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Methylation Status of *p16* gene in Iraqi Colorectal Cancer Patients

Wafaa Sabri Mahood[1] Mohammed Ibraheem Nadir[2] Khalid Tobal[3]

Basim Ahmed Asker[4]

[1]College of Education –Ibn AL-Haytham

[2]Genetic Engineering & Biotechnology Institute- University of Baghdad

[3] King's College London School of Medicine-Guys Hospital , London

[4]London College of Medicine, Unit of Digestive diseases, Baghdad

Abstract: The *CDKN2A* gene (encode cyclin dependent kinase inhibitor 2A) is considered as a tumour suppressor gene involved in cell-cycle control, and methylation in promoter regions of this gene is a frequent event in CRC patients as it associated with the loss of p16 protein expression in cancer cells leading to gene silence. The methylation status of the p16 gene was examined in 35 samples of Iraqi colorectal cancers (primary carcinomas) and the five samples of Iraqi bowel inflammation patients using Methyl Sensitive High Resolution Melting and the correlation between the methylation status and the clinicopathological findings was evaluated. The results show aberrant methylation of the *p16* gene was detected in 17 colorectal tumor samples out of the 35 (48.75%) primary colorectal carcinomas but there no change in methylation status was found, suggesting that the aberrant methylation of *p16* was frequently observed in Iraqi colorectal carcinomas. The clinicopathological data were then correlated with these results. Significant differences were observed with age ($p<0.01$), tumour location ($p<0.01$), moderately differentiated ($p<0.01$) and (T3) lymphatic invasion ($p<0.01$). This study provides evidence for hypermethylation status p16 gene in CRC patients, which may serve as useful information on CRC cancer progression.

Key words: Methlation, *P16,* Colorectal *CDKN2A***.**

مثيلة جين *P16* في مرضى اورام القولون والمستقيم العراقيين

وفاء صبري ماهود[1] محمد ابراهيم نادر[2] خالد طوبال[3] باسم احمد عسكر[4]

[1]كلية التربية – ابن الهيثم – جامعة بغداد

[2]معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا– جامعة بغداد

[3]كلية لندن الطبية الملكية– مستشفى كايز – لندن – بريطانيا

[4]مدينة الطب – مركز امراض الجهاز الهضمي – وزارة الصحة – بغداد – العراق

الخلاصة: يعد جين *CDKN2A* (الذي يشفر الى مثبط الكاينيز cyclin dependent kinase inhibitor 2A) جين كابح للسرطان والذي تتضمنه السيطرة على الدورة الخلوية، المثيلة بمناطق البادئ غالبا ما تحدث بمرضى اورام القولون والمستقيم وترتبط بفقدان التعبير عن البروتين *p16* في الخلايا السرطانية مما يؤدي الى كبت او اخماد الجين.

تم التحري عن حالة مثيلة جين *p16* في 35 عينة لمرضى اورام القولون والمستقيم العراقيين و 5 عينات لمرضى التهاب القولون باستخدام تقنية Methyl Sensitive High Resolution Melting وكذلك تم البحث في علاقة حالة المثيلة والصفات السريرية لهؤلاء المرضى. اظهرت النتائج مثيلة شاذة لجين *p16* في 17 من 35 عينة بنسبة 48.75% لعينات اورام القولون والمستقيم ولم يلاحظ اي تغيير بحالة المثيلة بعينات مرضى التهاب القولون مما يشير الى ان المثيلة الشاذة غالبا ما تحدث في مرضى اورام القولون والمستقيم العراقيين. بعد ذلك تم البحث بعلاقة الصفات السريرية مع هذه النتائج، وجدت اختلافات معنوية مع العمر ($p<0.01$) وموقع الورم ($p<0.01$) و ($p<0.01$) moderately differentiated وكذلك (T3) الغزو اللمفاوي للورم ($p<0.01$). هذه الدراسة تعطي ادلة على مثيلة عالية لجين p16 لمرضى اورام القولون والمستقيم العراقيين والتي ربما تفيد كدليل بتطور او تقدم اورام القولون.

Introduction

Colorectal cancer (CRC) arises due to genetic alterations through gene mutations and methylation and transforms colorectal epithelial cells into colorectal adenocarcinoma cell (1). Several studies have investigated the genetic and epigenetic events underlying the development of colorectal cancer (CRC), this is one of the world's leading common cancers (2).

Three major mechanisms affecting genes' functions in CRC have been described: microsatellite instability (MSI), chromosomal instability (CIN), and CpG island methylator phenotype (CIMP). More than one mechanism may occur in the same tumor. In MSI tumors, which account for 15% of CRCs, DNA mismatch repair genes are either mutated or methylated leading to tumors with a microsatellite instability phenotype (3). DNA methylation is the most well-studied epigenetic change, and provides a new generation of cancer biomarkers. DNA methylation occurs frequently in the promoter regions of tumour suppressor genes, and it plays an important role in tumour development (4,5). One study has concluded that many gene

promoter methylations result in transcriptional silencing, and so might be exploited as biomarkers for the early detection of CRC (6). p16 hypermethylation might be a predictive factor of poor prognosis in some surgically treated cancers (7). Iraq represent the sixth country incidence rates of colorectal cancer in Asia while India the first country followed by, Yemen, Bangladesh, Egypt and Pakistan (8). In this study we aimed to investigate of the p16INK4A gene methylation status of the promoter region and to describe the correlations between the methylation and clinical parameters in Iraqi colorectal cancer (CRC) patients.

Materials and Methods

Patients' Selection

A total of 35 colorectal cancer Iraqi patients who fulfilled the Modified Dukes classification of Astler and Coller, 1954. Those patients underwent to the Gastroenterology and Hepatology Diseases Center (Baghdad) between October, 2011 and June,2012. Samples include 15 male and 14 female with average age 52.5 years range (between 25 to 80 years), in addition to five of bowel inflammation patients also were evaluated for methylation inflammation . Ethical permission to conduct the research was obtained from these hospitals and from all participants in this study. Patients treated with radiotherapy or chemotherapy were excluded.

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.

DNA Isolation and Sodium Bisulphite Treatment

Genomic DNA was extracted from tissue samples using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol . The extraction of DNA were done in National Center for Early Detection of Cancer in Baghdad.The resulting DNA was sodium bisulphite modified in the Molecular Oncology Unite Guys Hospital / King' s College / London / UK using an (EpiTect® 96 Bisulfite, Qiagen). Methylated DNA control for Methyl sensitive PCR assays was generated using (M.SssI) CpG Methyltransferase (New England Biolabs).

Methylation-Sensitive HRM Assay

Detection of methylation were done in the Molecular Oncology Unite Guys Hospital / King' s College / London / UK. The promoter methylation status of p16 gene was determined using Methylation-Sensitive HRM assay as described previously by Wojdacz and Dobrovic, 2007(9) . The sequences of primers used for amplification of the promoter regions of p16 were previously described (10)

F- GGAGCCTTCGGCTGACTGGCTGGCC-3'.

R- AGCAGCGCCCGCACCTCCTCTA-5'.
A specific CpG-rich sequence in the p16 promoter region (from -21964733-21964800 bp 67 bp fragment containing 8 CpG sites). analyses were run according to the following conditions: 1 cycle of 95 ºC for 10 min, 2 cycles of 95 ºC for 10 s,60 ºC for 15 s,70 ºC for 20 s .2 cycles of 94 ºC for 10 s,58 ºC for 15 s,72 ºC for 20 s. 2 cycles of 94 ºC for 10 s,57 ºC for 15 s,72 ºC for 20 s. 45 cycles of 94 ºC for 10 s,56 ºC for 15 s,72 ºC for 20 s . Followed by an HRM steps, to prepare melting step of 95 ºC for 1min with Ramp Rate of temperature 4.4 and curve acquisition step of 40 ºC for 1 min with Ramp Rate 2.2.The melting data acquisition began step of 65 ºC for 1 s with Ramp Rate 1.The last step is melting data acquisition ended 95 ºC for 1 s with Ramp Rate 0.02. PCR reaction was performed in a 20 ml total volume 2 µl Buffer, 3.2µl MgCl2(25 mmol), 0.4 µl of the four (10mM dNTPs) , 0.2 µl Taq polymerase , 10.7 µl of distilled water,1µl of the Syto9 dye, primer mix 0.5 µl and 2 µl of bisulphite modified DNA, the work conducted in a Thermal Cycler (Light cycler 480 Rocche).Followed by sequencing reaction using Big Dye Terminator kit (Applied Biosystems, Foster City, CA) and the same forward primers . The sequencing reaction was analyzed using the (Verti 96 thermal cycler 9 Applied Biosystems). After product cleanup the sequencing analysis was done using the sequencer 3730.

Results and Discussion

The p16 is a member of the INK4A/ARF family of suppressors of cyclin-dependent kinases (CDKs) which plays an important role in the G1-S transition by binding to CDK4 and CDK6 and inhibiting the progression of the cell cycle (11,12). Epigenetic mechanisms play an important role in colon cancer and aberrant methylation of the p16 gene is commonly observed (13).

Characteristic of Samples

The methylation of P16 promoter in 40 patient including 35 colorectal cancer and 5 patients with inflammation ,was detected, the age on average (25-72) were enrolled in this study table (1), high percentage of tumor in age more than fifty years 23/35(77.14%). Sixteen of 35 (42.5%) patients were male and 19/35(54.28) were female. High percentage of patients with left site CRC 15/35 (42.85%) compared to right site tumour 6/35 (17.14%), while 13/35 (37.14%) with rectal site tumour. Larsson and Wolk, in 2006 (14) published in there review report to that high consumption of processed meat was associated with an increased risk of distal colon (left colon) cancer but not of proximal colon cancer.

The most of patients were with moderately differentiated adenocarcinoma 27/35 (77.14%) and 6/35(17.14) were with well differentiated adenocarcinoma.

Table (1): Characteristic feature of the colorectal tissue used in methylation analysis

Characteristic	Total no.	Percentage (%)
Age Yrs		
≤ 50	8	22.85
>50	27	77.14
Gender		
Male	16	45.71
Female	19	54.28
Site		
Right colon	6	17.14
Left colon	15	42.85
Rectum	13	37.14
Unknown	1	2.85
Differentiation		
Moderate	27	77.14
well	6	17.14
Tumour villous	2	5.71

Methylation analysis

CDKN2A promoter hypermethylation has been described in 12-51% of colorectal cancers and is often included in the panel of markers used to assess the CIMP phenotype (15).The MS-PCR was used detected the methylation status of p16 gene. High resolution melting relies on the use of high sensitivity florescence detection instrumentation, fully saturating intercalating dyes and software allowing the analysis of the melting profiles of PCR products (16). HRM was developed by Wojdacz and Dobrovic in 2007 (9) for discrimination between methylated and unmethylated sequences after bisulphite modification of the target DNA. Sodium bisulphite converts unmethylated cytosines to uracil and leaves methylated cytosines intact. Therefore, the PCR product derived from a methylated template will have a higher melting temperature than that from an unmethylated template and those differences can be resolved by melting analysis.

Colorectal cancers from 35 Iraqi patients were evaluated for methylation status. A stretch of 69 bases incorporating (8) CpG sites in the p16 proximal promoter was analyzed by primer which was used as shown in figure (1). P16 gene amplification were successfully determined by using MS-HRM of 40 samples.

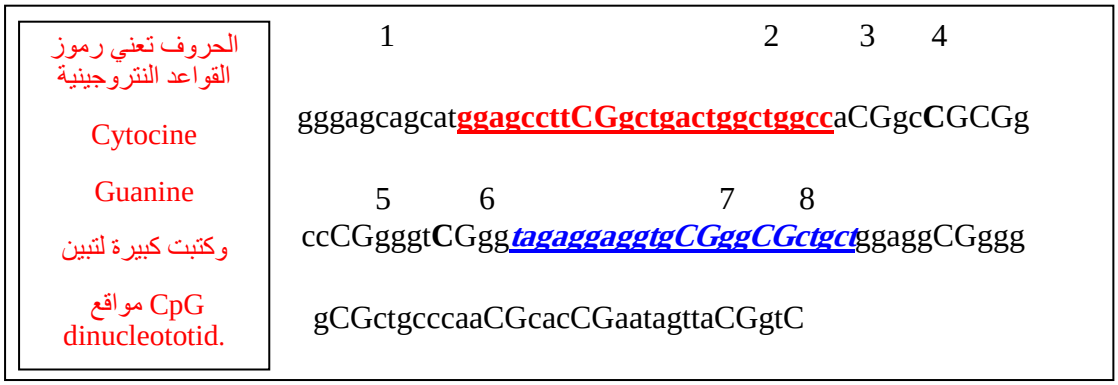

Figure (1): Map of the P16 promoter region and primer positions. The sequence numbered relative to the transcription start site for human P16. Characters in red indicate the forward primer binding sites for methylation-specific MS- HRM and character in blue indicate the reveres primer the , CG capital letter indicate the cytocine-guanine (CpG dinucleotid).

The normalize melting curve for p16 gene shown in figure (2) indicating the abnormal methylation. Cleanup the amplified product was done for some samples to confirm the result and detect the methylation by sequencing assay figure (3) . The methylation status of 5' CpG island of the p16 gene was detected in 17/35(48.57%) colorectal tumor samples.

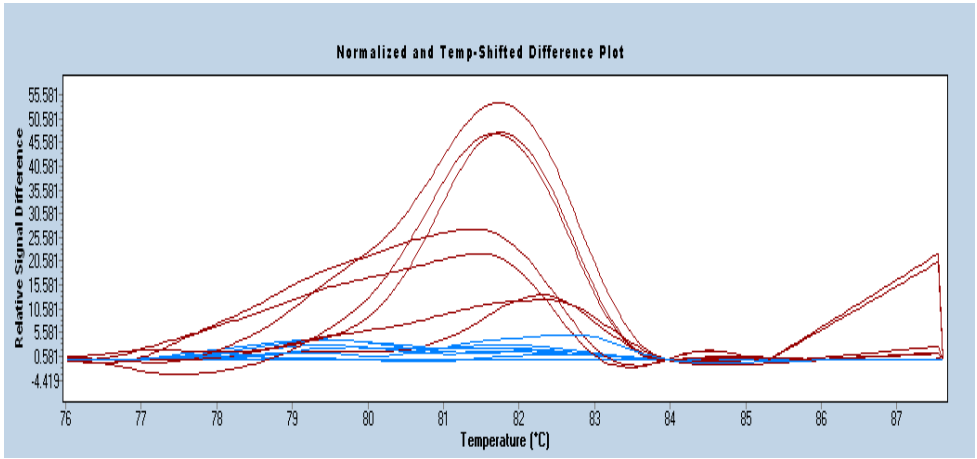

Figure (2): MS-HRM assay (normalized melting curves) methylated status are indicated in red color ,unmethylaed in blue color A:MLH1,B:BNIP3,C:P16 isolated from colorectal tissue.

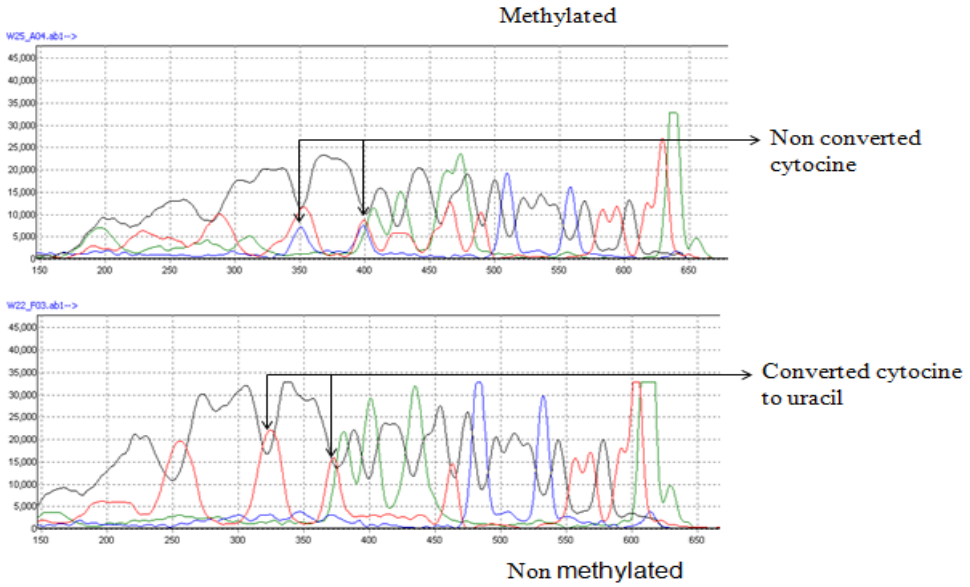

Figure (3): Sequencing analysis showing p16methylation at sites as pointer. Comparable between two samples, the upper non methylated sequencing and the lower revealing methylated CpGs sites,(Green-A, Red -Thymin , Blue-Cytocine. Black-Guanine).

and 18/35(51.42%) were unmethylated. This value is in agree with other reports (17,18) but lower than 66% in Kashmiri population (19), also there are studies reporting lower values in the literature (20; 21).

Published data indicated that the DNA methylation primarily influences the cytosine of symmetrical dinucleotide CpG in human (22) and the subsequent pattern of DNA methylation is transmitted through mitosis and maintained after DNA replication (23), and thereby aberrant CpG island methylation could promote the carcinogenesis.

The Relation of P16 gene Methylation and Clinicopathological Feature

Methylation of p16 gene promoter, for CRC patients, is a frequent even in various cancers.The relation of p16 methylation with clinicopathological variables in these patients, has yield discrepancies in the literature although methylation could not be associated to clinicopathological variables by some authors (24,25).These differences may be explained by the high sensitivity of the methods used in different studies. The use of primers and probes designed for the methylated sequences and application of nested PCR increases the specificity of the method markedly and makes it possible to detect low levels of methylated sequences (26). p16 has been associated to the following: Dukes' stage and lymphati (27; 28). Also to age, sex, tumor location, differentiation grade and histological type (28). The correlation between methylation status for p16 gene and clinicopathological

parameter in 35 colorectal cancer patient shown in table (1).

Table (1): Methylation status for P16 in relation to clinicopathological parameter in 35 colorectal cancer patient

Variable		No.%	P16 meth.	P16 unmeth.	P value
Total no.		**35**	**17(48.57)**	**13(51.42)**	
Gender	Male	16(46)	10(62.5)	6	0.355 NS
	Female	19(54)	10(52.63)	9	
Total no.		35			
Age	≤ 50	8(22.85)	2(25)	6(75)	0.0026 **
	>50	27(77.14)	15(55.55)	12(44).	
Tumor	Right	6(17.14)	3(50)	3(50)	
Location	Left	15(42.85)	7(46.66)	8(53.3)	0.0042 **
	Rectal	13(37.14)	6(46.15)	7(53.8)	
	unknown	1(8.57)	1(100)	-	
Differen.	Moderate.	27(77.14)	13(48.14)	14(51.8	0.0027 **
	well	6(17.14)	2(33.33)	4(66.6)	
	Tu. Vi.	2(6)	-	2(100)	
Duke s'Stage		**21**			
	B	12(57.14)	5(41.66)	7(58.33)	0.0437 *
	C	9(42.58)	6(66.66)	3(33.33)	
Frozen		**14**	-	-	
Invasion		**20**			
	T2	4(20)	2(50)	2	0.0036 **
	T3	14(70)	5(35.71)	9	
	T4	2(10)	2(100)	0	
Frozen		**15**			

* (P≤0.05), ** (P≤0.01).,meth.-methylated, unmeth.-unmethylated, Differen.-Differentiated ,Tu.Vi.-Tumour villus

The p16 gene promoter methylation was no association regarded gender (P-0.355), increased frequency of p16 methylation in age over fifty 15 of 27 (55.55%) , high association (P≤0.01) was seen. Depend on the tumour location ,differentiation and Dukes' Stage: frequency of methylation status was did not have different between colon and rectal, 3/6(50%), 7/15 (46.66%) and 6/13(46.15%) for right site, left site and rectum, respectively, although that there was high association (P≤0.01). Moreover, epigenetic inactivation of the p16 by methylation has been observed in 13/21 (48.14%) for moderately differentiated with high associated relation than well differentiated 2/6(33.33)% (P≤0.01). Low association in B and C stage 5/12(42%) and 6/9(66.66%) respectively, (P≤0.05).Also there was high significant association in p16 and invasion T3 , T2 (P≤0.01), 5/14(35.71%) and 2/4(50%) respectively.

These hypermethylated genes are not only probable pathogenic events in the polyp to cancer progression sequence, but are also neoplasm-specific molecular events that have the potential to be used as molecular markers for pre-malignant tumors in the colon. hMLH1 promoter methylation is an early phenomenon in comparison to polyp formation, while methylation of p16 and MGMT is correlated to the progression (29).

No correlation was found between p16 methylation clinicopathologic factors including age, gender or tumor location ,the researcher found that p16 gene methylation in colorectal cancers associated with Duke's staging and P16 methylation plays a role in the carcinogenes is of a subset of colorectal cancer, and it might be linked to poor prognosis (30). Methylation led to a strong negative effect on survival time (p - 0.0003, log-rank; this is additionally highlighted by a HR (95%CI) of 1.6 (1.2-2.1) indicating that patients with methylation-positive tumors have a 60% increased risk of death in comparison to methylation-negative patients (31).

5 CpG site-specific analysis P16 in normal tissue

Five samples from bowel inflammation patients also were evaluated for methylation, table (3) showed the characteristic feature. There is not any methylation was detcted in P16 gene, this is consist with Kang and his colleagues (2012)(32) whose found none in normal tissues were:

Table (3): The characteristic feature of the five normal specimence

Characteristic	Total no.	Percentage (%)
Age yrs (45-60)	5	
> 50	4	80
<50	1	20
Gender		
Male	1	20
Female	4	80

hypermethylation in p16 gene. Other report indicate that P16 methylation was observed in 45.3% of the normal tissue samples (33). Because of the small number which used as a normal cases to detect the P16 gene methylation status in this study, other study need to investigate the risk potential of those patients with colorectal cancer. In conclusion this study provides evidence for hypermethylation status of p16 gene in CRC tissues, which may serve as useful information on CRC cancer progression.

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