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Two Steps Methylation Specific PCR for Assessment of *APC* Promoter Methylation in Gastric Adenocarcinoma

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Abstract

Gastric Cancer (GC) is the second most common cancer in the world and a leading cause of cancer-related mortality. Methylation of promoter CpG islands (CGIs) belonging to tumor suppressor genes causes transcriptional silencing of their corresponding genes leading to carcinogenesis and other disorders. Adenomatous Polyposis Coli (*APC*) a tumor suppressor gene is inactivated by methylation of promoter region in CpG islands. In current study, methylation of CGIs in the promoter of *APC* was analyzed using two steps Methylation Specific PCR in 36 tumor tissue samples together with an equal number of normal tissues belonging to patients with confirmed diagnosis of gastric carcinoma. Methylation specific PCR (MSP) was positive for 28 out of 36 (77.77%) of the tumors. No correlation was found between *APC* hypermethylation and the age of patients regardless of the relevant cofactors in aberrant methylation. Our study showed that hypermethylation of the *APC* gene was frequent among our patients with gastric cancer. Although the *APC* hypermethylation has been proposed to be involved in the early stages of gastric carcinogenesis, but the *APC* gene alone, does not seem to be a reliable marker for the different stages of cancer prognosis. Thus, it is recommended to use a panel of genes for evaluating the CpG Island methylator phenotype (CIMP) phenomena as an early event in gastric cancer, both for tumor and serum samples.

Keywords: Methylation specific PCR (MSP); Gastric cancer (GC); Hypermethylation; CpG islands (CGIs); Adenomatous polyposis Coli (APC)

Introduction

Gastric Cancer (GC) is among the most common malignancies worldwide and remains a leading cause of cancer in Asia and certain European countries [1]. Being an endemic region with high incidence, GC in

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Iran is considered as an etiologic and management challenge for the health program. A range of genetic and epigenetic alterations are associated with GC [2-5]. It is now obvious that different genetic pathways lead to diffuse and intestinal types of gastric cancers which are the two common forms of GC [6] among which epigenetic alterations, such as DNA methylation of CpG islands are the most evident. In GCs of both histological types, multiple tumor suppressor genes including *APC, CDKN2A, GSTP1, CDH1, MGMT, MLH1, DAPK, THBS-1, RUNX3*, etc., have shown to be commonly methylated and all are as potential candidate molecular markers for these cancers, specially since methylation of some of these genes may occur early in gastric carcinogenesis [12-14]. On the other hand, in several instances promoter hypomethylation leads to activation of genes, such as *Maspin* and *Cyclin D2* which are involved in gastric carcinogenesis [8, 64]. Human telomerase reverse transcriptase gene *(hTERT)* is the first gene which has shown that methylation promoter region correlating with upregulation of gene in this cancer [7]. Hypermethylation of CpG islands is associated with silencing of genes and has been proposed as a mechanism for inactivation of tumor suppressor genes in carcinomas [9-11]. The reason for aberrant methylation is currently unknown. The *APC* gene was first identified in the germlines of individuals with Familial Adenomatous Polyposis (FAP)[15]and has been shown to play a role in the development of sporadic colorectal cancer [16, 17].Under normal conditions, APC protein binds to the β- catenin and maintains it at low level. Increased level of β-catenin activates growth-promoting genes such as *c-Myc* through interaction of β-catenin with *Tcf-4* complexes, which ultimately leads to loss of cellular growth control. Inactivation or reduction in the level of APC protein occurs via multiple mechanisms, including allelic loss, mutation, and by methylation of CpG islands in the promoter [18]which plays a role in the development of certain GCs. Somatic mutations and hypermethylation of *APC* has been described in gastric, esophageal, thyroid, endometrial, hepatic, breast, colorectal and pancreatic cancers [19-26]. Recently, hypermethylation of promoters and the 5' segment of genes have become recognized as an important epigenetic mechanism for inactivating genes. Thus evaluation of *APC* hypermethylation may influence future treatment regimens and specially assessment of tumor suppressor genes and their role in carcinogenesis. The purpose of the present study was to investigate the prevalence of *APC* promoter hypermethylation among some Iranian patients with GC and assess its applicability as a candidate molecular marker.

Materials and Methods

DNA Extraction

Tumor tissues along with normal adjacent tissues were dissected from gastric adenocarcinoma following the operation of patients with confirmed diagnosis of gastric carcinoma. Tissues were wrapped in sterile gas, suspended in liquid nitrogen and stored at -70°C till the time of DNA extraction. Additional 5 ml of whole blood were collected in EDTA (25mM) and kept frozen (under the same conditions as above). Genomic DNA of all samples were extracted from tissues and serum using the proteinase K digestion and phenol chloroform method, as described previously [27].

Bisulfite treatment of DNA Extracted genomic DNA was digested with the *Hind III* restriction enzyme for which no restriction site was found in the entire promoter and the *APC* gene. 1 µg of digested DNA was modified by the sodium bisulfite method [28]. Briefly denaturation was carried out for 5 min by boiling, followed by immediate chilling on ice. The procedure was then followed by equilibration against 0.3 M NaOH for 5 min on ice. Low melting agarose (2%)was added to samples (2V/V), 5 µl of which containing 100 ng of DNA was dropped into cooled mineral oil for the purpose of bead formation. Subsequently each bead was incubated in 100 µl of freshly prepared sodium bisulfide solution (2.5 M Sodium bisulfide, 125 mM Hydroquinone, pH 5) at 50°C for 4 h in the dark. Further treatment was stopped by washing beads with1 ml of TE buffer (10 mM Tris base 1mM EDTA pH 8)six times each time for 15 min. Subsequently desulphonation was carried out with 500 µl of 0.2 M NaOH (2x15 min)and neutralized with 1.5 V of 1M HCl. Ultimately, beads were washed with 1 ml of TE buffer followed by equilibrations against 1 ml of ddH_2O(2x15 min). Beads were used directly for PCR or kept at 4°C for at most four weeks.

Methylation Specific PCR

MSP was carried out by applying four sets of primers. The first two sets were used for amplification of promoter ending to longer product that was used for subsequent amplification that specifically differentiates the methylated, from the unmehylated CpG dinucleotides. The first two sets of primers were designed for the region of promoter without any CpG dinucleotides, while the other two sets of primers were designed for the internal region of the promoter with CpG dinucleotids. The sequences of the first two sets of primers were; 5'TTT GTT TGT TGG GGA TTG GGG

3' (forward) and 5'AAA CCC TAT ACC AAA AAA AAA CCA TC 3' (reverse) resulting in a 402 bp amplification product. The second set of primers were ere 5' GTT AGG GTT AGG TAG GTT GTG 3' (forward), and 5' AAA ACA ATA CAA AAA AAA ACC ACC TTC 3' (reverse)leading to a 320 bp product. PCR conditions consisted of 10 cycles of amplification with primary denaturation at 94°C for 50 sec, annealing at 62°C for 1 min (touchdown 0.2°C/cycle) and extension at 72°C for 1 min followed by 27 cycles at 94°C for 50 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min which was then followed by a 10 min final extension at 72°C. Agarose gel electrophoresis was used subsequently to detect the PCR products. Following the first round of amplification, the resulting PCR product was applied to a second round of PCR by using primers designed for specific amplification of methylated or unmethylated cytosines in the CpG dinucleotides. One of the two sets of primers designed for identification of unmethylated CpG dinucleotides in the secondary nested amplification reaction were; 5' GTG TTT TAT TGT GGA GTGTGG GTT 3' (sense)and 5' AAC CAA TCA ACA AAC TCC CAA CAA 3' (antisense), leading to a 111 bp product. The second set of primers that were used for identification of methylated CpGs were; 5'TAT TGC GGA GTG CGG GTC 3' (sense)and 5' TCA AG AAC TCC CGA CGA 3' (antisense), resulting in a 98 bp amplification product. MSP was performed in 50µl PCR reaction mixture containing 1X reaction buffer, 0.2mM of each dNTP, 6mM MgCl 2, 8mM β-mercaptoethanol, 0.8 µg/ml BSA, 8% DMSO, and 20 pmol of methylated or unmethylated specific primers and 1.2U of Taq polymerase. PCR conditions were composed of 94°C denaturation for 5 min, 10 cycles consisting of 50 sec denaturation at 94°C, 45 sec annealing at 53°C (Touchdown 0.3°C/cycle), 1 min of extension at 72°C followed by 25cycles of 50 sec denaturation at 94°C, 45 sec annealing at 52.5°C, 1 min extension at 72°C, and subsequently continued by 10 min of final extension at 72°C. Each set of primers (methylated or unmethylated specific primers) were used for amplification of tumor and normal tissue samples as well as negative and positive controls. In order to check the efficiency of bisulfite treatment, the β-actin promoter (NCBI accession No: 102630) was used as an internal control in a separate amplification. Amplification was carried out by applying; 5'TGG TGA TGG AGG TTT AGT AAG T 3' (sense) and 5'ACC CAA TAA AAC CTA CTC CTC CCT TAA 3' (antisense). PCR conditions were composed of 4 min of primary denaturation at 95 °C followed by 40 cycles of denaturation for 1 min at 94°C, 58 sec of annealing at 55.5°C, extension for 1 min at 72°C and a final extension of 10 min at 72°C. The 132bp PCR product was run on a 2% (w/v) agarose, stained with ethidium bromide and visualized by UV transluminator.

Results

Methylation specific PCR (MSP) differentiates unmethylated from methylated alleles based on sequence alterations produced following the bisulfite treatment of DNA, which converts unmethylated, but not methylated cytosines to uracil. Subsequent PCR using primers specific for either methylated or unmethylated cytosines in the CpG dinucleotides makes discrimination of methylated from unmethylated cytosines possible. In order to successfully amplify and analyze methylation status of the *APC* promoter, we designed a two-step procedure for amplification. Four sets of primers were designed based on the available APC promoter sequences in data bases (NCBI accession No: U02509). Following the bisulfite treatment two sets of primers were used for primary amplification in order to obtain sufficient product for subsequent amplification. This approach resulted in a more efficient methylation specific PCR. As a result, discrimination between these two conditions could accurately be carried out. The products of primary amplification were either 402 or 320 bp (Fig. 1).

The two sets of primers for primary amplification were designed against a part of *APC* promoter sequence devoid of any CpG dinucleotides. This adaptation eliminated interference of any methylated cytosine and also was a suitable mean for evaluating efficacy of

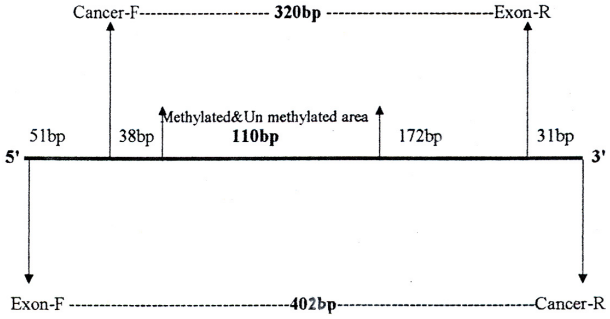

Figure 1. The plan of two steps methylation specific PCR profile of APC promoter. The product of primary amplification with primers designed for promoter sequence without CpG dinucleotide results to either 402 or 320 bp product which were used for the second nested amplification. The product of second amplification would either be 98 bp for methylated or 111 bp product for unmethylated CpG dinucleotides.

Table 1. Histological features of tissues and methylation status of *APC* promoter

Differentiation status	Total	Methylated	Unmethylated	Methylated/Unmethylated
Intestinal Type	20	11 (30.55%)	4 (11.11%)	5 (13.88%)
Diffuse Type	12	8 (22.22%)	3(8.33%)	1(2.77%)
Moderate	4	2 (5.55%)	1 (2.77%)	1 (2.77)
Total	36	21 (58.33%)	8 (22.22%)	7 (19.44%)

bisulfite treatment. For subsequent amplification, primers were designed against the internal part of the primary amplification product of the *APC* promoter which contains the highest number of CpG dinucleotides. PCR products from the second round of amplification were either 98 bp for the methylated or 111 bp in case of the unmethylated cytosines in CpG dinucleotides. Figure 1 represents schematically the length of PCR products and their relative position in the *APC* promoter.

Thirty six patients with regard to age, sex, clinical signs and symptoms, radiographic studies, site of tumorigenesis and pathological examinations including tumor differentiation status were included in this study (Table1). Patients with lung and liver metastasis and individuals who did not have the generals conditions required for undergoing surgery were omitted.

All the study protocols were approved by the editorial Commission of Science and Ethics at the National Institute of Genetic Engineering and Biotechnology. Figure 2 shows the 402 bp and 320 bp amplification products of primary amplifications either of which could be used for secondary PCR repeat. Because the primary amplification was carried out in a region of the *APC* promoter in which no CpG dinucleotides were present and if the bisulfite treatment was fully implemented, it was possible to amplify with the primers that were designed for this region of the *APC* promoter. Further verification was carried out by applying β-actin in the bisulfite treatment and subsequent MSP. Being a house keeping gene, the β-actin promoter exhibits unmethylated cytosines and, thus the complete conversion of cytosines either in the CpG dinucleotides or in isolated from to uracil should happen if the bisulfite treatment is adequate and efficient. Based on this assumption two sets of primers were designed for specific amplification of DNA extracted from normal blood samples and were used for either methylated or unmethylated promoters of β-actin (Fig. 3). Production of 132 bp PCR product following the application of primers to unmethylated cytosines further supported the appropriateness of the treatment.

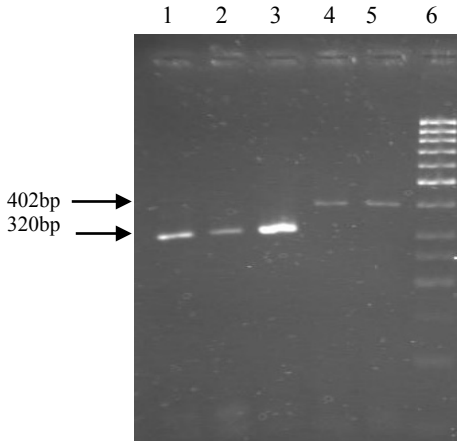

Figure 2. PCR Reaction for Gastric Adenocarcinoma samples and healthy control with two sets primers 320 and 402 bp fragments.1 and 2: Patients samples (320 bp fragment), 3: Healthy Control sample (320 bp fragment), 4 and 5: Patients samples (402 bp fragment).

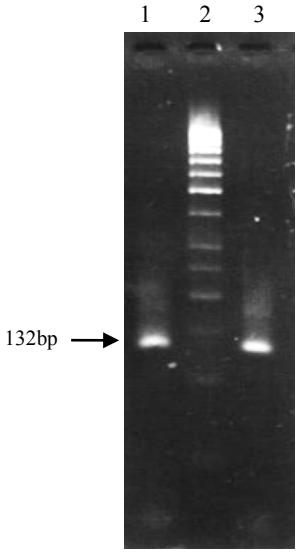

Figure 3. Amplification of ß-actin following to bisulfite treatment and application of primers designed for unmethylated cytosines in its promoter. From left; lane 1 amplification product of blood DNA of healthy donor, lane 2 DNA ladder [Fermentas 50 base pair)and lane 3 the same as lane 2 but DNA extracted from tissue of the patient.

We also used the universal methylated DNA (Chemicone, Germany) in bisulfite treatment and PCR amplification along with DNA extracted from tissues as well as DNA extracted from blood samples. Selective amplification of the 98 bp fragment and absence of an observable 111 bp amplification product further proved that the treatment was complete (Fig. 4). Applying extracted DNA from blood of the healthy donors and treatment with DNA methylase (Biolabs, England) was among further controls to evaluate whether treatment is appropriate and MSP has worked well (data not shown). Following such controls MSP was carried out on extracted DNA from the normal and tumor tissue samples and treated with bisulfite (Fig. 4). It was found that 21 (58.33%) out of 36 cases of gastric carcinoma were methylated. No methylation was found in normal tissues of patients (Controls). Comparing methylation status of cytosines in CpG dinucleotides in males with females, 9 males (42.86%) and 12 females (57.14%) with a mean age of 55.5 years old, showed 58.33 % hypermethylation for the *APC* promoter. Also, 8 out of males simultaneously showed both the methylated and unmethylated promoter. Our results initiate that may be the prevalence of the promoter hypermethylation is more frequent among gastric adenocarcinoma patients. We also found similar results in 6 available serum samples.

Discussion

Promoter methylation could be observed in many types of cancers leading to aberrant transcriptional silencing of tumor suppressor genes [29]. Our results showed that 28 (77.77%) out of 36 patients are methylated. This phenomenon has reliably been associated with gastric cancer development [30, 31] and plays a major etiologic role for many types of neoplasms [32-35]. Previous studies have shown that, in gastric cancer, hypermethylation of the promoter, progressively increases with histopathologic progression from chronic gastritis, intestinal metaplasia and adenoma to carcinoma [36, 37]. Mutation of *APC* has shown to play an important role in colorectal tumorogensis. *APC* encodes a large protein with multiple cellular functions and interactions; and its role in the WNT signaling pathway is specially well described. In a complicated pathway via β-catenin, stimulation of the transcription of *WNT* target genes such as *myc* and *cyclin D*, results in cell proliferation and oncongenesis [38]. Mutational changes in this gene would lead to alteration of signal transduction in the cell, differentiation, mediation of intercellular adhesion, stabilization of the cytoskeleton and possibly regulation of the cell cycle during apoptosis [22]. A marked difference has been reported with respect to *APC* mutation in GC by histological type. *APC* mutations are significantly frequent in intestinal type GC in comparison to those of the diffuse type GC [22]. The level of *APC* methylation was found to be different among various kinds of tumors and genetic backgrounds for example 45% in liposarcoma [39], while highly frequent (100%)in Barrett's esophagus patients [20], it is less apparent in esophageal squamous cell carcinoma (ESCC) [40]. Waki *et al.,* [2003] reported that approximately 50% of subjects fewer than 32 years of age with stomach cancer are methylated in *APC* [41]. Less than 10% of the *APC* promoter methylation was reported in colorectal tissue, lymphocytes of patients with synchronous colorectal adenoma, carcinoma and lung cancers [42-44]. In colorectal carcinoma the *APC* is methylated in 18% of sporadic tumors. Methylated *APC* promoter is frequently present both in primary gastric cancer and in adjacent non-cancerous mucosa [18,45]. Hypermethylation of *APC* is extremely uncommon (1%) in tumors of familial adenomatous polyposis. While in non-small-cell lung cancer results regarding *APC* methylation are divergent, probably because of difference in techniques and patient cohorts [46]. Numerous studies have shown that demethylation of whole of the human genome are corresponding to age cancer patients that it can make to human genome damage such as hypomethylation of promoter and mutation of oncogenes [47], Suzuki et al. [2004] showed that age related increase in demethylation is

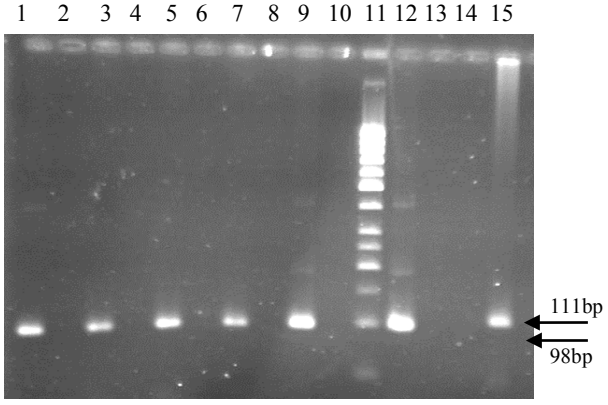

Figure 4. Turmeric samples with methylated primers (existence of 98 bp fragment for lane 1, 3, 5, 7 and 9) and unmethylated primer (absence of 111 bp fragment for lanes 2, 4, 6, 8 and 10). Universal methylated DNA (with 98 bp band and absence of 111 bp fragments with methylated and unmethylated perimers, for lanes 12 and 13, respectively). Healthy control DNA (with 111 bp fragment and absence of 98 bp band for lanes 14 and 15, respectively). Lane 11: DNA ladder marker.

associated with genomic damage in gastric cancer cells. While we were not able to find correlation between APC hypermethylation and age, which maybe it was due to our small sample size. We could not find any complete data related to the importance of the host factor such as H. Pylori, but several studies have shown that methylation in H. pylori positive patient is significantly higher level [63]. Nevertheless, other studies have shown that promoter hypermethylation is not the only cause of reduction in gene expression, thus other mechanisms may also be involved. Mutations in the *APC* were implicated in sporadic and familial colorectal neoplasia. The observation that the frequency of detectable *APC* mutations is similar in colonic adenomas and carcinomas (~60%) has suggested that the *APC* mutations may be an early or even the initiating event in the process of clonic carcinogenesis [51]. Promoter methylation has not certainly occurred in cancer; therefore, it is strongly recommend that the *APC* should not be included in gene panels used to study on hypermethylation role in gene silencing in colorectal cancer and pituitary adenomas [52-54]. This is because the methylation of *APC* has been frequently observed in sequential carcinogenic steps, as well as in normal gastric mucosae [40]. Aberrant methylation in noncancerous tissues occurs only in a fraction of cells, which is expected to be highly variable, and qualitative analysis of methylation does not seem suitable. Also, different CGIs and, even within them, different regions show different susceptibility to aberrant DNA methylation [55]. Interestingly, *APC* gene was commonly methylated in fully normal liver tissues (88.2%), and reduced methylation frequency was detected in cirrhotic liver and/or tissue in chronic hepatitis (21.6%) [56]. However, most literatures have shown that hypermethylation of markers such as *APC*, *P14ARF*, *hMLH1*, *WRN* and *CRPB1* are relatively frequent in gastric carcinoma compare with other genes [57]. In this study, *APC* promoter methylation was not restricted to tumor tissue but was also found in uninvolved tissues at lower level. Some studies have shown that these findings may point to the fact that the methylation is an independent process from the tumor development. Tamura [2004] have demonstrated that the methylation of the *APC* promoter is equally frequent in neoplastic and non-neoplastic gastric epithelia in GC [14]. *APC* promoter methylation is not restricted to tumor tissue but is also found in uninvolved tissues at even higher percentages (12 %-60%). Numerous normal gastric mucosa have shown the monoallelic methylation of APC promoter which maybe is due to the first shock of two hit shock hypothesis and probably shows LOH (loss of hetrozygosity) in APC locus in this cancer [61].These findings may point to the fact that the methylation occurs in early stages of gastric cancer but not during of development of tumor [58].We were also found hypermethylation in 77% of serum samples by the MSP method, but we were not able to rely on this part of our data, due to our small sample size. Nevertheless, serum DNA is a useful remote material for tumor biomarker, because a single-gene approach cannot obtain sufficient sensitivity and specificity to discriminate the existence of GC. Thus, more genes need to use a multiple marker approach for GC patients such as *CDH1*, *DAP- Kinase*, *GSTP1*, *P15* and *P16* [40, 59].

In summary, this study demonstrated that regardless of the relevant cofactor in aberrant methylation, hypermethylation of the *APC* gene was frequent among some Iranian patients with GC. Therefore, it is proposed that APC hypermethylation maybe used as one of the biomarkers in gastric carcinogenesis. Along with this gene, and using a panel of other genes, could evaluate in CIMP phenomena as one of the early molecular events in gastric carcinogenesis[60,62].

Our research grant was not enough for performing our study with the large sample size, therefore, confirmation of the results in this investigation, with a higher sample size, is highly recommended.

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