

PREVALENCE OF APC 1B PROMOTER DELETION IN A
COHORT OF MUTATION – NEGATIVE
FAMILIAL ADENOMATOUS
POLYPOSIS PATIENTS

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In Partial Fulfillment
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of Master of Science in Genetic Counseling

By
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CERTIFICATION OF APPROVAL

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DEDICATION

I would like to dedicate the time and effort I put into this research to my amazing family and friends who have provided me with endless support during the past two years of my graduate career. Making you proud was one of the driving forces in everything I have been able to accomplish.

ACKNOWLEDGEMENTS

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ABSTRACT

Familial adenomatous polyposis (FAP), estimated to occur in 1 in 8,000 to 1 in 10,000 individuals, is caused by mutations in the APC gene. Advances in technology have allowed for more mutations to be detected, providing a genetic etiology for the clinical diagnosis of hundreds to thousands of precancerous colon polyps. Traditionally, the use of sequencing had sufficed in detection of disease-causing mutations. However, it is now recognized that large rearrangements (deletions or duplications) involving the APC gene explain a subset of mutation-negative cases that are not easily detected by sequencing alone. Several recent studies have described families in which deletions involving the APC 1B promoter, resulting in a classic presentation of FAP, were detected using multiplex ligation-dependent probe amplification (MLPA) analysis. This study aimed to re-test mutation-negative individuals with a clinical diagnosis of FAP in hope of detecting and further characterizing deletions in the 1B promoter of the APC gene. Although no 1B promoter deletions were detected through this study, two previously undetected deletions in the APC gene were discovered. These two deletions highlight the value of re-referral for complete genetic testing to detect all possible disease-causing mutations. The results of this study support the need to retest mutation-negative individuals using new, advanced technologies to reveal previously missed genetic etiologies for polyposis.

INTRODUCTION

Background

The adenomatous polyposis coli (APC) gene has been extensively studied because both inherited and de novo mutations in the gene are known to cause familial adenomatous polyposis (FAP). The classic form of the condition is characterized by the occurrence of hundreds to thousands of precancerous, adenomatous polyps in the colon and rectum, which may occur in childhood, but usually appear in the second decade of life (Bisgaard et al. 1994). The incidence of classic FAP in the general population is approximately 1 in 8,000 to 1 in 10,000 live births (Bisgaard et al. 1994). Most cases of FAP coincide with a family history of colorectal cancer at younger ages due to the autosomal dominant inheritance of the condition. Therefore, anyone who carries a mutation in the APC gene has a 50% chance of passing it on to each of his or her children. The condition may also be the result of a de novo mutation in approximately 20-25% of cases (Bisgaard et al. 1994). Without proper treatment, nearly 100% progression to colorectal cancer occurs by age 35 to 40 years (Munck et al. 2011). Early detection of polyposis consisting of the hallmark pan-colonic carpeting of pre-cancerous, adenomatous polyps through colonoscopy allows for increased screening and/or surgical intervention before cancer forms. Total colectomy with ileorectal anastomosis or proctocolectomy with ileal-pouch-anal anastomosis are the recommended surgical procedures to reduce the risk for colorectal cancer in FAP individuals. However, residual risks may remain for any

rectal tissue not removed and there have been a few reported cases of cancer developing in the ileal pouch.

The APC gene is a tumor suppressor gene made up of 21 exons spanning a large region of chromosome 5q21 (Grodin et al. 1991). Over 1500 mutations in the APC gene have currently been identified (Kadiyska et al. 2013). The position of many of these mutations within the gene has been correlated with an expected range of presenting symptoms; including polyp burden and presence of extracolonic features (Fearnhead et al. 2001). However, family members with the exact same mutation may present with a different phenotype. The presence of less than 100 adenomatous polyps developing in the fourth and fifth decades of life accompanied by an increased risk for colon cancer has been characterized as attenuated FAP (AFAP) (Spirio et al. 1993). Generally, mutations occurring in the middle of the APC gene result in a more severe or classic FAP phenotype, while mutations occurring at either the 5' or 3' end of the gene result in an AFAP phenotype. Extracolonic features of FAP include: congenital hypertrophy of the retinal pigmented epithelium (CHRPE), osteomas, desmoid tumors, adenomatous polyps and cancer of the duodenum, epidermoid skin cysts, hepatoblastoma, fundic gland polyps, gastric cancer, dental anomalies, pancreatic cancer, and brain tumors (Wallis et al. 1999). Advances in genetic testing have allowed for early screening and prevention following detection of APC mutations in patients with adenomatous polyps and/or a known family history of FAP.

It has been previously understood that the vast majority of causative APC gene mutations are nonsense and frameshift mutations, resulting in a truncated protein product and abnormal function (Charames et al. 2008). Therefore, genetic testing for individuals with a clinical diagnosis was originally carried out with protein truncation testing. It became clear very early on that this technique had low sensitivity, and even in cases where a truncated protein was discovered, the specific genetic sequence change leading to the truncated protein remained elusive. Techniques have since evolved to include complete gene sequencing. Truncating germline mutations in the APC gene are detected in up to 90% of individuals with a classic presentation of FAP (Hegde et al. 2014). This leaves approximately 10% of families with a mutation-negative test result following sequence analysis.

A significant number of these mutation negative families are explained by large (exonic or whole-gene) deletions/duplications not readily detectable by sequence analysis. New research shows that deletions of the APC promoter region may also explain a proportion of mutation-negative families. Two promoters control expression of the gene. Promoter 1A has been historically known as the main promoter in which impaired protein expression has been linked to 18% of colorectal cancer and adenomatous tumors (Esteller et al. 2000). More recently, studies have focused on the implications of impaired protein expression linked to the 1B promoter. Protein expression from the 1B promoter has been found to be higher than that of the 1A promoter and germline inactivation of the 1B promoter has been deemed as disease-causing (Rohlin et al. 2011). One such study of a Manitoba Mennonite

kindred found a large novel germline deletion in the 1B promoter using linkage analysis and multiplex ligation-dependent probe amplification (MLPA) (Charames et al. 2008). Promoter deletions are thought to cause silencing of the tumor suppressor gene much like hypermethylation of the APC promoter. Decreased expression of APC may therefore also cause clinical symptoms of FAP. A second study reinforced this hypothesis by once again finding a deletion encompassing more than half of the 1B promoter in members of 54 families from the Swedish Polyposis Registry (Rohlin et al. 2011). Each of the families also showed decreased protein function as a result of the promoter deletion. A Bulgarian family study looking to improve upon the detection rate for mutations in the APC gene found deletions that encompassed the entire 1B promoter region. A three-generation family history consisting of three males clinically diagnosed with FAP, among unaffected family members, shared a novel deletion of the entire 1B promoter, which resulted in significantly reduced protein production by the tumor suppressor gene (Kadiyska et al. 2013). The most recent findings of the effect of a 1B promoter deletion on polyposis identified seven probands from seven families, all with a clinical diagnoses of FAP, who were also found to be identical by descent from a common [American] founder (Snow et al. 2014).

These reports in the literature have resulted in the recommendation that large deletion/duplication testing should also include analysis of the regulatory regions (specifically promoter 1B) of APC. In addition, individuals suspicious for an APC-associated polyposis condition with no deleterious APC mutation found, should be

evaluated for promoter 1B deletions if these mutations were not analyzed in the initial testing (Rohlin et al. 2011). Most mainstream testing laboratories are now including MLPA in the testing strategy for individuals with a clinical diagnosis of FAP. The next important step for providers will be to re-contact APC mutation-negative individuals with a clinical diagnosis to offer them the most up to date testing available.

Research Purpose

The purpose of this study was to determine the prevalence of deletions in the 1B promoter region of the APC gene in patients who were previously classified as mutation-negative in the University of California San Francisco (UCSF) polyposis registry. Genotype-phenotype correlations in those patients and families identified with a 1B promoter deletion were also assessed, as reports in the literature are conflicting. These goals are important for patient care since knowledge of a mutation associated with FAP in the family may lead to earlier identification of polyposis before polyps have had a chance to progress to a malignancy. Benefits of detecting a mutation may also relieve patients of the burden of uncertainty about the genetic cause of their condition, as well as providing information for future research into treatment options. As pointed out by Roy and Khandekar (2012), once the mutation has been identified in the proband, this information enables tailoring/personalizing cancer preventative strategies for other asymptomatic family members. Predictive testing of at-risk family members will allow for either the addition of increased

screening, prophylactic surgery, or in the case of a true negative, returning an individual's screening regimen to population level. The results of this study will add to the growing body of information about 1B promoter deletions and help promote the detection of family members with an increased risk of developing polyposis and/or colorectal cancer.

MATERIALS AND METHODS

Sampling

The UCSF Gastrointestinal Cancer Prevention Program maintains a registry of individuals who have undergone genetic testing for FAP either as a result of a clinical diagnosis or known family history of polyposis. As part of the UCSF consent process, every individual that is offered genetic testing is also offered the option to participate in a long-term follow up study and research program. One option included in the program is to donate a blood sample to be stored at the UCSF tissue core at Mt. Zion Hospital for future research. This allows the sample to be used in studies analyzing the utility of new genetic testing methods and for characterization of genetic conditions. Collecting samples from individuals with rare genetic disorders is especially important for the improvement of detection and classification methods.

The individuals in the UCSF polyposis registry were sorted according to the inclusion criteria determined for this study. Both electronic and paper medical records for patients were reviewed to ensure eligibility. Each individual had to meet the following inclusion criteria to be considered for the study:

- Clinical diagnosis of classical or attenuated FAP
- Colonoscopy screening with pathological confirmation of numerous adenomatous polyps
- Previous genetic testing with negative results for mutations in the APC gene in the presence of a clinical diagnosis

- Genetic testing for APC mutations, at either Ambry Genetics prior to September of 2011 or at Myriad Genetics prior to June of 2012, neither of which included MLPA testing

The initial cohort meeting the inclusion criteria totaled 46 individuals.

Samples without enough volume stored for successful DNA extraction were excluded from the study. Subjects with polyposis of a non-adenomatous or mixed type were also excluded. Finally, any subjects who already received MLPA testing through a CLIA approved lab that included the APC 1B promoter region were excluded. These exclusion criteria narrowed the list to 42 patient samples. However, the tissue core was unable to locate three banked samples, yielding a final cohort of 39 individuals.

Research approval for this study already existed through a previous UCSF IRB protocol that allowed for all future studies of this type to be conducted through the GI Cancer Prevention Program, including the use of patient medical records and stored samples. Patient consent to participate in the study was again verified through confirmation of the signed and dated form in their medical record. This study was also granted IRB approval through California State University Stanislaus in September of 2014 (Protocol #1415-011). Finalization of the study cohort coincided with notification of IRB approval. There were no foreseen risks to any patients involved in this study. All samples were previously de-identified in the storage system and can only be identified using an excel file key system under restricted access on the UCSF database server. Results were matched to the patient under strict confidentiality at the end of the study. Patients will be contacted with any new

information corresponding to their test results. Likewise, medical records were viewed for inclusion data and demographic information without an increased risk of breaching patient confidentiality.

The patients, who met the aforementioned inclusion criteria, were selected to have their stored buffy coat (white blood cells and neutrophils, which contain DNA) samples analyzed using MLPA. The samples had been in storage anywhere from within the current year to approximately 10 years. Samples were collected from the UCSF tissue core at Mt. Zion and shipped directly to the Huntington Medical Research Institute (HMRI) overnight on dry ice. Huntington Medical Research Institute is a CLIA certified, clinical genetics diagnostic lab of which Dr. Faye Eggerding is the clinical director. Once Dr. Eggerding received the samples, she extracted genomic DNA from thawed buffy coat and performed the MLPA as described below.

Extraction of DNA from Buffy Coat Archival Samples

Buffy coat samples were frozen for storage by lab technicians at the UCSF tissue core in 5% DMSO (dimethyl sulfoxide). Two samples were frozen without DMSO (FAP446CRE52 and FAP411MCS63). The variation in storage buffer depended on when the sample was stored ranging from less than one year ago to ten years ago. Samples were rapidly thawed, washed with phosphate buffered saline (PBS), and spun down for 5 minutes at 10,600 x G in an Eppendorf 5417C centrifuge. The supernatant was carefully removed so as not to disturb the small cell pellet at the

bottom of each tube. Samples were resuspended in 300 μ l of TE buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA) containing 0.5% SDS (sodium dodecyl sulfate) and 20 μ l of Proteinase K (Roche cat no. 03 115 844 001). Samples were then incubated at 55oC for at least several hours. DNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated by addition of sodium acetate to 0.3M and 2.5x volume of 100% ethanol. DNA precipitates were recovered by centrifugation at 14,000 rpm in a 5417C Eppendorf centrifuge, washed with 70% ethanol, and resuspended in TE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA).

DNA yield was determined by measuring absorbance at 260 nm (A_{260} of 1= 50 μ g/ml dsDNA). DNA purity was determined by the ratio of absorbance at 260 nm divided by that at 280 nm ($A_{260}/280$ should be between 1.7-2.0). Most samples had a DNA concentration between 300 to 900 ng/ μ l and a ratio of 1.8 to 1.9. These concentrations were within the acceptable range for successful analysis using MLPA techniques.

Detection of APC Gene Deletion or Duplication Rearrangements

Genomic DNA extracted from buffy coat samples was analyzed for large gene rearrangements (deletions/duplications) using the multiplex ligation probe amplification (MLPA) procedure described by the manufacturer (MRC Holland, P043-D1, Lot#D1-0513; MRC Holland MLPA reagents, EK5-FAM). Briefly, DNA samples (100 μ g and 150 μ g each) in 5 μ l of TE (10 mM Tris-HCl pH 8.2, 0.1 mM EDTA) were denatured for 5 minutes at 98oC. Samples were cooled to 25oC, 3 μ l of

hybridization buffer containing the APC probe mix was added to each sample and they were denatured for 1 minute at 95oC. They were then incubated for 18 hours at 60oC in an Eppendorf MasterCycler Pro. Following hybridization, the temperature was lowered to 54oC and 32 ul of ligase mix containing ligase-65 enzyme was added to each sample. Ligation was carried out at 54oC for 20 minutes followed by 5 minutes at 98oC to inactive the ligase enzyme. The ligation reaction was placed at room temperature and prepared for the PCR step. To each ligation reaction, 10 ul of polymerase mix containing FAM labeled PCR primers and SALSA DNA polymerase was added. PCR was carried out in an Eppendorf MasterCycler Pro with 35 cycles of 30 seconds at 95oC, 30 seconds at 60oC, 60 seconds at 72oC, and ending with 20-minute incubation at 72oC. FAM labeled PCR products were analyzed on an Applied Biosystems DNA sequencer using Genescan software.

Principles of MLPA and Analysis of MLPA Results

MLPA is a quantitative method based on probe hybridization and ligation followed by PCR amplification of the ligation products. MLPA can be used to determine the copy number of up to 50 or more DNA sequences in one reaction. Sample DNA is denatured and DNA sequences of interest (for example, gene exons or promoter regions) are interrogated by hybridization to two oligonucleotide probes that hybridize in juxtaposition (3' end of upstream probe is immediately adjacent to the 5' end of the downstream oligonucleotide). DNA ligase covalently joins the two juxtaposed oligonucleotides only if the nucleotides at their junction are perfectly

base-paired. Once ligated the probes can be amplified by PCR using a single primer pair. Each probe produces a PCR product with a unique length. The PCR amplicons are separated and quantified by electrophoresis.

The APC probe mix P043-D1 contains 43 different MLPA probes whose PCR products range in size from 130 to 472 base pairs. The probe mix contains 29 probes for the APC gene, 3 probes for the MUTYH gene and 11 reference probes. Each APC exon is represented by at least one probe and two probes detect the APC alternative promoter 1B in exon 1.

For MLPA data analysis, comparison of sample results with reference DNA samples analyzed at the same time is required. Each run contains normal control samples for the APC gene, a positive APC control (patients with a promoter 1B deletion), a no DNA control, and a no ligase control. Within sample normalization is done by dividing the peak area of each amplicon by the signal of all probes in the sample. To detect gene dosage differences, samples are normalized to the mean obtained with two control samples and results are calculated as allele copy number as compared to normal controls. A ratio of 1.0 is normal, a ratio of 0.5 indicates a heterozygous deletion, and a ratio of 1.5 indicates heterozygous duplication.

The UCSF polyposis registry currently houses a number of both positive and negative test results for individuals in the same families. The documentation of these individuals allowed for both a positive and a negative control sample to be sent along with the cohort samples for MLPA. The positive control sample for the cohort was obtained from an individual known to have a clinical diagnosis of FAP with a

clinically detected deletion of exons 14-15. This 29-year-old Hispanic woman was given a clinical diagnosis of FAP when she was 24. She underwent colectomy at age 25 and later developed papillary thyroid cancer. The pathology of papillary thyroid cancer has been associated with FAP in several studies. A male relative of this woman was used as a negative control for the study. The male individual had no history of symptoms related to polyposis. He had several normal screening colonoscopies, which he began receiving at an earlier age than general population recommendations as a result of his family history of colon cancer and polyposis. The patterns of the negative results for the remaining samples in this study were matched to the negative control to assure quality control and interpretation of results.

Data Analysis

Analysis of MLPA results began by creating two patient categories within the polyposis registry pertaining to the presence or absence of a known APC mutation. This process included results from this study as well as results from previous testing done in the clinic. Results showing the presence of a mutation were broken down into subcategories of whole or partial gene deletion/duplication, nonsense or frameshift mutation (resulting in protein truncation). Missense mutations may or may not affect protein production and function. The type of mutation was important information when analyzing genotype-phenotype relationships. Only results showing a partial or whole gene deletion or duplication were investigated for phenotypic symptoms. Information was then gathered from patient files for which a known

deletion or duplication was detected from previous studies in the registry. Categories included gender, age of onset of polyposis, severity of polyposis, pre- or post-status colectomy, presence of extra colonic features, and ethnicity.

Phenotypic characteristics presented by individuals harboring a partial gene deletion, of one to several exons, were compared to the phenotype represented by the one individual with a deletion of one entire APC allele and to the phenotype of the one individual known to have an APC 1B promoter deletion. Each of these genotypic and phenotypic results was then compared to the current knowledge found in the literature. Descriptive statistical analyses were performed on the demographic and phenotypic information for individuals with known APC deletions or duplications in the UCSF polyposis registry.

RESULTS

Interpretation of MLPA Results

Dr. Faye Eggerding, who has done countless clinical research on the APC mutations known to cause FAP, performed the MLPA assays and interpreted results. She reported that although high concentrations of gDNA were obtained, there were regions of variability seen in the MLPA results for all samples. These regions of variation were not present in her control samples. However, the same variation was found even in the positive control sent from the UCSF Tissue Core. Her interpretation of the variation is as follows.

The cause of the peak variability affecting both the APC probes and the control probes is not completely clear. Incomplete denaturation of sample DNA or inadequate DNA sample can produce false positive deletion results and variable peaks. The MLPA probe mix contains specific DNA denaturation control and DNA quality control fragments that indicate if sample DNA is sufficient and adequately denatured. In all cases these controls indicated the samples had been adequately denatured and that the quantity of sample DNA was appropriate.

The MLPA reaction is robust and reproducible; however, the tissue of origin, storage of the sample and method of DNA extraction can influence the MLPA peak pattern. The DNA extracted from the buffy coat samples was of high concentration and good quality (almost all samples had a 260/280 ratio of 1.9). Fragmentation of the DNA perhaps due to prolonged storage or multiple freeze thaw cycles should not

influence the relative MLPA peak signals. Sample DNA fragmentation should not significantly influence the relative probe signals (peak area of each probe amplification product divided by the peak area of all probe amplification products in the sample) because the target sequence of the MLPA probes is less than 100bp.

Impurities or contaminants in the DNA sample may affect the MLPA reaction, as the MLPA reaction may be more sensitive to certain impurities than a standard PCR reaction. Impurities in the DNA samples may decrease the activity of the polymerase during PCR and result in peak variation. Different MLPA probes can react differently to decreased polymerase activity; some probes are not affected while others may show a reduction in peak signal or an increase in relative peak signal. Reducing the amount of DNA sample used in the MLPA reaction and thereby diluting the contaminants may be helpful. Avoiding multiple freeze thaw cycles or prolonged storage may help in this situation.

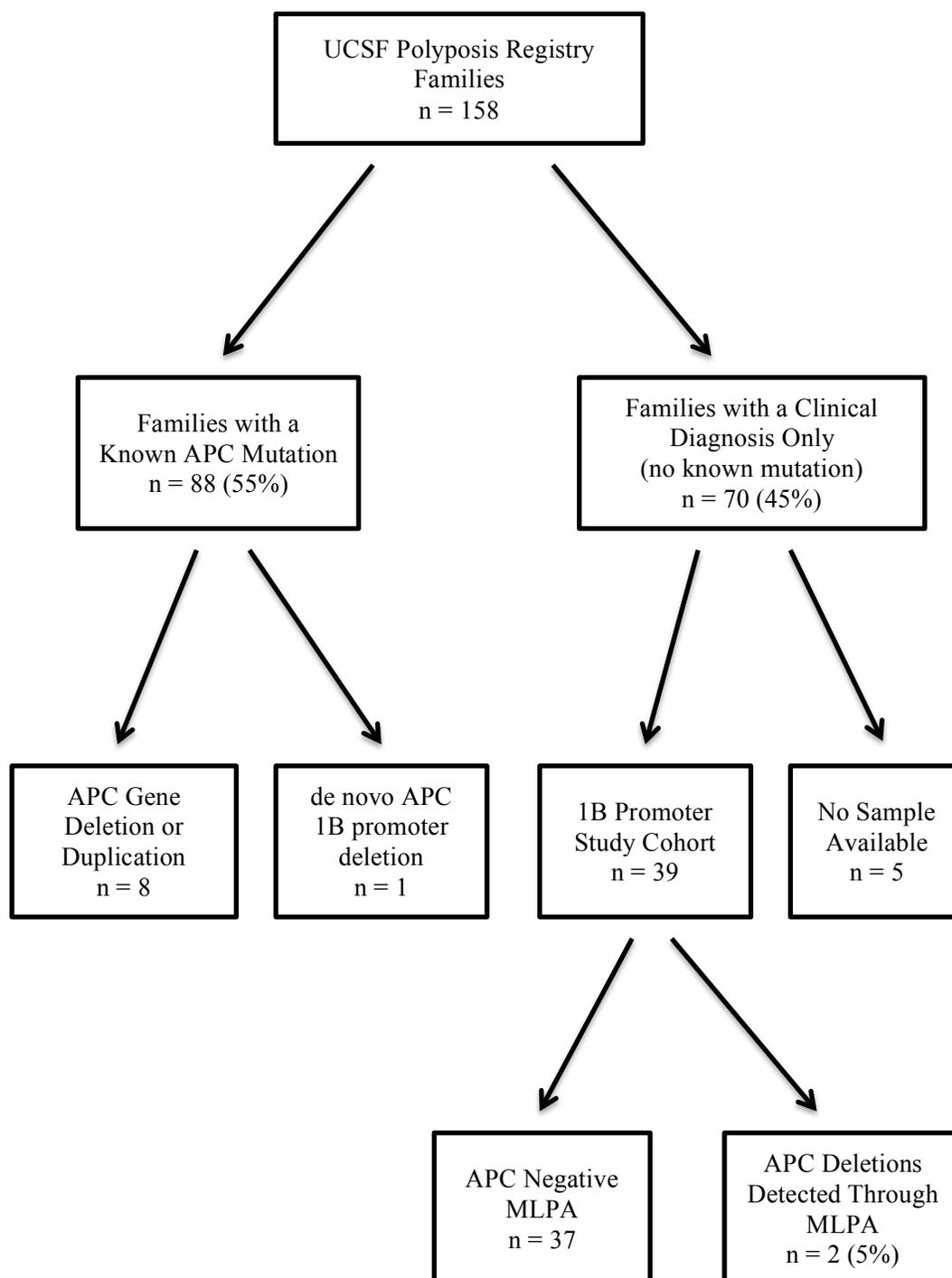
Detected Mutations

A total of 158 probands within the UCSF polyposis registry have been clinically diagnosed with colonic polyposis. However, only about half have received a positive genetic test result with a mutation in the APC gene, confirming the clinical diagnosis. Family members of these individuals were then able to receive single site testing for a known familial mutation. To date, most of the individuals within the cohort have undergone genetic testing in an attempt to further explain their clinical diagnosis. Results of previous testing may be inconclusive or incomplete depending

on the year and at which lab the testing was performed. Some individuals have yet to undergo full, clinical genetic testing and received the incomplete results through research testing. It is therefore important that these individuals be re-contacted to give them the option of receiving the most up to date testing available. There were originally 70 probands listed in the registry without a known APC mutation. Preliminary elimination narrowed the list down to 46 probands who were considered for this study and a subset of 39 individuals was chosen according to the aforementioned inclusion and exclusion criteria.

The flowchart in **Figure 1** depicts the breakdown of individuals within the UCSF polyposis registry. The right side of the flowchart represents how the cohort for this study was selected from the registry. The registry flowchart provides an example of how increasing knowledge and technological advances have enhanced the ability to detect mutations. Prior to this study, 88 of the 158 (55%) patients in the UCSF polyposis registry were informed that a mutation was found in their APC gene that explains the clinically diagnosed polyposis. The majority were base pair insertions or deletions that resulted in a frameshift, yielding a stop codon and therefore, truncated proteins. Five of the 88 individuals were found to have bi-allelic mutations in MUTYH detected that explained their attenuated-like phenotypic presentation of polyposis. Two individuals had a variant of uncertain significance (VUS) detected in the APC gene. These variants have yet to be classified as either benign or pathogenic. The relevant genetic testing laboratories will track these variants to update the literature and reports for individuals harboring such variants.

Eight individuals were found to have partial APC gene deletions, one individual was found to have a duplication, and one individual was recently found to have a deletion in the 1B promoter of the APC gene. The remaining 70 individuals either had a base pair insertion or deletion resulting in a nonsense or frameshift mutation.

Figure 1. UCSF polyposis registry flowchart

Demographics

The demographic, genotypic, and, phenotypic information for probands within the polyposis registry with an APC deletion or duplication detected prior to this study, as well as for the two individuals found to have deletions in this study, is listed in **Table 1**. Nine individuals were found to have an APC deletion and one individual was reported as having an APC duplication. The samples from two individuals, who had previously received mutation – negative genetic test results, were found to have APC deletions through the use of MLPA in this study. One had a deletion of exon 4 and the other had a deletion of the entire APC gene. These cases are a prime example of a now, well known, sub-group of individuals who required advanced genetic testing technology to detect the APC deletions responsible for their presentation of FAP.

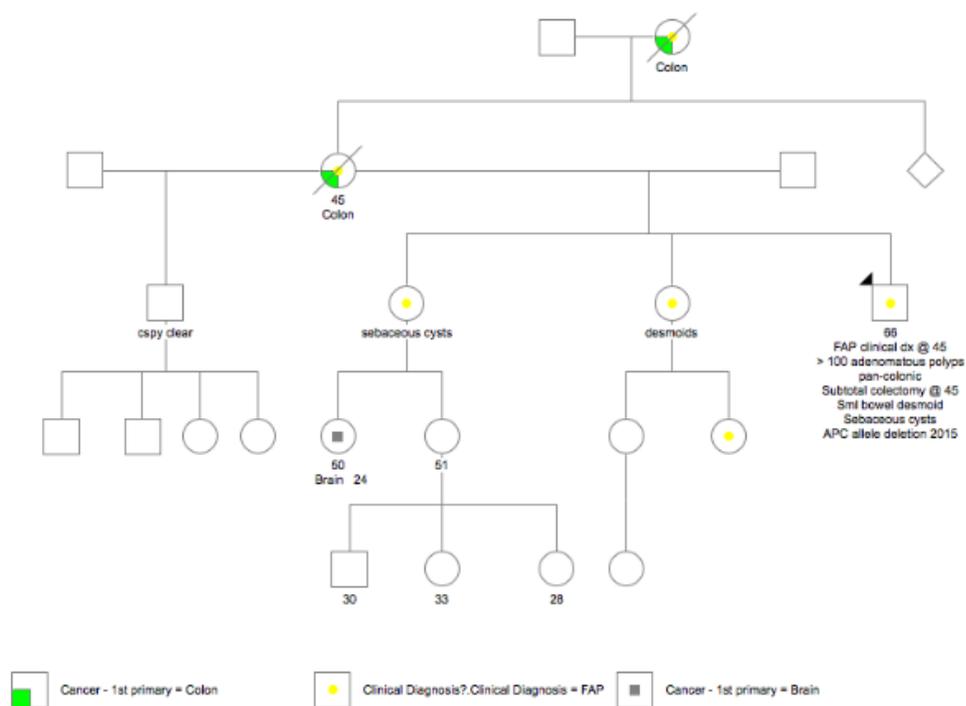
Table 1. Testing results and disease characteristics of all individuals in the UCSF polyposis registry with large rearrangements in APC
 *All individuals had pan-colonic polyposis. Sanger sequencing (Seq), Southern Blot (SB), Multiplex ligation-dependent probe amplification (MLPA), Next Generation (targeted) Sequencing (NextGenSeq)

Testing	Age of Diagnosis	Gender	APC Mutation	Ethnicity	Age of Colectomy	Other Features
Myriad Seq/SB	28	M	Deletion exon 15	Italian, Native American	32	Fundic gland & duodenal polyps
Myriad Seq	31	F	Deletion exons 4-5	Caucasian	N/A	Fundic gland & duodenal polyps
Myriad	12	F	Deletion exons 11-13	Hispanic	N/A	Fundic gland polyps
Myriad Seq/MLPA	36	F	Deletion of exon 4	Irish, Russian, Italian, Ashkenazi Jewish	36	Fundic gland & duodenal polyps
MLPA + APC 1B Promoter	45	M	Whole gene deletion	Portuguese	45	Small bowel desmoid tumor
MLPA + APC 1B Promoter	41	M	Deletion of exon 4	Hispanic (Mexico)	N/A	None
NextGen Seq	19	F	5'UTR deletion (1B Promoter)	Caucasian	19	None
Myriad Seq/SB	41	M	Duplication of exons 1-2	Hungarian, Russian	41	None
Myriad Seq/SB	24	F	Deletion of exons 14-15	Hispanic	25	Papillary thyroid cancer
NextGen Seq	23	F	Deletion of exons 11-13	Hispanic	No colectomy due to advanced disease state	Fundic gland polyps; jaw osteoma

One of the two individuals with a deletion found through this study is a 66-year-old man of Portuguese ancestry who was given a clinical diagnosis of FAP at the age of 45. He subsequently underwent subtotal colectomy and later developed a small bowel desmoid tumor. He was originally tested for mutations that were truncating in nature through a research study in 2000. As with most of the results of research studies, before clinical genetic testing was available, his results were inconclusive. He was found to have a deletion of an entire APC gene/allele through the use of MLPA in this study. This deletion now provides a clear, genetic etiology for his clinical diagnosis of polyposis.

The family history of this individual looks like that of a family with classic FAP being passed on in an autosomal dominant pattern (**Figure 2**). His paternal grandfather had colon cancer at an unknown age and polyposis. His father had early-onset colon cancer diagnosed at age 45 and polyposis. Lastly, both of his sisters and one niece also had polyposis and another niece had brain cancer at age 24. Each of these relatives may now receive more accurate genetic testing because of the APC deletion found through the use of newer technology. Family members without polyposis may also benefit from testing to rule out the colorectal cancer predisposition and return to population screening.

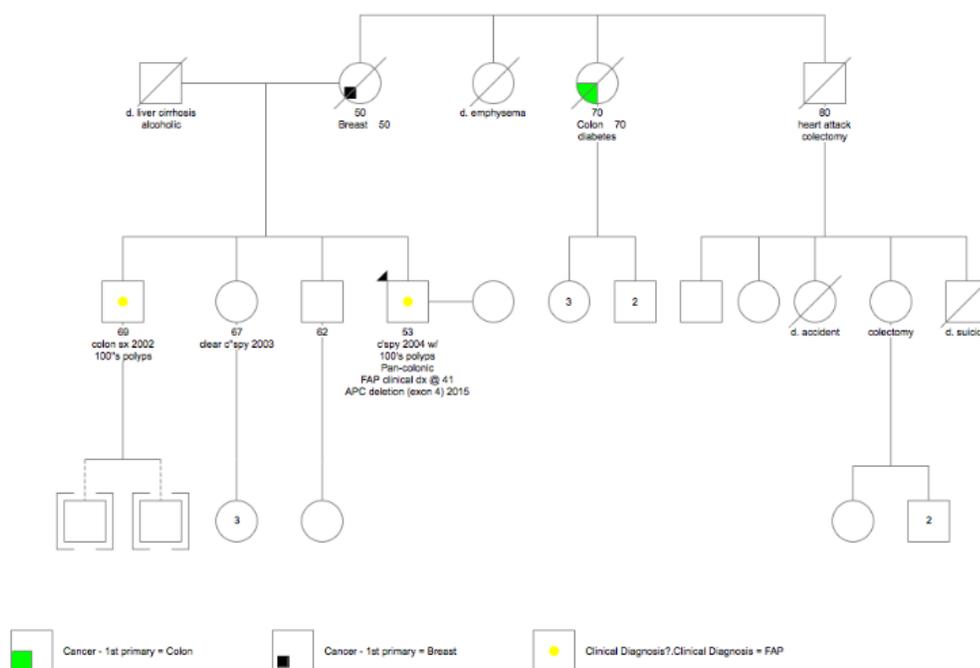
Figure 2. Family history of the individual with an APC whole gene deletion found through MLPA



The second individual is a 52-year-old Hispanic man who presented with polyposis at the age of 41 with no other symptoms of FAP. He was originally tested through Myriad Genetics in 2004 using Southern Blot, which was unable to detect a mutation in the APC gene. He was found to have a deletion of exon 4 through the use of MLPA in this study, which provides a genetic etiology for his clinical diagnosis.

The family history for this individual is less typical of FAP in that the only report of colon cancer is in the maternal aunt and occurred at a later age (**Figure 3**). His mother had breast cancer at a younger age of 50, but no reports of colon polyps. It is unclear where this APC mutation may have come from, but the proband's oldest brother also having had polyposis suggests an autosomal dominant inheritance pattern. His sister and second oldest brother follow increased screening guidelines with clear colonoscopies to date. Although the closely related family members have been recommended to undergo screening at younger ages, having a known familial mutation will now allow unaffected, negative family members to test and possibly reduce screening.

Figure 3. Family history of the individual with an APC exon 4 deletion found through MLPA

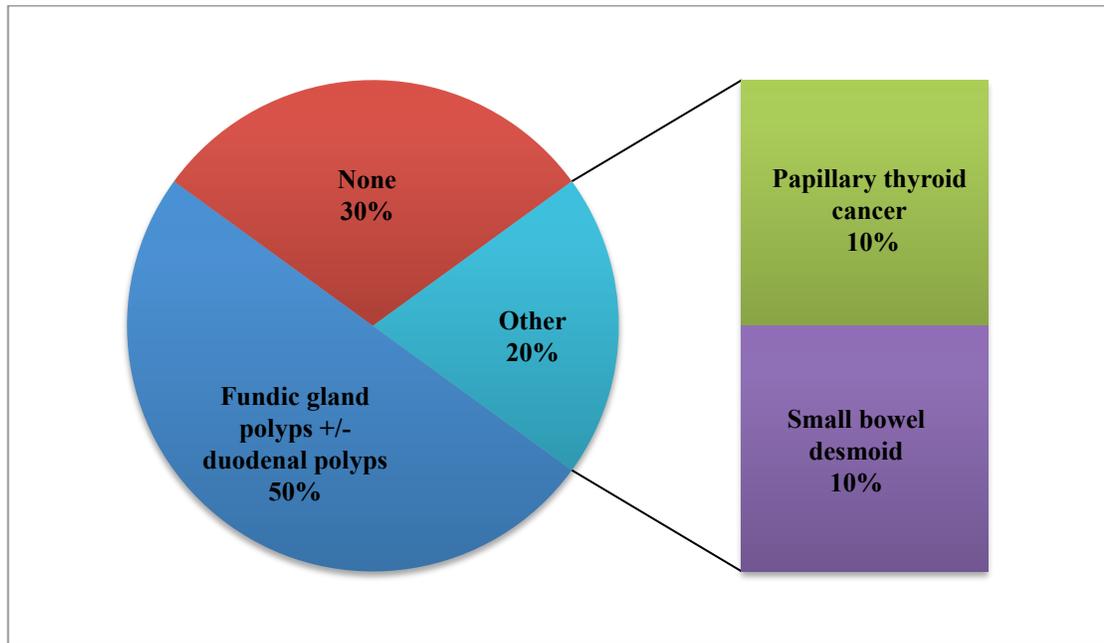


Genotype/Phenotype

The two individuals re-tested through this study, whom were found to carry previously undetected APC deletions, showed varying phenotypic presentations in comparison to the rest of the individuals with APC mutations in the polyposis registry in that they were slightly older than most. One individual had a deletion of APC exon 4, while the other had a deletion of an entire copy of the APC gene. However, neither of the reported phenotypes was outside the realm of those typically classified as being phenotypic descriptions of FAP.

All individuals considered for this study were tested within the UCSF polyposis registry and presented with greater than 100 adenomatous polyps lining the entire colon (pan-colonic). The classic presentation of a “carpet” of polyps of an innumerable burden was reported for this cohort. Individuals with less than 100 adenomatous polyps or polyps of mixed pathology were originally excluded from the study cohort. Most of the individuals underwent complete colectomy or proctocolectomy within one year of receiving a diagnosis. Notes were not found for colectomy procedures on three individuals and one individual presented with late-stage, metastatic disease and colectomy was not expected to benefit the individual. The most common phenotype with the registry was a combination of fundic gland polyps with or without a combination of duodenal polyps. Three of the individuals had no further extracolonic phenotypic presentations. One individual presented with papillary thyroid cancer at a later age in life (**Figure 4**).

Figure 4. Extracolonic features (n = 10)



Overall, the ethnic ancestry for individuals in the polyposis registry reflects the ethnic diversity found in the bay area of California. Individuals who received genetic testing under the umbrella of the polyposis registry represented countries from across the globe. The most common ethnicity for mutation-positive individuals found within the registry was Hispanic, specifically individuals with ancestors originating from Mexico. Caucasian ancestry, by definition of the individual, was the second most common. Further analyses for correlations between ethnic background and clinical diagnosis of polyposis will be conducted on the cohort in the future.

Descriptive statistics were conducted on phenotypic and demographic mutations in the polyposis registry from the previous Genzyme Genetics collaboration, and clinical testing (**Table 2**). Overall, mutation-positive females in the polyposis registry were more numerous than mutation-positive males, 60% to 40%, respectively. People who identify as being of Hispanic ancestry made up the majority of the population, followed by North American (Caucasian) ancestry. Ancestry labeled “other” included reported ancestries of Italian, Native American, Irish, Russian, Ashkenazi Jewish, Portuguese, and Hungarian descent. The average age of diagnosis for individuals with a known APC mutation was 30 years old and the average age for individuals to undergo complete or procto-colectomy was 33 years old. These statistics are merely descriptive, as the positive cohort for large rearrangements only was not big enough ($n = 10$) to run analyses that would be statistically significant.

There is currently scarce literature addressing genotype/phenotype specific to deletions and duplications in APC. Research on similar populations to that of this study is needed, and a direct comparison may be possible sometime in the future. Had the cohort included more deletion-positive individuals, further statistical analyses may have been able to be conducted. Although the cohort of individuals with deletions was too small to run more in-depth statistical analyses, the descriptive statistic results may lend to better characterization of individuals within the cohort.

Table 2. Descriptive statistical analyses of cohort demographic information

	n	Proportions
Gender		
Male	4	40%
Female	6	60%
Ethnicity		
Hispanic	4	40%
Caucasian	2	20%
Other	4	40%
	n	Years
Average age of Diagnosis	10	30
Average age at Colectomy	6	33

DISCUSSION

1B Promoter Cohort

This study aimed to determine the prevalence of APC 1B promoter deletions in a cohort of mutation – negative individuals within the UCSF polyposis registry who have been clinically diagnosed with FAP. Most of the individuals in the cohort have been tested with previous genetic testing that included sequencing and Southern Blot, but were tested before the most recent technologies were incorporated. The probands selected for this study received follow-up testing using the technique of MLPA for deletion/duplication analysis performed on stored buffy coat samples. Current studies hope to use the MLPA technology to detect mutations that encompass the APC 1B promoter region, which have been shown to disrupt function of the gene. The use of MLPA analysis in this study yielded two previously undetected deletions in the APC gene itself. Previous testing for these two individuals included Southern Blot conducted at CLIA certified laboratories before the technique of MLPA had been incorporated into regular analyses. Comparisons of genotype and phenotype were therefore made between the two APC deletions detected in this 1B promoter study, APC deletions detected during a previous research collaboration with Genzyme Genetics, and the APC deletions, single duplication, and APC 1B promoter deletion detected through clinical genetic testing. Although the deletions found in this study were not in the APC 1B promoter region, they emphasize the significance of undergoing complete genetic analysis.

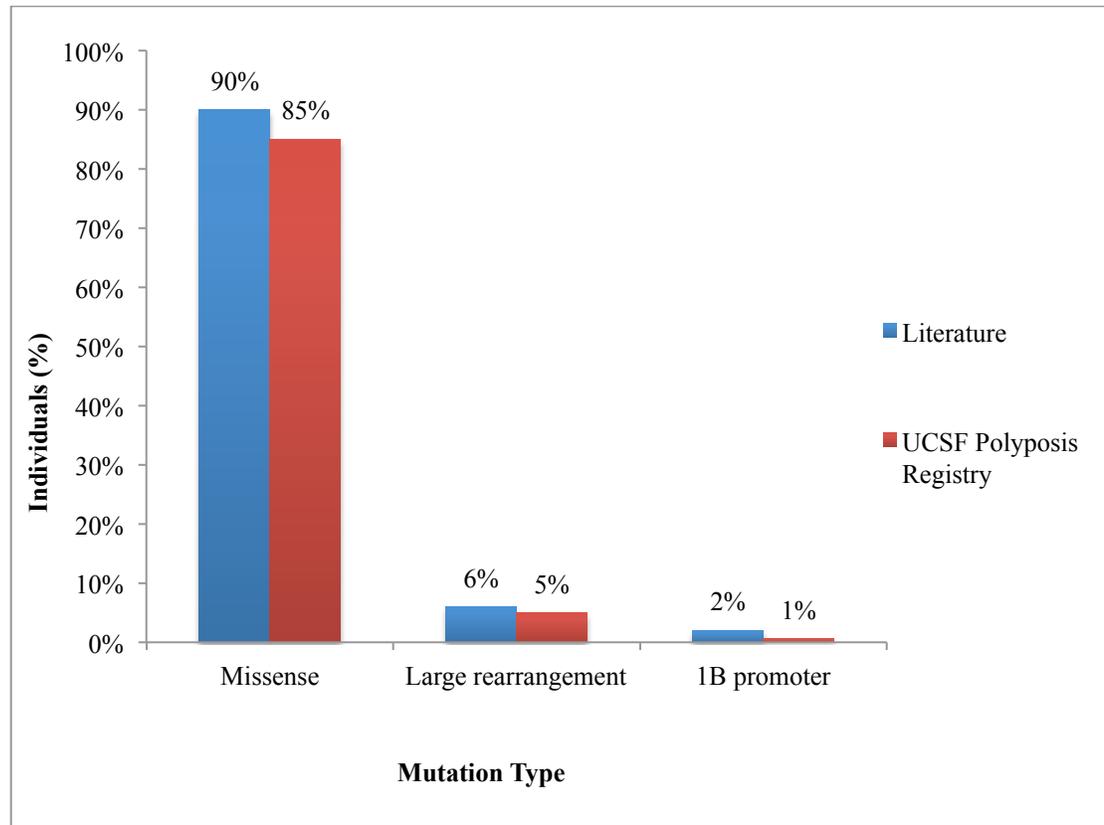
APC Mutation Findings in the UCSF Polyposis Registry

Overall, 8-12% of polyposis diagnoses, characterized by having pan-colonic polyposis of 100 polyps or greater, have either a partial or whole APC gene deletion (Michils et al. 2005). This information has been added to previous knowledge gathered from genetic testing for mutations in internationally referenced databases. The detection rate for mutations leading to inactivation of the APC gene has increased greatly with advances in technology. Nonsense, frameshift, and splice-site mutations make up a large percentage, 80-90%, of mutations detected in individuals with a clinical diagnosis of FAP. A comparison of the findings of this research study demonstrates that approximately 85% of the mutations in the UCSF polyposis registry were also frameshift or splice-site mutations (**Figure 5**).

This study added to the existing data regarding the percentage of partial or whole APC gene deletions that contribute to polyposis. Since 10 large rearrangements were detected in the 158 families in the registry, this cohort represents approximately 6% of polyposis diagnoses having a large rearrangement. This also aligns with what has been found in the literature in that approximately 6% of large deletions or duplications that were previously missed through protein truncation testing, sequencing, and mutation scanning have been detected using MLPA (Nielsen et al, 2007). The results of this study were similar to the literature in that two out of 39 (5%) individuals that had previously been described as mutation-negative were found to have deletions through the use of MLPA for detection of large

rearrangements. Likewise, the single 1B promoter deletion that was found through clinical testing of an individual at UCSF represents 1% (1:99) of the total mutations detected in the polyposis registry. The literature reports that 1B promoter deletions are thought to represent approximately 2% of all cases of FAP (Rohlin et al, 2011). Even at two percent, the number of individuals that may benefit from the use of this data and technology may be significant for future preventative measures.

Figure 5. Comparison of the percentage of mutations (by type) comprising the literature reports versus the entire polyposis registry



Out of the 158 families in the polyposis registry thus far, eight individuals were found to have partial deletions involving exons in the APC gene. Deletions ranged from one exon to three consecutive exons. Several individuals and families have been documented in the published literature as having the same deletions of exons and whole gene deletions. The phenotype for the partial gene deletions ranged from no extracolonic features to the presence of possibly harmful extracolonic features including fundic gland polyps, duodenal polyps, one case of a jaw osteoma, and one case of papillary thyroid cancer. Additionally, one individual was found to carry an entire APC gene deletion that had previously gone undetected through use of previous methods. The phenotype reported for the one whole APC gene deletion included the only case of a desmoid tumor. No correlations can be made between the individuals of such a small cohort. However, reports in the literature do support that fundic gland polyps, desmoid tumors, and duodenal polyps are some of the most common phenotypic presentations of classic FAP resulting from large rearrangements. None of the individuals found to carry APC deletions in the UCSF polyposis registry presented with symptoms previously unreported in classic forms of FAP.

Overall, approximately 50% of individuals with FAP will develop gastric polyps of either adenomatous or hyperplastic pathology (Burt, 2003). Small bowel polyps are also a common feature of FAP. It has been estimated that 50-90% of individuals with FAP will develop small bowel polyps anywhere from the second to third portion of the duodenum (Kadmon et al, 2001). There are currently variable

estimations of the prevalence of thyroid cancer in the FAP population. Current estimations range from 2.6-12%, which have been detected through the use of ultrasound for screening in cohorts of varying sample size (Jarrar et al, 2011). Records for thyroid screening were not included as a part of the focus of this study. However, one individual out of 10 (10%) appears to be in line with the reported prevalence. Desmoid tumors may occur after surgical intervention, specifically colectomy, and are common in the abdominal area. It has been estimated that desmoid tumors occur in 10%-30% of individuals with FAP, which is increased 800 fold over the risk for desmoid tumors in the general population (Nieuwenhuis et al, 2011). One individual out of the 10 (10%) deletion/duplication-positive individuals in the polyposis registry was reported as having a desmoid tumor, which is in line with the literature. Surgical intervention to remove these benign tumors is usually postponed until they cause complications or increased risk for problems from pressing on nearby tissues or organs.

Detection of Large Rearrangements

Technological advancements in the techniques used for detection of disease-causing mutations have rapidly evolved over the past decade. Sequencing was the first technology available at a clinical level and currently approximately 80-90% of APC mutations may be detected using this method (Hedge et al. 2014). These mutations are mainly truncating in nature. Next, Southern Blot was incorporated into the testing pipeline of many clinical laboratories. Myriad Genetics added the

technology, which detects large rearrangements (deletions and duplications), in August of 2004. The addition of Southern Blot increased mutation detection rates for APC to over 90% (Michils et al. 2005). However, this still allowed approximately 10% of large rearrangements to remain undetected. The use of MLPA in clinical genetic tests has been shown to boost the detection rate an additional 8-12% (Nielsen et al. 2007).

The addition of MLPA technology has also been shown to increase the detection rate of large rearrangements within the UCSF polyposis registry. Data collected on detection rates within the registry with addition of each technology shows a higher sensitivity with incorporation of MLPA compared to Southern Blot (**Table 3**). The individual with a whole APC gene deletion missed by Southern Blot, but detected using MLPA, gives a perfect example of the inability of Southern Blot to pick up even some of the largest gene deletions. The switch to MLPA for deletion/duplication analysis boosted the detection rate to 75%. This is not as high as the literature reports, but is significant in that it detects a broader type of mutation than sequencing and Southern Blot analyses. Therefore, it is paramount that the APC 1B promoter be included as a part of the MLPA genetic testing kit on a regular basis to increase the pick-up rate for these deletions.

Table 3. Detection of disease-causing mutations in APC over time

	Detection Rate in Polyposis Registry	Percentage of Mutations Detected by Method ¹	Year Method was Added for APC Testing
Sequencing	4/5 (80%)	80-90%	< 2004
Sequencing + Southern Blot (SB)	33/52 (63%)	> 90%	2004
Sequencing + MLPA (no 1B)	14/24 (75%)	*	2007
Sequencing + MLPA + APC 1B Promoter	3/3 (100%)	**	2011 (Ambry) 2012 (Myriad)
Sequencing + array CGH + 1B Promoter	2/2 (100%)	N/A	2013 (Myriad)

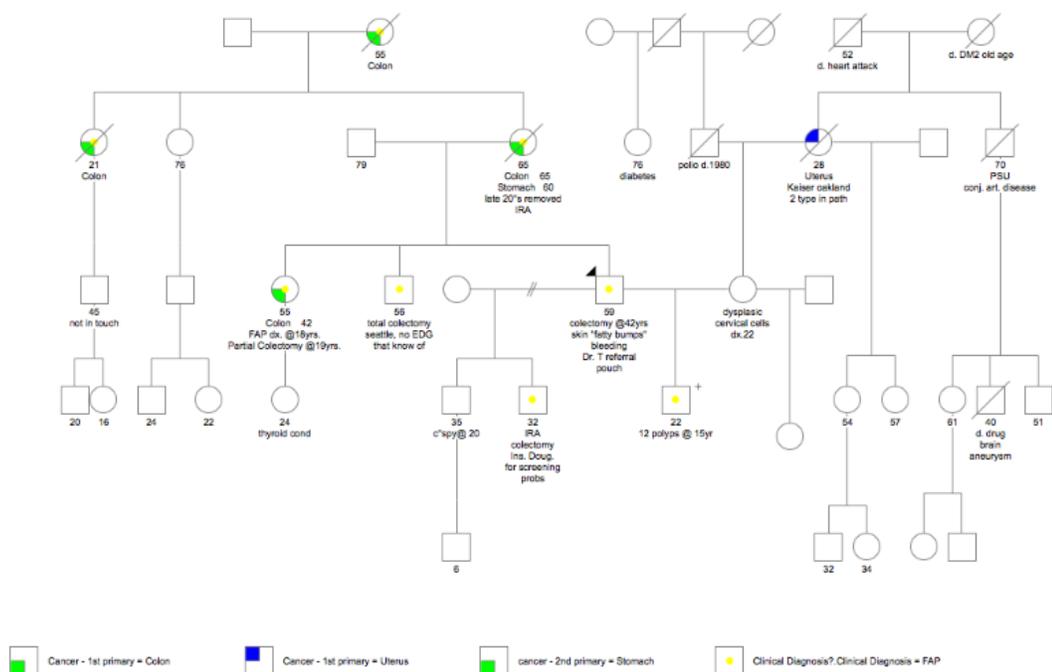
Note: ¹As reported in the literature. The addition of *MLPA and then **MLPA with the APC 1B promoter to detect large rearrangements increased the detection rate by ~8-10% and then ~1-2%, respectively.

One family in the UCSF Polyposis Registry serves as a perfect example of the clinical utility of re-contacting mutation-negative individuals for updated, complete testing. This family presents with a strong history of polyposis and colon cancer (**Figure 6**). The first individual, referred to as the proband, to receive genetic testing in the family is a 59-year-old male who was clinically diagnosed with FAP at 42. He subsequently underwent colectomy. Genetic testing was performed through a research study in collaboration with Genzyme Genetics. Protein truncation testing alone was used for mutation detection. A result of inconclusive or incomplete was given to this individual and he has yet to revisit the option of completing the testing. Interestingly, a series of genetic testing for two of his three sons did yield an APC duplication of exons 1-2. His second oldest son was the first to pursue genetic testing. He was clinically diagnosed with FAP at the age of 19 and had a colectomy soon after. Genetic testing was performed through Myriad Genetics in 2003 using sequencing only. No mutation was detected. The family's journey for an answer continued when the proband's third son through a second marriage was clinically diagnosed with FAP at age 15. He underwent a total colectomy at age 17. Genetic testing was performed through Myriad Genetics in 2009, which used sequencing and Southern Blot. The duplication of exons 1-2 was detected and a genetic etiology for the polyposis in the family was finally provided. The proband's oldest son had two clear colonoscopies at age 29 and 31, but decided to have genetic testing for a more definitive answer. He tested as a true negative through Ambry Genetics in 2011. The example provided by the results of genetic testing performed by different

technologies in this family highlights the importance of bringing clinically diagnosed, mutation-negative individuals back in for complete testing.

Figure 6. Family history of individuals tested using three different technologies

* An example of the benefit of re-testing in clinically diagnosed, mutation-negative individuals and families



Disseminating Results

Results of this study will be shared with the individuals for which new APC deletions were discovered. As previously mentioned, individuals in the polyposis registry who provided samples for research also agreed to be a part of the long-term follow-up study, which allows them to be re-contacted for any reason. The most common reasons for re-contact include, updates regarding testing technology or interpretation of results, inquiries about new cancer diagnoses within the family, and updated screening and prevention guidelines that are directly related to a known familial mutation. This is especially important for these two individuals, as these new results may have a significant impact on their care, as well as testing options for at-risk family members. Although current recommendations for clinical diagnoses of FAP include yearly colonoscopy, along with other screening for phenotypic symptoms, an actual genetic etiology may provide further evidence for insurance coverage for the specific individual. Family members will also benefit from this information because an actual mutation has been identified, which can be tested through a simple, and inexpensive single-site genetic test. Single site testing for the mutation in other family members will help the family determine who should receive increased screening, if found to carry the same mutation, or a return to population-based screening if found to be a true negative. If an individual in the family tests negative for the known mutation, but has had a colorectal cancer diagnosis, the individual may be considered to be a phenocopy. In this specific case, the specific oncology care team involved in the individual's care would provide screening

recommendations. Individuals within the cohort who remain APC mutation-negative after the results of this study will not be contacted, as another negative result will not have an impact on their care, and may in fact cause more anxiety or confusion within the families because an etiology for their clinical diagnosis has yet to be found. These individuals, however, will have their files updated to include negative MPLA results so that they may be considered for future studies if and when the knowledge of causative APC mutations grows and technology advances.

The detection of two APC deletions that were missed by previous testing techniques highlights the use of MLPA as a valuable addition to the suite of available genetic testing procedures. Since large rearrangements have been found in approximately 2% of all FAP cases, it is imperative that we continue to search for additional mutations that contribute to disease presentation in this population. New advances have provided the opportunity to re-evaluate previously mutation-negative FAP cohorts for newly detected deletions that provide an etiology for disease. Therefore, this research adds further support for re-evaluation of mutation – negative individuals that have received a clinical diagnosis of FAP.

CONCLUSION

The technology of MLPA has been slowly incorporated into the methods of the major genetic testing laboratories within that past few years. As a result, more individuals with disease-causing mutations, such as partial and whole gene deletions in APC, have begun to be detected. The results of this study expand the list of large rearrangements in a registry of polyposis patients. A total of 9 deletions and one duplication in the APC gene have been detected to date within the UCSF polyposis registry. Two of these mutations were detected as a result of this study, which were missed during clinical evaluation using sequencing and Southern Blot techniques. It may therefore be approximated that 5-6% (2/39) of mutation-negative individuals with a clinical diagnosis of FAP may in fact have a large deletion in the APC gene that could explain their symptoms and be valuable information for the testing of other at-risk family members.

The previous case example of the FAP family, in addition to the whole gene deletion found through this study, provide further evidence of the value of re-testing using MLPA. In both cases, large rearrangements had been missed through testing with older technologies. The fact that Southern Blot missed an entire gene deletion for the individual included in this study, which was then detected using MLPA, shows the increased sensitivity of deletion/duplication analysis. Finding genetic etiologies for polyposis in individuals and large families improves clinical care on two levels. At the patient level, complete genetic testing provides an answer for both

affected and at-risk individuals within these polyposis families. Anyone testing positive will be followed more closely and in turn have a better life expectancy outcome. Those who test negative will avoid increased screening regimens and have peace of mind that they cannot pass the condition on to their children. At a clinical level complete genetic testing saves healthcare dollars and resources spent on high-risk screening for individuals who may actually be true negatives. Combined with the results of this study, finding that approximately 6% of the mutation-negative individuals had a large rearrangement picked up through deletion/duplication testing (MLPA), should make more clinicians consider re-referral for complete genetic testing for mutation-negative individuals with a clinical diagnosis of FAP.

Overall, the more information and evidence that is gleaned from studies such as this, the better equipped genetics teams will be when evaluating and recommending screening and prophylactic treatment for those with a known family history of polyposis. A broader knowledge of how to detect the widest breadth of mutation types will allow further characterization of the possible phenotypes that may present with changes in each location along the APC gene.

Limitations Of The Study

One limitation of the study is that the cohort is made up of patients seen exclusively at the UCSF Mt. Zion medical center. Although a decent sample size was obtained from this population, the results may not be transferable to other populations with different demographics. Results may only be representative of patients seen at a

large, academic research institution, which is a regional referral center for patients with polyposis. The results may not apply to members of private corporations within the same demographic area, such as Kaiser. Comparing the individuals with known APC gene or promoter deletions or duplications to those detected at other institutions, both statewide and nationally, would allow for more in-depth statistical analyses.

The buffer used in sample preparation and storage, including multiple freeze thaw cycles, at the UCSF Tissue Core appears to have negatively impacted sample quality. While the purity measures obtained by HMRI suggested a quality reasonable for clinical diagnostic testing, the MLPA results were not as clean as expected. Other programs at UCSF have reported similar problems with DNA quality and quantity.

Future Studies

One thing to consider would be that the individuals in the registry may have numerous barriers preventing them from supplying blood samples. Some of these barriers involve location of the patient, current state of health, insurance coverage, and willingness to participate in further research. The increased cost and lab availability may also render buccal samples as the most feasible. Use of buccal samples would also help to remove the barriers of distance and health status, as the kits could be mailed directly to the individual and then back to UCSF. The caveat to this method would be making certain that enough DNA would be obtained. Buccal samples have a higher failure rate than whole blood, but are usually adequate for targeted testing, such as MLPA. Therefore, if the buccal kits could guarantee enough

DNA for testing, a good follow up study would be to re-test patients who remain mutation-negative from this study with fresh samples and the utilization of the newest technology.

Another enhancement to the current study would be to include more individuals with attenuated polyposis, i.e. patients with between 20-100 polyps, as the phenotype associated with large rearrangements has yet to be delineated. In fact, there is conflicting evidence in the literature regarding phenotype in patients with APC promoter 1B deletions. Some have reported a more attenuated phenotype, while others have reported a classic phenotype.

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