A Protocol for Genetic Evaluation of Patients with Multiple Colorectal Adenomas and without Evidence of *APC* Gene Mutation

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ABSTRACT: **Background:** Patients with multiple (< 100) colorectal adenomatous polyps are at increased risk for colorectal cancer. Genetic evaluation of those patients who test negative for *APC* gene mutation is both a clinical and economic burden but is critical for counseling and surveillance. In Israel, this is confounded by the fact that national health insurance does not fully cover genetic evaluation of *APC* gene exon 16.

Objectives: To perform a comprehensive genetic evaluation of *APC* gene mutation-negative polyposis patients with the aim of developing a future evaluation protocol.

Methods: Genetic analyses were performed in 29 *APC* gene mutation-negative Jewish individuals with 5 to ≥ 40 colonic adenomas who did not fulfill Amsterdam (clinical) criteria for Lynch syndrome. Analyses included completion of *APC* gene exon 16 sequencing, analysis for *APC* gene copy number variations (deletions or duplications), *MUTYH* gene sequencing, and microsatellite instability in CRC patients fulfilling "Bethesda" (laboratory investigation) criteria for Lynch syndrome.

Results: Completion of *APC* gene exon 16 sequencing revealed one patient with the E1317Q polymorphism. All were normal by *APC* multiplex ligation-dependent probe amplification analysis. Pathogenic *MUTYH* mutations were found in three patients, all of North African origin; two additional patients had variants of unknown significance. One of six patients with Bethesda-positive criteria was MSI-High with immunohistology consistent with *MLH1* mutation.

Conclusions: Based on this small but well-characterized cohort with multiple colorectal adenomas, Lynch syndrome needs to be excluded if there are compatible criteria; otherwise *MUTYH* sequencing is probably the first step in evaluating *APC*-negative patients, especially for Jews of North African descent. Completing *APC* exon 16 sequencing and copy number variations analysis should probably be the last evaluations.

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KEY WORDS: adenomas, *APC*-negative polyposis, colorectal cancer, *MUTYH*

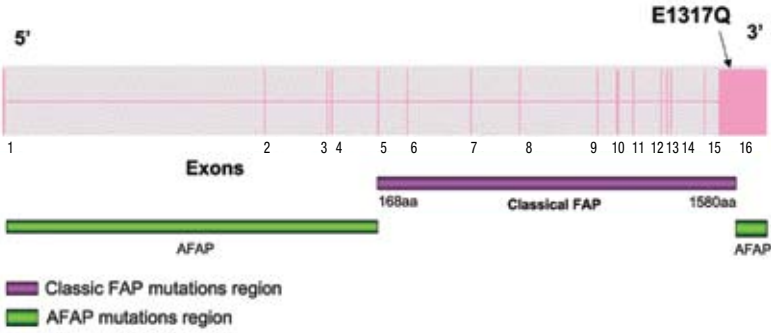
It is estimated that approximately 30% of all colorectal cancer cases are due to genetic predisposition and are frequently associated with the presence of polyps as a main or minor manifestation of disease [1]. Autosomal dominant conditions associated with a particularly high CRC risk include Lynch syndrome and the adenomatous polyposis syndromes comprising familial adenomatous polyposis and *MUTYH*-associated polyposis. Another group is the non-adenomatous polyposis syndromes that include familial juvenile, hyperplastic and mixed polyposis syndromes [1].

Often the first step in genetic evaluation of individuals with multiple (usually defined as > 5) colorectal adenomatous polyps is exclusion of FAP by *APC* gene sequencing. In Israel, national health insurance finances almost complete *APC* gene sequencing (exons 1–15, but exon 16 only up to 2500 base pairs from 5'). Exon 16 is large, and sequencing it is expensive and rarely demonstrates a pathogenic mutation [Figure 1] [2,3].

Further genetic evaluation of patients with *APC* gene-negative multiple adenomas is both an economic and clinical burden. However, it is crucial because the outcome influences patients' surveillance and intervention, as well as genetic consultation and clinical surveillance for at-risk family members. Additional genetic testing is expensive since the current national medical insurance, known in Israel as the "health basket," does not pay for other genetic tests that are needed for evaluation of multiple adenomas. These include full sequencing of the *APC* gene, *APC* analysis for deletions or duplications by MLPA, and analysis for mutations in the *MUTYH* gene and mismatch repair genes (for Lynch syndrome). The costs could reach several thousand dollars and still not be diagnostic. Table 1 presents the genetic differential diagnosis of *APC*-negative multiple adenomas.

CRC = colorectal cancer
MIS = microsatelite instabity
FAP = familial adenomatous polyposis
MLPA = multiplex ligation-dependent probe amplification

Figure 1. Schematic representation of the *APC* gene showing the relative large size of exon 16, the common mutation sites for attenuated familial adenomatous polyposis (AFAP) and site of the E1317Q polymorphism. The *APC* gene has 16 exons (exon 1 is not a coding exon). Exons 2–15 have 653 amino acids (aa) and exon 16 has 2,190 aa. Mutations contributing to classical FAP occur between exon 5 and the 3' portion of exon 16 (aa158–aa1580), whereas those associated with AFAP tend to cluster in the extreme 5' portion of the gene (aa1–aa157) and the 3' portion of exon 16 distal to codon 1900 (aa1901–aa2843) (not shown) [2,3].



MSI = microsatellite testing, IHC = immunohistochemistry for mismatch genes, MLPA = multiplex ligation-dependent probe amplification.

Table 1. Genetic and clinical differential diagnosis of APC-negative multiple adenomas

Etiology	Genetic syndrome	Inheritance	Responsible gene(s)
Incomplete *APC* gene sequencing	FAP or AFAP	Autosomal dominant	*APC* gene (5q21-22)
APC gene deletion or duplication	FAP or AFAP	Autosomal dominant	*APC* gene (5q21-22)
MUTYH polyposis	MAP	Autosomal recessive	*MUTYH* gene (1p32.1-34.3)
Undiagnosed Lynch syndrome	Lynch	Autosomal dominant	Mismatch repair genes: *MSH2* (2p21-22), *MLH1* (3p21.3), *MSH6* (2p16), *PMS2* (7p22)
Incorrect pathology diagnosis	Mixed or hyperplastic or juvenile polyposis, etc.	Autosomal dominant	*CRAC1* (15q13-14), *BMPR1A* (10q22.3), *SMAD4* (18q21.1)
Unknown	?	?	?

FAP = familial adenomatous polyposis, AFAP = attenuated familial adenomatous polyposis, MAP = multiple adenomatous polyposis

Patients with classical FAP develop hundreds of adenomatous polyps in the rectum and colon, usually starting in the teenage years. If not treated, most patients develop CRC by age 40 [4]. However, it is now recognized that some patients develop only a few (< 100) adenomatous polyps, and later in life; this is described as "attenuated" FAP. When untreated, CRC occurs a decade later than in FAP [4]. However, various studies have shown an *APC* gene mutation in only 5–15% of such cases [5].

In 2002, *MUTYH* gene-associated polyposis was described in patients with a clinical diagnosis of classic FAP or AFAP and no detectable *APC* gene mutations, but a recessive mode of inheritance [4,6-9]. MAP patients harbor biallelic mutations (mutations on both gene copies, one from each parent) in *MUTYH*, a base-excision repair gene (also referred to as the *MYH* gene) [10]. It has been reported that 1% of Caucasians carry one of the two most common *MUTYH* mutations – Y179C and G396D [4,10]. However, in many European and non-European countries there are also ethnic-specific mutations [4,11]. The clinical diagnosis is further confounded by the recent description of non-adenomatous polyps and extracolonic tumors occurring in patients with *MUTYH* mutation [12,13]. In addition, heterozygosity (having a single mutation in only one gene copy) is probably related to an increased risk for cancer [4,14,15].

Although multiple adenomas are not usually considered part of Lynch syndrome, they do appear in the precancer phase [16]. For this reason, in its differential diagnosis, FAP needs to be excluded [17,18]. Clinical criteria commonly known as the "Amsterdam" criteria or "Bethesda" criteria for laboratory investigation should raise the suspicion of Lynch syndrome and the diagnosis should be confirmed by genetic testing [1,18,19].

Because of the clinical importance for the patient and family to have a definitive genetic diagnosis, we performed a comprehensive genetic evaluation of a cohort of *APC* gene-negative multiple adenoma patients in order to propose guidelines for an efficient and least costly genetic evaluation.

PATIENTS AND METHODS

This clinical study was performed in a tertiary medical center and was approved by the local Institutional Review Board.

The study population comprised 29 Jewish individuals who did not have clinical "Amsterdam" criteria for Lynch syndrome and were referred for clinical and genetic evaluation. All participants had multiple adenomas, with or without CRC, and normal (but incomplete) *APC* gene sequencing. Their pathology was reviewed by a gastrointestinal pathologist and their cancer pedigree and personal history were reviewed for the possibility of unrecognized Lynch syndrome. The data obtained for each participant included their country of origin and that of both parents, family history of colorectal polyps and any cancer, mode of inheritance, number of colorectal adenomas, history of CRC and age at adenomas/CRC detection.

Regarding genetic analysis, after obtaining informed consent, DNA was extracted from peripheral blood. DNA was analyzed for: completion of *APC* gene exon 16 sequencing, *APC* gene analysis for deletions/duplications by the MLPA technique, and *MUTYH* gene sequencing. Microsatellite instability was evaluated in DNA extracted from the tumor specimens of patients with CRCs that met the revised Bethesda criteria for Lynch syndrome [1].

AFAP = attenuated FAP

MAP = *MUTYH*-associated polyposis

RESULTS

The study population comprised 29 patients, 52% males and 48% females, with a mean age of 53 years (range 19–76, median 58). Table 2 summarizes their demographic information, clinical data and genetic findings. Neoplasia inheritance was dominant in 7 patients, recessive in 14 and unknown in 8; 12 patients reported having CRC in a first-degree relative.

CRC had occurred in seven patients; six were in the left colon and six of them fulfilled the revised Bethesda criteria for Lynch syndrome [1,19]. Six patients, all with CRC, had 6–10 adenomas; 5 patients, one of them with CRC, had 11–19 adenomas; and 18 patients had ≥ 20 adenomas.

Table 2. Demographic and clinical data for patients, grouped by CRC and numbers of adenomas: 6–10, 11–19, ≥ 20

Patient no.	Gender	Age (yrs)	Descent	Family history	Inheritance	Consanguinity	Cancer/ site	No. of polyps	Polyp type	Genetic findings
1	F	46	Persia	Pancreas 2nd deg	Recessive	None	**Sigmoid**	7	Adenoma	None
2	F	40	Algeria	CRC 2nd deg; lung 1st deg	Recessive	None	**Rectum**	8	Adenoma+hp	*MUTYH* polymorphisms: IVS6+35A/G, IVS12-27C/T *MUTYH* variant: L417M
3	F	42	Turkey	CRC 2nd deg	Recessive	2nd cousins	**Ascending**	8	Adenoma	*MUTYH* polymorphism:Q335H ***MSI-H, MLH1* on IHC**
4	F	45	Algeria	CRC 1st deg	Recessive	Distant cousins	**Rectum**	10	Adenoma	None
5	M	76	Iraq	CRC 1st deg	Recessive	None	**Rectum**	10	Adenoma	None
6	F	43	Tunisia	Pancreas 2nd deg	Recessive	None	**Rectum**	10	Adenoma+hp	None
7	F	37	Poland/Romania	None	Unknown	None	**Rectum**	15	Adenoma	*MUTYH* polymorphism: Q335H
8	M	75	Poland	CRC 1st deg	Dominant	None	None	› 12	Adenoma	None
9	F	40	Iraq	CRC 1st deg	Dominant	None	None	15	Adenoma	None
10	M	61	Yemen	None	Unknown	None	None	15	Adenoma	None
11	F	52	Poland/ Czechoslovakia	Lung 1st deg	Recessive	None	None	› 17	Adenoma	*MUTYH* polymorphisms: Q335H, IVS6+35A/G
12	F	63	Morocco	CRC 1st deg	Recessive	2nd cousins	None	20	Adenoma	None
13	M	61	Poland/Romania	CRC 2nd deg	Unknown	None	None	› 20	Adenoma+hp	*MUTYH* polymorphism: Q335H
14	M	63	Syria	None	Unknown	None	None	› 20	Adenoma+hp	*MUTYH* polymorphisms: Q335H, IVS6+35A/G
15	F	67	Morocco	CRC 1st deg	Recessive	None	None	› 20	Adenoma	***MUTYH* mutations** G396D/Y179C
16	F	47	Egypt	None	Unknown	None	None	25	Adenoma	None
17	M	76	Russia	CRC 2nd deg	Recessive	None	None	30	adenoma	*MUTYH* polymorphisms: Q335H, IVS6+35A/G
18	M	64	Morocco	None	Unknown	None	None	› 30	adenoma	***MUTYH* mutation 1186-1187insGG;** *MUTYH* polymorphism: V22M
19	M	72	Russia	None	Recessive	None	None	› 35	adenoma+hp	None
20	M	58	Morocco	None	Recessive	None	None	› 40	adenoma	***MUTYH* mutations:** G396D/G396D
21	F	22	Romania	CRC 1st deg	Dominant	None	None	› 40	adenoma	*MUTYH* polymorphism: Q335H
22	M	60	Russia	CRC 1st deg	Dominant	None	None	› 40	adenoma	***APC* variant:** E1317Q
23	F	26	Morocco/Yemen	Breast, brain 2nd deg	Recessive	None	None	› 40	adenoma+hp	*MUTYH* polymorphism: V22M
24	M	40	Romania	None	Unknown	None	None	› 40	adenoma+hp	None
25	M	72	Uzbekistan	CRC, brain, stomach 1st deg	Dominant	2nd cousins	None	› 40	adenoma	None
26	M	62	Syria	CRC 1st deg	Recessive	None	None	› 40	adenoma+hp	*MUTYH* polymorphism: Q335H *MUTYH* variant: S512F
27	F	50	Poland/Ukraine	CRC 1st deg	Dominant	None	None	› 40	adenoma	None
28	M	19	Morocco	None	Unknown	None	None	› 40	adenoma+hp	None
29	M	60	Poland		Dominant	None	None	50	adenoma	*MUTYH* polymorphism: IVS4+56 G/A

CRC = colorectal cancer, hp = hyperplastic polyp, MSI-H = high microsatellite instability, IHC = immunohistochemistry
Bold signifies known mutations

Complete *APC* gene exon 16 sequencing revealed a polymorphism (E1317Q) in a single patient of European origin [5,20]. *APC* gene MLPA analysis was found to be normal in all cases. One patient, who was of Middle Eastern origin, of six with Bethesda criteria-positive CRCs was MSI-High and had immunohistology consistent with a mutated *MLH1* gene.

MUTYH analysis revealed pathogenic mutations in three patients. One patient was homozygous for the G396D mutation, another patient was a compound heterozygote for the G396D and Y179C mutations, and a third patient was found to carry a mutation causing a premature stop codon (1186–1187insGG). These patients were all of North African origin and had > 20 adenomatous polyps. Age at detection was between 58 and 67 years; one had an unknown mode of inheritance while two had recessive inheritance.

Two other patients of non-European origin had monoallelic *MUTYH* variants of unknown significance and a recessive mode of inheritance. One, with the S512F variant, had multiple adenomas; the other with the L417M variant had CRC. Both also had known *MUTYH* polymorphisms on the second *MUTYH* allele. Twelve patients had *MUTYH* polymorphisms; 5 of them had more than one polymorphism.

Overall, a genetic perturbation was found in 15 of the 29 patients. There appeared to be a greater likelihood of detecting a genetic abnormality in patients with ≥ 15 adenomas. These included mutations, variants and polymorphisms of uncertain clinical significance.

DISCUSSION

In this study we performed a complete molecular genetic evaluation of 29 Jewish individuals with < 100 colorectal adenomatous polyps with or without CRC who had had near-complete normal *APC* gene sequencing. The aim of the study was to assess the utility of this evaluation as measured by mutation detection and its actual cost.

Based on this small cohort, we found that completion of *APC* gene exon 16 sequencing further than 2500 base pairs from 5' only detected the weakly pathogenic polymorphism (E1317Q) in one patient. Its clinical significance for CRC is controversial, but it has been associated with risk for multiple adenomas in Jews, particularly Jews of non-European origin [5,20]. The performance of *APC* gene MLPA analysis for deletions/duplications did not detect any abnormalities. Our results are similar to other reports in the literature showing a low yield of *APC* gene mutations or deletions/duplications in such cases [21].

However, in our study we identified three patients carrying pathogenic *MUTYH* mutations, two biallelic and one monoallelic, leading to a premature stop codon. Furthermore, two other patients were found to harbor *MUTYH* variants of unknown clinical significance, possibly representing novel mismatch mutations. These findings suggest that *MUTYH* analysis should probably be the first step in the genetic evaluation of patients with *APC* gene-negative multiple adenomas. Since two mutations (G396D and Y179C) are responsible for as much as 85% of *MUTYH* mutations in Caucasians [4,9,13,15], it is reasonable to begin the molecular analysis with their assessment, and to perform complete *MUTYH* sequencing only if neither of these mutations is detected or if the patient is a heterozygous carrier of only one mutation. Even though the clinical syndrome of MAP is established for biallelic *MUTYH* carriers, there is some clinical evidence for increased CRC risk even in heterozygous carriers [4,14,15].

Interestingly, although our cohort was relatively small, the three patients carrying pathogenic mutations in the *MUTYH* gene were all Jews of Moroccan origin. The two patients carrying monoallelic *MUTYH* variants were also of non-European origin. These findings might have clinical implications, especially for the recommended molecular evaluation of non-European Jewish patients with multiple colorectal adenomas. The prevalence of *MUTYH* mutations in Israel and the possibility of ethnic-specific mutations are currently being researched.

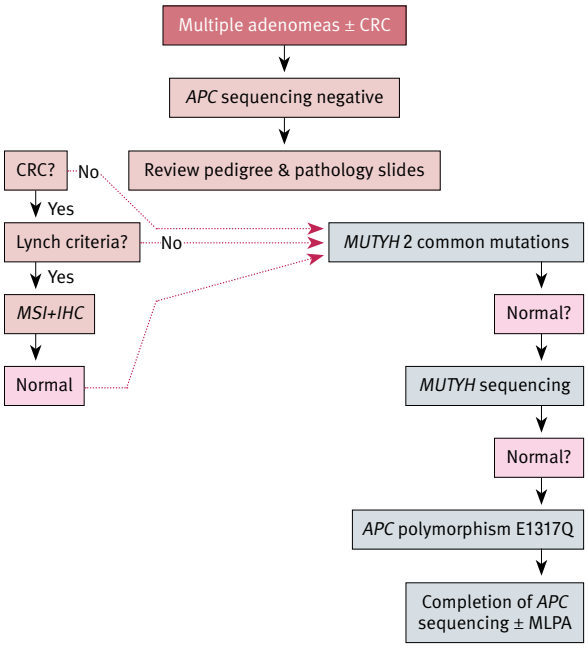
If CRC is associated with a cancer pedigree compatible with Lynch syndrome (Amsterdam or Bethesda criteria) [1], it is recommended that microsatellite instability be tested for and/or immunohistochemical staining be performed for the four mismatch repair gene products (*MSH2, MLH1, MSH6* and *PMS2*).

Multiple colorectal adenomatous polyps appear to include a heterogeneous group of disorders comprised, in part, by various genetic syndromes. As the genetic evaluation is expensive it should follow an established protocol. Our suggested clinical approach for the genetic evaluation of multiple colorectal adenomas is summarized in Figure 2. The CRC, polyp and colonic pathology slides should be reviewed by an expert pathologist to look for specific histologic findings [22]. This means (excluding the diagnosis of non-adenomatous polyposis, and presence of intramucosal adenomas in FAP) identifying histologic features occurring in Lynch syndrome tumors (e.g., intraepithelial lymphocytes, Crohn's-like reaction, and mucinous medullary pattern).

In our experience, a crude calculation (based on costs quoted by Israeli laboratories) shows that complete genetic evaluation for one patient might cost as much as 9500 NIS (4 NIS = 1$): *APC* gene sequencing (exons 1–15 plus 5' exon 16) approximately 4000 NIS, two *MUTYH* mutations approx 700 NIS, *MUTYH* sequencing approx 1300 NIS, *APC* gene MLPA approx 500 NIS, and MSI+IHC approx 3000 NIS), emphasizing the need for a prior genetic consultation by an oncogenetics counselor.

IHC = immunohistochemical staining

Figure 2. Suggested clinical approach for the genetic evaluation of patients with multiple colorectal adenomas but no *APC* gene mutation, after first reviewing the pathology and cancer pedigree.



The strength of this study is the detailed clinical and genetic characterization of our cohort. A limitation is the small size of the study population, which is due to the expenses involved, but adequate for the conclusions given above.

In summary, we studied a cohort of *APC* gene mutation-negative patients with multiple colorectal adenomas, with or without CRC, and found that after excluding Lynch syndrome by clinical-pathological criteria, testing for *MUTYH* gene mutations was the most rewarding investigation, especially for patients of non-European origin. Examining *APC* gene exon 16 for the E1317Q variant and completing its sequencing and *APC* MLPA was relatively unrewarding, as is MSI in most such cases not fulfilling Lynch syndrome criteria.

Note added in Press:

A recent publication showed that *APC* allelic imbalance can cause FAP in a small number of *APC* and *MUTYH* mutation-negative patients and should be examined; see Castellsague E et al., *Gastroenterology* 2010; 129: 439-47.

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