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RESEARCH ARTICLE

DNA Methylation Pattern of Early Genes of Epstein Barr Virus Associated with Gastric Carcinoma in Group of Iraqi Patients

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Abstract

DNA methylation is one of the epigenetic changes that may affect cell functions, which associated with several steps of cancer progression. Gastric carcinoma is endemic in Iraq with highly association of Epstein Barr Virus infections. Epigenetic changes for gastric carcinoma diagnosis are very important to early diagnose the disease also consider as biomarker to search of the progression of cancer. So detection of methylation of early genes in Epstein barr virus associated gastric carcinoma is very useful to confirm the diagnosis of the disease. The first step is detection of the virus in the fresh and embedded tissues, then search of the methylation pattern of the early genes of the virus that expressed several important proteins that effect the life cycle if the virus also the progression of the disease using conventional polymerase chain reaction. The virus was detected in 20% of tissue samples, while the methylation state was positive in 87.5% in tissues affected with the virus. Highly percentage of the methylation in the early genes of the virus may affect the virus infection which will affect the progression of the cancer.

Keywords: DNA methylation, Epigenetic change, Epstein barr virus, virus early genes, gastric carcinoma.

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INTRODUCTION

Epstein Barr virus (EBV) is a ubiquitous herpes DNA virus with an incidence of approximately 90% in the general population and may remain asymptomatic for the whole life of the patient[1]. The major targets of EBV infections are the B lymphocytes and epithelial cells. EBV was associated with several human tumors, such as oral cancer, lymphomas and gastric cancer[2]. The EBV type that infects gastric tissue and may cause cancer is a critical type with an incidence of nearly 12% of all gastric carcinomas in the general population. The virus considered one of the causative agents of gastric cancer, and has a major effect in oncogenic persistent[3].

The gene expressions of the EPV play an important role in the tumor progression. The virus has three latency stages: stage one, two and three, by the patterns of the expression of the virus genes. In gastric carcinoma latency stage one or two were responsible of latent infection, which produce several viral oncogenic genes[4].Viral genes expression modifies relying on the state of the tumors and the tissue of origin[5]. Additionally, the latent gene stage could be inhibiting by methylation. This methylation might be a reaction of the host immune system versus obtrusive DNA; however, the methylation state could be useful to the virus by let it fleeing from the host immune system[3]. Epigenetic alteration such as DNA methylation has important role in the regulation of the transcription of that DNA and this may lead to the progression of the tumorigenesis[6].

Early genes of the virus such as Bam HI-A rightward ORF one (BARF1) and Bam HI-H rightward ORF one (BHRF1), have very important function in the pathogenesis of this virus, as BARF1 encode (c-fms) which is an oncogene protein and BHRF1 encodes the (Bcl-2) which is an antiapoptotic gene. BARF1 plays a major role in the lytic cycle of the virus and it considered an oncogene agent in cancer cases playing a critical function in the progression of cancer in the stomach[7]. The oncogenic effect of this ORF could convert the epithelial cells and induce the production of (Bcl-2), this situation make cells permanence in unsuitable states[8].

Methylation pattern of the BARF1 promoter was confirm in different cell lines in epithelial caners, and the CpGs of this promoter were methylated mostly. This suggests that BARF1 duplication must ride methylation-induced inhibition[9]. In the lytic stage of the EPV, BHRF1 has a critical function in the replication cycle and viral particles liberation, as it produce the oncogene Bcl-2, which has an important function in the viral pathogenesis. The transcription of BHRF1 indicates a potential service in the preservation of the persistent of infection of this virus[10,11].

In this study, I select number of gastric carcinoma samples to survey the methylation pattern of early genes BARF1 and BHRF1 and their role in effecting the production of corresponding genes.

MATERIALS AND METHODS

A total of 140 fresh gastric and paraffin embedded tissue samples were included in this study, one hundred of these samples from gastric carcinoma patients (60 samples were fresh gastric tissue, and the other forty samples were taken from patients blocks as paraffin embedded tissue) as patients group, while the control group was 40 fresh gastric biopsies obtained from apparently healthy people whom visit the hospital suffering from gastric ulcers so they considered negative control as this group was not diagnosed with any type of cancer, all samples were precede to the series examination and tests of this study, starting from the extraction of the DNA until the final step of the examination.

DNA was extracted from both paraffin embedded and fresh tissues using the standard method and according to the manufacture instruction of the two kits were used. QIAamp DNA of formalin fixed paraffin embedded tissue kit used to obtain the DNA from embedded samples while the DNA of the fresh samples was extracted by QIAamp fresh tissue kit.

Then the quality and integrity of the DNA was checked using housekeeping genes , GAPDH primer gene, samples gave positive results after PCR amplification with this primer set as the program of Rameshkumar et al.[12] and produce bands of 240 bp considered of good quality and further complete the other tests of this study, while other samples which gave negative results in this amplification were excluded from the study as the quality of these samples was bad, the number of excluded samples was 20 (six from fresh patients

and 14 from the paraffin embedded tissues).

Then PCR amplification was done to detect the EBV, the master mix was prepared with total volume 25 µl per one reaction containing 10 *p*mol forward and reverse primers, 2.5 U Go Taq ® DNA polymerase, 200 µM of each dNTP, 1X of 5 X Green Go Taq reaction buffer, then nuclease free water was added until the volume reach 23 µl, finally 2 µl of genomic DNA was added, the primers were directed to conserved regions of EPV encoding capsid protein gp220 and EPV nuclear antigen (EPNA1), the primer sets sequences were illustrated in table 1.

DNA Bisulfite Treatment and Methylation Analysis

Samples show positivity in the previous two amplifications was further continuing to bisulfite modification of DNA according to Sodium bisulfite modification of DNA[14] In 50 µl water, add 2 µl of genomic DNA to 6 µl of NaOH at 38°C for 9 minutes, then incubation conditions was 32 µl of 12 mM hydroquinone and 530 µl of sodium bisulfite at 52°C for 15 hour in dark conditions. Then, purification of DNA with nuclear free water using the Cleanup Kit. Finally DNA incubated at 25 for 7 minutes; precipitation of DNA with 2 µl of glycogen, washed with 70% ethanol; then resuspended in distilled water.

Table 1. EPV primer sets and the product size[13]

EPV primer sets	Sequence	Product size
gp220 forward	GGCTGGTGTCACCTGTGTTTA	239 bp
Reverse	CCTTAGGAGGAACAAGTCCC	
EPNA1 forward	GTCATCATCATCCGGGTCTC	269 bp
Reverse	TTCGGGTTGGAACCTCCTTG	

After treatment and modification of DNA, methylation pattern of BARF1 and BHRF1 genes was detected using methylation specific polymerase chain reaction, using the primer sets that illustrated in table 2.

Then methylation specific PCR products were resolved on two percentage gel, the results examined by comparing the product sizes of each sample with the product size of each primer sets, if a band present with 142 bp by using methylation primer set of BARF1 gene this indicate positive results for methylation to this gene, additionally the un methylated primer sets for the same gene was of 142 bp product size also and if the band appear this means negative results and there was no methylation state in the BARF1 gene, while the other gene results was readied by the appearance of a band of 218 bp for the methylation primer set of BHRF1 means positive results and the methylation state was appear, and the appearance of a band of 220 bp for un methylated primer set means negative results and there was no methylation in the BHFR1 gene.

Table 2. Sequences of the primer sets for the methylation specific polymerase chain reaction (MSP) used in this study[15]

BARF1	Methylation	Forward	GTTGGATTTAGTTATTTTGTCGTTC
		Reverse	TTATCATATAAACCTAAAACCCGTA
BARF1	Un methylation	Forward	GTTGGATTTAGTTATTTTGTTGTTTG
		Reverse	TTATCATATAAACCTAAAACCCATA
BHRF1	Methylation	Forward	TTTGTATATTTGGTTAGTTGATCGA
		Reverse	CGAAACGTAATACTTCCTAAAAACG
BHRF1	Un methylation	Forward	TTTGTATATTTGGTTAGTTGATTGA
		Reverse	CCCAAAACATAATACTTCCTAAAAACA

RESULTS

A total of 140 samples were submitted to check the quality of their DNA; 20 samples were excluded because the quality of their DNA was low as demonstrated in table 3.

Epstein Barr virus positive results was detected in 24/120 of the samples which represented 20%, the virus was detected in 18 sample in the fresh samples group, and detected in 6 samples in the paraffin embedded tissue samples group, these results was be demonstrated in the table 4.

The methylation state appear in high incidence in the samples that was detected with the EBV, as the methylation was detected in 87.5 % from the cases, these results was demonstrated in table 5.

DISCUSSION

Epigenetic changes such as DNA methylation are recognized as an important mechanism in cancer initiation and progression[16]. Epstein Barr Virus infection induces increased genome-wide gene methylation, resulting in

Table 3. Classification of samples included in this study

Patients group				Total number of patients samples after exclusion	Control group
Fresh samples		Paraffin embedded tissue			
Number of samples	Number of excluded samples	Number of samples	Number of excluded samples		
60	6	40	14	80	40

Table 4. Detection of the Epstein Barr Virus

		Positive	%	Negative	%	Total
Patients group	Fresh samples	18	33.3	36	66.7	54
	Paraffin embedded samples	6	23.1	20	76.9	26
Control group		0	0.0	40	100	40
Total		24	20	96	80	120

Table 5. Methylation status in EBV associated gastric carcinoma

Patients group	Positive (methylated)	%	Negative (non-methylated)	%	Total
Fresh samples	16	88.9	2	11.1	18
Paraffin embedded samples	5	83.3	1	16.7	6
Total	21	87.5	3	12.5	24

the formation of a unique type with high CpG methylation in tumor cells. Given its important functions in cancer initiation and progression, DNA methylation is being explored as a biomarker for cancer[17].

The open reading frame BARF1 is a potent oncogene in EBV carcinomas and has a variety of important biological functions in the pathogenic and carcinogenic mechanism of this virus[18] BARF1 had been reported to be expressed in EBV-positive gastric cancer tissues during latency and was considered as oncogenic, parallel to the more widely studied latent membrane protein 1 (LMP1)[19]. Especially in EBV-positive GC, BARF1 is expressed in the absence of LMP1, possibly functioning as an EBV oncogene in this disease and playing an important function for developing of gastric carcinoma caused by EBV[20]. Expression of BARF1 gene during lytic replication is regulated by the immediate early proteins BRLF1 and is independent of the promoter methylation status[21]. The methylation status of BARF1 promoter may play an important role in cancer progression during latency infection of the virus. The open reading frame BHRF1 similar to Bcl-2 in its function as it inhibits apoptosis of the B lymphocytes and epithelial cells. In vitro studies showed that BHRF1 could enhance cell resistance to apoptosis and cell death caused by many external factors, such as foreign virus infection[22]. In this study, I explored the CpG methylation profiles of EBV early genes in the gastric cancers caused by this virus after bisulfite treatment using conventional PCR.

The result of the methylation of the early genes of EPV in this study considered high with an incidence of 87.5 %, this result was higher than the result of the methylation in the same early genes in a study done in China using quantitative real time PCR, these differences in the result may be because of the geographical distribution, also the difference in the molecular technique that used in both studies as the conventional PCR was used in this study.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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