time in Iraqi population. An understanding of these alterations will give insight into prognostic significance and preventive strategies for colorectal cancer patients in Iraq.

MATERIALS AND METHODS

Ethics statement

The Al Mustaqbal University College Review Board and the Institutional Review Board of Qadisiyah University approved this study. Consent was also obtained from the patients before the samples were collected.

Study cohort

The cohort participants included 43 CRC patients. The participants in this study were selected from CRC patients admitted to the Gastroenterology and Hepatology Diseases Center of teaching and private hospitals in Baghdad, Iraq between October 2012 and March 2016. Permission for the study was obtained from the hospitals. Each participant also gave informed consent. The CRC patients enrolled in this study were selected with the help of surgeons in the above-mentioned hospitals. The participants in the cohort study were 51.2% (22/43) male and 48.8% (21/43) female. The participants ranged in age from 21-82; the average age was 54. Clinical data for all study subjects was collected through direct interviews with the patients and by investigating their hospital records. The control group consisted of normal tissues taken from the same patients; 35 resection margins were histopathologically proven to be free from malignancy. An immunohistochemical (IHC) staining protocol was applied to paraffin embedded sections of the normal and CRC tissues in order to estimate the expression level of CDKN2A (p16/Ink4a).

Immunohistochemistry procedures

The paraffin embedded tissue samples (the CRC tissues and the control tissues) were cut into samples 5 µm thick and then placed on positively charged slides and left overnight to dry at room temperature. The slides were placed vertically in a drying incubator (hot air incubator) at 65º C and left overnight. They were then immersed in several solutions at room temperature for five minutes each: first xylene, then absolute ethanol, than a solution of 95% ethanol, then 70% ethanol, and finally double-distilled water.(Van Triest et al., 2000)

The slides were autoclaved for two minutes at 121º C in an epitope retrieval solution. Then the slides were removed and placed in a diluted washing buffer jar for five minutes after cooling.(Bartek et al., 1999) 2 to 3 drop of peroxidase block was apply to each sample, then the slide was place in a humid chambers and was incubate at room ^{0}C in 30 minutes. One hundred µl of the diluted primary antibody (US biological, USA) or negative control reagent was applied to the sections, and they were then returned to a humid chamber and incubated at 37ºC for one hour. After that, the slides were gently rinsed with washing buffer and placed in a fresh washing buffer bath for one minute. 1 to 2 drops of bio-tinylated link secondary antibody were applied to the sections, and then the slides were placed in the humid chamber and incubated at 37ºC for 30 minutes. After this, one to two drops of the streptavidin-HRP reagent were applied, and the slides were placed in the humid chamber again and incubated at 37ºC for 30 minutes. After the slides were rinsed, a DAB-

substrate chromogen solution was applied to the slides, which were then incubated in darkness at room temperature for 20 minutes. They were then counter-stained with Mayer's heamatoxylin stain for one minute. The sections were dehydrated by immersing the slides in jars of ethanol and then xylene.

Evaluation of the immunostaining

We counted the stained cells with the assistance of a histopathologist, who helped us avoid the non-tumorous areas in each section. Each set of test conditions included two types of control specimens: a positive control tissue and a negative control (containing monoclonal mouse IgG2a anti-aspergillus niger glucose oxidase), which contained an antibody that exhibits no specific reactivity with human tissues.

The reactivity was evaluated by counting the total number of all positive and negative cell nuclei in several randomly selected fields in each section. Tumor reactivity was expressed as the marker percent (i.e., the number of stained tumor cells per 1000 cells in each section). More than 1000 cells were evaluated under a 40x high-power field, and the percentage of positive cells was calculated.(Nakamura et al., 1998) The p16INK4a intensity of positivity score was indicated as follows.(Zhao et al., 2003) An expression of less than 5% was considered negative and was the cutoff value, 5-25% expression is indicated by (+), 26-50% expression by (++), 51-75% expression by (+++), and more than 75% expression by (++++).

PCR

A KAPA Express Extract Manual DNA Extraction Kit (Kapabiosystems, South Africa) was used to extract DNA from the paraffin embedded tissue samples, according to the manufacturer's instructions. To amplify the p16 gene, a specifically designed primer set was used (forward 5'-GACTTCAGGGGTGCCACATT-3'; reverse 5'-CGTAACTATTCGGTGCGTTG-3') to obtain DNA fragments of 391 bp. This newly designed primer was validated, including testing for inclusivity and exclusivity. Amplification was performed using a SureCycler 8800 Thermal Cycler (Agilent Technologies, USA) in a final volume of 50 µL, which contained 100-300 ng DNA, 2.5 mM MgCl2, 5 µl primers, and 25 µL 1X KAPA2G Robust Hot Start Ready Mix (Kapabiosystems, Cape Town, South Africa). The theromocycling conditions were as follows: Initial denaturation was performed at 94°C for 3 min, followed by 32 cycles of denaturation at 94°C for 18s. The samples were annealed at 53°C for 22s and then extended at 72°C for 22s, with a final extension at 72°C for 10 min. The PCR products were detected through electrophoresis on a 1% agarose gel containing ethidium bromide, which was placed in a TBE buffer and run at a constant voltage (100 V) for approximately 50 min. The DNA was visualized using a VISION Gel Documentation System (Scie-Plas, UK).

Sequencing

The p16-positive specimens (as determined by PCR) were sequenced. For the sequencing analysis, PCR products were purified using a StrataPrep DNA Gel Extraction Kit (Agilent Technologies, USA); sequencing was performed at the National Instrumentation Center for Environmental Management (NICEM), College of Agriculture and Life Sciences, Seoul National University (South Korea). Sequencing reactions were performed using both forward and reverse primers in a total reaction volume of 10 µL (ABI BigDYE V3.1 Ready-Reaction Kit; Applied Biosystems, USA), according to the manufacturer's recommendations. The samples were analyzed on a 3730XL DNA Analyzer (Applied Biosystems, USA). Forward and reverse complementary sequences were aligned using ApE (A Plasmid Editor) software (v2.0.49, 2015), and the results were submitted to GenBank and analyzed via a BLAST search (http://blast.ncbi.nlm.nih.gov/) on the GenBank database.

RESULTS

The immunoexpression of p16INK4a in the thirty-five normal colorectal sections were below the used cutoff value (< 5%) and were therefore regarded as negative. The descriptive statistic of the normal colorectal tissue immunoexpression for p16INK4a is shown in Table 1.

Thirty-two of the 43 CRC patients (Table 2) had positive reactions for p16INK4a. The Mann-Whitney test was used (Table 3) to detect the degree of significance of the immunoexpression of p16INK4a in CRN and CRC tissue. Its expression in CRC tissue was compared to its expression in CRN tissue using a p value of <0.001.

Point mutation in ORF encoding p16

A DNA fragments spanning 372-391 bp in the ORF encoding p16 was successfully amplified from the CRN and CRC sections under study (Figure-1). Multiple sequence alignment (MSA) of sequences of p16 from the CRC/CRN sections and ref|NM_001195132.1 (a reference sequence of p16 ORF retrieved from GenBank) revealed only a one-point mutation (G/A) at a certain locus in the p16 ORF. The G allele was present in both the healthy and the reference sequences. However, the A allele was present in the p16 sequences of the CRC sections.

Table 1: Descriptive Statistics of the Immunoexpression of p16INK4a Expression in Colorectal Normal Tissue (CRN) and Colorectal Cancer (CRC).

Descriptive data for p16INK4a		
Markers	**CRN n = 35**	**CRC n = 46**
Mean	1.91	31.93
Std. Error of Mean	0.24	4.50
Std. Deviation	1.42	29.53

Table 2: Percentages of the Immunoexpression of p16INK4a in Colorectal Cancer (CRC) Patients.

p16INK4a	Positive	%	Negative	%
Total (n=43)	32	74.4	11	25.6

Table 3: Mean Ranks of the Immunoexpression of p16INK4a in Colorectal Normal Tissue (CRN) and Colorectal Cancer (CRC), According to the Mann-Whitney Test.

Marker	CR group	No.	Mean ranks	P value
p16INK4a	CRN	35	27.13	<0.001
	CRC	43	49.57	

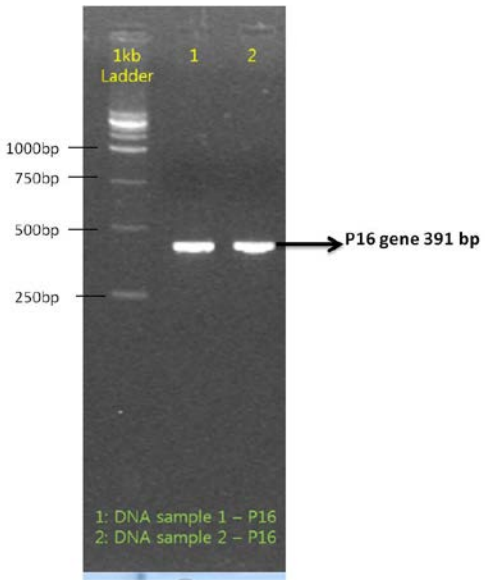

Figure-1, DNA fragments in the ORF encoding p16 was successfully amplified from the CRN and CRC sections under study.

DISCUSSION

We found that 14.3% of the normal colorectal tissue had no immunoexpression, and the rest had less than 5% immunoexpression, which was regarded as negative. All the p16INK4a-positive cells showed nuclear immunoexpression of p16INK4a, and some of the tumor cells showed residual p16INK4a immunoexpression in cytoplasm. The rest of the cells (those with negative expression) showed minimal staining or none at all. This supports the findings of another study by Palmqvist et al.(Palmqvist et al., 2000) Palmqvist conducted an immunohistochemistry (IHC) on colorectal tissue and found that all normal samples were essentially p16INK4a negative, with only few scattered positive cells along the crypt axis with no specificity. Other studies have obtained similar results.(Jung et al., 2001) A study on methylation of p16 detected it in 53% of tumors, while it occurred in only 18% of normal colonic mucosa.(Krakowczyk et al., 2008) The methylation of p16INK4a causes gene silencing and depresses its function as a tumor suppressor in colorectal tumors.(Schneider-Stock et al., 2003) Aggressive and poorly differentiated tumors have been found to be associated with methylated p16INK4a.(Ramirez et al., 2008)

In contrast, this study found that 74.4% of CRC samples reacted positively after IHC staining, most with a high immunoexpression score. The most common alteration was an increase in the p16INK4a protein immunoexpression, which occurred in 92% of the adenomas and 91% of the adenocarcinoma.(Arber et al., 1999) This can be explained by the fact that p16INK4a is a tumor-suppressor protein and therefore increases in colorectal cells with extreme proliferation rates in an attempt to slow down the cell cycle rate. The presence of p16INK4a genetic alterations are also associated with shorter survival in CRC patients.(Esteller et al., 2001)

In order to determine the possible effect of point mutation on the level of p16 expression in the CRC and CRN sections under study, it was necessary to investigate the sequence of the p16 ORF. The data in this study imply the presence of point mutation (G/A) in certain loci in the p16 ORF in cancerous and normal tissues. Most previous studies have focused on the possible role of hyper-methylation in the promoter region of p16 on the level of p16 expression in normal and cancerous tissues.(Ye et al., 2005; Psofaki et al., 2010; Lee et al., 2011; Li et al., 2015) Only one previous study examined the possible role of point mutations in p16 ORF on the level of its expression.(Trzeciak et al., 2001) This study did not find a possible correlation between the presence of point mutations and low levels of p16 expression in normal or cancerous tissues.(Trzeciak et al., 2001)

This is the first investigation in the Iraqi patients in which p16 gene has been analyzed at both genetic and protein expression level in CRC. It was limited to by the size of the cohort and confined to patients admitted to the Gastroenterology and Hepatology Diseases Center of teaching and private hospitals in Baghdad region only. Therefore, a solid conclusion cannot be drawn from the current data. However, a major limitation is the small sample size used in the present study, Nevertheless, many other studies used smaller size studies and reached good conlcusions.

CONCLUSIONS

A high to moderate level of p16 expression in CRC sections, along with a lack of expression in CRN sections, would suggest that p16 plays a role in CRC carcinogenesis. The absence of a high number of point mutations in the p16 gene in the CRC sections and the high to moderate level of p16 expression in the CRC sections highlight the need for further research investigating the methylation status in cpG islands included in the p16 promoter. Moreover, a detailed study on the possible role of point mutations in the p16 ORF should be conducted with a larger sample and be correlated with two factors: the hyper-methylation status of the p16 promoter and p16 expression levels.

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