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CpG Island Methylator Phenotype (CIMP) Correlation with Clinical and Morphological Feature of Colorectal Cancer in Iraq patients

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Abstract

In colorectal cancer CpG islands of tumor suppressor genes is thought to be an important mechanism in human carcinogenesis, has been shown to occur early in colorectal carcinogenesis, purpose of this study early detection of tumorigenic pathway of CRC by used molecular mothed. Total of 47 patients with colorectal carcinoma were collected among of these patients 26 (55%) males and 21(45%) females, with a range age from 37 years to 72 years, mean age (54.5 year), CIMP $_{high}$ was show 36.17%, while the CIMP $_{low}$ 25.53% and CIMP $_{neg.}$ 38.29%, with a cutoff value of 2 from 5 genes. CIMP was associated with female in 42.85% comparted with male 30.76%, while related with age, CIMP $_{high}$ was highly expression in patients with group two Group 1≥50 41.37% versus 27.77% of group one Group 1≤50, but close associated with poorly differentiated 47.05%, followed by moderate differentiated 29.41%, compared with well differentiated 23.52%, and CIMP $_{high}$ more frequency (52.94%) in right site compared with left site and rectum 35.29%, 11.76% respectively, while in mucinous 75% compared with non-mucinous 29.78%, classical panel of CIMP the highly repeated was shown in Hmlh1 with 34%, followed by p16 with 31.91%, while the MINT2 was reveled 29.78%, and less percentage was recorded in both MINT1 and MINT31 with 25.53% and 23.40% respectively. The rate of methylation loci, CIMP $_{high}$ shown close association between CIMP with female old patient's right site mucinous and poorly differentiated of CRC specimen.

Introduction

Colorectal cancer is one of the most frequent malignancies in the Western world. Worldwide, approximately 1, 2 million people developed colorectal cancer in 2008 and the disease related mortality was about 36% (1) (2) The disease affects slightly more men than women and sporadic colon cancer is considered to be a disease of the elderly with a median age at diagnosis of 70 years (3). More than 90% of the colorectal cancers occur sporadically, which means that affected patients do not have a family history of colon cancer (4).

Epigenetic changes are modifications of the genome heritable during cell division that do not involve a change in DNA sequence (5). Since its discovery in 1983, the epigenetics of human cancer has been in the shadows of human cancer genetics. But this area has become increasingly visible with a growing understanding of specific epigenetic mechanisms and their role in cancer, including hypomethylation, hypermethylation, loss of imprinting and chromatin modification (6).

It is also alternative mechanism for carcinogenesis. Most of the CRCs have epigenetic alterations e.g. DNA methylation and histone modification and these coexist with the classic genetic changes (7).

The term DNA methylation refers to the methylation of cytosine residues (5-methylcytosine) at CpG sites found throughout

the genome (8). These epigenetic alterations are characteristically clustered in so-called CpG islands in gene promoter regions, and hypo and hypermethylation of these regions are related to activation and inhibition of transcription, respectively. This type of gene regulation is essential to cell differentiation as well as embryological development (9). Epigenetic mechanisms, while not resulting in DNA sequence alterations, are involved in the activation of oncogenes and in the inactivation of tumor suppressor genes. DNA hypermethylation modifies cytosine nucleotides in the CpG islands (10).

CIMP shows a great deal of overlap with MSI tumors, and are observed to be inversely correlated to the most common genetic phenotype, chromosome aberrations (11).

Cancer-related hypermethylation of promoter regions leads to transcriptional silencing of the gene. A growing number of cancer-related genes are found to be methylated in their promoter region. Many tumor suppressor genes seen mutated in familial cancer syndromes have been discovered to be methylationally silenced in sporadic cancers (12).

In 1999, Toyota *et al.,* described a subgroup of CRCs that showed frequent promoter hypermethylation, which they called CIMP short for the CpG Island Methylator Phenotype (13)

The discovery of CIMP led to the proposal of a tumorigenic pathway of CRC driven by promoter hypermethylation and hence epigenetic, rather than genetic, inactivation of tumor suppressor genes (14).

Material and methods

Specimens, the sample collection from GIT center, Baghdad hospital and private hospitals. In period from 1-4-2013 to 1-2-2014, which more than three biopsies were obtained from grossly tumor areas, and surgery specimens obtain after surgery tumor removal both specimens from surgery and biopsies were fixed with 10% buffered formalinized saline, for preparation the paraffin embedded tissue blocks to histological molecular diagnostic methods DNA extraction from FFEP.

Histological evaluation specimens, slides from fixed paraffin embedded tissue blocks were stained with haematoxylin – eosin stain and subsequently evaluated by an experienced pathologist. DNA extraction from FFPF, QIAamp DNA FFPE tissue kit, (50) reaction Mineute columns, kits. DNA evaluation by Nano drop For an A260/A280 value of 1.5, the percentage of protein in the DNA preparation, for good PCR-SSP results, DNA is required with an A260/A280 quotient of 1.6 or greater.

The sections of tumor tissue should contain more than 50% of neoplastic cells (15) in order to avoid false negatives. Sodium bisulfite treatment of genomic DNA, (A), the procedure is based on the chemical reaction of single-stranded DNA with sodium bisulfite (HSO3 _) at low pH and high temperatures. The chemistry of each reaction step is as follows: sulfonation at the carbon-6 position of cytosine, irreversible hydrolytic deamination at the carbon-4 position to generate uracil sulfonate, and, finally, subsequent desulfonation under alkaline conditions to generate uracil. Methylation at the carbon-5 position impedes sulfonation at the carbon-6 position in the first reaction step. Although 5-methylcytosine can react with bisulfite, this reaction is extremely slow, and the equilibrium favors 5-methylcytosine rather than thymine (the deaminated product of 5-methylcytosine). Of note is that subsequent purification is necessary to remove bisulfite salts and other chemicals used in the procedure. (B), The sodium bisulfite treatment converts unmethylated cytosines of the original DNA sequence to uracil, whereas methylated cytosines remain as cytosine. The CpG dinucleotide is the methylation target in human cells (bold) (17). EpiTect Fast DNA Bisulfite Conversion Kits now provide a very fast and streamlined procedure for efficient conversion and purification of DNA tissue samples Furthermore, the bisulfite thermal cycling program provides an optimized series of incubation steps necessary for thermal DNA denaturation and subsequent sulfonation and cytosine deamination, enabling high cytosine conversion rates of over 99%. Desulfonation, the final step in chemical conversion of cytosines, is achieved by a convenient on-column step included in the purification procedure.

Methylation specific PCR Master mix EpiTect® MSP For highly accurate methylation-specific PCR without optimization. Control DNA EpiTect® cat. No. 59655 Control DNA (human), methylated and bisulfite converted (100), For 100 control reactions for methylation analysis. Control reactions should be performed when undertaking methylation analysis (e.g., methylation specific PCR MSP) to ensure that the PCR primers are specific for the detection of methylated bisulfite converted or unmethylated bisulfite converted DNA. Use 1 µl (10 ng) of each control DNA for every PCR reaction. Kit recommend using the EpiTect MSP Kit or EpiTect MethyLight PCR Kit for highly specific and reliable methylation-specific PCR results. Control DNA EpiTect® cat. No. 59665. Control DNA (human), unmethylated and bisulfite converted (100). For PCR analysis, kit recommend the use of 10 ng of each control DNA for every PCR reaction. Kit recommend using the EpiTect MSP Kit for highly specific and reliable unmethylation-specific PCR results.

Sequences of control primer *COL2A1* Collagen 2A1*

COL2A1 (the collagen 2A1 gene) was used to normalize for the amount of input bisulfite-converted DNA (17). To normalize for the amount of bisulfite-treated DNA present in the reactions, the bisulfite specific amplification of the housekeeping gene COL2A1a reference reaction, *Collagen 2A1* COL2A1 was run for all tumor samples. COL2A1 sequences are short, interspersed, repeated sequences that are present in the human genome. Moreover, the COL2A1 reaction is

designed from a COL2A1 sequence depleted of CpG dinucleotides by evolutionary deamination, and is hence methylation independent. To normalize results for the efficacy of PCR amplification, a reaction with presumably fully methylated reference DNA (human genomic DNA methylation from Qagin was run for each gene.

Program of different MSP panel

From each primers (10 pmol), and bisulfite-modified DNA (30 –50 ng) in a final volume of 25$\mu\ell$. Positive and negative control: for each MSP reaction, used Control DNA EpiTect, use 1 µl (10 ng) as positive and or distilled water (without template DNA) negative controls, respectively.

The classic CIMP marker panel, consisting of *hMLH1, p16, MINT1, MINT2,* and *MINT31,* Primer sequences and PCR conditions of all 5 loci for both methylated and unmethylated forms are shown in Table 1. Primers (10 pmol each), and bisulfite-modified DNA (30–50 ng) in a final volume of 25. Amplifications were carried out in a thermal cycler for 33 cycles Denaturation 98ºC 5 – minutes (40 seconds at 95_C, 50 seconds at variable temperatures according to primer, and 50 seconds at 72_C) and were given a final 10- minute extension. Polymerase chain reaction products were electrophoresed in 2.5% agarose gels and visualized under ultraviolet illumination after ethidium bromide staining.

Results

Sex and age distribution, total of 47 patients with colorectal carcinoma were collected among of these patients 26(55%) males and 21(45%) females figure (1), with a range age from 37 years to 72 years, mean age (54.5 year), with 1:1.2 ratio between female and male. The patients' age were classified into two group first 50≥ years (38.29%), second group 50≤ years 29 (61.70).

Morphological differentiation, as regards with grades of colorectal carcinoma, it was observed that (14.89%) cases of well differentiated, (53.19%), moderate differentiated and (53.19%), and (31.91%) poorly differentiated.

Site of tumor: Patients with CRC were classified according to the site of tumor location as shown in, right site consistent

Table 1. The Primer Sequences

Gene or Locus	Sequence (5–3)	Size, bp	AT C	Gen Bank No	Location, bP
P16-mF P16-mR	TTATTAGAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCGTAA	150	65	AF527803	19907–20055
P16-uF P16-uR	TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA	151	60	AF527803	19907–20056
hMLH1 mF hMLH1-mR	TATATCGTTCGTAGTATTCGTGT TCCGACCCGAATAAACCCAA	153	60	AY217549	1294–1446
hMLH1-uF hMLH1-uR	TTTTGATGTAGATGTTTTATTAGGGTTGT ACCACCTCATCATAACTACCCACA	124	60	AY217549	1248–1371
MINT1-mF MINT1-mR	GGGTTGAGGTTTTTTGTTAGC CTACTTCGCCTAACCTAACG	102	64	AC026774	44415–44314
MINT1-uF MINT1-uR	GGGGTTGAGGTTTTTTGTTAGT TTCACAACCTCAAATCTACTTCA	118	55	AC026774	44416–44299
MINT2-mF MINT2-mR	TTGTTAAAGTGTTGAGTTCGTC AATAACGACGATTCCGTACG	90	60	AF135502	465–554
MINT2-uF MINT2-uR	GGTGTTGTTAAATGTAAATAATTTG AAAAAAAAACACCTAAAACTCA	88	58	AF135502	5–92
MINT31-mF MINT31-mR	TGTTGGGGAAGTGTTTTTCGGC CGAAAACGAAACGCCGCG	84	60	AF135531	588–654
MINT31-uF MINT31-uR	GAATTGAGATGATTTTAATTTTTTGT CTAAAACCATCACCCCTAAACA	105	64	AF135531	352–456

(51.06 %) of all cases and patients, with left site (36.17 %) and (12.76 %) in the rectum from all cases.

Assessment of CIMP

For all colorectal carcinoma examined, the rate of methylation loci, CIMP $_{high}$ shown in 36.17%, while the CIMP $_{low}$ and the CIMP $_{neg}$ were registered in 25.53%, and 38.29% . The internal control of methylation collagen2 was appeared in 90 bp .The confirm that the methylation locus was indeed tumor specific analysis in normal mucosa was performed from cancer in this study.

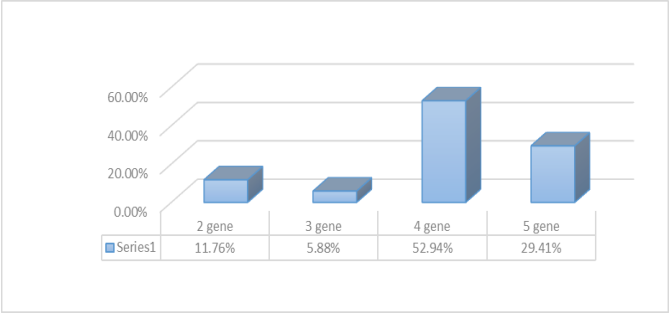

Fig. 1. CIMP $_{high}$ repeated gene. **Chi=**12.157 p=0.007

In this classical panel of CIMP the highly repeated gene show in hMLH1 was frequency with 34% percentage the hMLh1M and hMLh1 un M showed in (124bp and 153 bp) respectively the second gene repeated in p16 with 31.91%, P16 M and P16unM reveled in (160 and 161 bp) respectively, while MINT2 registered in 29.78% the MINT2 M and MINT2 un M appeared in (90bp and 88bp), the less percentage in MINT1 with 25.53%, MINT1 M and MINT1un M was appeared (102 bp and 188bp), while 23.40% in MINT31, the MINT31M and MINT31 unM appeared in (105bp and 84bp), while in CIMP

Table 2. The CIMP distribution in CRC

Gene and loci	Total	CIMP + 17	CIMP L 12	CIMP – 18	Total	P value
LMLh1	19 (40.42)	16 (34.04)	3 (6.38)	28 (59.57)	47	0.000046
P16	18 (38.29)	15 (31.91)	3 (6.38)	29 (61.70)	47	0.00002
MINT1	16 (34.04)	12 (25.53)	4 (8.51)	31 (65.95)	47	0.000004
MINT2	15 (31.91)	14 (29.78)	1 (2.1)	32 (68.08)	47	<0.001
MINT31	12 (25.53)	11 (23.40)	1 (2.1)	34 (72.34)	47	0

Table. 3. CIMP $_{high}$, CIMP $_{low}$ and CIMP $_{neg.}$ related with sex.

No.	Sex	CIMP $_{high}$ No / %	CIMP $_{low}$ No / %	CIMP $_{neg.}$ No / %	Total	P value	X^2
1.	Female	9 (42.85)	6 (28.57)	6 (28.57)	21	0.526	1.286
2.	Male	8 (30.76)	6 (23.07)	12 (46.15)	26	0.199	3.231
	Total	17	12	18	47	0.462	1.544
	P value	0.732	0	0.046			
	X2	0.118	0	4.000			

Table 4. CIMP $_{high}$, CIMP $_{low}$ and CIMP $_{neg}$ related with age.

No.	Age groups	CIMP $_{high}$ NO/%	CIMP $_{low}$ NO/%	CIMP $_{neg.}$ NO/%	Total	P value	X^2
1.	Group1≥50	5(27.77)	5(27.77)	8(44.44)	18	0.472	1.500
2.	Group2≤50	12(41.37)	7(24.13)	10(34.48)	29	0.374	1.966
	Total	17	12	18	47	0.633	0.913
	P value	5.765	0.667	0.444			
	X2	0.016	0.414	0.505			

low the highly percentage in MINT1 8.51% followed by hMLH1 and P16 6.38% in both and less percentage in MINT2 and MINT31 with 2.12% in both, Table 2

Repeated methylation loci in CIMP high, CIMP high related with more than two genes expression, the 4 genes repeated in this panel recorded high frequency with 52.94%, following by 5 genes repeated 29.41% and less frequency appeared in 2 gene and 3 gene repeated 11.76% and 5.88% respectively Figure 1.

CIMP related with sex, CIMP(high and low) was influenced by sex, it was more common in female 42.85%, 28.57%, respectively than male 30.76%, 23.07% respectively but there was no differences for CIMP neg. with no significant difference as shown in, Table 3.

CIMP Related with age, CIMP high was influenced by age, CIMP high was highly expressed groups two (41.37%) vs (27.77%) of group one, while in CIMP neg the highly percentage was recorded in the group one (44.44%) comported with (34.48%) in group two, Table 4.

CIMP Related with morphological feature, CIMP high showed close association with poorly differentiated CRC specimens 47.05%, followed by moderately differentiated (29.41%), compared with well differentiated (23.52%). CIMP low and CIMP neg reveled highly percentage 66.66% in moderately differentiated in for both, Table 5.

Table 5. CIMP related with morphological feature in CRC patients.

No.	Grade	CIMP high NO/%	CIMP low NO/%	CIMP neg. NO/%	Total	P value	X^2
1.	well	4(23.52)	2(16.66)	1(5.55)	7	0.223	3.000
2.	moderate	5(29.41)	8(66.66	12(66.66)	25	0.109	4.440
3.	Poor	8(47.05)	2(16.66)	5(27.77)	15	0.067	5.400
	Total	17	12	18	47	0.138	6.954
	P-value	0.318	0.011	0.0003			
	X^2	2.294	9.000	15.500			

Table.6 CIMP high, CIMP low and CIMP neg related with site of tumor

No	Site of tumor	CIMP high NO/%	CIMP low NO/%	CIMP neg. NO/%	Total	P value	X^2
1.	Right	9(52.94)	5(41.66)	10(55.55)	24	0.269	2.625
2.	Left	6(35.29)	6(50)	5(27.77)	17	0.916	0.176
	Rectum	2(11.76)	1(8.33)	3(16.66)	6	0.472	1.500
	Total	17	12	18	47	0.792	1.692
	P-value	0.038	0.072	0.039			
	X^2	6.529	5.250	6.500			

CIMP Related with mucinous and non- mucinous of tumor, CIMP $_{high}$ was appeared in high percentage of mucinous cases (75%) compared with non-mucinous (29.78%), with significant difference,.

CIMP Related with site of tumor

CIMP reveled association with site of tumor, CIMP $_{high}$ were more frequent (52.94%) in right site compared with left site and rectum 35.29%, 11.76% respectively, while in CIMP $_{low}$ 50% recorded in left, but CIMP $_{neg}$ was shown 55. 55% in right site, Table 6, with no significant difference.

Discussion

CpG island methylation phenotype CIMP.

In this study the CIMP $_{high}$ was shown 36.17%, while the CIMP $_{low}$ 25.53% and CIMP $_{neg.}$ 38.29%, with a cutoff value of 2 out of 5 genes. CIMP is detected in approximately 30–40% colon cancers (13) (18) (19) (20) (21). Which were companied with the results we gained in colorectal cancers 32% (22), while CIMP $_{high}$ was observed in (29.6%) (23). Where are of less percentage was detected in 24.0% of patients (24) while (25%) were CIMP $_{high}$ (25) and 24% CIMP $_{high}$ positive of sporadic CRC, (26) was observed CIMP $_{high}$ 23.1% and 24.0% respectively (27) (28). Some study was appeared less rate of methylation status CIMP $_{high}$ 18.9%, and 13.9% respectively in study of (29) (30), while CIMP $_{high}$ 16.7% in (31).

In this study used different criteria CIMP was associated with female was more common in 42.85% comparted with male 30.76%, Clinicopathological features previously reported to be associated with CIMP positive epigenotype include CIMP+ tumors were significantly associated with female gender (14) (20) (31) (32), while realted with age, CIMP $_{high}$ was highly expression in patients with age group two 41.37% versus 27.77% of group one. In the whole population, CIMP-High was clearly associated with older age (31), the presence of CpG island methylation correlated with the occurrence of tumors in the elderly (14) (20) (23) (25) (34).

In addition, cancer-specific DNA methylation is more frequent than age-related DNA methylation in a subclass of CIMP-positive CRCs (34), age-related epigenetic defects have been proposed as potential sources of the field defect in colon carcinogenesis (35) (36). CIMP is associated with poor survival in advanced colorectal cancer patients. CIMP cancers seem to have distinct clinical characteristics (more common in proximal tumors (14) (22) (27) (33) (37) (38)

CIMP $_{high}$ close association with poorly differentiated 47.05%, followed by moderate differentiated 29.41%, compared with well differentiated 23.52%, our results was same line with (37) (23) (33) (14) (22) (27) that was found to be associated significantly with poorly differentiated CRC tumor. In our study was showed mucinous 75% compared with non-mucinous 29.78%, CIMP tumors have a characteristic phenotype with a specific histology (mucinous) features the close relationship with among high level methylation status of multiple loci (CIMP), in sporadic CRCs with mucinous and non-mucinous histology (29) (20) (33) (34) (36) (37), while significantly higher percentages of mucinous CRC had CIMP (30) (26).

CIMP $_{high}$ more frequancy (52.94%) in right site compared with left site and rectum 35.29%, 11.76% respectively. It has been suggested that proximal and distal CRCs show differences in epidemiological incidence, morphology and molecular biological characteristics (39) (40). Many studies have demonstrated close associations between nutrition and DNA methylation changes in human cancers of the colon and other tissue types (41) (42) (43).

Lifestyle factors, such as diet, smoking, physical activity, and body weight management, are known to constitute the majority of cancer causes. Epigenetics has been widely proposed as a main mechanism that mediates the reversible effects of dietary and lifestyle factors on carcinogenesis (44). Other candidate bioactive food components include alcohol and other key nutritional factors of one-carbon metabolism, polyphenols and flavonoids in green tea, phytoestrogen, and lycopene. Some data also support a link of DNA methylation with physical activity and energy balance (44). Effects of dietary and lifestyle exposures on DNA methylation may be additionally modified by common genetic variants, environmental carcinogens, and infectious agents, an aspect that remains largely unexplored (44). The dietary factors assessed, dietary fiber appeared to have the greatest impact on CIMP-high tumors; however, fiber was also associated with reduced risk of CIMP-low tumors (45). Since folate is a source of methyl group, the potential of folate exposure to alter DNA methylation is subject of many previous studies. Several studies indicate a 20–40% reduction in the risk of CRC with the highest dietary folate supplementation (46) (47). Several dietary factors, including folate, methionine, vitamins B12 and B6, are involved either directly or indirectly in DNA methylation (48). In addition, absorption of water from the stool increases the risk of exposure to higher concentrations of exogenous substances that may act as epimutagenes, proposed environmental factors that can affect the epigenetic status of genes (49) (50).

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