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Heteroduplex Analysis of Causative APC Mutations in FAP Patients Referred for Genetic Counseling in Mashhad Ghaem Hospital, Iran

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Since the isolation and characterization of the adenomatous polyposis coli (APC) gene in 1991, none of the currently available techniques were able to detect 100% of the germline mutations. In the present study we have used Heteroduplex Analysis (HA) and followed by DNA sequencing to test in an exhaustive analysis of entire APC coding region in a group of 26 unrelated Khorasanies patients with familial adenomatous polyposis (FAP). Sixtheen causative mutations (61.5%) were detected. This study shows that HA is less sensitive for single base pair substitutions, whereas mutations related to insertions or deletions are easier to detect by the HA method. Since more than 70% of APC mutations are small deletion (66%) or small insertions (6%), therefore HA is particularly suitable for the APC mutation detection.

*Keywords:*APC gene, Heteroduplex analysis, Mutation, Khorasan province

1. Background

Familial adenomatous polyposis (FAP) is a dominantly inherited disorder characterized by the formation of hundreds to thousands of colorectal adenomas by the second or third decade of life (1-4). Without surgical treatment the presentation of malignant tumors inevitably occurs, usually before the age of 40 years, emphasizing the importance of an early detection by screening of all risk family members (1). The identification and characterization of the adenomatous polyposis coli (APC) gene in 1991 has facilitated the direct identification of germline mutations responsible for FAP (5-7). Knowledge of the specific APC mutation is of great importance for the presymptomatic diagnosis of FAP. Indeed, once the specific disease-causing mutation has been identified in a family, the need for annual endoscopic examination can be omitted in those who test negative, whereas the surveillance has to be improved by a closer follow-up by a yearly sigmoidoscopy beginning after the childhood, in those who test positive. To perform the mutation analysis of the APC gene, a large number of well-established techniques are currently available including RNase protection assay (8), single stand conformation analysis (9), hetroduplex analysis (10), denaturing gradient gel electrophoresis (11), protein truncation test (12), colorimetric assay (13), chemical cleavage of mismatch (14) and dHPLC (15). Overall, frequency of identifying the causative mutation ranges from 21% to 82% (16-18). The main reasons for this low detection rate are that mutations in FAP have an extremely heterogeneous spectrum and are scattered all along the 15 exons of the APC gene, which is a rather large gene with an open reading frame of 8535 nucleotides coding for a predicted final protein product of 2843 amino acids with a mass of 311 KDa (6, 7). Another reason is the sensitivity of the mutation detection technique used itself (19). The remaining reasons for the lack of identification of the causative mutation are the possibility of clinical misdiagnosis of FAP, the lack of exploration of regulatory elements influencing APC expression and the possibility of non-allelic genetic heterogeneity in FAP (16, 17). For the genetic counseling of FAP families, Cottrell et al (1992) (9) advocated a hierarchical approach to the genetic analysis, starting with a search for the APC gene mutations, followed by linkage studies using closely linked markers. In the same way, we proposed a strategy for APC mutation screening for the first time in Mashhad, Ghaem hospital, Iran. First a rapid screening of a large area of APC by analyzing the 5' half of exon 15, second, an analysis of APC exons 1-14, third, a screening of the remaining part of APC exon 15.

2. Objectives

In the present study we have used Heteroduplex Analysis (HA) and followed by DNA sequencing to test in an exhaustive analysis of entire APC coding region in a group of 26 unrelated Khorasanies patients with familial adenomatous polyposis (FAP).

3. Material and Methods

The FAP patients of this study belong to 26 unrelated Khorasani family's referred to gastrointestinal depart-

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ments in Mashhad Ghaem and Razavi hospitals. All patients had clinically and pathologically confirmed FAP.

3.1. Genomic DNA Preparation

At the time of genetic counseling, blood samples (5ml) were collected into EDTA anticoagulant. Genomic DNA was extracted according to the saturated NaCl deproteinization method described by Miller et al (1988) (20).

3.2. Polymerase Chain Reaction

The first 14 exons and the 23 fragments of exon 15 of the APC gene were amplified in order to screen the entire coding region for mutations. PCR was performed using primers pairs previously described by Groden et al. (1991) (7). All reactions were carried out in a 50µl reaction mixture containing 25 pmol of each appropriate primer, 5 µl of a buffer containing 100mM, Tris HCl, Ph 8.8, 500Mm potassium chloride, 15 Mm magnesium chloride and 0.1% Triton X-100, 200 mM each dNTP, 1.0 unit Taq polymerase (cinagen, Iran) and 200-500 ng of genomic DNA.The PCR was carried out in an automatic thermal cycler (Techne) denatured for four minutes at 94°C, followed by 30 to 35 repeated cycles of one minute denaturing at 94°C, one minute at recommended annealing temperature and one minute elongation at 72°C. The PCR products were checked on a 2% agarose gel.

3.3. Heteroduplex Analysis

To ensure that the maximum yield of heteroduplex DNA was obtained, the samples amplified by PCR were heated to 94°C for four minutes and allowed to cool slowly to room temperature over a period of at least 50 minutes. 20 µl of each sample were then mixed with 4 µl of loading buffer (30% glycerol, 0.05 % bromophenol blue, 0.05% xylene cyanol, 60 mM EDTA), and applied to a hydrolink MDE gel (AT Biochem). Electrophoresis was performed for 16 hours at 200V (10V/cm). Gels were stained by ethidium bromide and photographed under UV light.

3.4. DNA Sequencing

Any PCR product showing an abnormal mobility shift pattern on HA, segregating with the FAP disease in the family was directly sequenced. The primer used for template prepration and amplification conditions were described by Groden et al. (1991) (7). The PCR products were purified using the Agarose GelExtract mini Kit (5 prime GmbH, Hamburg, Germany) according to the manufacturer's instructions. Direct sequencing was performed by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia).

3.5. APC Gene Mutation Analysis

The nucleotide and deduced amino acid sequences were compared with reference sequences of the APC gene available at the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4. Results

Among these 26 unrelated Khorasani patients, we have identified 16 causative mutations (61.5%), which are summarized in Table 1. We have screened 100% of the total coding sequence of the APC gene by HA and found 30 typical heteroduplex patterns. The mobility shift was related to the causative mutation in 16 patients (16/26, 61.5%). There were 9 deletions of 1 to 5 bp and 2 single bp insertion, all were frameshift mutation with a stop codon downstream leading to a predicted truncated protein product. The 14 remaining heteroduplex pattern were all related to a silent polymorphism, an A to G substitution at codon 545 of exon 13 for 11 patients, a C to T substitution at codon 2402 of exonic fragment 15-R for two patients and a C to G substitution at codon 2622 of exonic fragment 15-U for one patient.

Table 1. Detection and Characterization of Germline Mutations of the APC Gene in 26 Unrelated FAP Patients

Family	HA	Exon	Codon	Nucleotide	Mutation
1	+	8	301	901	C > T
2	-	-	-	-	-
3	+	15-I	1554	4661	A ins
4	+	15-E	1146	3438	C del
5	-	-	-	-	-
6	+	4	156	466	T ins
7	+	15-E	1061	3182	AA del
8	+	15-G	1305	3914	AAAT del
9	+	15-G	1308	3923	AAAAG del
10	-	-	-	-	-
11	+	8	301	901	C > T

12	+	8	301	901	C > T
13	-	-	-	-	-
14	+	15-E	1113	3337	C > T
15	+	15-G	1308	3923	AAAAG del
16	+	15-G	1365	4093	GGTG del
17	-	-	-	-	-
18	+	11	472	1417	C > T
19	-	-	-	-	-
20	-	-	-	-	-
21	+	4	169	505	GATA del
22	-	-	-	-	-
23	-	-	-	-	-
24	+	15-H	1416	4248	C del
25	-	-	-	-	-
26	+	15-N	1981	5941	A del

5. Discussion

The detection and characterization of single nucleotide variation in genomic DNA still represents a common technical obstacle in the analysis of large gene such as the APC gene. Actually, of over 1000 FAP patients reported in the world literature, the overall frequency of identifying the causative mutation is only 30% (19-21) and ranges from 21% (22) to 82% (13).

At the present time, there are many mutation screening techniques available. Among the most commonly used techniques, some are less sensitive but easy to use, some are not suitable for screening large numbers of individuals, because of their complexity. We therefore need to establish an appropriate stepwise program of a logical and time-saving approach to the molecular analysis of FAP. This approach has to be based on the sensitivity and on the simplicity to use of the different techniques. Because of their rapidity and simplicity, we decided to use the HA for the mutation detectability rate as a first step of our screening program. The detectability rates for HA were 35-64%. In the present study the mutation detection reached 61.5% and was in concordance with rates previously published in the world literature (21). Among the generally advocated reasons for this rather low detectability rate, like clinical misdiagnosis of FAP, the lack of exploration of regulatory elements influencing APC expression and the possibility of non-allelic heterogeneity in FAP, the sensitivity and the robustness of the mutation detection technique employed could be a key feature. Bhattacharyya and Lilley (1989) (23) have shown that insertions or deletions lead to looped-out bases in double stranded DNA resulting in bulges. Insertions or deletions created very stable heteroduplexes, with rigidly defined structures, exhibiting large gel retardations consistent with high bent strictures. Therefore, mutations related to insertions or deletions are easier to detect the HA method. Since more than 70% of APC mutations are small deletion (66%) or small insertions (6%) (24), these are particularly suited to HA. The 11 mutations we have detected by HA were small insertions or deletions. Another time-saving development of HA is the use of multiplex PCR assay followed by HA on agarose minigel after a short electrophoresis of four hours. Cama et al. (1995) (25) have used this strategy to detect the three more frequently occurring mutations of the APC gene, which are deletions at codons 1061, 1068 and 1309. This single step genetic diagnosis is reliable for insertions or deletions of more than 3 bp, but fails to detect smaller or single base pair mutations (26). For the remaining patients, co-segregation analysis may represent an alternative efficient method for presymptomatic diagnosis.

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