Juan, I-Cheng

PhD Thesis

The MYH Gene Status in Patients with Sporadic Colorectal Polyps

Wielkopolskie Centrum Onkologii w Poznaniu
Poznan University of Medical Sciences Poland.

ZaKład Immunologii Nowotworow.

Promotor: Dr hab. Med. Piotr Wysocki

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Table of Content

LIST OF ABBREVIATIONS.. I
DEFINITION OF TERMS IV
ABSTRACT XI
1. INTRODUCTION 1
1.1. THE COLON 1
1.2. COLORECTAL POLYPS 1
1.2.1. CLASSIFICATION OF COLORECTAL POLYPS 2
1.2.2. SERRATED ADENOMA 2
1.2.2.1. Hyperplastic polyps 2
1.2.2.2. Sessile serrated adenomas 2
1.2.2.3. Traditional serrated adenomas 3
1.2.2.4. Mixed hyperplastic adenomatous polyps 3
1.2.3. RISK FOR COLORECTAL CANCER 3
1.2.3.1. Age 3
1.2.3.2. Gender 3
1.2.3.3. Race 4
1.2.3.4. Location 4
1.2.3.5. Lifestyle 4
1.2.3.5.1. Cigarette smoking 4
1.2.3.5.2. Alcohol consumption 5
1.2.3.5.3. Fatty foods 5
1.2.3.5.4. Being overweight 5
1.2.3.6. Other disease conditions 5
1.2.4. MANAGEMENT OF COLORECTAL POLYPS 6
1.3. DEVELOPMENT OF COLORECTAL CANCER 6
1.3.1. CHANGES IN APOPTOSIS REGULATION/TUMOR SUPPRESSOR GENES 6
1.3.1.1. The *APC* gene 7
1.3.1.1.1. FAP 7
1.3.1.1.2. AFAP 7
1.3.1.2. The *p53* gene 8
1.3.2. CHANGES IN DNA METHYLATION 8
1.3.2.1. CpG island methylator phenotype (CIMP) 8
1.3.2.2. O-6-Methylguanine DNA methyltransferase (MGMT) 8
1.3.3. CHANGES IN RAS-RAF-MAPK PATHWAY 9
1.3.3.1. The *KRAS* mutation 9
1.3.3.2. The *BRAF* mutation 9
1.3.4. ABNORMALITIES IN MICROSATELLITE INSTABILITY (MSI) AND DNA

MISMATCH REPAIR (*MMR*) GENES 9
1.3.4.1. Lynch syndrome/HNPCC 10
1.3.5. ABNORMALITIES IN BASE-EXCISION-REPAIR (*BER*) GENES 10
1.4. COLORECTAL CANCER 10
2. THE *MYH* GENE 17
2.1. GENERAL INFORMATION ABOUT *MYH* GENE 17
2.1.1. DISEASES-CAUSING MUTATIONS OF THE *MYH* GENE AND THEIR CORRESPONDING PROTEINS 17
2.2. *MYH*-ASSOCIATED POLYPOSIS (MAP) 18
2.3. *MYH* GENE MUTATIONS AND COLORECTAL CANCER 18
2.3.1. INDICATIONS FOR *MYH* GENE TESTING 19
5. Parents diagnosed to be *Myh* gene mutation carriers.[47] 19
2.4. LOH ANALYSIS 19
3. THE MAIN AIM 22
4. MATERIALS AND METHODS 23
4.1. STUDY GROUPS 23
4.1.1. CRITERIA FOR COLONSCOPY 23
4.1.2. POLYPS AND BLOOD 23
4.1.3. CONTROL GROUP 23
4.2. REAGENTS 23
4.3. METHODS 28
4.3.1. DNA ISOLATION 28
4.3.1.1. DNA from polyp tissue 28
4.3.1.2. DNA from blood tissue 29
4.3.2. PCR-SSCP 29
4.3.2.1. Confirmation of DNA isolation and determination of the best reaction temperature 29
4.3.2.2. PCR-SSCP analysis 30
4.3.2.2.1. Labeled primer with isotope 30
4.3.2.2.2. PCR reaction 31
4.3.2.2.3. Electrophoresis ....
4.3.3. DNA PURIFICATION 32
4.3.3.1. Column purification (QIA quick purification) 33
4.3.3.2. T-easy ligation 34
4.3.3.3. Bacterial competent JM 109 transformation 34
4.3.3.4. Isolation of the plasmid DNA 35
4.3.4. DNA SEQUENCING 35
4.3.5. LOH ANALYSIS 36

4.3.6. STATISTICAL ANALYSIS 38
5. RESULTS 42
5.1. GENERAL INFORMATION ABOUT THE STUDY GROUP 42
5.2. RESULTS FOR THE DNA ISOLATION AND TEMPERATURE TESTS 42
5.3. RESULTS FOR PCR-SSCP 43
5.4. RESULTS FOR THE DNA SEQUENCING ANALYSIS 44
5.4.1. *MYH* EXON 2 FORWARD 44
5.4.2. *MYH* EXON 12 FORWARD 45
5.5. LOH ANALYSIS 46
5.6. STATISTICAL ANALYSIS 47
5.6.1. FREQUENCY DISTRIBUTIONS OF SELECTED VARIABLES FOR THE V22M *Myh* (Val22Met) POLYMORPHISM 47
5.6.2. FREQUENCY DISTRIBUTIONS OF SELECTED VARIABLES FOR THE Q324H *Myh*(Gln324His) POLYMORPHISM 47
5.6.3. GENOTYPE DISTRIBUTIONS AND ALLELE FREQUENCIES OF THE V22M *Myh* (Val22Met) POLYMORPHISM FOR GENES OF PATIENTS WITH COLON POLYPS AND THE CONTROL GROUP 48
5.6.4. GENOTYPE DISTRIBUTIONS AND ALLELE FREQUENCIES OF THE Q324H *Myh* (Gln324His) POLYMORPHISM FOR GENES OF PATIENTS WITH COLON POLYPS AND THE CONTROL GROUP 49
5.6.5. THE ASSOCIATION BETWEEN THE V22M *Myh* (Val 22Met) AND THE Q324H *Myh* (Gln324His) POLYMORPHISM IN THE STUDY GROUP 49
5.6.6. THE ASSOCIATION BETWEEN THE V22M *Myh*(Val 22Met) AND THE Q324H *Myh* (Gln324His) POLYMORPHISM IN THE CONTROL GROUP 49
5.6.7. THE ASSOCIATION BETWEEN CANCER FAMILY HISTORY AND V22M *Myh*(Val22Met) POLYMORPHISM 50
5.6.8. THE ASSOCIATION BETWEEN FAMILY HISTORY FOR CANCER AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 50
5.6.9. THE ASSOCIATION BETWEEN ADVANCED COLORECTAL POLYPS AND THE V22M *Myh* (Val22Met) POLYMORPHISM 51
5.6.10. THE ASSOCIATION BETWEEN ADVANCED COLORECTAL POLYPS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 52
5.6.11. THE ASSOCIATION BETWEEN GENDER AND THE V22M *Myh* (Val22Met) POLYMORPHISM 52
5.6.12. THE ASSOCIATION BETWEEN GENDER AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 53
5.6.13. THE ASSOCIATION BETWEEN CIGARETTE SMOKING AND THE V22M *Myh* (Val22Met) POLYMORPHISM 53

5.6.14. THE ASSOCIATION BETWEEN CIGARETTE SMOKING AND THE Q324H *Myh*(Gln324His) POLYMORPHISM 53
5.6.15. CIGARETTE SMOKING AND THE RISK FOR ADVANCED COLORECTAL POLYPS 54
5.6.16. CIGARETTE SMOKING AND THE RISK OF OCCURRENCE OF COLORECTAL POLYPS GREATER THAN 10 mm IN DIAMETER 54
5.6.17. THE ASSOCIATION BETWEEN AGE AND THE V22M *Myh* (Val22Met) POLYMORPHISM 54
5.6.18. THE ASSOCIATION BETWEEN AGE AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 55
5.6.19. THE ASSOCIATION BETWEEN AGE AND THE RISK OF DEVELOPING ADVANCED HIGH-RISK COLORECTAL POLYPS 55
5.6.20. THE ASSOCIATION BETWEEN AGE AND SIZE OF POLYPS 56
5.6.21. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS AND ADENOMAS AND THE V22M *Myh* (Val22Met) POLYMORPHISM 56
5.6.22. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 56
5.6.23. THE ASSOCIATION BETWEEN HYPERPLASTIC AND ADENOMA POLYPS AND THE V22M *Myh* (Val22Met) POLYMORPHISM IN SMOKING PATIENTS 57
5.6.24. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE V22M *Myh* (Val22Met) POLYMORPHISM IN NON-SMOKING PATIENTS 58
5.6.25. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM IN SMOKING PATIENTS 58
5.6.26. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE Q324H *Myh* (Gln324Hid) POLYMORPHISM IN NON-SMOKING PATIENTS 59
5.6.27. THE ASSOCIATION BETWEEN CRC PATIENTS AND OTHERWISE HEALTHY PATIENTS DIAGNOSED WITH POLYPS AND THE V22M *Myh* (Val22Met) POLYMORPHISM 59
5.6.28. THE ASSOCIATION BETWEEN CRC PATIENTS AND OTHERWISE HEALTHY PATIENTS DIAGNOSED WITH POLYPS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 60
5.6.29. THE ASSOCIATIONS OF V22M *Myh* (Val22Met) POLYMORPHISM IN POLYPS-POSITIVE PATIENTS BETWEEN THE PRESNECE OR ABSENCE OF FAMILY HITORY OF COLORECTAL CANCER 61

5.6.30. THE ASSOCIATIONS OF Q324H *Myh* (Gln324His) POLYMORPHISM IN POLYPS-POSITIVE PATIENTS BETWEEN POSITIVE AND NEGATIVE FAMILY HISTORY OF CANCER 61
5.6.31. THE ASSOCIATION BETWEEN BODY MASS INDEX AND THE V22M *Myh* (Val22Met) POLYMORPHISM........ 62
5.6.32. THE ASSOCIATION BETWEEN BODY MASS INDEX AND THE Q324H *Myh* (Gln324His) POLYMORPHISM 63
6. DISCUSSION 78
7. CONCLUSION 84
8. ACKNOWLEDGEMENTS........ 85
9. REFERRENCE 86
10. QUESTIONNAIRE........ 96

Table Index

Table 1-1 Histopathologic Features of Different Degrees of Cellular Dysplasia. 12
Table 1-2 Three Main Types of Hyperplastic Polyps........ 13
Table 2-1 Disease-Causing Mutations of the *Myh* Gene and Their Corresponding Proteins. 19
Table 4-1 Twelve Exons for the *Myh* Gene........ 38
Table 5-1 Frequency distributions of selected variables for the V22M *Myh* (Val22Met) polymorphism 63
Table 5-2 Frequency distributions of selected variables for the Q324H *Myh* (Gln324His) polymorphism........ 64
Table 5-3 Genotype distributions and allele frequencies of the V22M *Myh* (Val22Met) polymorphism for genes of patients with colon polyps and the control group........ 64
Table 5-4 Genotype distributions and allele frequencies of the Q324H *Myh* (Gln324His) polymorphism for genes of patients from the study and the control groups........ 65
Table 5-5 The association between the V22M *Myh* (Val22Met) and the Q324H *Myh* (Gln324His) polymorphisms in the study group 65

Table 5-6 The association between the V22M *Myh* (Val22Met) and Q324H *Myh* (Gln324His) polymorphisms in the control group 66
Table 5-7 The association between cancer family history and the V22M *Myh* (Val22Met) polymorphism 66
Table 5-8 The association between family history for cancer and the Q324H *Myh* (Gln324His) polymorphism 66
Table 5-9 The association between advanced colorectal polyps and the V22M *Myh* (Val22Met) polymorphism 67
Table 5-10 The association between advanced colorectal polyps and the Q324H *Myh* (Gln324His) polymorphism 67
Table 5-11 The association between gender and the V22M *Myh* (Val22Met) polymorphism 68
Table 5-12 The association between gender and the Q324H *Myh* (Gln324His) polymorphism 68
Table 5-13 The association between cigarette smoking and the V22M *Myh* (Val22Met) polymorphism 68
Table 5-14 The association between cigarette smoking and the Q324H *Myh* (Gln324His) polymorphism 69
Table 5-15 Cigarette smoking and the risk of advanced colorectal polyps 69
Table 5-16 Cigarette smoking and the risk of colon polyps greater than 10 mm in size 70
Table 5-17 The associations between age and the V22M *Myh* (Val22Met) polymorphism 70
Table 5-18 The associations between age and the Q324H *Myh* (Gln324His) polymorphism 70
Table 5-19 The associations between age and the risk of occurrence of advanced colorectal polyps 71
Table 5-20 The associations between age and polyp size 71
Table 5-21 The associations between hyperplastic and adenoma polyps and the V22M *Myh* (Val22Met) polymorphism 71
Table 5-22 The associations between hyperplastic polyps or adenomas and the Q324H *Myh* (Gln324His) polymorphism 72
Table 5-23 The associations between hyperplastic polyps or adenomas and the V22M *Myh* (Val22Met) polymorphism in smoking patients 72
Table 5-24 The associations between hyperplastic polyps or adenomas and the V22M *Myh* (Val22Met) polymorphism in non-smoking patients 72
Table 5-25 The associations between hyperplastic polyps or adenomas and the Q324H *Myh* (Gln324His) polymorphism in smoking patients 73

Table 5-26 The associations between hyperplastic polyps or adenomas and the Q324H *Myh* (Gln324His) polymorphism in non-smoking patients 73
Table 5-27 The associations between CRC- or non-CRC patients diagnosed with polyps and the V22M *Myh* (Val22Met) polymorphism 74
Table 5-28 The associations between polyps in CRC patients or otherwise healthy patients and the Q324H *Myh* (Gln324His) polymorphism.................74
Table 5-29 The associations of V22M *Myh* (Val22Met) polymorphism in polyps-positive patients between positive and negative family history for CRC. 75
Table 5-30 The associations of Q324H *Myh* (Gln324His) polymorphism in polyps-positive patients between positive and negative family history for CRC. 76
Table 5-31 The association between body mass index (BMI) and the V22M *Myh* (Val22Met) polymorphism 76
Table 5-32 The association between body mass index (BMI) and the Q324H *Myh* (Gln324His) polymorphism..76

Figure Index

Figure 1-1 Recommendations for Colonoscopy Examination 14
Figure 1-2 Pathways for Cancer Formation...15
Figure 2-1 Functions of *BER* genes...20
Figure 2-2 Results for Accumulation *Myh* Gene Defects in Different Genes. 21
Figure 4-1 DNA Ladder for 100 bp (Promega) 41

LIST OF ABBREVIATIONS

8-oxoG - 8-oxoguanine
A - Adenine
AFAP - Attenuated family adenomatous polyposis
APC - Adenomatosis polyposis coli
APS - Ammonium persulfate
BER **–**Base excision repair
BMI – Body mass index
BRAF - B-Raf proto-oncogene serine/threonine-protein kinase
Bp – Base pair
C - Cytosine
CA IN T (V, TV) - Carcinoma in tubular (villous, tubulo-villous) adenoma
CHRPE - Congenital hypertrophy of the retinal pigment epithelium
CI - Confidence interval
CIMP - CpG island methylator phenotype
CRC – Colorectal cancer
D - Aspartic acid
del – deletion
E - Glutamic acid
EDTA - Ethylenediaminetetraacetic acid
F - Phenylalanine
FAP - Familial adenomatous polyposis
G - Guanine
g - gram
ˇg – gravity
Gln - Glutamine
H - Histidine
His - Histidine
hMYH - Human Homolog of the MutY Repair Protein
HNPCC - Hereditary nonpolyposis colorectal cancer
IVS1 + 5G/C - Point mutation in intron 1 splice donor site causing intron 1 retention resulting in nuclear mRNA degradation
L - Leucine
LOH –Loss of heterozygosity
M - Methionine

M – Mole
MAP –*MYH* associated polyposis
MAPK-ERK pathways - a signal transduction pathway that transfers signaling from surface growth factor receptors to nucleus regulating cellular processes such as proliferation, survival and gene expression.
Met - Methionine
Mg^{2+} - Magnesium
MGMT - O-6-methylguanine-DNA methyltransferase.
mg — milligram
MHAP - Mixed hyperplastic adenomatous polyps
ml – milliliter
MLH1 - MutL homolog 1 gene, which is located at chromosome 3 and highly associated with HNPCC
MM - millimeter
mM – millimole
mm - millimeter
MMR – Mismatch repair
mRNA – Messenger Ribonucleic acid
MSI - Microsatellite instability
MTH1 - MutT homolog 1
MutYH – mutY Homolog gene also known as *MYH* gene.
Myc – It codes for a protein which is a gene transcription factor.
ng - nanogram
OGG1 - 8-oxoguanine glycosylase
OR - odds ratios
P - Proline
PCR - Polymerase chain reaction
PCR-SSCP - PCR-Single strand conformational polymorphism
Q - Glutamine
R - Arginine
S - Serine
SSA - Sessile serrated adenoma
SSCP - Single strand conformational polymorphism
T - Thymine
TH - Tubular adenoma high-grade dysplasia
TL - Tubular adenoma low-grade dysplasia
TSA - Traditional serrated adenoma

TVH - Tubular-villous high-degree dysplasia

TVL - Tubular-villous adenoma low-grade dysplasia

u - unit

USG – Ultrasonography

V - Valine

Val – Valine

VH - Villous adenoma high-degree dysplasia

VL - Villous adenoma low- grade dysplasia

χ^2 – Chai-square

Y- Tyrosine

µl – microliter

µM – micromole

DEFINITION OF TERMS

Allele — A shortened form of allelomorph. It is an alternative form of a single or a specific gene. Both alleles of a gene have the same characteristics but the production and function of each allele may be different.

Autosomal dominant gene — Only one autosomal dominant gene is needed to perform its functions. At least half of the offspring will inherit the same genetic trait. If this dominant trait is involved with disease conditions, this disease will be seen in every generation.

Autosomal recessive gene — The condition only appears when an individual acquires two recessive forms of an autosomal gene. If both parents are carriers, 25% of their offspring will be affected, 50% of their offspring will become carriers and 25% of their offspring will show the dominant trait. Skipped generation inheritance can be seen in autosomal recessive traits.

Carcinoma in situ — The carcinoma cells are confined in the space where the cancerous changes began with no further spreading. It is considered as an early stage tumor.

Codon — The codon is a tri-nucleotide sequence. After transcription to the RNA, each codon is translated into a specific amino acid. The AUG codon corresponds to the amino acid methionine and serves as start codon. The UAA, UGA and UAG codons are stop codons and do not correspond to amino acids.

Colonization — Bacterial or fungal species which populate an area, whether on growth plates or in living organisms. In vitro, colonization of these pathogens on growth plates allows physicians to confirm diagnoses and to test for the

sensitivities of pathogens to drug treatments.

Column purification

Nucleic acids have a tendency to attach or become absorbed onto solid surfaces depending on the pH and salt concentrations. The addition of samples containing nucleic acids and buffers into columns containing thin layers of silica membranes forces the mixtures through the silica membranes. The nucleic acids will be held by the silica membrane. Diluted water can change the pH and salt concentrations and wash out the nucleic acids.

DNA denaturation

Also known as DNA melting. It is the procedure of opening up the double helix structure of DNA by breaking the hydrogen bonds between strands. The denaturation temperature depends on the CG content of the DNA. The higher the content of GC, the higher the temperature is needed. Transforming double-helix DNA into single-stranded DNA enables fragments of interest to be analyzed.

Dysplasia

Alteration in cellular maturation. Anisocytosis, poikilocytosis, hyperchromatism and presence of mitotic figures are four major characteristics of dysplasia. Cells vary in size, shape and maturation stage. It is a precancerous lesion. The chance of dysplasia lesions progressing to cancerous lesions depends on the degree of dysplasia.

Electrophoresis

This is the use of electrical power to drive the components of interest through a gel. Due to the different sizes of the components, the distances moved on the gel will be different. Particular fragments can be removed for further study. It is a widely used technique for the analysis of DNA, RNA and proteins.

Exon

This is the actual fragment that is transcribed from DNA to RNA which corresponds to amino acid formation.

Gene — Genes are the hereditary material which holds the information for building and maintaining living organisms. Genes are passed down through generations.

Gene repair — During replication and transcription, mistakes happen all the time. To maintain the integrity of genes, various enzymes correct mistakes at different stages of replication and transcription. The gene repair process can prevent fetal mutations or diseases which cause mutations to happen and pass into further generations.

Genotype — This is the constitution of genes in living organisms, from single cells and organs to whole individuals. It is not completely related to the outside appearances of living organisms. People with the same genotype may differ in appearance. People with the same appearances may have different genotypes. This is the portion of genetic material that passes down through generations.

Genetic consultation — Genetic consultation is offered to individuals with inherited family diseases. Through testing, particular genes based upon family histories and the risk of developing the same disease can be detected. Screening protocols and treatment schedules can be planned in advance. It is a way of preventing patients with genetic family diseases from becoming ill. It is very important for the assessment of the risk of passing the same disease-causing gene to further generations.

Heterozygotes — In diploid or polyploid organisms, chromosomes have different alleles of a gene at the same locus.

Homozytogotes — Chromosomes have the same alleles of a gene at the same locus.

Hyperplasia — An increase in cell numbers in a tissue or an organ without changes in cellular maturation. It is a response to excess stimulation and presents as enlargement of the organ. This

is a pre-neoplasm/pre-benign tumor lesion. Despite the malignancy potency, the large size of the organ demands treatments.

Intron The DNA fragments which are not transcribed into RNA and are not related to amino acid formation. Gene switching is located at the introns. Changing the switching status is highly disease related.

Kinase Kinase is involved in the process of phosphorylation and the enzymes for kinase reactions are phosphotransferases. The main function of kinase reactions is to transfer phosphate groups to target proteins, which are usually enzymatic proteins. Kinase changes the function, location and interactions of the target protein with other proteins.

Locus The specific location of a gene on a chromosome.

Ligation The process of combining two fragments of DNA. Ligation enzymes can recognize the same sequence of two DNA fragments and create the joining point. The same ligation reaction can occur in different species.

Metaplasia Changes in the type of cells, replacing the original cell populations. This is a reversible process and is usually induced as a response to environmental hazards. The degree of cellular maturation stays intact. The malignancy potential of metaplasia is low and cells usually return to the normal cell type shortly after the hazards disappear.

Missense mutation A point mutation in the gene sequence. The mutation produces different amino acids and further changes the protein structure and function.

Mutant Genetic changes that cause a cell, an organ or an individual to present with new characteristics or genetic traits that are not seen in wild types.

Mutation Changes in gene sequences caused by radiation, UV light, oxidative damage or other mutagenic materials. These changes may or may not cause changes in cellular functions or outside appearances. The majority of mutations are neutral.

Neoplasm An abnormal mass resulting from the abnormal proliferation of cells. The cells can be fully matured, poorly distinguished or somewhere in between. According to the cellular characteristics, neoplasms can be classified as benign, pre-malignant or malignant.

Nonsense mutation A point mutation that causes premature termination of amino acid production. The mutation produces a stop codon in the middle of the sequence and stops further amino acid translation. It can change the function and structure of the protein.

Oncogene Gene with a potential to turn normal cells into tumor cells. In normal situations the expression of oncogenes is depressed. When mutations occur in these genes or the depression mechanisms disappear, cellular growth is no longer under control and progresses to form neoplastic cells.

PCR This is a technique of amplifying DNA fragments. Thermal cycles are applied to replicate DNA copies. Primers are needed to induce replication. Replication is based on DNA templates; any mutations on the templates can be amplified many times for further investigation.

Phenotype The external appearance. It is influenced by inherited genotypes and non-hereditary environmental factors. People with the same genotype but with different environmental exposures may show totally different phenotypes. Changes in environmental factors may change the phenotype.

Plasmid DNA	Mainly found in bacteria, the plasmid contains extra DNA material besides the regular chromosomal DNA. The genetic material in the plasmid is related to drug resistance but does not affect the normal growth of bacteria. By using the rapid reproductive characteristics of bacteria, large quantities of DNA fragments of interest can be produced following their insertion into the plasmid DNA. The bacterial vector system came from this concept.
Polymorphism	Where two or more different phenotypes exist in one specific population in a specific area at a particular time point. Polymorphism is due to different adaptations to environmental conditions and this biological diversity ensures that populations will not become totally diminished under extreme environmental changes.
Promoter area	This is an area located upstream of the transcriptional start site of a specific gene. It facilitates the transcription of this particular gene.
Sequencing	Determines the nucleotide order of a specific DNA fragment. The comparison of experimental sequencing with wild type sequences enables mutation sites to be found and the type of mutation to be determined.
SSCP	Single-stranded DNA can be analyzed using gel electrophoresis. This is a technique to determine differences between single strands of DNA after the same experimental treatments. Mutation fragments can be discovered using this process.
Transcription	Decoding DNA to RNA codes.
Translation	Production of proteins from RNA codes to amino acids.
Transversion	Purines substitute pyrimidines or pyrimidines replace purines in a DNA sequence. Transversion causes huge changes in the order of amino acid and structural

differences in proteins. Transversion can be caused by oxidative damage, ionization and alkylating agents.

Vector This is a vehicle for transferring foreign DNA fragments into other cells. The plasmid is one type of vector systems.

Wild type The naturally occurring phenotype of a species is called the wild type. For genetic analysis, the wild-type gene is referred to as the standard gene. Other genes from the same locus which are different from wild-type genes are called mutant genes.

ABSTRACT

Introduction: Colorectal polyps are common in men and their incidence increases with age. Various mechanisms are responsible for the development of colorectal polyps but more and more attention is being focused on the genetic background of this phenomenon. Recently, several authors reported the correlation of colorectal polyps with mutations of the *Myh* gene. In this study, *Myh* gene status was evaluated in patients with newly diagnosed colorectal polyps.

Subjects and materials: Between the years 2004-2008, 235 patients who underwent endoscopic examination at Wielkopolskie Centrum Onkologii were diagnosed with colorectal polyps. Blood and polyp tissue were obtained from all of these patients. The control group comprised 347 healthy subjects from whom blood samples were available. From polyp and blood samples, DNA was extracted and analyzed for presence of *Myh* gene mutations and loss of heterozygosity (LOH). Statistical analyses of the relationships between lifestyle and occurrence of *MYH* polymorphism were performed.

Results: Within the study population - 35 V22M gene polymorphisms and 73 Q324H gene polymorphisms were found. The association between V22M and Q324H polymorphisms in the control group and study groups were both statistically significant (P=0.00061 in control group, P=0.005 in study group). There was no significant difference in the frequency of analyzed polymorphisms between the study and control group. The study revealed that advanced polyps tended to occur below the age of 60. Gender, smoking, alcohol consumption, overweight and obesity were not related to an increased risk of colorectal polyps.

Conclusions: The study demonstrated that in the Polish population genetic variations of the *Myh* gene are not associated with the development of colorectal polyps.

1. INTRODUCTION

1.1. THE COLON

The colon, the ending part of human gastrointestinal tract, is covered by the peritoneum except for the distal part of the colon and rectum. It is about 100-150 cm in length and its main functions are water and salt absorption and solid feces formation.[1] There are two main types of cells in the colon: absorptive cells and mucus-secreting goblet cells. They are straight tubular glands or crypts and extend to the muscular mucosa. Goblet cells are located in the base of the glands, whereas the absorptive cells mostly occur at the luminal surface. Another important group of cells are the stem cells, which are located at the base of the gland and continually replace the epithelium.[1,2] Apart from mucus-secretion and absorption, the colon can be considered as a defense organ since lymphocytes and plasma cells flow through the lamina propria. Inside the colon lumen are numerous bacteria which are responsible for a small amount of cellulose digestion and fat-soluble vitamin absorption.[2]

The absorptive cells are at the top of the glands and contain numerous cryptal cells. Apoptosis constantly occurs at the top of the crypts and these cells are replaced by newly formed cells from the stem cells. Stem cells are also responsible for repairing damaged crypt cells[1]. Normal replacement period is 2-3 days. According to this kind of cell arrangement and character, the location of modified cells is very important. If the modified cells reside only at the top of crypts, it means that solely the matured cells are affected and this situation is defined as low-grade dysplasia. If the modified cells are located at the bottom of crypts, the possibility of stem cell invasion is high, and this is defined as high-grade dysplasia. A modified cell refers to changes in cell types, from columnar to other types of cells; and changes in cell differentiation, from well to poorly defined cells and changes in nuclear integrity, from small, dense nucleus to large, loose nucleus.

1.2. COLORECTAL POLYPS

A colorectal polyp is a tumor mass that grows into the lumen of the colon. It starts

as a small sessile lesion without a stalk. The development of a stalked or a pedunculated polyp is due to the traction of the mass.[3, 4, 5]

1.2.1. CLASSIFICATION OF COLORECTAL POLYPS

1. Changes without dysplasia, polyps present as normal mucosa cells, polyps present as inflamed, hyperplastic polyps and sessile serrated adenoma (SSA)

2. Changes with low-grade dysplasia, tubular adenoma low-grade dysplasia (TL), villous adenoma low-grade dysplasia (VL), tubular-villous adenoma low-grade dysplasia (TVL) and traditional serrated adenoma (TSA)

3. Changes with high-grade dysplasia, tubular adenoma high-grade dysplasia (TH), villous adenoma high-degree dysplasia (VH), tubular-villous high-degree dysplasia (TVH) and carcinoma in tubular (villous, tubulo-villous) adenoma CA IN T (V, TV) (Table 1-1)

1.2.2. SERRATED ADENOMA

Serrated adenomas are defined as serrated hyperplastic polyps with adenoma changes or with dysplasia.[5,6] Serrated adenomas are subdivided into true hyperplastic polyps, sessile serrated adenomas, traditional serrated adenomas and mixed hyperplastic adenomatous polyps by their histological features.[6,7] They were classified as HPs before the year 2003.[5,8]

1.2.2.1. Hyperplastic polyps

The hyperplastic polyp (incidence- 80%-90%) is defined as a serrated polyp with regular proliferation.[9,10] There are three subtypes in this category: microvesicular type, goblet cell type and mucin-poor type (Table 1-2).[11]

1.2.2.2. Sessile serrated adenomas

Nucleus changes of Sessile serrated adenomas (SSA) (incidence - 10%-15%) are usually found at the middle/upper portion of crypts with irregular distribution of goblet cells.[2,9] Ninety percent of SSA lesions are

positive for BRAF mutations[11] and nearly 70% of the lesions are positive for CIMP mutations.[2]

1.2.2.3. Traditional serrated adenomas

The majority of traditional serrated adenomas (TSA) (incidence- 2%-3%) are pendunculated with uniform populations of abnormal epithelial cells, which are eosinophilic in appearance with an elongated nucleus.[2] Twenty to thirty-six percent of TSA lesions are positive for BRAF mutations.[12]

1.2.2.4. Mixed hyperplastic adenomatous polyps

Mixed hyperplastic adenomatous polyps (MHAP) (incidence - 1%-2%) are adenomas appearing with more than one of the histological characteristics mentioned above.[2,10] All of these lesions are positive for BRAF mutations.[12]

1.2.3. RISK FOR COLORECTAL CANCER

1.2.3.1. Age

Age is the one of the best defined risk factors for colorectal polyps. In fact, more than 50% of patients who are diagnosed with colon polyps are aged 60 or older. The majority of these patients have had no previous syndromes. It is believed that more than 90% of people aged above 60 years have one or more colorectal polyps.[13]

Various kinds of polyps have different prevalence at a particular age. If polyps are found in patients' early life, such as the teens or mid twenties, familial polyposis is highly possible and these patients are at much higher risk of developing adenomas and colorectal cancer. Radical surgery (colectomy) is often needed and genetic consultation is recommended. If polyps are found at the age above 60 and size and number of polyps are within the normal range, polyptectomy is required and follow up is recommended.

1.2.3.2. Gender

There is no influence of gender on the incidence of colorectal polyps. The incidence of colorectal polyps is the same for males and females. However, numerous studies have indicated that larger polyps tend to be found more often in males and that the conversion of polyps into adenocarcinomas is more frequent in males.[13,14]

1.2.3.3. Race

There is no significant influence of race on the development of colon polyps, but larger polyps are more often found in black people and more black people develop colorectal cancer due to the lack of regular medical follow up. However, the progression of polyps into adenocarcinomas occurs faster in white populations. There are no such comparisons between western and eastern populations.[15,16,17]

1.2.3.4. Location

Polyps are found throughout the colon but it has been observed that larger polyps (>10 mm) are more often located at the left side of the colon, from the middle of the transverse colon to the sigmoid. Small polyps (6-10 mm) and diminutive polyps (<6 mm) are more often found at the right side of the colon, from the celiac colon to the middle of the transverse colon. The bigger the size, the higher the risk of developing colorectal cancer.[18,19,20]

1.2.3.5. Lifestyle

1.2.3.5.1. Cigarette smoking

Cigarette smoking is a known risk factor for colorectal cancers. Thousands of carcinogens are contained in a cigarette and cigarette smoking increases the risk of all kinds of cancers. Colon polyps are known to be the precursor for colon cancers; an increased risk of developing colorectal polyps increases the risk of cancer development.[21] Cigarette smoking is often combined with coffee consumption, alcohol consumption and sometimes with fat intake.

The amount and duration of cigarette smoking are important risk factors. Research has shown that the more packs/year of cigarette smoking the higher the risk for developing colon polyps. The majority of those polyps are hyperplastic polyps.[21,22]

1.2.3.5.2. Alcohol consumption

Alcohol consumption alone cannot be identified as an independent risk factor for colorectal polyps. The amount of alcohol intake plays an important role, either increasing or decreasing the risk. Ten grams of alcohol per week decreases the risk of colorectal polyps by 15%.[23] It is also believed that excessive alcohol intake is one of the risk factors for colon cancers.

1.2.3.5.3. Fatty foods

Fatty acids and bile acids are toxic to the colorectal epithelium. An excess amount of fatty acids accumulated in colorectal areas is a risk factor for colorectal polyps and cancer.[24]

1.2.3.5.4. Being overweight

Obesity is associated with the formation of colorectal polyps. A single study demonstrated that in patients aged 30 or above, one unit increase of the BMI index increased the risk of developing colorectal polyps by 1%.[25]

1.2.3.6. Other disease conditions

Patients with chronic inflammatory bowel diseases, especially ulcerative colitis, are at a higher risk of developing colorectal cancers. There is a 2% increase of the risk after 10 years of active colitis, 8% after 20 years and 18% after 30 years. Chromosomal instability, MSI and hypermethylation, found in colorectal cancer, are also found in ulcerative colitis patients.[26] Ninety percent of colorectal cancers originate from colorectal polyps[3] and chromosomal instability, MSI and hypermethylation are frequently found in colorectal polyps.[27,28] Chronic active inflammatory bowel diseases may also participate in polyp

development.

Patients with allergies show a decreased risk of colorectal cancers. It is believed that allergies alter the patient's immune system and changes the response to particular molecular changes in cells, resulting in a decreased risk of colorectal carcinoma.[29]

1.2.4. MANAGEMENT OF COLORECTAL POLYPS

For patients with no clinical signs and syndromes indicated for colonoscopy examination, no previous colorectal polyp removal and no family history of colorectal cancers, regular colonoscopy screening should be started at age of 50 (Figure 1-1). If no polyps or other abnormal tissues are found during colonoscopy examination, the next colonoscopy examination should be done in next 3 years. If polyps or cancerous tissues are found during the exam, they must be excised and analyzed by histopathologists. If the histopathologic reports indicate cancer tissues, start cancer treatment should be started according to specific oncologic guidelines.

For patients with a positive family history for polyps, colonoscopy should be performed at an early age. For patients with a positive FAP, colonoscopy should be started in their twenties. All polyps should be removed by colonoscopy if they are manageable with a colonoscope; otherwise, a surgery is required. The standard recommended procedure for FAP patients is total colectomy. Partial colectomy should be performed on patients with Lynch syndrome. In patients with such genetic disorders colonoscopy should be performed every year.

1.3. DEVELOPMENT OF COLORECTAL CANCER

A neoplasm or a tumor is defined as excessive and uncontrolled growth and spread of abnormal cells. These cells are structurally and biologically different from their original ones. Many genes are involved in reducing carcinoma development. Mutations of these control genes cause uncontrolled cellular proliferation and cancer development (Figure 1-2).

1.3.1. CHANGES IN APOPTOSIS REGULATION/TUMOR

SUPPRESSOR GENES

1.3.1.1. The *APC* gene

The *APC* gene is a tumor suppressor gene located on chromosome 5q21. If mutations cause the loss of the *APC* protein activates, *Myc* proto-oncogene expression and uncontrolled cellular growth begins.[30]

1.3.1.1.1. FAP

Familial adenomatous polyposis (FAP) is an autosomal dominant disease which is characterized by hundreds to thousands of adenoma polyps in the colorectal region.[4,30,31,32] FAP accounts for around 1 in 8300 to 1 in 1400 birth[30] and around 0.1-1% of all colorectal carcinomas.[27,31] The *APC* mutations are found in 80%-85% of FAP patients.[33] Patients develop FAP in their earlier decades and without treatment almost 100% of FAP patients develop colorectal cancer before the age of 40.[29,31,32,34] Genetic consultation[35] and subsequent colectomy[32] represent a standard care in patients with FAP.

1.3.1.1.2. AFAP

Attenuated family adenomatous polyposis (AFAP) is an autosomal dominant disease characterized by less than 100 polyps in the colon and a later disease development compared to FAP patients.[27,31,35,36] Mutations occur at distinguished parts of the *APC* gene, for example, the 3' end mutation on exon 9 of the *APC* gene presents with fewer polyps than the 5' end mutation on exon 9 of *APC* gene.[31,35]

The *APC* mutations are only found in 10% of AFAP patients.[33] Despite fewer clinical syndromes and the later development of the disease, if untreated 80% of AFAP patients will ultimately develop colorectal cancer by the age of 45-55 years.[35] Colonoscopy-based removal of polyps is recommended for patients with fewer polyps and must be followed by regular colonoscopy. For patient with multiple, large polyps, surgical treatment is recommended.[35]

1.3.1.2. The *p53* gene

The *p*53 gene is a tumor suppressor gene and its presence is used to distinguish between adenomas and adenocarcinomas. Mutations of the *p*53 gene have rarely been detected in adenomas but they are present in 75% of adenocarcinomas. The *p*53 gene is translated into the p53 protein, which arrests cells in the G_1 phase of the cell cycle and induces apoptosis. The concentration of the p53 protein was found to be higher in DNA-damaged cells. The cellular growth can not be stopped with the loss of the *p*53 gene.[37,38]

1.3.2. CHANGES IN DNA METHYLATION

DNA methylation is one of the mechanisms that activates or silences gene expression.[2] The activation of tumor promoter genes or the silencing of tumor suppressor genes both result ultimately in tumor growth.[6] The *MLH1* and *MGMT* genes are usually inactivated by DNA methylation.

1.3.2.1. CpG island methylator phenotype (CIMP)

The CpG island is located at the promoter area, where CG sequence repeats in front of a gene. Under normal conditions, this area is generally unmethylated. Hypermethylation of the CpG island at the promoter area may turn the following gene expression off. Thirty to fifty percent of colorectal cancers and other kinds of cancers are found positive for hypermethylation at the CpG island. Aberrant methylation by DNA methyltransferase restores the gene expression.[2,6,10]

1.3.2.2. O-6-Methylguanine DNA methyltransferase (MGMT)

The main function of MGMT, which is produced by the *MGMT* gene, is DNA repair. The expression of MGMT protects genes from G:C to A:T transition. MGMT removes O6-methylguanine, which is pro-mutagenic and causes G:C to A:T transition in DNA.[39] Inactivation of *MGMT* genes causes accumulations of O6-methylguanine in growth regulating genes, such as the *KRAS* and *p53* genes.[40] Hypermethylation of the CpG island of *MGMT* turns off *MGMT* gene expression and causes other gene mutations.[27,39,40]

1.3.3. CHANGES IN RAS-RAF-MAPK PATHWAY

There are two genes in the RAS-RAF-MAPK pathway - *KRAS* and *BRAF*. The pathway is extremely important for tumor formation. Extracellular growth factors are mediated via this pathway into nucleus where it leads ultimately to activation of various cellular processes. Mutations in the *KRAS* and *BRAF* genes result in the constant transduction of signals and efficiently stimulate malignant features of tumor cells. The *KRAS* and *BRAF* genes mutations are mutually exclusive in colorectal cancer.[41] The *KRAS* and *BRAF* genes mutations appear to correlate with different pathways.

1.3.3.1. The *KRAS* mutation

The KRAS mutations are associated with MGMT methylation. Loss of *MGMT* functions leads to G:C accumulation in the *KRAS* gene and loss of *KRAS* functions.[42] The *KRAS* mutations are closely related to hyperplastic polyps.

1.3.3.2. The *BRAF* mutation

The *BRAF* mutations in colorectal cancer are strongly associated with microstabilite instability (MSI) and DNA methylation abnormalities. Although MSI is also associated with HNPCC, *BRAF* mutations do not occur in HNPCC. The *BRAF* mutations are closely related to SSA (90%), and MHAP (100%).[2,12]

1.3.4. ABNORMALITIES IN MICROSATELLITE INSTABILITY (MSI) AND DNA MISMATCH REPAIR (*MMR*) GENES

The DNA mismatch repair genes are genes which recognize and correct DNA replication errors, including mispaired nucleotides, small insertions and deletions.[43] The failure of *MMR* function leads to the accumulation of uncorrected, small instability sequences, called microsatellite instability, in the genome.[44] Microsatellite instability is the hallmark of defective *MMR* genes, and it appears in approximately 10%-20% of all colorectal cancers and almost 90%-95% of HNPCC cases.[28,45] The more the accumulation of MSI, the

higher risk of future development of colorectal carcinoma: a high-MSI was found in 15% of colorectal cancers.[28]

Up to date, six kinds of *MMR* genes (*MSH2, MLH1, MSH3, MSH6, PMS1* and *PMS2*) have been identified. Germline mutation of *MLH1* and *MSH2* can be found in 60% of hereditary nonpolyposis colorectal cancer (HNPCC) cases.[46]

1.3.4.1. Lynch syndrome/HNPCC

Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominant disease, which accounts for 3%-5% of all colorectal cancers. Sixty to eighty percent of HNPCC cases have either mutations of *MLH1* or *MSH2* genes and 40%-60% of patients with HNPCC are at increased risk of endometrial cancer.[47] In patients with Lynch syndrome colonoscopy cannot distinguish Lynch-associated lesions from sporadic colon adenomas and cancers. Compared to FAP patients, in Lynch syndrome adenomas and subsequent cancers develop at later age. Patients with mutations of *MMR* genes are also at high risk of development of endometrial, ovarian, renal cell and gastric cancers. Patients diagnosed with Lynch syndrome must be examined annually (colonoscopy, ultrasonography of abdomen, transvaginal ultrasonography) starting at age 25-30. Upon diagnosis of a localized colorectal cancer partial colectomy is usually performed.[35]

1.3.5. ABNORMALITIES IN BASE-EXCISION-REPAIR (*BER*) GENES

Base-excision-repair genes are the *MutYH* gene, the *OGG1* gene and the *MTH1* gene and only a defect in the *MutYH* gene is discovered in associate with colorectal polyps and cancers. Oxidation damage is the potential mechanism for DNA damage and causes G:C to T:A transversion. The *MutYH* gene plays an important role in excision of mismatched adenine.[36] As mentioned previously, high MSI is a major cause for gene mutation and cancer development. Some patients with stable MSI also develop colorectal polyps and cancers. Among these patients, 33% are found to have decreased levels of MYH protein. The *MYH* gene mutation is also a risk factor for colorectal polyps and cancer.[48]

1.4. COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer in the world and the second most common cancer in western societies.[4,17] There are more than 148,000 new cases each year in the United States of America and this type of cancer caused more than 55,000 deaths in 2006.[47] Males are more prone to develop CRC than females, despite the equal possibility for colorectal polyps in both genders.[17] The previous belief that Asian populations had a lower risk of developing colorectal cancer has now changed since CRC incidence is growing rapidly in many Asian areas,[49] such as Japan and Taiwan.

Fortunately, CRC is believed to be a preventable disease.[13] More than 90% of CRCs originate from benign colorectal adenomas. Colorectal polyps are believed to be precancerous lesions. The risk factors for developing colorectal polyps represent simultaneously also risk factors for developing CRC. Colonoscopy screening is currently the best method of prevention of CRC.[50]

The risk factors for developing CRC are multifactorial, including environmental and genetic factors.[24] Twenty-five percent of CRC patients have a family history of either CRC or other cancers.[34] Three to six percent of CRC cases are diagnosed from known Mendelian syndromes, such as FAP, HNPCC and MAP.[45,51] Besides colonoscopy screening, genetic consultation is also important for patients with family history of CRC or with particular genetic syndromes.

Table 1-1 Histopathologic Features of Different Degrees of Cellular Dysplasia

Low-grade dysplasia	High-grade dysplasia	Non-invasive carcinoma (carcinoma in situ)
Mild to moderate dysplasia in old nomenclature.	Severe dysplasia in old nomenclature.	
Spindle-shaped epithelium with hyperchromatic nuclei	Spindle-shaped epithelium with hyperchromatic, moderately pleomorphic nuclei.	Marked pleomorphic cells with swollen, large vesicular nuclei
Small chromatin particles	Irregular with angular chromatin particles.	Bridges of nucleolus- associated chromatin reaching angular chromatin deposits both in the nucleus and along the nuclear membrane.
Regular nuclear membrane	Irregular nuclear membrane	Large nucleus with disrupted polarity and atypical mitoses. Irregular notched nucleus nuclear membrane.
Stratified nuclei do not surpass the deeper half of the epithelial thickness.	Stratified nuclei surpass the superficial half of the epithelium and may reach the luminal epithelial border.	Budding or branching crypts or tubules with epithelial septa, back-to-back glands and cribriform growth. The glands are often arrayed obliquely to the basement membrane.

[1,2,10,12]

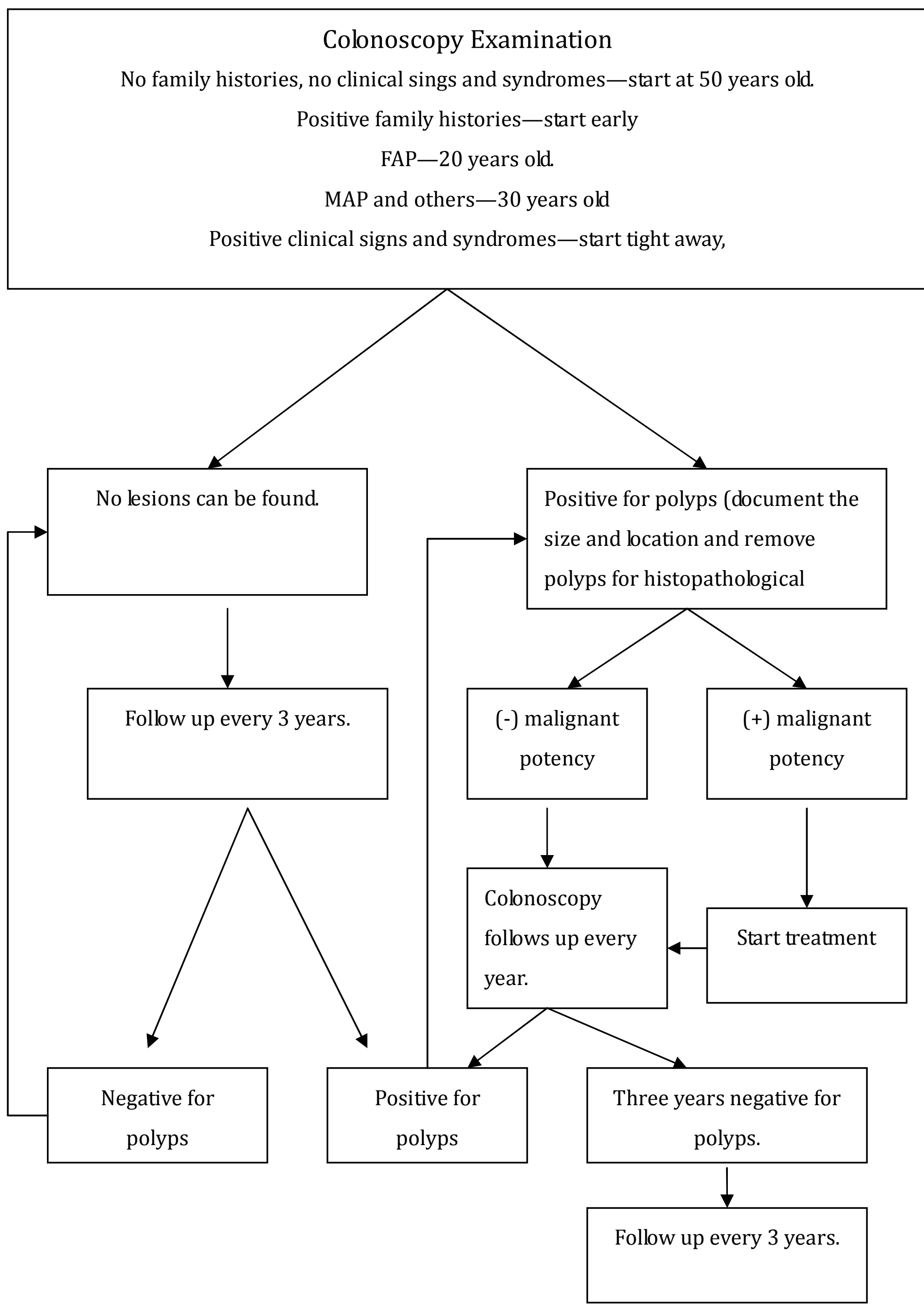
Komentarz:

Table 1-2 Three Main Types of Hyperplastic Polyps

Mucin-microvesicular polyps	Goblet cell-rich polyps	Mucin-poor polyps
Most common		Least common
The number of goblet cells is the greatest of the three, but is decreased compared with normal colonic mucosa		Goblet cells are decreased or absent in this type.
Large portion of proliferation; up to one-half of the crypt.	The proliferation is much less than the mucin-microvesicular type, limited to the superficial one-third of the mucosa, or even to its surface	
Nuclear atypia and stratification are rare.	Minimal nuclear atypia or stratification	Prominent nuclear atypia
Focally thickening of the basement membrane.	Basement membrane is thickened among almost all polyps in this type.	
		Associated with hyperplasia of clear neuroendocrine cells.
54% with KRAS mutation	67% with BRAF mutation 68% with CIMP	75-82% with BRAF mutation 92% CIMP

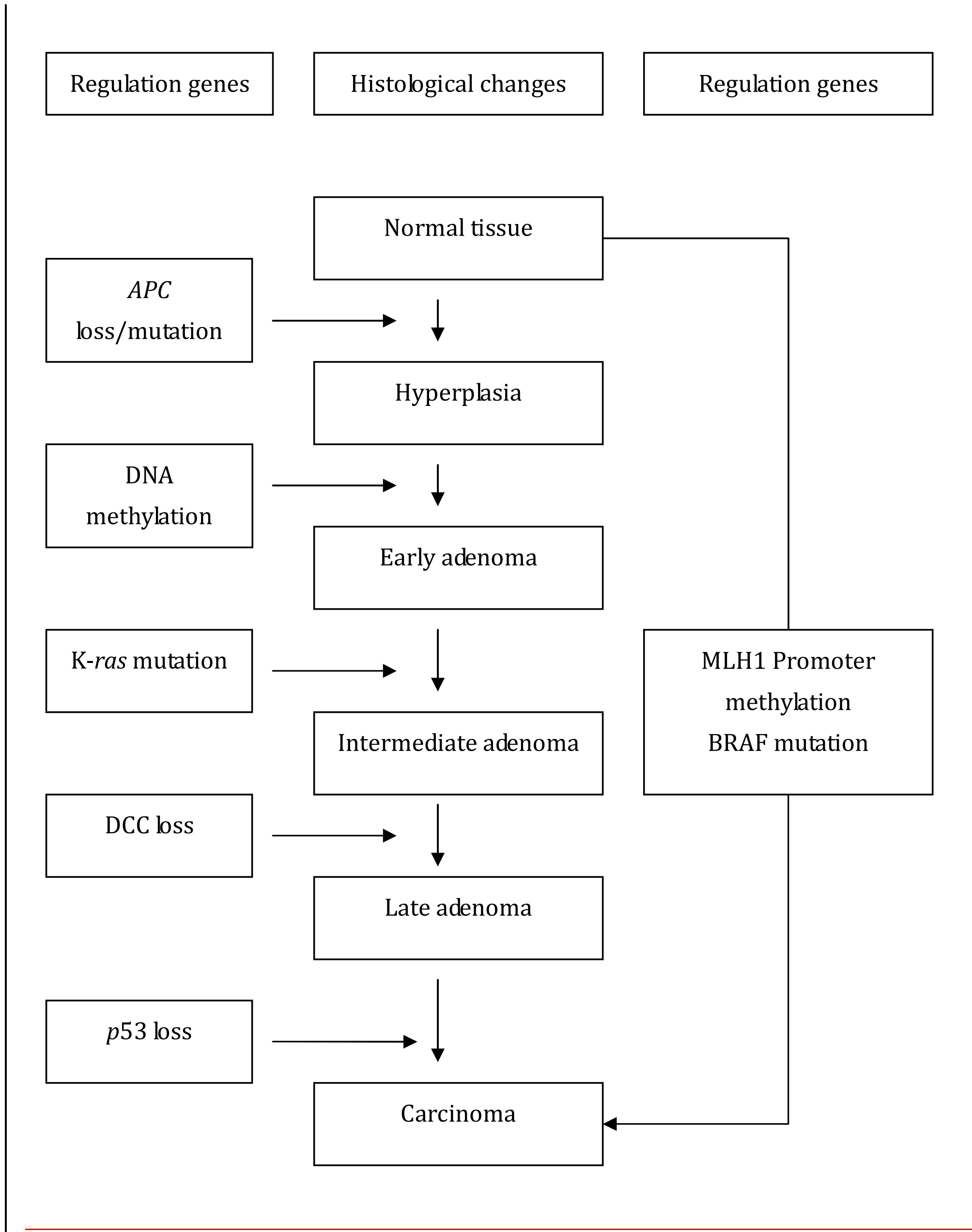
[9,10,11,52]

Figure 1-1 Recommendations for Colonoscopy Examination



13,53

Figure 1-2 Pathways for Cancer Formation


Regulation genes
Histological changes
Regulation genes
Normal tissue
APC
loss/mutation
Hyperplasia
DNA
methylation
Early adenoma
K-ras mutation
MLH1 Promoter
methylation
BRAF mutation
Intermediate adenoma
DCC loss
Late adenoma
p53 loss
Carcinoma

2. THE *MYH* GENE

2.1. GENERAL INFORMATION ABOUT *MYH* GENE

The *Myh* gene is located on chromosome 1P 32-34. It contains 12588 bp of DNA codons and is divided into 16 exons. There are 1838 bp of DNA which can be transcribed into mRNA and subsequently 546 amino acids are translated into the Myh protein- hMYH located in the nucleus and mitochondria. The Myh protein is an enzyme which excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired with guanine, cytosine or 8-oxoG (7,8-dihydro-8-oxoguanine) – typical major oxidative-damaged-DNA lesions.[36,54] If unrepaired, such phenomenon leads to the G:C to T:A transversion. Therefore, the *Myh* gene is classified as a *BER* (base-excision-repair) gene.

There are three *BER* genes, the *Myh*, *OGG1* and *MTH1* genes which encode MutY, MutM and MutT proteins, respectively. The main function of BER proteins is removal of oxidative damaged DNA and prevention of mutations. The MutM protein removes 8-oxoG from duplex DNA and MutT hydrolyses 8-oxoG to prevent damaged codons from further replication. Up to date, only the *Myh* gene mutation has been found to be related to colorectal polyps and colorectal cancers.[5,33,36,55,57] (Figure 2-1)

2.1.1. DISEASES-CAUSING MUTATIONS OF THE *MYH* GENE AND THEIR CORRESPONDING PROTEINS

Many mutations are known to be associated with increased risk of colorectal polyps and colorectal cancers and even an increase risk of other cancers. Different mutations are involved in different mechanisms of cancer development, including influence on the transcription rate,[48,56] changes in signal pathways in mitochondria and nuclei,[48,58] nonsense and missense mutations, mutations of the *Myh* gene that prevent the mismatch site being recognized and therefore cannot be corrected,[5,48] changes in the glycosylase function and an increased risk of developing endometrial cancer. (Table 2-1)

2.2. *MYH*-ASSOCIATED POLYPOSIS (MAP)

The association between *Myh* gene mutations and colorectal polyps and cancers was first reported by Nadal Al-Tassan et al. (2002). It was found in a family with adenoma polyposis without *APC* gene mutations or MSI in *MMR* genes.[5] *Myh* gene mutations were consistently found in this family. Biallelic *Myh* mutations have since been identified in approximately 20%-25% of FAP and AFAP patients, who are not *APC* gene mutations carriers, and in 1.4% of all cases of adenomatous polyposis.[4,36,59] This syndrome is defined as *Myh*-associated polyposis (MAP).

Myh-associated polyposis is an autosomal recessive syndrome. Mutations of *Myh* gene result in an accumulation of G:C toT:A transversions in many related genes. Research has found that many patients with MAP showed accumulation of transversions in growth regulatory genes, such as the *APC* gene and the *K-ras* gene.[4,7,60] (figure 2-2). Accumulation of G:C to T:A transversions in these genes causes further dysfunction of these genes and increases the risk of colorectal polyps and cancer development. The Y165C and G382D mutations were the first identified *Myh* gene mutations and account for approximately 80% of Caucasian MAP patients.[48,61] The mutation incidence of *Myh* gene is various in different races.[48] (Figure 2-2)

The clinical manifestation of MAP is similar to that of AFAP syndromes, with lower numbers of polyps found in the colorectal region, usually less than 100 polyps, although some reports have shown MAP patients with more than 100 polyps.[62] The polyps develop at later age, compared with FAP patients. As it has a recessive inheritance pathway, many patients develop sporadic colorectal polyps and some of them develop colorectal cancer even without polyp formation.[54]

2.3. *MYH* GENE MUTATIONS AND COLORECTAL CANCER

As the well known characteristic and obvious family history of FAP, the incident for FAP develops into colorectal cancer decreases dramatically. *Myh*-associated polyposis is now an important risk factor for the development of colorectal cancer. Inactivation of the *Myh* gene increases the risk of sporadic colorectal cancers. The *Myh* gene mutations are found in 20%-25% of FAP and AFAP patients without *APC* gene mutations[4,36] and a 33% microsatellite stable CRC shows an increase of

8-OG accumulation with a decrease of MYH proteins,[48] and accounts for 0.4% -0.75% of all colorectal cancers.[33]

Although MAP is inherited recessively, mono-allelic mutations of the *Myh* gene show a slight increase in the risk of developing colorectal cancers. Patients carrying a mono-allelic *Myh* mutation have an increased risk for colorectal cancer by ~3 fold,[47,48] and patents with bi-allelic *Myh* mutations have an increased risk of 50-300 fold of developing colorectal cancers.[33,48,63] Due to its insignificant family history and less symptomatic clinical features, around 50% of patients with MAP will be diagnosed with CRC.[47]

2.3.1. INDICATIONS FOR *MYH* GENE TESTING

1. *APC* gene-negative individuals with features of classic FAP/AFAP
2. Autosomal recessive inheritance of CRC or multiple adenomas
3. Brothers or sisters with known *Myh* mutation
4. Young onset of CRC with negative *MMR* gene mutation
5. Parents diagnosed to be *Myh* gene mutation carriers.[47]

2.4. LOH ANALYSIS

Loss of heterozygosity (LOH) is a somatic deletion of small fragments of DNA in a currently active allele which causes dysfunction of a particular gene, while the other allele is inactivate in nature. It usually happens in heterogeneity tumor suppressor genes,[61,64] such as *MMR* genes,[44] *MGMT* genes and *Myh* genes. Loss of heterozygosity is a prognostic factor for cancer development and progression.[28]

Table 2-1 Disease-Causing Mutations of the *Myh* Gene and Their Corresponding Proteins.

Intron/exon	Mutations and corresponding proteins.	Outcome of polymorphism
Intron 1	IVS1 + 5G/C	Decrease in transcription rate of up to 30%.[48,56]
Exon 2	P18L (55C→T) V22M (66G→A)	Changed signal binding site at mitochondria.[48,58]

Exon 3	Y90X (270 C→A)	Nonsense mutation.
Exon 7	Y165C (494 A→G)	Failure to recognize the sites of mismatch.[5,47]
Exon 8	V220M (658 G→A)	Missense changes; changes at the catalytic binding site of express protein.[4,65]
Exon 12	Q324R (971 A→G)	
Exon 12	Q324H (972G→C)	Associated with increased risk of endometrial cancer.[66]
Exon 13	G382D (1145G→C)	Changes the catalytic core of glycosylase.[5,48]
Exon 13	L406M (1216C→A)	
Exon 14	E466X (1396 G→T)	A missense mutation with unknown defect in DNA.[67]
Exon 14	466delE (1395_1397delGGA)	
Exon 16	S501F (1502 C→T)	Involved in nuclear signal pathway.[48,57]

[4,48,56,58,67,68]

Figure 2-1 Functions of *BER* genes.

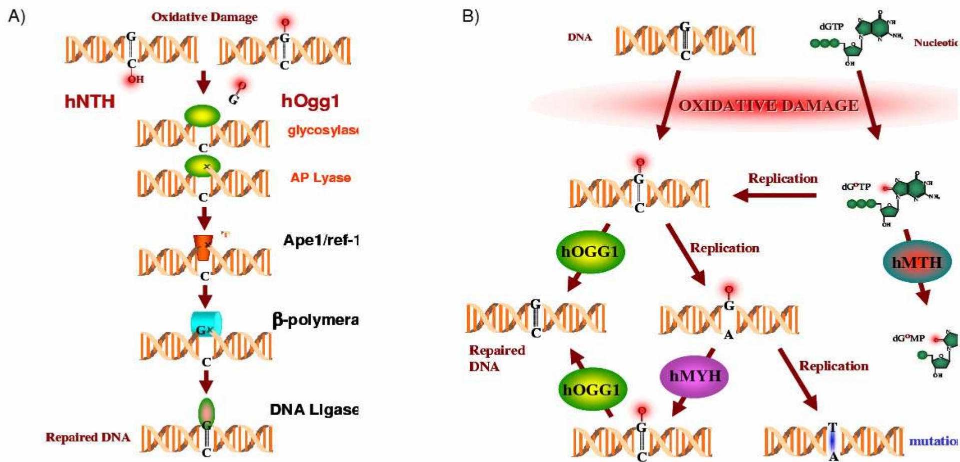


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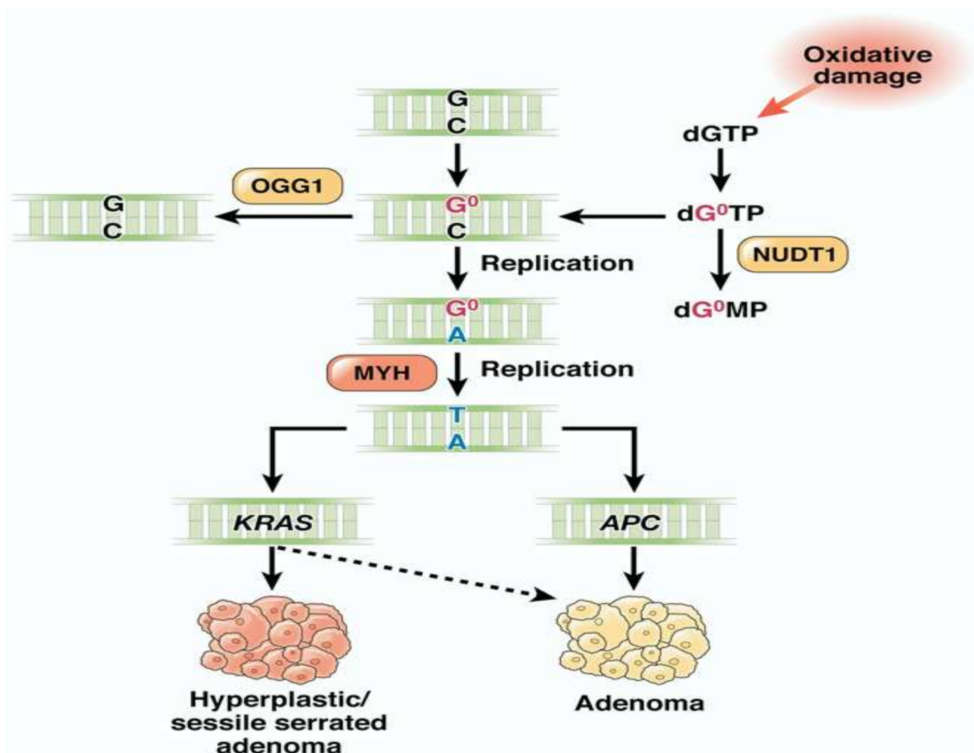
Figure 2-2 Results for Accumulation *Myh* Gene Defects in Different Genes.



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3. THE MAIN AIM

Specific mutations of the *Myh* gene are known to be related to colorectal cancers. The incidence of different *Myh* gene mutations varies between different races. The *Myh* gene status in the Polish population has never been explored. One has not demonstrated whether *Myh* gene mutations may also increase the risk of colorectal polyps. Since early screening of some colorectal cancer-related genes is already applied in high risk patients, there is still no consensus as to whether analysis of *Myh* gene status should also be included.

PARTICULAR AIMS

1) determination of the *Myh* gene variations in the Polish population
2) determination of the relationship between variations of *Myh* gene and the incidence of colorectal polyps and colorectal cancers
3) determination of the relationship between different hazardous lifestyles and habits and the incidence of colorectal polyps and colorectal cancers
4) determination of the relationship between different hazardous lifestyles and habits and particular *Myh* gene mutations.
5) determination of the rationale for of early *Myh* gene screening, if the *Myh* gene mutation variants are involved in the progression of colorectal polyps to colorectal cancers.

4. MATERIALS AND METHODS

4.1. STUDY GROUPS

The study group comprised 235 patients who underwent colonoscopy at the Wielkopolska Cancer Centrum in years 2004-2008. From these patients tissue samples (polyps) and blood were collected. The control group comprised 347 healthy people without any specific medical history who have not undergone colonoscopy. From control patients only blood was collected and sent to our laboratory for genetic analysis. The study was approved by local bioethical committee at the University of Medical Sciences. All patients participating in the study have signed an informed consent.

4.1.1. CRITERIA FOR COLONSCOPY

1. Presence of particular symptoms such as abdominal discomfort, bloody stools, changes in bowel habits and positive for occult blood in stools. 2. History of polyps. 3. History of CRC – follow-up colonoscopy. The study group comprised solely patients from whom colorectal polyps were obtained.

4.1.2. POLYPS AND BLOOD

Each removed polyp was divided into two parts, one was used for pathologic evaluation and another, along with whole blood, was used for genetic analysis. Particular information related to patients' age, gender, lifestyle, previous medical history and family history, was gathered using specific questionnaires. (Sec. 9)

4.1.3. CONTROL GROUP

4.2. REAGENTS

1 Agar (Gibco BRL)

2 Agarose (Promega)

3 Acrylamide (sigma)

4 Ammonium persulphate-APS (Sigma)

5 Bacterial component JM109 (Promega)

6 Bisacrylamide (Sigma)

7 Boric acid (Sigma)

8 Bromophenol blue power (Sigma)

9 Buffer

- Buffer for coralload PCR reaction contain 15 mM Mg^{2+} (Qiagen)
- Buffer for DNA sequencing 5× concentration (Promega)
- Buffer for electrophoresis :
  - buffer TAE 1× concentration
  - buffer TBE 1× concentration
- Buffer for DNA isolation-Wizard Genomic DNA Purification Kit (Promega):
  - Cell Lysis Solution
  - DNA rehydration solution
  - Nuclei Lysis solution
  - Protein precipitation solution
- Buffer for kinase 10× concentration (Promega)
- Buffer for ligation T4 2× concentration (Promega)
- Buffer for DNA mini-isolation Minipre Kit (Qiagen)
  - Buffer P1
  - Buffer P2
  - Buffer P3
- Buffer for DNA filtration (Qiagen)
  - Buffer QG
  - Buffer PE
  - Buffer EB
- Buffer for PCR reaction containing 15 mM Mg^{2+} (Qiagen)
- Buffer Q-Solution for PCR reaction (Qiagen)

10 dATP 100 mM (Promega)

11 dCTP 100 mM (Promega)

12 dGTP 100 mM (promega)

13 Dithanolamine (FLUKA Biochemika)

14 DNA ladder for 100 bp (Promega) (Figure 4-1)

15 DNA rehydration solution (Promega)

16 DNA Sequencing kit-DNA Cycle Sequencing System (Promega)

- ddA Nucleotide mix
- ddC Nucleotide mix
- ddG Nucleotide mix
- ddT Nucleotide mix
- DNA sequencing 5× buffer
- DNA sequencing stop solution
- Taq polymerase for DNA sequencing 5 u/µl (Promega)

17 dTTP 100 mM (promega)

18 EDTA (Sigma)

19 Enzyme

- Kinase T4 10 u/µl (Promega)
- Ligase DNA T4 3 u/µl (Promega)
- Taq polymerase for DNA 5 u/µl (Qiagen)
- Taq polymerase for DNA sequencing Taq 5 u/µl (Promega)

20 Ethanol (POCH)

21 Ethidium bromide (Sigma)

22 Formamide (Sigma)

23 Growth medium

- LB
- LB agar
- S.O.C (Invitrogen)

24 Glacial acetic acid (POCH)

25 Glycerol (Sigma)

26 Isopropanol (POCH)

27 Isotope [γ-^{32}P] ATP 110TB q/mmol; 3000 Ci/mmol (GE HEALTH CARE)

28 Luria broth (Sigma)

29 Penicillin G soda salt 1663 u/mg (Sigma)

30 Tetramethylethylenediamine (TEMED) 99% (Sigma)

31 Tris (Sigma)

32 Urea (Sigma)

33 Vector: pGEM®-T Easy 50 ng/μl (Promega)

34 Xylene cynade (Sigma)

Preparation for bacterial growth medium and plates

1. Preparation of LB medium
   25 g Luria broth
   Add water to 1000 ml
2. Preparation of LB plate
   250 ml of LB solution
   3 g of agar

Preparation of buffer for electrophoresis

1. 50× TAE
   242 g Tris
   57.1 ml glacial acetic acid
   100 ml 0.5M EDTA (pH 8)
   Add water to 1000 ml
2. 1× TAE
   20 ml 50× TAE
   980 ml water

10 µl of ethidium bromide

3. 10x TBE

   54 g Tris

   27.5 g boric acid

   20 ml 0.5 M EDTA (pH 8)

   Add water to 1000 ml

Preparation of 10 mM dNTP

   2.5 µl dATP

   2.5 µl dCTP

   2.5 µl dGTP

   2.5 µl dTTP

   Add water to 100 µl

Preparation of 40% PAG solution

   190 g acrylamide

   10 g bisacrylamide

   Add water to 500 ml

Preparation of 95% formamide dye

   47.5 ml of 99% formamide

   2.5 ml water

   A few drops of dithanolamine and xylene cynade

Preparation of gel for electrophoresis

1. 2% agarose gel

   2.0 g of agarose

   Add 1× TAE up to 100 ml

2. SSCP gel

   12 ml 40% PAG solution

   16 ml 50% glycerol

   8 ml 10× TBE

   Add water to 80 ml

3. DNA sequencing gel

   6% polyacrylamide gel with 7.5M urea

   1.5 g bisacrylamide

   28.5 g acrylaminde

   225 g urea

50 ml 10× TBE
Add water to 500 ml.

4. LOH analysis gel
   6% polyacrylamide gel with 7.5M urea
   1.5 g bisacrylamide
   28.5 g acrylaminde
   225 g urea
   50 ml 10× TBE
   Add water to 500 ml.

4.3. METHODS

4.3.1. DNA ISOLATION

4.3.1.1. DNA from polyp tissue

The isolation of DNA was performed according to the instructions of the Wizard Genomic DNA Purification kit.

Nuclei Lysis solution, at a volume of 600 µl, and 120 µl of 0.5M EDTA (pH>8) were mixed in a centrifuge tube on ice and then 0.5-1 cm of the polyp sample was mixed with 600 µl EDTA/Nuclei Lysis solution. To digest the polyp sample, 120 µl of proteinase K was added to the mixture which was then incubated at 55 °C overnight until the polyp was totally digested. Then 200 µl of Protein Precipitation Solution at room temperature was added and the mixture was vortexed vigorously at a high speed for 20 s. The sample was chilled on ice for 5 min before being centrifuged for 4 min at 13,000-16,000ˇ*g*. The supernatant containing the DNA was removed and placed into a new tube where it was mixed with 600 µl of isopropanol. The tube was gently inverted until the white thread-like strands of DNA appeared. The tube was centrifuged to remove the supernatant. The remaining DNA at the bottom of the tube was washed with with 600 µl of 70% ethanol. This was centrifuged again and the supernatant removed. The washed DNA was air dried and 100 µl of DNA rehydration solution was added. The DNA was stored at 2-8 °C. The amount of DNA was about 10 ng/µl.

4.3.1.2. DNA from blood tissue

The isolation of DNA was performed according to the instructions of the Wizard Genomic DNA Purification kit.

A volume of 300 µl of blood was mixed with 900 µl of Cell Lysis Solution. The mixture was incubated for 10 minutes at room temperature and inverted 5-6 times during incubation. It was then centrifuged at 13,000-16,000ˇ*g* and the supernatant was discarded. Vortex The centrifuge tube was vortexed and the sediment was allowed to re-suspend before adding 300 µl of Nuclei Lysis Solution into the re-suspend solution. The mixture was pipetted several times before adding 300 µl of Protein Precipitation Solution into the mixture and vortexing again. The mixture was centrifuged at 13000-16000ˇ*g* at room temperature for 3 min and the clean supernatant was removed and placed into a new tube. A volume of 300 µl isopropanol was added into the tube and mixed gently until the white band appeared. The tube was centrifuged and the supernatant was removed. The remaining DNA at the bottom of the tube was washed with 600 µl of 70% ethanol, centrifuged again and the supernatant was removed. The washed DNA was air dried and 100 µl of DNA rehydration solution was added before storing at 2-8 °C. The amount of DNA was about 10 ng/µl.

4.3.2. PCR-SSCP

4.3.2.1. Confirmation of DNA isolation and determination of the best reaction temperature

In order to confirm the success of the DNA isolation and to find out the best PCR temperature for each exon, PCR was performed. Four DNA samples were randomly chosen from either the blood or polyp samples

for each exon. The PCR was performed PCR at 45 °C, 50 °C, 55 °C, 60 °C

and 65 °C. (Table 4-1)

The amount used for the PCR reaction was 25 µl: 5 µl of DNA and 20 µl of the master mix. A volume of 500 µl master mix was prepared for each exon and the reaction was carried out at different temperatures.

Reagents	Master mix	Concentration in each reaction tube.
Colarload PCR buffer 10×	50 µl	1×
5× Q solution	100 µl	1×
Forward Starter150 µM	5 µl	1.5 µM
Reverse Starter150 µM	5 µl	1.5 µM
dNTP 10 mM	5 µl	0.1 mM
Taq polymerase 5 u/µl	5 µl	0.05 u/µl
DNA samples ~10 ng/µl	100 µl	~2 ng/µl
Water	230 µl	

Thirty-five cycles of the PCR reaction followed the programmed temperatures below.

94 °C for 5 min - denaturation

94 °C for 15 s - denaturation Tested temperature (45 °C , 50 °C, 55 °C,60 °C and 65 °C) for 15 s - annealing 72 °C for 15 s - elongation	35 cycles

72 °C for 5 min - continuous elongation

After the PCR reaction was completed, 5 µl of each sample was checked in 2% agarose gel together with the 100 bp DNA ladder. The temperature with the most obvious bands indicated the appearance of the DNA and the best temperature for the PCR reaction.

4.3.2.2. PCR-SSCP analysis

4.3.2.2.1. Labeled primer with isotope

For the PCR-SSCP analysis, starters were labelled on 5'end with [gama-32P]ATP (3000 Ci/mmol, Amersham). After being labelled, they served as primers for the SSCP reaction, and 10 μl of primers were prepared for each exon.

Reagents	Mix	Concentration of mix
Forward Starter150 μM	1 μl	15 μM
Reverese Starter150 μM	1 μl	15 μM
Kinase 10 u/μl	1 μl	1 u/μl
Kinase buffer 10×	1 μl	1×
[gama-32P]ATP 3000 Ci/mmol	2 μl	
Water	4 μl	

Kinase reaction: 37 °C for 35 min and followed by 85 °C for 10 min.

4.3.2.2.2. PCR reaction

The volume used for the PCR reaction was 5 μl, including 4 μl of master mix and 1 μl of each DNA sample. Thirty-five cycles of PCR were performed at the best reaction temperature for the exon. Fifty samples were tested using 10 μl of primers and 250 μl of the master mix.

Reagents	Master mix	Concentration in each tube
PCR buffer 10×	25 μl	1×
5× Q solution	50 ul	1×
Primers	10 μl	
dNTPs 10 mM	2.5 μl	0.1 mM
Taq polymerase 5 u/μl	2.5 μl	0.05 u/μl
DNA ~ 10 ng/μl	50 μl	~3 ng/μl
Water	110 μl	

Ninety-five percent formamide dye was added at a volume of 20 µl and the sample were denaturated at 95 °C for 5 min. The samples were then ready for electrophoresis.

4.3.2.2.3. Electrophoresis

Solutions	Concentration in the gel
12 ml of 40% PAG solution	6% of PAG
16 ml of 50% glycerol	10% of glycerol
8 ml of 10× TBE	1× TBE
Add water to 80 ml	

The solution was gently mixed and 600 µl 10× APS and 60 µl of TEMED were added before loading the solution in between two glass plates. The space between the two glass plates was about 0.3 mm. The solution solidified at room temperature, forming the electrophoresis gel. The glass plates containing the gel in between them were moved to the electrophoresis machine and 1× TBE buffer was placed onto the top and bottom of the gel. A volume of 5 µl of each denaturated sample was loaded onto the combs of the gel. A maximum of 49 samples could be run in one gel. The voltage of the electrophoresis was 250V, overnight. The gel was placed onto Whatman 3MM paper, and the gel was dried and autoradiographed.

4.3.3. DNA PURIFICATION

1 After autoradiography there were different bands on the X-ray film which showed different patterns of mobility.

2 The corresponding sites of those different mobility bands on the gel were cut and marked as mutations.

3 One regular mobility band on the gel was also removed and marked as wild type.

4 The cut pieces of gel were soaked in water overnight. The DNA material on the gel dissolved into the water.

5 Re-amplification:

The same exon as used for the PCR-SSCP was used for the re-amplified PCR for mutations and wild type. The amount used in the PCR reaction was 25 µl: 5 µl of DNA material in the water and 20 µl of the master mix.
A volume of 150 µl master mix was used for 5 mutations and 1 wild type sample.

Reagents	Master mix	Concentration in each reaction tube
Colarload PCR buffer 10x	15 µl	1×
5× Q solution	30 µl	1×
Forward Starter150 µM	1.5 µl	1.5 µM
Reverse Starter150 µM	1.5 µl	1.5 µM
dNTP 10 mM	1.5 µl	0.1 mM
Taq polymerase 5 u/µl	1.5 µl	0.05 u/µl
DNA samples ~10 ng/µl	30 µl	~2 ng/µl
Water	69 µl	

The PCR reaction was performed at the best reaction temperature of the exon for 35 cycles. All of the PCR results were loaded onto 2% agarose gel and electrophoresiswas carried out at 100 mV. Each band on the gel was cut and prepared for DNA purification.

4.3.3.1. Column purification (QIA quick purification)

The cut agarose gels which contained the DNA material were placed into the centrifuge tube and 4 volumes of QG solution to 1 volume of DNA sample was added and incubated at 55 °C until the DNA sample was completely dissolved. The dissolved mixture was transferred into a spin column with a 2 ml collecting tube below and centrifuged for 30-60 s at 5000ˇ*g*. The solution in the collecting tube was discarded and the spin column was washed with 500 µl QG and quickly centrifuged. Following this, 500 µl of the wash buffer PE was added to the spin column and centrifuged for 2 min at 5000ˇ*g* followed by 30 s at 13,000-16,000ˇ*g*. The collecting tube was replaced with the centrifuge tube and the spin column was centrifuged with the centrifuge tube for 1 min at 13000-16000ˇ*g*. The centrifuge tubes were discarded, the new

tube was placed under the spin column and 30 µl of water was added to the centre of the spin column and centrifuged for 2 min at 13000-16000˘*g*.

The amount of DNA materials in the tubes were usually enough for DNA sequencing. Sometimes the DNA materials were just not enough, and the bacterial vector system was used to enhance the number of DNA fragments needed.

4.3.3.2. T-easy ligation

2× Rapid ligation buffer	5 µl
pGEM®-T Easy 50 ng/µl vector	1 µl
Purified DNA from column purification	3 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl

To ligate the purified DNA from column purification, the reagents were prepared as described above and left overnight at 4 °C.

4.3.3.3. Bacterial competent JM 109 transformation

For this procedure, the ligated purified DNA was inserted into bacterial plasmid DNA.

Each ligated sample (10 µl) was placed into one 50 µl vial of One Shot Cells Performed and incubated on ice for 30 min. Then the mixture was

heated at 42 °C for exactly 30 s and placed on ice. A volume of 250 µl of

SOC solution was added to each vial and incubated at 37 °C for 1 h with 225 rpm shaking. Penicillin G was applied to the LB agar plate prior to spreading the transformed cells onto the agar, where 100 µl from the transformation vial was applied. One transformed vial was sufficient for three agar plates. The plates were inverted and incubated at 37 °C overnight.

The surviving bacterial colonies looked like white spots on the agar

plates. Three to five colonies were chosen and placed into tubes containing 2 ml of LB medium. These bacterial growth tubes were

incubated overnight at 37°C with gentle shaking.

4.3.3.4. Isolation of the plasmid DNA

A volume of 1.5 ml of the LB medium was removed and placed into a centrifuge tube and centrifuged for 5 min at 6000˘*g*. The supernatant was discarded and 50 µl of the P1 buffer was added. The mixture was pipetted at room temperature; after 5 minutes, 150 µl of the P2 buffer was added at room temperature and left for 5 min. Then, 150 µl of the P3 buffer was added to the mixture on ice and gently mixed for 5 min.

The mixture was centrifuged at 4 °C for 10 min at 13000-16000˘*g* and the supernatant was collected. This procedure was repeated 2-3 times until there were no white sediments in the supernatant.

A volume of 400 µl isopropanol was added to the supernatant at 4 °C and gently mixed until the white DNA bands appeared. The mixture was centrifuged and the sediments washed with 70% ethanol. This was centrifuged again, the supernatant was removed and the DNA sediment was allowed to air dry before 30 µl of water was added and the DNA was stored at 2-4 °C.

4.3.4. DNA SEQUENCING

The forward template or reversed template of each exon served as the starter for DNA sequencing. The starter concentration required for sequencing was 1 µM: 1 µl of 15 µM DNA was diluted in 149 µl of water. The starters were labelled with [gama-32P] ATP 3000 Ci/mmol.

Reagents	Amounts	Concentration.
10× kinase buffer	1 µl	1×
Kinase 10 u/µl	1 µl	1 u/µl
Forward starters or reverse starters.	5 µl	0.5 µM

[gama-32P] ATP 3000 Ci/mmol.	2 µl	
Water	1 µl	

The DNA samples were either from the column purified DNA or isolated from the bacterial vector system. The master mix for DNA sequencing was as follows:

Reagents	Amounts	Concentration
5× DNA sequencing buffer	5 µl	1.39×
Taq polymerase for DNA sequencing 5 u/µl	1 µl	0.28 u/µl
Labelled primer for forward reaction or reverse reaction	2 µl	
Purified DNA	3 µl	
Water	7 µl	

The master mix was divided into four microtubes at a volume of 4 µl and 2 µl of one of the dNTPs (ddATP, ddCTP, ddGTP or ddTTP) was added to each microtube.

The PCR was performed at the best temperature for the specific exon for 35 cycles. A volume of 3 µl of sequencing stop buffer was added to each microtube and denaturation took place at at 95 °C for 5 min. Then 6% polyacrylamide gel with 7.5M urea mixed with 600µl of 10× APS and 60 µl of 99% TEMED were added. The mixture was evenly placed between two glass plates and allowed to solidify. The running buffer was 1× TBE and it was placed onto the top and bottom of the gel. A pre-run at 1200 V for 30 min was needed. Then, 3 µl of each reaction mixture was loaded according to the sequence ACGT. Electrophoresis was performed at 2000 V for around one and a half hours. The gel was placed onto Whatman 3MM paper, dried and then autoradiographed.

4.3.5. LOH ANALYSIS

Patients with different mobility bands on the PCR-SSCP were indicated by the LOH analysis, which was performed for both the blood and polyp samples. Two templates (below) were labelled with [gama-32P] ATP 3000 Ci/mmol.

D1S451	Forward: 5'-ATCATGGGACTTTGCAGC

	Reverse: 5’-ATGGCCTGGCATAGTGTG
D1S2677	Forward: 5’-GTTCTGGAGTTTGCCTCTAA Reverse: 5’-AGCTGTGATTGTNCCACT

Reagents	Mix	Concentration of the mix
Forward Starter150 μM	1 μl	15 μM
Reverese starter 150 μM	1 μl	15 μM
Kinase 10 u/μl	1 μl	1 u/μl
Kinase buffer 10×	1 μl	1x
[gama-32P]ATP 3000 Ci/mmol	2 μl	
Water	4 μl	

The reaction volume was 5 μl: 4 μl of master mix and 1 μl of DNA. The master mix was sufficient for 50 samples (250 μl).

Reagents	Master mix	Concentration in each tube.
PCR buffer 10×	25 μl	1×
5× Q solution	50 ul	1×
Primers	10 μl	
dNTPs 10 mM	2.5 μl	0.1 mM
Taq polymerase 5 u/μl	2.5 μl	0.05 u/μl
DNA ~ 10 ng/μl	50 μl	~3 ng/μl
Water	110 μl	

The PCR reaction was performed at 55 °C for both D1S451 and D1S2677, for 35 cycles.

94 °C for 5 min - denaturation

Steps	
94 °C for 15 s - denaturation 55 °C for 15 s - annealing 72 °C for 15 s - elongation	35 cycles

72 °C for 5 min - continuous elongation

A volume of 20 μl of 95% formamide dye was added to 5 μl of PCR reagents. Denaturation took place at 95 °C for 5 min. Electrophoresis was performed

using 6% polyacrylamide gel with 7.5M urea mixed with 600 µl of 10× APS and 60 µl of 99% TEMED. A pre-run at 1200 V was performed before loading 5 µl onto each comb and running at 2000 V for one and a half hours. The gel was placed onto Whatman 3MM paper, dried and autoradiographed.

4.3.6. STATISTICAL ANALYSIS

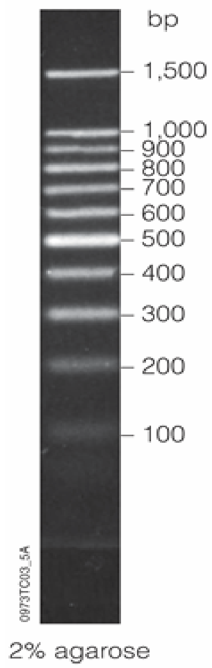
The Pearson's chi-squared (χ^2) and Fisher's exact tests were used to test the differences in genotype and allele distribution, between the patients and control subjects, respectively. Statistical analyses were performed using Statistica v.7.1 software (Statsoft, USA). For polymorphisms containing less than five observations per cell, the Fisher-Freeman-Halton exact test was performed using StatsDirect statistical software v.2.6.2. Logistic regression was employed to calculate the odds ratios (OR) at a 95% confidence interval (95% CI) and also to calculate the interactions. The odds ratios were calculated using a demonstration version of GraphPad InStat 2. Linkage disequilibrium analysis of the *Myh* and *Myh* gene polymorphisms was performed using the free online software Haploview version 4.0. All values were two-sided (http://www.broad.mit.edu/mpg/haploview/index.php).

Table 4-1 Twelve Exons for the *Myh* Gene

Exon 1	Forward: 5’-CAGAGCGCAGAGGCTTTGAAGG Reverse: 5’-CTGAACGGAAGTTCGACCCATC	60 °C
Exon 2	Forward: 5’-AATTTGGCCTCATTGTGACTGA Reverse: 5’-AATCTGCCTTTCATGGCCAATG	55 °C
Exon 3	Forward: 5’-CACAGGCTGCTGTGTCCCAAGA Reverse: 5’-CCCACCCACTGTCCCTGCTCCT	60 °C
Exon 45	Forward: 5’-AACTCCTCATCTGGGGTTGCAT Reverse: 5’-GGTCTGACCCATGACCCTTCCC	60 °C
Exon 67	Forward: 5’-ACCACCTTCACCCTTGACCTTG Reverse: 5’-ACCCAAGACTCCTGGGTTCCTA	50 °C
Exon 8	Forward: 5’-GGAACCCAGGAGTCTTGGGTGT Reverse: 5’-AAGGAGGCTGGGCACGCACAAA	55°C
Exon 9	Forward: 5’-TTTGTGCGTGCCCAGCCTCCTT Reverse: 5’-TGCTGTGAAGCAGAGCTCCTTT	55 °C

Exon 10	Forward: 5’-AAAGGAGCTCTGCTTCACAGCA Reverse: 5’-CACTCCTTAGGACTTCTCACTG	65 °C
Exon 11	Forward: 5’-GTAAGCCTACTGGGGAAGGGG Reverse: 5’-GCAGAATCTTACTCAGGTTAG	50 °C
Exon 12	Forward: 5’-GCCCTCTTGGCTTGAGTAGGGT Reverse: 5’-TCTCTTGTTACTCATGCCACTG	60 °C
Exon 13	Forward: 5’-AGGGAATCGGCAGCTGAGGCCT Reverse: 5’-AAAAGCCAACATCCTTGGCTAT	60 °C
Exon 14	Forward: 5’-TATATCCACAGGCCTATTTGAA Reverse: 5’-ATATTCATGTAGAACATGTAGG	55 °C
Exon 15	Forward: 5’-GACATGAAGTTAAGGGCAGAAC Reverse: 5’-TGTTCACCCAGACATTCGTTAG	55 °C
Exon 16	Forward: 5’-AACTACAAGGCCTCCCTCCTTCCA Reverse: 5’-AACAACAGGATTCTCAGGGAATG	60 °C

Figure 4-1 DNA Ladder for 100 bp (Promega)



5. RESULTS

5.1. GENERAL INFORMATION ABOUT THE STUDY GROUP

The study group comprised 126 males (51.43%) and 119 females (48.57%). The mean age of the study group was 59.57 years old. The study group was divided into two further groups, (i) age 60 or below and (ii) above 60 years of age, for the purpose of statistic analysis. Accordingly, 114 patients (45.53%) were 60 years old or younger and 113 (53.47%) were aged above 60 years. Seventy-seven patients (31.42%) were smokers and 168 (66.14%) were non-smokers; 131 (53.91%) patients had a positive family history of cancer of any kind and 112 (46.10%) had a negative family history of cancer; 27 (11.54%) patients had been previously treated for CRC or colorectal polyps; 30 (12.82%) patients were diagnosed with CRC at the moment of polyp removal. Among the studied population 76 patients (31.15%) were of normal body weight (BMI<25) and 168 (68.85%) were obese (BMI>25).

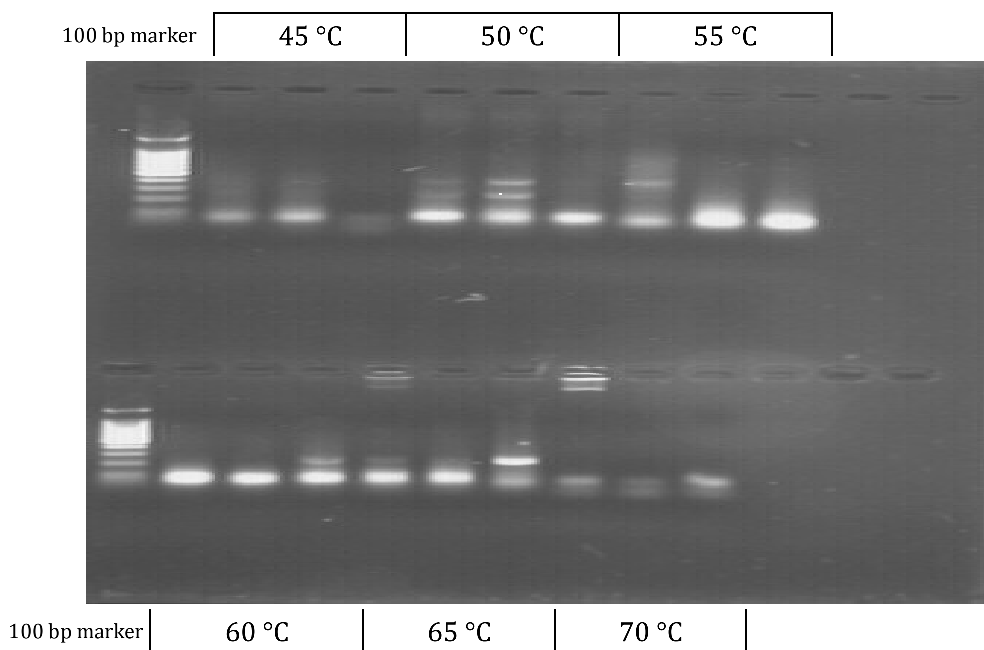
5.2. RESULTS FOR THE DNA ISOLATION AND TEMPERATURE TESTS

Determination of the best reaction temperature for each exon based on the observation of the lightest band on the electrophoresis gel under UV light.

Exon 1	60 °C	Exon 8	55 °C	Exon 13	60 °C

Exon 2	55 °C	Exon 9	55 °C	Exon 14	55 °C
Exon 3	60 °C	Exon 10	65 °C	Exon 15	55 °C

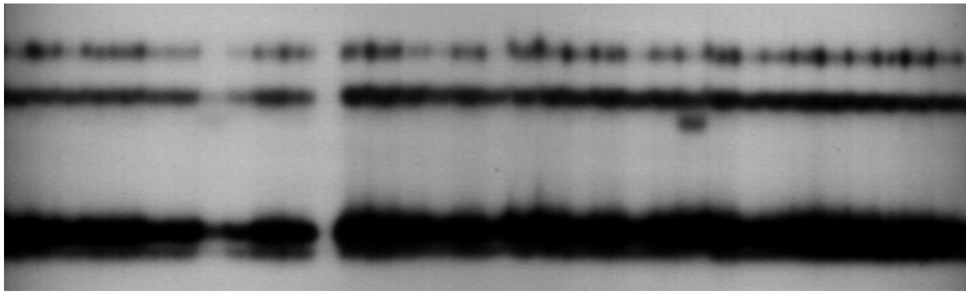
The *Myh* exon 1 temperature test



For Exon 1, the most visible bands were at the temperature of 60 °C, and this temperature was used for the PRC-SSCP and sequencing of exon 1.

5.3. RESULTS FOR PCR-SSCP

The *Myh* exon 2 PCR-SSCP

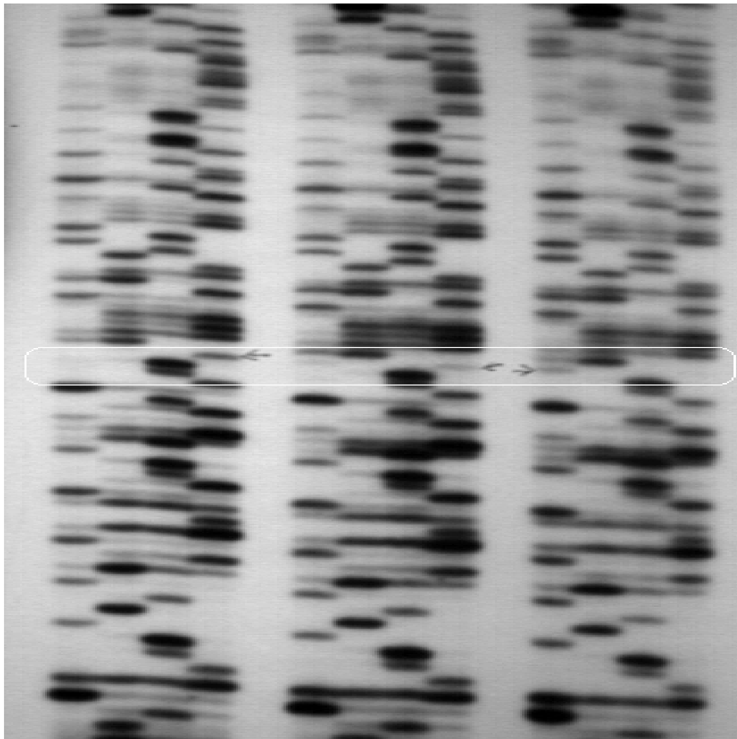


Wild-type bands are bands that are similar to the most of the other bands; in this case it meant patients with the same fragment for the *Myh* exon 2. Mutation bands are bands which show different mobilities to wild-type bands. All of the mutation bands and one wild-type band were collected for DNA sequencing analysis.

5.4. RESULTS FOR THE DNA SEQUENCING ANALYSIS

5.4.1. *MYH* EXON 2 FORWARD

Wild type	Mutation 1	Mutation 2
A T C G	A T C G	A T C G



Reading the result from the bottom to the top:

Myh exon 2 wild-type reading

6686 gccat catgaggaag ccacgagcag ccGtgggaag tggtcacagg aagcaggcag

6741 ccagccagga aggga

Myh exon 2 mutation bands reading:

6686 gccat catgaggaag ccacgagcag ccAtgggaag tggtcacagg aagcaggcag

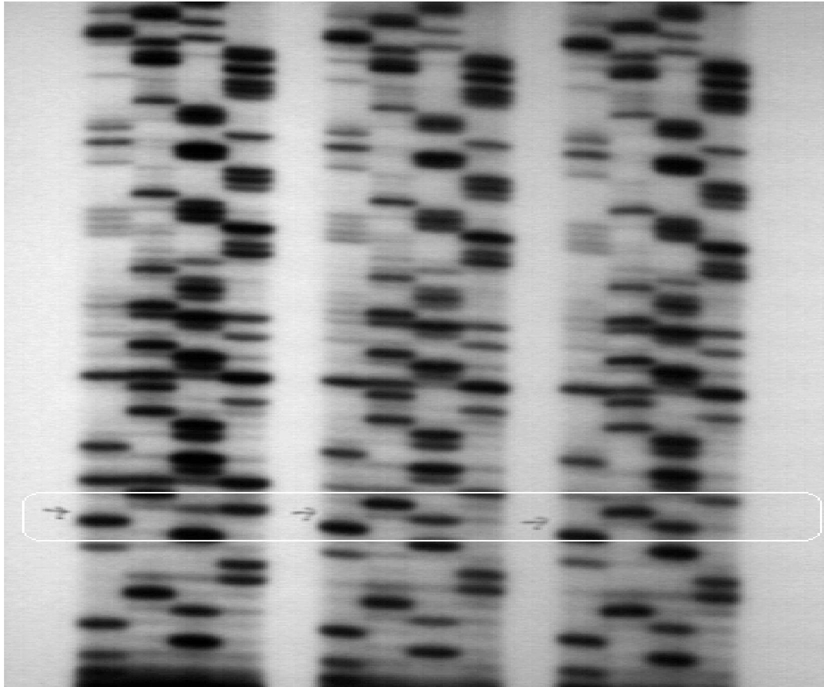
6741 ccagccagga aggga

This G to A conversion changed the number 22 protein from valine to methionine, (V22M).

5.4.2. *MYH* EXON 12 FORWARD

Wild type   Mutation 1   Mutation 2

A   T   C   G   A   T   C   G   A   T   C   G



Myh exon 12 wild type reading:
9354 acactgg aca**G**tgccac ctgtgcctgc ctccctcgga gccctgggac cagaccctgg 9411 gagtggtcaa

Myh exon 12 mutation reading:
9354 acactgg aca**C**tgccac ctgtgcctgc ctccctcgga gccctgggac cagaccctgg 9411 gagtggtcaa

The correspondent protein change was protein 324, glutamine to histamine (Q324H).

Instead of the most common mutations, Y165C and G382D, for white populations, the Polish population showed a different pattern of *Myh* gene mutations. The V22M and Q324H were more frequent in the Polish population. We found no Y165C and G382D mutations in our experiment.

5.5. LOH ANALYSIS

LOH analysis was performed on every mutated sample of polyps and blood. No

LOH was found in the study.

5.6. STATISTICAL ANALYSIS

5.6.1. FREQUENCY DISTRIBUTIONS OF SELECTED VARIABLES FOR THE V22M *Myh* (Val22Met) POLYMORPHISM

There were 35 patients with V22M polymorphisms in the study group, 19 of these patients were aged 60 or below and the rest 16 patients were above 60 years. Statistical analysis showed no significant difference in the distribution of V22M polymorphism between both age groups. The P-value was 0.672 for group age 60 or below and 0.6978 for group above 60 years old (Table 5-1).

In next analysis 18 V22M polymorphisms were found in males and 17 in females. There was no significant difference in occurrence of V22M polymorphism in relation to gender. The P-value was 0.9885 for male patients and 0.9881 for female patients.

Ten smoking patients with the V22M polymorphism were found, whereas lack of the V22M polymorphism was found among 77 smokers. The V22M polymorphism was found in 25 out of 150 non-smoking patients. Statistic analysis showed no significant difference in V22M polymorphism distribution between smoking and non-smoking patients. The P-value for non-smoking patients was 0.9727 and for smoking patients it was 0.9117.

The V22M polymorphism was found in 19 out of 151 patients who consumed alcohol regularly and in 16 out of 93 patients who never drank. There was no statistical difference in distribution of the V22M polymorphism between drinking and non-drinking patients. The P-value for drinking patients was 0.7305 and for non-drinking patients it was 0.6278.

5.6.2. FREQUENCY DISTRIBUTIONS OF SELECTED VARIABLES FOR THE Q324H *Myh*(Gln324His) POLYMORPHISM

The Q324H polymorphism occurred in 31/107 patients whose age were 60 or below and in 42/122 patients aged above 60 years. There was no statistical

significance in the distribution of Q324H polymorphism between both age groups. The P-value was 0.6817 and 0.7151 for ≤60 and > 60 populations, respectively (Table 5-2).

Regarding the gender, 40/121 male patients and 33/105 female patients were positive for the Q324H polymorphism. Statistical analysis showed no differences in Q324H distribution between male and female patients. The P-value for male was 0.9169 and for female - 0.9059.

For differences in smoking habit, 53/157 non-smoking patients and 20/72 smoking patients were found to have the Q324H polymorphism. There was no statistical significant in difference in distribution of Q324H polymorphisms between smoking and non-smoking patients. The P-value for non-smokers was 0.7882 and for 0.6097 for smokers.

The Q324H polymorphism was found in 45/144 patients, who regularly consumed alcohol and in 28/85 of non-drinking patients. There was no significant Q324H distribution difference between drinking and non-drinking patients. The P-value for the drinking group was 0.9900 and for the non-drinking group it was 0.9665.

5.6.3. GENOTYPE DISTRIBUTIONS AND ALLELE FREQUENCIES OF THE V22M *Myh* (Val22Met) POLYMORPHISM FOR GENES OF PATIENTS WITH COLON POLYPS AND THE CONTROL GROUP

Regarding the genotype, in the study group the 22 Val/Val genotype was detected in 209 patients and in 35 patients the 22 Val/Met genotype was revealed. In the control group 271 individuals were diagnosed with the 22 Val/Val genotype and 39 individuals with the 22 Val/Met genotype. The difference in distributions of 22 Val/Val and 22 Val/Met between the study and the control groups were not statistically significant. The P-value for 22 Val/Val was 0.9209 and for 22 Val/Met it was 0.6858 (Table 5-3).

The analysis of particular alleles revealed 453 Val alleles and 35 Met alleles in the study group and 581 Val alleles and 39 Met alleles in the control group. Statistical analysis showed no significant differences in the distributions of these alleles between the study group and the control group. The P-value for

Val alleles was 0.9482 and for the Met alleles it was 0.6710.

5.6.4. GENOTYPE DISTRIBUTIONS AND ALLELE FREQUENCIES OF THE Q324H *Myh* (Gln324His) POLYMORPHISM FOR GENES OF PATIENTS WITH COLON POLYPS AND THE CONTROL GROUP

For the genotype, in the study group 157 patients were diagnosed with the 324Gln/Gln genotype and 73 patients with the 324 Gln/His polymorphism. In the control group, in 268 individuals the 324 Gln/Gln genotype and in 79 patients the 324 Gln/His genotype were detected. There were no statistically significant differences in distributions of 324 Gln/Gln and 324 Gln/His between the study and control groups. The P-value for 324 Gln/Gln in the study and control groups was 0.3829 and for 324 Gln/His it was 0.0846 (Table 5-4).

For the alleles, 387 Gln alleles and 73 His alleles were found in the study group and 615 Gln alleles and 79 His alleles were found in the control group. Statistical analysis showed no significant differences in the distributions of these alleles between the study and the control groups. The P-value for the Val alleles in the study and control groups was 0.5870 and for His alleles was 0.0661.

5.6.5. THE ASSOCIATION BETWEEN THE V22M *Myh* (Val 22Met) AND THE Q324H *Myh* (Gln324His) POLYMORPHISM IN THE STUDY GROUP

In the study group, there were 209 wild-type genotypes and 35 Val/Met polymorphisms in exons 2. In exon 12, 157 wild-type genotypes and 73 Gln/His polymorphisms were found. There was no significant association between wild type of these two exons. The P-value was 0.1205. The association of the heterozygote mutation between V22M and Q324H was statistically significant. The P-value was 0.0005 (Table 5-5).

5.6.6. THE ASSOCIATION BETWEEN THE V22M *Myh*(Val 22Met) AND THE Q324H *Myh* (Gln324His) POLYMORPHISM IN THE CONTROL GROUP

In the control group, 271 wild-type genotypes and 39 Val/Met polymorphisms were found in exon 2. In the same group in exon 12, 268 wild-type genotypes and 79 Gln/His polymorphisms were found. There was no statistically significant difference in wild type association between these two exons. The P-value was 0.3139. The association of heterozygote mutation between V22M and Q324H was statistically significant. The P-vale was 0.0061 (Table 5-6).

5.6.7. THE ASSOCIATION BETWEEN CANCER FAMILY HISTORY AND V22M *Myh*(Val22Met) POLYMORPHISM

The family history of cancer is believed to be a risk factor for colorectal polyps. In our experiment, 131 patients were positive and 112 patients were negative for a family history of cancer. Regarding the genotype, 114/131 patients were wild type without mutation, and 17/131 patients were positive for the 22 Val/Met polymorphism. Furthermore, 94/112 patients were wild type and 18/112 patients were positive for the V22M polymorphism, in patients with a negative family history of cancer. The association between family history of cancer and the V22M polymorphism distribution was not statistically significant. The P-value for wild-type genotype between the group of patients with positive family history for cancer and the group with negative family history for cancer was 0.9235. The P-value for the V22M polymorphism between the groups of patients with positive and negative family history for cancer was 0.6814 (Table 5-7).

Analysis of the distribution of alleles revealed 245 Val alleles and 17 Met alleles in the group of patients with positive family history for cancer. 206 Val alleles and 18 Met alleles were found among patients with negative family history for cancer. No statistically significant differences were found in the between family cancer history and the distribution of particular alleles. The P-value for Val allele distribution between the positive groups with positive and negative family history for cancer was 0.9509. The P-value for Met allele distribution between both groups was 0.6628.

5.6.8. THE ASSOCIATION BETWEEN FAMILY HISTORY FOR CANCER AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

Of the studied patients in 122 cases, positive histories for cancer have been found and in 106 patients the incidence of cancer in families was not demonstrated. Genotype analysis revealed that 88/122 patients were wild type without mutation and 34/122 patients were positive for the 324 Gln/His polymorphism. Furthermore, among patients with negative family history for cancer 67/106 patients were wild type and 39/106 patients were positive for the Q324H polymorphism. There were no statistically significant differences in the Q324H polymorphism distribution between both groups. The P-value for wild type between both groups was 0.5991. The P-value for the Q324H polymorphism between both groups was 0.3969 (Table 5-8).

In further studies 210 Gln alleles, 34 His alleles and 173 Gln alleles, 39 Hist alleles were found in patients with positive and negative family history of cancer, respectively. No statistically significant correlation between family history of cancer and the distribution of alleles was found. The P-value for the Gln allele between the positive family history of cancer group and the negative family history of cancer group was 0.7542. The P-value for the Hist allele between both groups was 0.3295.

5.6.9. THE ASSOCIATION BETWEEN ADVANCED COLORECTAL POLYPS AND THE V22M *Myh* (Val22Met) POLYMORPHISM

There were 224 patients whose polyps were defined as low risk for the development of colorectal cancers and 20 patients with advanced polyps. In the current study one has tried to determine whether there were any associations between the V22M polymorphism and the particular polyp type. In patients with low-risk polyps, 191 polyps were wild type and 33 polyps were V22M polymorphism. In patients with advanced polyps, 18 polyps were wild type and 2 were V22M polymorphism. The statistical analysis showed no significant association between the V22M polymorphism and the type of polyp. The P-value for the wild-type genotype between the low and high risk polyps was 0.8736. The P-value for the V22M polymorphism between the low and high risk polyps was 0.8168 (Table 5-9).

The study found 415 Val alleles and 33 Met alleles in the low risk polyp group and 38 Val alleles and 2 Met alleles in the advanced polyps group. There was no statistically significant difference in the distribution of Met alleles and the

advanced polyps. The P-value for the distribution of the Val allele between the low risk polyps and the advanced polyps was 0.9151. The P-value for the distribution of the Met allele between the low risk and high risk polyps was 0.8414.

5.6.10. THE ASSOCIATION BETWEEN ADVANCED COLORECTAL POLYPS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

There were 211 polyps defined as low risk and 19 polyps defined as high risk; 140/211 patients with low-risk polyps had wild-type genotypes and 71/140 patients had the Q324H polymorphism. Among patients with high-risk polyps, 16/18 patients had wild-type genotypes and 2/18 patients had the Q324H polymorphism. The association between the Q324H polymorphism and the advanced polyp type was not statistically significant. The P-value for the wild-type genotype between the low risk polyps and the advanced polyps was 0.5284. The P-value for the Q324H polymorphism between the low risk polyps and the advanced polyps was 0.2070 (Table 5-10).

In the current study 351 Gln alleles and 71 His alleles were found among patients with low risk polyps and 34 Gln alleles and 2 His alleles were found among patients with high-risk polyps. There was no statistically significant difference between the distribution of Met alleles and the advanced polyps. The P-value for the distribution of the Gln allele between the low and high-risk polyps was 0.7013. The P-value for the distribution of the His allele between the low and high-risk polyps was 0.1829.

5.6.11. THE ASSOCIATION BETWEEN GENDER AND THE V22M *Myh* (Val22Met) POLYMORPHISM

There were 102/ 119 female patients with wild-type genome and 17/119 female patients with the heterozygote V22M polymorphism; 107 of 125 male patients were wild type and 18/125 male patients had the heterozygote V22M polymorphism. Statistical analysis showed no significant association between the V22M polymorphism and gender. The P-value for the wild-type genotype between female and male patients was 0.9944 and the P-value for the heterozygote mutation between female and male patients was 0.9824 (Table 5-11).

5.6.12. THE ASSOCIATION BETWEEN GENDER AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

There were 75/108 female patients with wild-type genome and 33/108 female patients with the heterozygote Q324H polymorphism; 81 of 121 male patients were wild type and 40/121 male patients had the heterozygote Q324H polymorphism. Statistical analysis showed no significant association between the Q324H polymorphism and gender. The P-value for the wild-type genotype between female and male patients was 0.9421 and the P-value for the heterozygote mutation between female and male patients was 0.8752 (Table 5-12).

5.6.13. THE ASSOCIATION BETWEEN CIGARETTE SMOKING AND THE V22M *Myh* (Val22Met) POLYMORPHISM

Genotype analysis found that 67/77 smoking patients had the 22 Val/Val genotype and 10/77 smoking patients had the 22 Val/Met genotype. For non-smoking patients, 142/167 patients had the 22 Val/Val genotype and 25/147 had the 22 Val/Met genotype. There was no statistical significance in the association between the V22M polymorphism and smoking. The P-value for the association between the 22 Val/Val genotype and the smoking and non-smoking groups was 0.9898. The P-value for the association between the 22 Val/Met genotype and the smoking and non-smoking groups was 0.8717 (Table 5-13).

The allele analysis found that 114 Val alleles and 10 Met alleles were in the smoking group and 309 Val alleles and 25 Met alleles were in the non-smoking group. The association between the Met allele and smoking was not statistically significant. The P-value for the Val allele distribution between the smoking and non-smoking groups was 0.9953. The P-value for the Met allele distribution between smoking and non-smoking groups was 0.8578.

5.6.14. THE ASSOCIATION BETWEEN CIGARETTE SMOKING AND THE Q324H *Myh*(Gln324His) POLYMORPHISM

The genotype analysis found that 52 of 72 smoking patients had the 324 Gln/Gln genotype while 20 of 72 smoking patients had the 324 Gln/His

genotype. For non-smoking patients, 104 of 157 had the 324 Gln/Gln genotype and 53 of 157 had the 324 Gin/His genotype. There was no statistical significance in the association between the Q324H polymorphism and smoking. The P-value for the 324 Gln/Gln genotype in association with the smoking and non-smoking groups was 0.7802. The P-value of the 324 Gln/His genotype in association with the smoking and non-smoking groups was 0.6117 (Table 5-14).

The allele analysis found 112 Gln alleles and 20 His alleles in the smoking group and 261 Gln alleles and 53 His alleles in the non-smoking group. The association between the His allele and smoking was not statistically significant. The P-value for the distribution of the Gln allele between the smoking and non-smoking groups was 0.9571. The P-value for the distribution of the His allele between the smoking and non-smoking groups was 0.5768.

5.6.15. CIGARETTE SMOKING AND THE RISK FOR ADVANCED COLORECTAL POLYPS

Of the 77 smoking patients, 3 were diagnosed with advanced (high-risk) colorectal polyps and 74 with low risk colorectal polyps. For the non-smoking patients, 17 were diagnosed with advanced colorectal polyps and 151 with low risk colorectal polyps. Statistic analysis showed an insignificant association between smoking and the incidence of high-risk polyps. The P-value was 0.2031 (Table 5-15).

5.6.16. CIGARETTE SMOKING AND THE RISK OF OCCURRENCE OF COLORECTAL POLYPS GREATER THAN 10 mm IN DIAMETER

Of the 77 smoking patients, 73 had polyps below 10 mm in diameter and 3 had polyps bigger than 10 mm. Out of the 168 non-smoking patients, 157 had polyps less than 10 mm in diameter and 11 had polyps bigger than 10 mm. The association between smoking and a polyp size greater than 10 mm was statistically insignificant. The P-value was 0.7828 (Table 5-16).

5.6.17. THE ASSOCIATION BETWEEN AGE AND THE V22M *Myh* (Val22Met) POLYMORPHISM

There were 114 patients below age 60 and 140 patients at age 60 or above; 95 patients, who was younger than 60 years old, had wild-type genotypes and 19 patients at this group had the V22M polymorphism; 114 patients, who were 60 years old or above, had wild-type genotypes and the rest 15 patients in this group had the V22M polymorphism. The associations between age <60 and ≥ 60 and the V22M polymorphism were not statistically significant. The P-value was 0.5112 (Table 5-17).

There were 209 Val alleles and 19 Met alleles in the group below 60 years and 244 Val alleles and 16 Met alleles in the group of 60 years old or above. Statistical analysis showed no significant difference in the association of Met alleles distribution and age. The P-value was 0.4898.

5.6.18. THE ASSOCIATION BETWEEN AGE AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

There were 107 patients below 60 years old and 122 patients aged 60 or above; 76 patients, who were younger than 60 years old, had wild-type genotypes and 31 patients aged below 60 had the Q324H polymorphism; 80 patients aged 60 or above had wild-type genotypes and 42 patients at this group had the Q324H polymorphism. The associations between age <60 and ≥60 years and the Q324H polymorphism were not statistically significant. The P-value was 0.6162 (Table 5-18).

There were 183 Gln alleles and 31 His alleles in the group of patients aged below 60 years and 202 Gln alleles and 42 His alleles in the group of patients aged 60 or above. Statistic analysis showed no difference between the distribution of Met alleles and age. The P-value was 0.5813.

5.6.19. THE ASSOCIATION BETWEEN AGE AND THE RISK OF DEVELOPING ADVANCED HIGH-RISK COLORECTAL POLYPS

In the studied population, 17/131 patients with advanced polyps were aged below 60 years and 3/114 patients with advanced polyps were aged 60 years or above. Statistical analysis showed a significant difference between the occurrence of advanced colorectal polyps and age. The P-value was 0.0085.

Younger patients were at higher risk of developing advanced colorectal polyps (Table 5-19).

5.6.20. THE ASSOCIATION BETWEEN AGE AND SIZE OF POLYPS

In the group of patients aged below 60 years, 119 of 131 patients had polyps <10 mm in diameter and 12/131 patients had polyps larger than 10 mm. In the group of patients who are 60 years old or above, 111 of 114 patients had polyps <10 mm in diameter and 3/114 patients had polyps larger than 10 mm. There was a trend toward association of age >60 years old and polyps >10 mm, however, the P-value was 0.0602, (Table 5-20).

5.6.21. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS AND ADENOMAS AND THE V22M *Myh* (Val22Met) POLYMORPHISM

There were 92 patients with hyperplastic polyps and 147 patients with adenomas; 80 of patients with hyperplastic polyps had wild-type genotypes, while 12 patients with hyperplastic polyps had the 22 Val/Met polymorphism. In the group of patients with adenomas, 124 of 147 patients had wild-type adenomas and 23 patients had adenomas with the 22 Val/Met polymorphism. There was no statistical significance regarding the associations between 22 Val/Val and hyperplastic polyps or adenomas. The P-value was 0.9541. There was also no statistical significance regarding the associations between the V22M polymorphism in the genotype and hyperplastic polyps or adenomas. The P-value was 0.7698 (Table 5-21).

There were 172 Val alleles and 12 Met alleles in the patients with hyperplastic polyps and 271 Val alleles and 23 Met alleles in patients with adenomas. There was no statistically significant correlation between the occurrence of Val alleles and hyperplastic polyps or adenomas. The P-value was 0.9714. The associations between Met alleles and hyperplastic polyps or adenomas were also statistically insignificant. The P-value was 0.7532.

5.6.22. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

There were 85 patients with hyperplastic polyps and 139 with adenomas; 54 of patients with hyperplastic polyps had wild-type genotypes and 31 patients had the 324 Gln/His polymorphism. In the group of patients with adenomas, 98 cases had wild-type adenomas and 41 cases had adenomas with the 324 Gln/His polymorphism. There was no statistically significant correlation between wild-type genotypes and hyperplastic polyps or adenomas. The P-value was 0.7127. There was also no statistically significant correlation between the Q324H polymorphism in the genotype and hyperplastic polyps or adenomas. The P-value was 0.5262 (Table 5-22).

There were 139 Gln alleles and 31 His alleles in patients with hyperplastic polyps and 237 Gln alleles and 41 His alleles in patients with adenomas. The occurrence of Gln alleles between hyperplastic polyps or adenomas had no statistical significance. The P-value was 0.8248. The associations between His alleles and hyperplastic polyps or adenomas were statistically insignificant. The P-value was 0.4864.

5.6.23. THE ASSOCIATION BETWEEN HYPERPLASTIC AND ADENOMA POLYPS AND THE V22M *Myh* (Val22Met) POLYMORPHISM IN SMOKING PATIENTS

The current study included 77 patients who were smokers, 35 of them had hyperplastic polyps and 42 others had adenomas. Of the patients with hyperplastic polyps, 33 patients had wild-type genotypes and the other 2 had the V22M polymorphism. Of the patients with adenomas, 34 of them had the wild-type genotype and 8 others had the V22M polymorphism. Statistic analysis showed no significant associations between wild-type genotype and hyperplastic polyps or adenomas in smoking patients. The P-value was 0.6665. There was also no statistically significant association between the V22M polymorphism and hyperplastic polyps or adenomas in smoking patients. The P-value was 0.2590 (Table 5-23).

In the smoking patients who were diagnosed with hyperplastic polyps, there were 68 Val alleles and 2 Met alleles; 76 Val alleles and 8 Met alleles were found in smoking patients diagnosed with adenomas. No statistically significant correlation was found between Val alleles and hyperplastic polyps or adenomas in smoking patients. The P-value was 0.8496. There was also no

statistically significant correlation between Mel alleles and hyperplastic polyps or adenomas in patients who smoked. The P-value was 0.2139.

5.6.24. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE V22M *Myh* (Val22Met) POLYMORPHISM IN NON-SMOKING PATIENTS

There were 162 non-smoking patients in the study group, 57 of them were diagnosed with hyperplastic polyps and 105 were with adenomas. Of the patients with hyperplastic polyps, 47 had wild-type genotypes and 10 had the V22M polymorphism. Of the patients with adenomas, 90 had the wild-type genotype and 15 had the V22M polymorphism. Statistical analysis showed no significant association between the wild-type genotype and hyperplastic polyps or adenomas in non-smoking patients. The P-value was 0.9704. There was also no statistically significant association between the V22M polymorphism and hyperplastic polyps or adenomas in non-smoking patients. The P-value was 0.8078 (Table 5-24).

In non-smoking patients diagnosed with hyperplastic polyps, there were 104 Val alleles and 10 Met alleles and 195 Val alleles and 15 Met alleles were found in non-smoking patients diagnosed with adenomas. No statistically significant association was found between Val alleles and hyperplastic polyps or adenomas in non-smoking patients. The P-value was 0.9831. Similarly, no statistically significant association was found between Mel alleles and hyperplastic polyps or adenomas in non-smoking patients. The P-value was 0.7889.

5.6.25. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM IN SMOKING PATIENTS

In the study group there were 72 smoking patients, of whom 34 were diagnosed with hyperplastic polyps and 38 with adenomas. Of the patients with hyperplastic polyps, 23 had wild-type genotypes and 11 the Q324H polymorphism. Of the patients with adenomas, 29 had the wild-type genotype and 9 the Q324H polymorphism. Statistic analysis showed no significant association between wild-type genotype and hyperplastic polyps or adenomas in smoking patients. The P-value was 0.8829. Similarly, no significant

association was found between the Q324H polymorphism and hyperplastic polyps or adenomas in smoking patients. The P-value was 0.7168 (Table 5-25).

In smoking patients diagnosed with hyperplastic polyps, 57 Gln alleles and 11 His alleles were found and 67 Gln alleles and 9 His alleles were found in smoking patients diagnosed with adenomas. No statistically significant correlation was found between Gln alleles and hyperplastic polyps or adenomas. The P-value was 0.9343. There was also no statistically significant association between His alleles and hyperplastic polyps or adenomas in smoking patients. The P-value was 0.6792.

5.6.26. THE ASSOCIATION BETWEEN HYPERPLASTIC POLYPS OR ADENOMAS AND THE Q324H *Myh* (Gln324Hid) POLYMORPHISM IN NON-SMOKING PATIENTS

Among 162 non-smoking patients in the study group, 51 were diagnosed with hyperplastic polyps and 101 were with adenomas. Among the patients with hyperplastic polyps, 31 had wild-type genotypes and others were with Q324H polymorphism. Of the patients with adenomas, 69 had the wild-type genotype and 32 had the Q324H polymorphism. Statistical analysis showed no significant association between the wild-type genotype and hyperplastic polyps or adenomas in non-smoking patients. The P-value was 0.7751. Also, no significant association was found between the Q324H polymorphism and hyperplastic polyps or adenomas in non-smoking patients. The P-value was 0.6363 (Table 5-26).

In the non-smoking patients diagnosed with hyperplastic polyps there were 82 Gln alleles and 20 His alleles and in the non-smoking patients diagnosed with adenomas 170 Gln alleles and 32 His alleles were found. No statistically significant correlation was found between Gln alleles and hyperplastic polyps or adenomas in the non-smoking patients. The P-value was 0.8712. Similarly, no statistically significant association was found between His alleles and hyperplastic polyps or adenomas. The P-value was 0.5953.

5.6.27. THE ASSOCIATION BETWEEN CRC PATIENTS AND OTHERWISE HEALTHY PATIENTS DIAGNOSED WITH POLYPS AND THE V22M *Myh* (Val22Met)

POLYMORPHISM

Thirty patients were diagnosed with colorectal cancer during the study, and 214 patients were diagnosed solely with colorectal polyps. Among the patients with colorectal cancer, 25 had the wild-type genotype and 5 had the V22M polymorphism. Among patients diagnosed with polyps, 184 had the wild-type genotype and 30 had the V22M polymorphism. No statistically significant association between the wild-type genotype and CRC or non-CRC patients. The P-value was 0.9138. Also, no statistically significant association was found between the V22M polymorphism and CRC-and non-CRC patients with polyps. The P-value was 0.9524.

There were 55 Val alleles and 5 Met alleles found in CRC patients diagnosed with polyps and 398 Val alleles and 30 Met alleles were found in non-CRC patients with polyps. Statistical analysis showed no significant difference between the frequency of Val alleles and CRC or non-CRC patients diagnosed with polyps. The P-value was 0.9426. No significant difference can be found between Met alleles and CRC or non-CRC patients diagnosed with polyps, either. The P-value was 0.9365.

5.6.28. THE ASSOCIATION BETWEEN CRC PATIENTS AND OTHERWISE HEALTHY PATIENTS DIAGNOSED WITH POLYPS AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

Among 28 patients diagnosed with CRC 201 patients with only polyps in our experiment. For the patients with colorectal cancer, 19 had the wild-type genotype and 9 had the Q324H polymorphism. Of the patients with only polyps, 137 had the wild-type genotype and 64 had the Q324H polymorphism. No statistical significance of wild-type genotype was found between the colorectal cancer patients with polyps and non-colorectal cancer patients with polyps. The P-value was 0.9011. Also, no statistical significance of Q324H polymorphism was found between colorectal cancer patients with polyps and non-colorectal cancer patients with polyps. The P-value was 0.8964 (Table 5-28).

There were 47 Gln alleles and 9 His alleles found in colorectal cancer patients with polyps and 338 Gln alleles and 44 His alleles were found in non-colorectal

cancer patients with polyps. Statistical analysis showed no significant difference of Gln alleles between colorectal cancer patients with polyps and non-colorectal cancer patients with polyps. The P-value was 0.9932. No statistical difference of His alleles was found between colorectal cancer patients with polyps and non-colorectal cancer patients with polyps. The P-value was 0.9804.

5.6.29. THE ASSOCIATIONS OF V22M *Myh* (Val22Met) POLYMORPHISM IN POLYPS-POSITIVE PATIENTS BETWEEN THE PRESNECE OR ABSENCE OF FAMILY HITORY OF COLORECTAL CANCER

There were 27 patients with positive family history of colorectal cancer and 217 patients whose family history was negative for this type of cancer. There were 25/27 patients with the 22 Val/Val genotype and 2/27 with the 22 Val/Met genotype. Among patients with negative family history 184 had the wild-type genotype and 33 had the V22M genotype. The occurrence of the wild-type genotype between patients with a family history of colorectal cancer and patients without such a family history was not significantly different. The P-value was 0.8804. The occurrence of the V22M genotype between patients with a positive family history for colorectal cancer and patients with a negative family history did not differ significantly. The P-value was 0.5003 (Table 5-29).

There were 52 Val alleles and 2 Met alleles found in the patients diagnosed with polyps with a positive family history of CRC. There were 401 Val alleles and 33 Met alleles found in patients with polyps who had a negative family history of CRC. No significant difference was found between the occurrence of Val alleles and positive or negative family history of CRC in patients diagnosed with polyps. The P-value was 0.9225. There was also no significant difference between the frequency of Met alleles and positive or negative family history of CRC in patients diagnosed with polyps. The P-value was 0.4802.

5.6.30. THE ASSOCIATIONS OF Q324H *Myh* (Gln324His) POLYMORPHISM IN POLYPS-POSITIVE PATIENTS BETWEEN POSITIVE AND NEGATIVE FAMILY HISTORY OF CANCER

There were 16 polyps-positive patients with family history of CRC who had the 324 Gin/Gin genotype and 10 who had the 324 Gln/His genotype. Among patients with negative family CRC history, 140 had the wild-type genotype and 63 had the Q324H genotype. The occurrence of the wild-type genotype among polyps-positive patients between positive or negative family history for CRC and was not significantly different. The P-value was 0.8630. The difference in occurrence of the Q324H genotype among polyps-positive patients between positive or negative family CRC history was also statistically insignificant. The P-value was 0.7406 (Table 5-30).

There were 42 alleles of Gln and 10 alleles of His found in polyps-positive patients with positive family CRC history and 343 Gln alleles and 63 His alleles found in the polyps-positive patients with negative family history for CRC . No significant difference was found between the occurrences of His or Gln alleles among patients with positive or negative family history for CRC. The P-value was 0.9247 and 0.7016, respectively.

5.6.31. THE ASSOCIATION BETWEEN BODY MASS INDEX AND THE V22M *Myh* (Val22Met) POLYMORPHISM

The body mass index (BMI) of 76 patients was within the normal range (BMI<25) and 168 patients were defined as being overweight or obese according to the BMI (BMI>25). In the group of patients with normal BMI group, 67 patients had the wild-type genotype and 9 patients had the V22M polymorphism. In the population of overweight or obese patients, 142 patients had the wild-type genotype and 26 patients had the V22M polymorphism. There was no statistically significant association between the occurrence of the wild-type genotype in the normal and the overweight/obese group. The P-value was 0.9153. The occurrence of the V22M polymorphism either showed no significant association with particular BMI group. The P-value was 0.6479 (Table 5-31).

There were 143 Val alleles and 9 Met alleles found in the normal BMI group and 310 Val alleles and 26 Met alleles found in the abnormal BMI group. No statistically significant difference was found between the Val alleles in the normal and abnormal BMI groups. The P-value was 0.9455. There was also no significant difference between the occurrences of the Met alleles in

particular BMI groups. The P-value was 0.6290.

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5.6.32. THE ASSOCIATION BETWEEN BODY MASS INDEX AND THE Q324H *Myh* (Gln324His) POLYMORPHISM

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The BMI of 69 patients was within the normal range (BMI<25) and 160 patient were defined as overweight or obese according to their BMI (BMI>25). In the normal BMI group, 49 patients presented with the wild-type genotype and 20 with the Q324H polymorphism. In the overweight or obese group, 107 patients presented with the wild-type genotype and 53 with the Q324H polymorphism. There was no statistical significant association between the occurrence of the wild-type genotype in the group of patients with normal BMI and increased BMI. The P-value was 0.8771. The occurrence of the Q324H polymorphism also showed no statistically significant difference between the BMI<25 and the BMI >25 groups. The P-value was 07652 (Table 5-32).

There were 118 Gln alleles and 20 His alleles found in the group of patients with normal BMI and 267 Gln alleles and 53 His alleles among patients with increased BMI. No statistically significant difference in the occurrence of Gln alleles between the groups with normal and increased BMI. The P-value was 0.9300. There was also no statistical difference of His Alleles found between in the BMI<25 and BMI>25 groups. The P-value was 0.7364.

Table 5-1 Frequency distributions of selected variables for the V22M *Myh* (Val22Met) polymorphism

Variables	V22M *Myh* positive cases n= 35	V22M *Myh* negative cases n= 209	P	OR, (95% CI)
age ≤60	19	95	0.6792	1.194 (0.6495 to 2.196)
age >60	16	114	0.6978	0.8381(0.4445 to 1.580)
male	18	107	0.9885	1.005 (0.5434 to 1.857)
female	17	102	0.9881	0.9952 (0.5321 to

				1.861)
non-smoker	25	142	0.9727	1.051 (0.6030 to 1.833)
smoker	10	67	0.9117	0.8913 (0.4189 to 1.896)
drinker	19	132	0.7305	0.8595 (0.4719 to 1.566)
non-drinker	16	77	0.6278	1.241 (0.6499 to 2.369)

Table 5-2 Frequency distributions of selected variables for the Q324H *Myh* (Gln324His) polymorphism

Variables	Q324H *Myh* positive cases n= 73	Q324H *Myh* negative cases n= 156	P	OR, (95% CI)
age ≤60	31	76	0.6817	0.8717 (0.5277 to 1.440)
age >60	42	80	0.7151	1.122 (0.7043 to 1.787)
male	40	81	0.9169	1.055 (0.6596 to 1.688)
female	33	75	0.9059	0.9403 (0.5732 to 1.542)
non-smoker	53	104	0.7822	1.089 (0.7069 to 1.678)
smoker	20	52	0.6097	0.8219 (0.4574 to 1.477)
drinker	45	99	0.9900	0.9714 (0.6200 to 1.522)
non-drinker	28	57	0.9655	1.050 (0.6173 to 1.785)

Table 5-3 Genotype distributions and allele frequencies of the V22M *Myh* (Val22Met) polymorphism for genes of patients with colon polyps and the control group

Gene	Polymorphism	Cases[a] (n=244)	Controls[a] (n=310)	P[b]	OR, P, (95% CI) [c]
	Genotypes				
	22 Val/Val	209	271	0.9209	0.9798 (0.7658 to 1.254)
	22 Met/Met	0	0		
	22 Val/Met	35	39	0.6858	1,140 (0.7011 to 1.854)
	Alleles				
	Val	453	581	0.9482	0.9906 (0.8351 to 1.175)
	Met	35	39	0.6710	1.140 (0.7115 to 1.827)
	Met allele	0.0717	0.0629	0.5603	

Table 5-4 Genotype distributions and allele frequencies of the Q324H *Myh* (Gln324His) polymorphism for genes of patients from the study and the control groups

Gene	Polymorphism	Cases[a] (n=230)	Controls[a] (n=347)	P[b]	OR, P, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	157	268	0.3829	0.8838 (0.6828 to 1.144)
	324 His/His	0	0		
	324 Gln/His	73	79	0.0846	1.394 (0.9733 to 1.997)
	Alleles				
	Gln	387	615	0.5870	0.9494 (0.7982 to 1.129)
	His	73	79	0.0661	1.394 (0.9929 to 1.957)
	His allele	0.158	0.114	0.135	

Table 5-5 The association between the V22M *Myh* (Val22Met) and the Q324H *Myh* (Gln324His) polymorphisms in the study group

Genotypes	V22M *Myh* (Val22Met) n=244	Q324H *Myh* (Gln324His) n= 230	P	OR, (95% CI)
Wild -type	209	157	0.1205	1.255 (0.9536 to 1.651)
Homozygote (mutant)	0	0	-	-

Heterozygote (mutant)	35	73	**0.0005**	0.4519 (0.2906 to 0.7028)

Table 5-6 The association between the V22M *Myh* (Val22Met) and Q324H *Myh* (Gln324His) polymorphisms in the control group

Genotypes	V22M *Myh* (Val22Met) n=310	Q324H *Myh* (Gln324His) n= 347	P	OR, (95% CI)
Wild type	271	268	0.3139	1.132 (0.9011 to 1.422)
Homozygote (mutant)	0	0	-	-
Heterozygote (mutant)	39	79	**0.0061**	0.5526 (0.3656 to 0.8353)

Table 5-7 The association between cancer family history and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	Positive family history for cancer n=131	Negative family history for cancer n=112	P[b]	OR, (95% CI) [c]
	Genotypes				
	22 Val/Val	114	94	0.9235	1.037 (0.7149 to 1.504)
	22 Met/Met	0	0	-	-
	22 Val/Met	17	18	0.6814	0.8075 (0.3972 to 1.641)
	Alleles				
	Val	245	206	0.9509	1.017 (0.7862 to 1.315)
	Met	17	18	0.6628	0.8075 (0.4064 to 1.604)
	Met allele	0.0649	0.0803	0.5130	

Table 5-8 The association between family history for cancer and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	Positive family history n=122	Negative family history n=106	Pb	OR, p, (95% CI) c
	Genotypes				
	324 Gln/Gln	88	67	0.5991	1.141(0.7567 to 1.721)
	324 His/His	0	0	-	-
	324 Gln/His	34	39	0.3696	0.7575 (0.4465 to 1.285)
	Alleles				
	Gln	210	173	0.7542	1.055 (0.8029 to 1.385)
	His	34	39	0.3295	0.7575 (0.4615 to 1.243)
	His allele	0.139	0.184	0.1917	

Table 5-9 The association between advanced colorectal polyps and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	Polyps with low risk of colon cancer n=224	Advanced colorectal polyps n=20	Pb	OR, P, (95% CI) c
	Genotypes				
	22 Val/Val	191	18	0.8736	0.9474 (0.4870 to 1.843)
	22 Met/Met	0	0	-	-
	22 Val/Met	33	2	0.8616	1.473 (0.3290 to 6.597)
	Alleles				
	Val	415	38	0.9151	0.9751(0.6133 to 1.550)
	Met	33	2	0.8414	1.473 (0.3408 to 6.368)
	Met allele	0.074	0.050	0.5740	

Table 5-10 The association between advanced colorectal polyps and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	Low risk polyps n=211	High-risk polyps n=18	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	140	16	0.5284	0.7464 (0.3682 to 1.513)
	324 His/His	0	0	-	-
	324 Gln/His	71	2	0.2070	3.028 (0.6854 to 13.381)
	Alleles				
	Gln	351	34	0.7013	0.8807 (0.5397 to 1.437)
	His	71	2	0.1829	3.028 (0.7130 to 12.862)
	His allele	0.168	0.055	0.0759	

Table 5-11 The association between gender and the V22M *Myh* (Val22Met) polymorphism

Genotypes V22M *Myh*	Female n=119	Male n=125	P	OR, (95% CI)
Wild type	102	107	0.9944	1.001 (0.6919 to 1.449)
Homozygote	0	0	-	-
Heterozygote	17	18	0.9824	0.9921 (0.4882 to 2.016)

Table 5-12 The association between gender and the Q324H *Myh* (Gln324His) polymorphism

Genotypes Q324H *Myh*	Female n=108	Male n=121	P	OR, (95% CI)
Wild type	75	81	0.9421	1.037 (0.6902 to 1.559)
Homozygote	0	0	-	-
Heterozygote	33	40	0.8752	0.9243 (0.5446 to 1.569)

Table 5-13 The association between cigarette smoking and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	Smokers n=77	Non- smokers n= 167	P[b]	OR, (95% CI) [c]
	Genotypes				
	22 Val/Val	67	142	0.9898	1.023 (0.6882 to 1.522)
	22 Met/Met	0	0	-	-
	22 Val/Met	10	25	0.8717	0.8675 (0.3970 to 1.896)
	Alleles				
	Val	144	309	0.9953	1.011 (0.7678 to 1.331)
	Met	10	25	0.8578	0.8675 (0.4065 to 1.851)
	Met allele	0.0649	0.0749	0.5496	

Table 5-14 The association between cigarette smoking and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	Smokers n= 72	Non- smokers n= 157	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	52	104	0.7802	1.090 (0.7061 to 1.683)
	324 His/His	0	0	-	-
	324 Gln/His	20	53	0.6117	0.8229 (0.4583 to 1.477)
	Alleles				
	Gln	122	261	0.9571	1.019 (0.7613 to 1.365)
	His	20	53	0.5768	0.8229 (0.4743 to 1.428)
	His allele	0.138	0.169	0.4004	

Table 5-15 Cigarette smoking and the risk of advanced colorectal polyps

Smoking status	Smoker	Non-smoker	P	OR, (95% CI)
Advanced colorectal polyps	3 (3.89%)	17 (10.12%)	0.2031	2.597 (0.7389-9.129)
Polyps with low risk of colon cancer	74 (96.11%)	151 (89.88%)	0.7672	0.9352 (0.6348-1.378)

Table 5-16 Cigarette smoking and the risk of colon polyps greater than 10 mm in size

Smoking status	Smoker	Non-smoker	P	OR, (95% CI)
Polyps ≤ 10 mm	73 (94.81%)	157 (93.45%)	1.000	0.9857 (0.6693-1.452)
Polyps >10 mm	4 (5.19%)	11 (6.55%)	0.7828	1.260 (0.3888-4.086)

Table 5-17 The associations between age and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	Age <60 n=114	Age ≥60 n=130	P[b]	OR, (95% CI)[c]
	Genotypes				
	22 Val/Val	95	114	0.8610	0.9503 (0.6559 to 1.377)
	22 Met/Met	0	0		
	22 Val/Met	19	16	0,5112	1.354 (0.6650 to 2.758)
	Alleles				
	Val	209	244	0,9091	0.9768 (0.7558 to 1.262)
	Met	19	16	0,4898	1.354 (0.6802 to 2.696)
	Met allele	0.0833	0.0615	0.3521	

Table 5-18 The associations between age and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	Age <60 n=107	Age ≥60 n= 122	P[b]	OR, (95% CI)[c]
	Genotypes				
	324 Gln/Gln	76	80	0.7791	1.083 (0.7207 to 1.628)
	324 His/His	0	0	-	-
	324 Gln/His	31	42	0.6162	0.8416 (0.4944 to 1.432)
	Alleles				
	Gln	183	202	0.8692	1.033 (0.7873 to 1.355)
	His	31	42	0.5813	0.8416 (0.5108 to 1.386)
	His allele	0.1448	0.1721	0.4262	

Table 5-19 The associations between age and the risk of occurrence of advanced colorectal polyps

Polyps	Age <60 n=131	Age ≥60 n=114	P	OR, (95% CI)
Advanced colorectal polyps	17 (12.98%)	3 (2.63%)	**0.0085**	4.931 (1.408- 17.265)
Polyps with low risk of colon cancer	114 (87.02%)	111 (97.37%)	0.5796	0.8937 (0.622 - 1.284)

Table 5-20 The associations between age and polyp size

Polyp size	Age <60 n=131	Age ≥60 n=114	P	OR, (95% CI)
Polyps ≤10 mm	119 (90.84%)	111 (97.37%)	0.7140	0.9329 (0.6506-1.338)
Polyps >10 mm	12 (9.16%)	3 (2.63%)	*0.0602*	3.481 (0.958-12.647)

Table 5-21 The associations between hyperplastic and adenoma polyps and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	Hyperplastic polyps n=92	Adenomas n=147	P[b]	OR, (95% CI)[c]
	Genotypes				
	22 Val/Val	80	124	0.9541	1.031(0.7026 to 1.512)
	22 Met/Met	0	0	-	-
	22 Val/Met	12	23	0.7698	0.8336 (0.3957 to 1.756)
	Alleles				
	Val	172	271	0.9714	1.014 (0.7777 to 1.322)
	Met	12	23	0.7532	0.8336 (0.4050 to 1.716)
	Met allele	0.0652	0.0782	0.5957	

Table 5-22 The associations between hyperplastic polyps or adenomas and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	Hyperplastic polyps n=85	Adenomas n=139	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	54	98	0.7127	0.9011 (0.5873 to 1.383)
	324 His/His	0	0	-	-
	324 Gln/His	31	41	0.5262	1.236 (0.7212 to 2.120)
	Alleles				
	Gln	139	237	0.8284	0.9591 (0.7224 to 1.273)
	His	31	41	0.4864	1.236 (0.7468 to 2.047)
	His allele	0.182	0.147	0.3277	

Table 5-23 The associations between hyperplastic polyps or adenomas and the V22M *Myh* (Val22Met) polymorphism in smoking patients

Gene	Polymorphism	Hyperplastic polyps n= 35	Adenomas n=42	P[b]	OR, (95% CI) [c]
	Genotypes				
	22 Val/Val	33	34	0.6665	1.220 (0.6350 to 2.345)
	22 Met/Met	0	0	-	-
	22 Val/Met	2	8	0.2590	0.3143 (0.06268 to 1.576)
	Alleles				
	Val	68	76	0.8496	1.074 (0.6807 to 1.694)
	Met	2	8	0.2139	0.3000 (0.06167 to 1.459)
	Met allele	0.0285	0.0952	0.0963	

Table 5-24 The associations between hyperplastic polyps or adenomas and the V22M *Myh* (Val22Met) polymorphism in non-smoking patients

Gene	Polymorphism	Hyperplastic polyps n=57	Adenomas n=105	P[b]	OR, (95% CI) [c]
	Genotypes				
	22 Val/Val	47	90	0.9704	0.9620 (0.5964 to 1.552)
	22 Met/Met	0	0	-	-
	22 Val/Met	10	15	0.8078	1.228 (0.5182 to 2.911)
	Alleles				
	Val	104	195	0.9831	0.9825 (0.7065 to 1.366)
	Met	10	15	0.7889	1.228 (0.5343 to 2.823)
	Met allele	0.0877	0.0714	0.5998	

Table 5-25 The associations between hyperplastic polyps or adenomas and the Q324H *Myh* (Gln324His) polymorphism in smoking patients

Gene	Polymorphism	Hyperplastic polyps n=34	Adenomas n=38	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	23	29	0.8829	0.8829 (0.4328 to 1.815)
	324 His/His	0	0	-	-
	324 Gln/His	11	9	0.7168	1.366 (0.5049 to 3.696)
	Alleles				
	Gln	57	67	0.9343	0.9508 (0.5875 to 1.539)
	His	11	9	0.6792	1.366 (0.5336 to 3.497)
	His allele	0.162	0.118	0.4471	

Table 5-26 The associations between hyperplastic polyps or adenomas and the Q324H *Myh* (Gln324His) polymorphism in non-smoking patients

Gene	Polymorphism	Hyperplastic polyps n=51	Adenomas n=101	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	31	69	0.7751	0.8897 (0.5178 to 1,529)
	324 His/His	0	0	-	-
	324 Gln/His	20	32	0.6363	1.238 (0.6446 to 2.377)
	Alleles				
	Gln	82	170	0.8712	0.9552 (0.6696 to 1,363)
	His	20	32	0.5953	1.238 (0.6743 to 2.272)
	His allele	0.196	0.158	0.4064	

Table 5-27 The associations between CRC- or non-CRC patients diagnosed with polyps and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	Polyps in CRC patients (n=30)	Polyps in otherwise healthy patients (n=214)	P[b]	OR, (95% CI) [c]
	Genotypes				
	22 Val/Val	25	184	0.9138	0.9692 (0.5501 to 1.707)
	22 Met/Met	0	0	-	-
	22 Val/Met	5	30	0.9524	1.189 (0.4282 to 3.301)
	Alleles				
	Val	55	398	0.9426	0.9858 (0.6670 to 1.457)
	Met	5	30	0.9365	1.189 (0.4441 to 3.183)
	Met allele	0.0833	0.0701	0.7107	

Table 5-28 The associations between polyps in CRC patients or otherwise healthy patients and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	Polyps in CRC patients (n=28)	Polyps in otherwise healthy patients (n=201)	P[b]	OR, (95% CI)[c]
	Genotypes				
	324 Gln/Gln	19	137	0.9011	1.040 (0.5589 to 1.936)
	324 His/His	0	0	-	-
	324 Gln/His	9	64	0.8964	1.055 (0.4731 to 2.351)
	Alleles				
	Gln	47	338	0.9932	0.9982 (0.6599 to 1.510)
	His	9	64	0.9804	1.009 (0.4760 to 2.141)
	His allele	0.161	0.159	0.9694	

Table 5-29 The associations of V22M *Myh* (Val22Met) polymorphism in polyps-positive patients between positive and negative family history for CRC.

Gene	Polymorphism	Polyps in patients with positive family history for CRC (n=27)	Polyps in patients with negative family history for CRC (n=217)	P[b]	OR, (95% CI)[c]
	Genotypes				
	22 Val/Val	25	184	0.8804	1.092 (0.6123 to 1.947)
	22 Met/Met	0	0	-	-
	22 Val/Met	2	33	0.5003	0.4871(0.1106 to 2.145)
	Alleles				
	Val	52	401	0.9225	1.042(0.6956 to 1.562)
	Met	2	33	0.4802	0.4871(0.1136 to 2.088)
	Met allele	0.037	0.076	0.2953	

Table 5-30 The associations of Q324H *Myh* (Gln324His) polymorphism in polyps-positive patients between positive and negative family history for CRC.

Gene	Polymorphism	Polyp patients with positive family history for CRC (n=26)	Polyp patients with negative family history for CRC (n=203)	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	16	140	0.8630	0.8923 (0.4616 to 1.725)
	324 His/His	0	0	-	-
	324 Gln/His	10	63	0.7406	1.239 (0.5668 to 2.710)
	Alleles				
	Gln	42	343	0.9247	0.9560 (0.6211 to 1.472)
	His	10	63	0.7016	1.239(0.5990 to 2.564)
	His allele	0.192	0.155	0.5820	

Table 5-31 The association between body mass index (BMI) and the V22M *Myh* (Val22Met) polymorphism

Gene	Polymorphism	BMI<25 (n=76)	BMI>25 (n=168)	P[b]	OR, (95% CI) [c]
	Genotypes				
	22 Val/Val	67	142	0.9153	1.043 (0.7010 to 1.552
	22 Met/Met	0	0	-	-
	22 Val/Met	9	26	0.6479	0.7652(0.3420 to 1.712)
	Alleles				
	Val	143	310	0.9455	1.020 (0.7740 to 1.343)
	Met	9	26	0.6290	0.7652 (0.3500 to 1.673)
	Met allele	0.059	0.077	0.4748	

Table 5-32 The association between body mass index (BMI) and the Q324H *Myh* (Gln324His) polymorphism

Gene	Polymorphism	BMI<25 (n=69)	BMI>25 (n=160)	P[b]	OR, (95% CI) [c]
	Genotypes				
	324 Gln/Gln	49	107	0.8771	1.062 (0.6835 to 1.650)
	324 His/His	0	0	-	-
	324 Gln/His	20	53	0.7652	0.8750 (0.4866 to 1.574)
	Alleles				
	Gln	118	267	0.9300	1.025 (0.7633 to 1.376)
	His	20	53	0.7364	0.8750 (0.5039 to 1.519)
	His allele	0.145	0.166	0.5738	

6. DISCUSSION

Instead of the most commonly found *Myh* mutations in white populations which are the Y165C and G382D polymorphisms, the Polish population tends to have V22M and Q324H polymorphisms. The difference in the type of *Myh* mutations between the Polish population and the whole Caucasian populations is quite significant. The roles of the Y165C and G382D polymorphisms in the development of colorectal polyps and colorectal cancer are well defined. If the V22M and Q324H polymorphisms are involved in colorectal polyp and colorectal cancer development, the mechanisms responsible for development of colorectal polyp and colorectal cancer in the Polish population may be different.

There was no significant difference in the frequency of analyzed *Myh* polymorphisms between the study and control group. This indicates that both polymorphisms found in the Polish population do not modify the risk of development of colorectal polyps or cancer. The V22M and Q324H polymorphisms have not been demonstrated to be involved in the development of colorectal polyps.

During the study 30 patients were diagnosed with colorectal cancer while undergoing prophylactic colonoscopy. Genetic analysis of these colorectal cancer patients did not show increased frequency of V22M and Q324H polymorphisms compared to patients diagnosed solely with colorectal polyps. This observation is in accordance with results of our study which demonstrated that V22M and Q324H polymorphisms are not involved in the progression of colorectal polyps to colorectal cancer. Additionally, the V22M and Q324H polymorphisms in the Polish population are not involved in the transition from normal colorectal tissue to colorectal polyps.

The *Myh* gene causes diseases via an autosomal recessive inheritance pathway. Theoretically, it causes diseases only when homozygous recessive genes occur. In a study of Fay Kastrinos et al and Takeshi Kambara et al, heterozygous mutations of the *Myh* gene were found to increase the risk of colorectal cancer by about 3 fold, whereas homozygous mutations of the *Myh* gene increased this risk by about 50-100 folds.[47,48] In the current study, homozygous mutations were not discovered. Both, the V22M and Q324H polymorphisms ware heterozygous mutations. No statistically significant increase in V22M or Q324H polymorphisms in polyp or

cancerous patients was found. The results indicate that heterozygous mutations of V22M and Q324H do not increase the risk of colorectal polyps and cancers.

No LOH was found in patients with V22M or Q324H polymorphisms. The LOH is designed to localize small genetic modifications that alter functions of particular genes. The negative LOH result means that the active *Myh* allele was still functional. It also explains why there was no increase in the incidence of colorectal polyps or colorectal cancer even though V22M and Q324H polymorphisms were found.

The close association between the V22M and Q324H polymorphisms indicates that the V22M polymorphism may cause the Q324H polymorphism. The result of the V22M polymorphism is a change in the signal binding site on the mitochondrial membrane.[48,58] Does this signal change cause further genetic changes in other exons? According to results of the current study, the occurrence of the V22M and Q324H polymorphisms seems to be closely related. The exact mechanism of this phenomenon is unknown. However, this close relationship between both polymorphisms is not involved in the development of colorectal polyps or cancer.

The Q324H polymorphism is supposed to be associated with extracolonic lesions, especially with endometrial cancers.[66] In the current study, in patients with family history of various cancers, a difference in the frequency of the V22M polymorphism or the Q324H polymorphism was not shown. In our female study group, no endometrial cancer was diagnosed with colorectal polyps. No second tumor was found in patients diagnosed with colorectal polyps. The association between Q324H polymorphism and extracolonic cancers is not obvious in Polish population.

Male population has been reported to have an increased incidence of colorectal cancer compared to females.[13,14] Colorectal polyps are the precancerous lesions, and the males are assumed to have more polyps than females. More genetic variations might be discovered in the male population. In the current study, two *Myh* polymorphisms, V22M and Q324H were equally distributed between both genders.

In the histopathological classification of colorectal polyps, class I and II are defined as low risk polyps, and class III is defined as advanced polyps. There were no statistically significant differences in the appearance of polyps among different age groups in the present study. However, in the younger patients (<60 years old), there was a higher tendency to develop advanced colorectal polyps. Colorectal polyps can

be found in the majority of people aged above 60 years. Most of those polyps are benign, with no malignant tendency, and usually no symptoms are associated with colorectal polyps.

Hyperplastic polyps and adenomatous polyps are thought to have different developmental pathways. The accumulation of *Myh* gene mutations in the K-ras gene leads to development of hyperplastic polyps[55] and the accumulation of *Myh* gene mutations in *APC* leads to development of adenomatous polyps.[55] The frequencies of the V22M and Q324H polymorphisms were not significantly different between the hyperplastic polyps and adenomatous polyps studied in the current experiment. Both polymorphisms were not involved in the development of these particular polyps.

Only 5% of colorectal cancers have a particular genetic bacground.[28,30,33,45,48,51] Most patients with genetic defects develop polyps early in life and these polyps convert into colorectal cancer at younger age than is usually observed in otherwise healthy patients. According to results of the current study, the difference in V22M and Q324H polymorphisms between high and low risk colorectal polyps was statistically insignificant. These two polymorphisms were not associated with the development of advanced polyps.

More polyps with a tendency of malignant transformation were found in Polish patients aged below 60 years. . The V22M and Q324H polymorphisms were not shown to lead to early development of advanced colorectal polyps. Thus the recommended age for starting prophylactic colonoscopy in otherwise healthy Polish population should be below 50 years.

Smoking is a well-known risk factor for various cancer types, including colorectal cancer. Since 90%of colorectal cancers originate from colorectal polyps it was important to evaluate whether smoking was a risk factor for the development of colorectal polyps. In the current study, there was no statistically significant relationship between smoking and the development of colorectal polyps or colorectal cancer. Some researchers reported that smoking is a risk factor for the development of hyperplastic polyps.[21] There is no predisposition for the development of hyperplastic polyps or other kinds of colorectal polyps in patients who smoke. There is no significant relationship between smoking and the size of the polyps. The larger the polyps are the greater possibility for malignant transformation. Smoking was not shown to be related to the development of large

polyps.

Smoking is one of many important environmental secondary hits for cancer genes.[22] Small mutations in cancer genes are called first hits. People with first hits in the cancer genes may not have an increased risk of cancer development. But when combined with environmental secondary hits, the risk of cancer increases.[69] Smoking does not increase the frequency of V22M and Q324H polymorphisms in patients with colorectal polyps or in patients with colorectal cancer. Smoking is not an environmental secondary hit in V22M or Q324H polymorphisms. Smoking has been demonstrated to be neither a risk factor for colorectal polyps nor for colorectal cancer.

Fatty acids are believed to be a main source of the oxidative stress mediators in the body.[25] The excess intake of fatty foods and overweight has been reported to increase the risk of colorectal cancer. The oxidative damage of normal genes increases the risk of cellular changes and the development of cancer. The overweight patients (BMI>25) were not at increased risk of colorectal polyps development and did not demonstrate increased frequency of V22M and Q324H polymorphisms. Overweight also was not demonstrated to cause the *Myh* gene polymorphism and probably it is not a risk factor for the development of colorectal polyps and colorectal cancer.

The inheritance pathway of some cancers, such as breast cancer has been well established.[70] Genetic defects can be passed on to further generations. A family history of cancer is always a very important indication for cancer screening at an early age. Genetic screening has become more and more popular in patients with family histories of particular cancers. In the current study, the incidence of V22M and Q324H polymorphisms showed was not significantly different between between groups of patients with positive or negative family history for cancer. Thus, no inheritance pathways were indicated in the V22M and Q324H polymorphisms.

Genetic mutations can cause accumulation of carcinogens throughout life and can ultimately lead to cancer development in older patients. Cancers are generally believed to be an old age disease, although the incidence of some types of cancer is increasing at earlier ages. In our study, cells with V22M and Q324H polymorphisms do not alter the ability to repair oxidative DNA damages even in older cells. The risk for younger patients to develop advanced colorectal polyps is higher than elder patients in Polish population, the frequencies of these two polymorphisms were

statistic insignificant between the different ages.

The exposure to environmental hazards is an important factor responsible for environmental secondary hits on cancer genes.[22,69] A long period and high frequency of exposure to environmental hazards increase the possibility of activation of oncogenes and inhibition of suppressor genes which ultimately lead to development of cancers. The degree of first hit genetic mutations and the amounts of smoking or alcohol consumption are known risk factors for cancer development. There are many environmental secondary hits, such as radiation, alcohol consumption, lifestyle, habits and other environmental hazards.

The current study compared frequencies of V22M and Q324H polymorphisms in patients who had smoking and alcohol drinking habits with patients who had no smoking or alcohol consumption habits. These two lifestyle choices have been reported to be the most common lifestyle hazards for inducing secondary hits on genetic variations. The equal appearance of these two polymorphisms in these two groups of patients indicated that the combination of cigarettes and alcohol did not increase the frequency of *Myh* gene variations found in our study.

Age, gender, being overweight, cigarette smoking and alcohol consumption do not increase the frequencies of V22M and Q324H polymorphisms in Polish population. Additionally, these two polymorphisms do not increase the risk of development of colorectal polyps or cancer. Morphologically, the relationship between these factors and colorectal polyps and cancer is well established. These factors increase the risk of both colorectal polyps and cancer. However, at the molecular level, the genes which are affected by these factors have not been determined.

The association between V22M and Q324H polymorphisms and incidence of colorectal polyps and cancer is not statistic significant. The early development of advanced polyps in the Polish population indicates that other genetic variations must also be involved. The genetic variations involved in the development of colorectal polyps and cancers may not be located only in the *Myh* gene exons. Or, the development of colorectal polyps and cancers in the Polish population might be mediated by other genetic pathways, such as involving *MGMT* or *MMR* gene mutations. Changes in *Myh* introns have been discovered and some of these variations were found to modulate gene translation and transcription, altering the normal gene functions.[48,56]

Genetic counseling is believed to represent a good preventive method for inherited cancers. Genetic counseling can prevent the progress of precancerous lesions into cancer. Such an approach prevents from cancer incidence by thorough cancer screening at an early age and removal of all possible precancerous lesions. FAP patients with *APC* gene mutations and patients with *BRCA1* or *BRCA2* germline gene mutations are examples of cancer prevention by genetic counseling. In the current study, *Myh* gene variations were not related to occurrence of colorectal polyps or cancer in the Polish population. This observation excludes *Myh* gene screening and subsequent genetic counseling as a preventive measure for the development of colorectal polyps or cancer.

7. CONCLUSION

1. The incidence of the most common *Myh* gene variations (V22M and Q324H) was not related with increased frequency of colorectal polyps.
2. The V22M and Q324H *Myh* gene polymorphisms were not associated with increased incidence of neoplastic transformation of colorectal polyps.
3. A close relationship between V22M and Q324H polymorphisms was found but it role has been not elucidated.
4. In the studied population, advanced, high-risk colorectal polyps were more frequent in younger patients aged below 60 years,
5. Age, gender, overweight, smoking or alcohol consumption were not shown to be associated with increased the frequency of V22M and Q323H polymorphisms.

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9. REFERRENCE

1. Dale C. Snover, M.D., Jeremy R. Jass, M.D., Cecilia Fenoglio-Preiser, M.D., and Kenneth P. Batts, M.D. Serrated Polyps of Large Intestine, A Morphology and Molecular Review of an Evolving Concept. American Journal of Clinical Pathology., 124: 380-391, 2005
2. Amy E. Noffsinger. Serrated Polyps and Colorectal Cancer: New Pathway to Malignancy. Annual Review of Pathology: Mechanisms of Diseases., 4: 343-364, 2009.
3. Andrew J. Aldridge and Jay N. L. Simson. Histological Assessment of Colorectal Adenomas by Size. Are Polyps Less than 10 mm in Size Clinically Important? European Journal of Surgery., 167: 777-781, 2001.
4. Jérémie H. Lefevre, M.D., Christelle M. Rodrigue, M.D., Najat Mourra, M.D., Malika Bennis, M.D., Jean-François Flejou, M.D., Rolland Parc, M.D. FACS., Emmanuel Tiret, M.D., Christian Gespach, Ph.D., and Yann R. Parc, M.D. Ph.D. Implication of *MYH* in Colorectal Polyposis. Annals of Surgery., 244: 874-880, 2006.
5. Yasser H. Shaib, M.D., Ehab Rabaa, M.D., and Tahir Qaseem, M.D. The Site Distribution and Characteristics of Colorectal Adenomas in Hispanics: A Comparative Study. The Ameircan Journal of Gastroenterology., 97(8): 2100-2012.2002.
6. Łukasz Krakowczyk, Joanna K.Strzelcyk, Brygida Adamek, Marzena Zalewska-Ziob, Jerzy Arendt, Stanisław Półtorak, Bogusław Maciejewski, and Andrzej Wiczkowski. Methylation of the *MGMT* and *p16* Genes in Sporadic Colorectal Carcinoma and Corresponding Normal Colonic Mucosa. Medical Science Monitor., 14(10): 219-225, 2008.
7. Mark A. Jenkins, Marina E. Croitoru, Neerav Monga, Sean P. Cleary, Michelle Cotterchio, John L. Hopper, and Steven Gallinger. Risk of Colorectal Cancer in Monoallelic and Biallelic Carriers of *MYH* mutations: A population-Based case-Family Study. Cancer Epidemiology, Biomarkers and Prevention., 15(2): 312-314, 2006.

8. Mary Beth Terry, Alfred I. Meugut, Roberd M. Bostick, John D. Potter, Robert W. Gaile, and Cecilia M. Fenoglio-Preiser. Reliability in the Classification of Advanced Colorectal Adenomas. Cancer Epidemiology, Biomarkers & Prevention., 11: 660-663, 2002.

9. Angel Ferrández, M.D., Wade Samowitz, M.D., James A. DiSario, M.D., and Randall W. Burt, M.D. Phenotypic Characteristics and Risk of Cancer Development in Hyperplastic Polyposis: Case Series and Literature Review. American Journal of Gastroenterology., 99: 2012-2018, 2004.

10. Carlos A. Rubio, Gabriella Nesi, Lucca MEsserini, Gian Carlo Zampi, Koichi Mandai, Masayuki Itabashi, and Kaiyo Takubo. The Vienna Classification Applied to Colorectal Adenomas. Journal of Gastroenterology and Hepatology., 21: 1697-1703, 2006.

11. Emina Torlakovic, M.D., Eva Skovlund, Ph.D., Dale C. Snover, M.D., Goran Torlakovic, M.D., and Jahn M. Nesland. M.D., Ph.D. Morphologic Reappraisal of Serrated Colorectal Polyps. The American Journal of Surgical Pathology., 27(1): 65-81, 2003.

12. N.J. Carr, H. Mahajan, K.L. Tan, N.J. Hawkins, and R.L. Ward. Serrated and Non-Serrated Polyps of the Colorectum: Their Prevalence in an Unselected Case Series and Correlation of BRAF Mutation Analysis with the Diagnosis of Sessile Serrated Adenoma. Journal of Clinical Pathology., 62:516-518, 2009.

13. Gavin C. Harewood. M.D., M,S,c and Garrett O. Lawlor, M.B. Incident Rated of Colonic Neoplasia According to Age and Gender: Implications for Surveillance Colonoscopy Intervals. Journal of Clinical Gastroenterology., 39(10): 894-899, 2005.

14. Shanmugarajah Rajendra. Jacqueline J. Ho, and John Arokiasamy. Risk of Colorectal Adenomas in the Multiethnic Asia Patient Population: Race Does Not Matter. Journal of Gastroenterology and Hepatology., 20: 51-55, 2005.

15. David A. Lieberman, M.D., Jeniffer L. Holub, M.P.H., Matthew D. Moraves, B.S., Glenn M. Eisen, M.D.Ph.D., Dawn Peters, Ph.D., and Cynthia D. Morris, Ph.D. Prevalence of Colon Polyps Detected by Colonoscopy Screening in Asymptomatic Black and White Patients. Journal of the American Medical Association., 300(12):1417-1422, 2008.

16. Hemant K. Roy, M.D., and Laura K Bianchi, M.D. Colorectal Cancer Risk: Black, White or Shades of Gray? Journal of the American Medical Association., 300(12):1459-1461, 2008.

17. Kimberly M. Kelly, Ph.D., Stephanie L. Dickinson, MAS, Cecilia R. Degraffinreid, MHS., Cathy M Tatum, MS., and Electra D Paskett, Ph.D. MSPH. Colorectal Cancer Screening in 3 Racial Groups. American Journal of Health Behavior., 31(5):502-513, 2007.

18. Nadal Al-Tassan, Nikolas H. Chmiel. Julie Maynard, Nick Fleming, Alison L. Livingston, Geraint T. Williams, Angela K. Hoges, D. Rhodri Davies, Shelia S. David, Julian R.. Sampson and Jeremy P. Cehadle. Inherited Variants of *MYH* Associated with Somatic G:C to T:A Mutations in Colorectal Tumors. Nature Genetics., 30:227-232, 2002.

19. Hakau, Unal, Haldun Selcuk, Hale, Golcan, Emin Tore, Aylin Sar, Murat Korkmaz, Banu Bilezikci, Beyhan Demirhan, Gurden Gur and Ugur Yilmaz. Malignant Risk of Small Polyps and Related Factors. Digestion Diseases Sciences., 52:2796-2799, 2007.

20. Yasuhiro Oono, Kuangi Fu, Hisashi Nakamura, Yosuke Iriguchi, Akihiko Yamamuta, Yasuhiro Tomino, Johji Oda, Masaru Mizutani, Satoshi Takayanagi, Daisuke Kishi, Tomoaki Shinohara, Kozo Yamada, Jun Matumoto, and Kazuhiro Imamura. Progression of a Sessile Serrated adenoma to an Early Invasive Cancer within 8 Months. Digestive Diseases and Sciences., 54: 906-909, 2009.

21. Shelia Hoar Zahnm, ScD, Pierluigi Cocco, MD, and Aaron Blair, PhD. Tobacoo Smoking as a Risk Factor for Colon Polyps. American Journal for Public Health.,81(7): 846-849, 1991.

22. Pramod Joseph, M.D., Rajeev Jayadevan, M.D., M.R.C.P., Ajit Kokkat, M.D., Mario Ricci, M.D., Nejat Kiyici, M.D., Edward Norkus, Ph.D., Tanaya Nayak, M.D. Cigarrete Smoking-An Independent Risk Factor for Colon Polyps. The American Journal of Gastroenterology., 98(9):S118, 2003.

23. Rajeev Jayadevan, Pramod Joseph, Tanaya Nayak, Ajit Kokkat, Mario Ricci, Nejat Ktyici, Edward Norkus, Hifary Hertan, Cs Pitchumoni. Does Alcohol Consumption Protect Against Colon Polyps? AGA Abstract., A169.

24. Peter R. Holt, MD. Dairy Foods and Prevention of Colon Cancer: Human Studies. Journal of the American College of Nutrition., 18(5): 379S-391S, 1999.

25. Ali Siddiqui A Helene N. Pena Sahdala A Hector E. Nazario A, Amar Mahgoub A Mahir Patel A Daisha Cipher A, Stuart Spechler. Obesity Is Associated with an Increased

Prevalence of Advanced Adenomatous Colon Polyps in a Male Veteran Population. Digestive diseases and sciences., 54: 1560–1564,2009

26. R.J.L.F. Loffeld. Colorectal Adenomas in Patients Presenting with Inflammatory Bowel Disease. The Netherlands Journal of Medicine., 67(1): 21-24, 2009.

27. Coral V.A. Wynter, Tajeshi Kambara, Michael D. Walsh, Barbara A. Leggett, Joanne Young and Jeremy R. Jass. DNA Methylation Patterns in Adenomas from FAP, Multiple Adenoma and Sporadic Colorectal Carcinoma Patients. International Journal of Cancer., 118: 907-915, 2006.

28. K. Ishiguro, T. Yoshida, H. Yagishita, Y. Numata, and T. Okayasu. Epithelial and Stromal Genetic Instability Contributes to Genesis of Colorectal Adenomas. Gut., 55: 695-702 2006.

29. E, Negri, C. Bosetti, C. La Vecchia, F. Levi, F. Tomei and S. Franceschi. Allergy and Other Selected Diseases and Risk of Colorectal Cancer. European Journal of Cancer., 35(13): 1838-1841, 1999.

30. Elizabeth Galf, Dani Bercovich, and Paul Rozen. Familial Adenomatous Polyposis. Orphanet Journal of Rare Diseases., 4(22): 1-23, 2009.

31. Durno, Carol A. and Gallinger, Steven. Genetic Predisposition of Colorectal Cancer: New Pieces in the Pediatric Puzzle. Journal of Pediatric Gastroenterology and Nutrition., 43(1): 5-15, 2006

32. Rosa Valanzano, M.D., Ferfinando Ficari, M.D., Maria Cristina Curia, Ph.D., Gitana Aceto, PhD., Serena Veschi, Ph.D., Alessandro Cama, M.D., Pasquale Battista, M.D., and Francesco Tonelli, M.D. Balance Between Endoscopic and Genetic Information in the Choice of Ilerectal Anatomosis for Familiar Adenomatous Polyposis. Journal of Surgical Oncology., 95:28-33, 2007.

33. Guillaume Bouguen, M.S., Sylvain Manfredi, M.D., Martine Blayau, M.D., Catherine Dugast, M.D., Bruno Buecher, M.D., Dominique Bonneau, Ph.D.,Laurent Siproudhis, Ph.D., Véronique David, Ph.D., and Jean-François Bretagne, Ph.D. Colorectal Adenomatous Polyposis Associated with *MYH* Mutations: Genotype and Phenotype Characteristics. Diseases of the Colon & Rectum., 50:1612-1617, 2007.

34. Jérémie H. Lefevre, M.D., Yann Parc, M.D. Ph.D., Magali Svrcek, M.D., Chrystelle Colas, M.D., Conor Shields, M.D., Jean-François Flejou, M.D., Rolland Parc, M.D. FACS., and Emmanuel Tiret, M.D. *APC*, *MYH*, and Correlation Genotype-Phenotype in Colorectal Polyposis. Annals of Surgical Oncology., 16:871-877, 2009.

35. Amanda Gammon, Wendy Kohlmann, and Randal Burt. Can We Identify the High-Risk Patients to Be Screened? A Genetic Approach. Digestion., 76: 7-19, 2007.

36. J.R. Sampson, S. Jones, S. Dolwani and J.P. Cheadle. *MutYH (MYH)* and Colorectal Cancer. Biochemical Society Transactions., 33(4): 679-683, 2005.

37. Gert U. Auer, Kerstin M. Heselmeyer, Rüdiger G. Steinbeck, Eva Munck-Wikland., and Anders D. Zetterberg. The Relationship between Aneuploidy and *p53* Overexpression during Genesis of Colorectal Adenocarcinoma. Virchowa Archiv.,424: 343-347, 1994.

38. Jeremy R Jass, Kelli G Biden, Margaret C Cummings, Lisa A Simms, Michael Walsh, Estelle Schoch, Stephen J Meltzer, Caroline Wright, Jeffrey Searle, Joanne Young, and Barbara A Leggett. Characterization of a Subtype of Colorectal Cancer Combining Features of the Suppressor and Mild Mutator Pathways. Journal of Clinical Pathology., 52: 455-460, 1999.

39. Takeshi Nagasaka., Ajay Goel, Kenji Notohara, Takaomi Takahata, Hiromi Sasamoto, Takuyuki Uchida, Naoshi Nishida, Noriaki tanaka, Clement Richard Boland and Nagahide Matsubara. Methylation Pattern of the O^6-Methylguanine-DNA Methyltransferase Gene in Colon during Progressive Colorectal Tumorgenesis. International Journal of Cancer., 122:2429-2436, 2008.

40. Shuji Ogino, Aditi Hazra, Gregory J. Tranah, Gregory J. Kirkner, Takako Kawasaki, Katsuhiko Nosho, Mutsuko Ohnish, Yuko Suemoto, Jeffrey A. Meyerhardt, David J. Hunter, and Charles S. Fuchs. *MGMT* Germline Polymorphism is Associated with Somatic *MGMT* Promoter Methylation and Gene Silencing in Colorectal Cancer. Carcinogenesis., 28(9):1985-1990, 2007.

41. Friedemann Honecker, Hendrik Wermann, Frank Mayer, Ad J.M.Gills, Hans Stoop, Ruud J.L.M. van Gurp, Karin Oechsle, Ewout Steyerberg, Jörg Th. Hartmann, Winand N.M. Dinjens, J. Wolter Oosterhuis, Carsten Bokemeyer, and Leender H.J.Looijenga. Microsatellite Instability, Mismatch Repair Deficiency and BRAF Mutation in

Sformatowano: Niemiecki (Niemcy)

Treatment-Resistant Germ Cell Tumors. Journal of Clinical Oncology., 27(13): 2129-2136, 2009.

42. S. de Vogel, M.P. Weijenberg, J.G. Herman, K.A.D. Wouters, A.F.P. de Goeij, P.A. van den Brandt, A.P. de Brűine, and M. van Engeland. *MGMT* and MLH1 Promoter Methylation Versus *APC*, KRAS and BRAF gene Mutations in Colorectal Cancer: Indications for Distinct Pathways and Sequence of Events. Annals of Oncology., 20(7):1216-1222, 2009.

43. Emily J. Greenspan, Jennifer L. Cry, Devon C. Pleau, Joel Levine, Thiruchandurai V. Rajan, and Christopher D. Heinen. Microsatellite Instability in Aberrant Crypt Foci from Patients without Concurrent Colon Cancer. Carcinogenesis., 28(4); 769-776, 2007.

44. Ana M Ferreira, Helga Westers, Sónia Sousa, Ying Wu, Renée C Niessen, Maran Olderode-Berends, Tineke van der Sluis, Peter TW Reuvekamp, Raquel Seruca, Jan H Kleibeuker, Harry Hokkema, Rolf H Sijmons and Robert MW Hofstra. Mononucleotide Precedes Dinucleotide Repeat Instability during Colorectal Tumor Development in Lynch Syndrome Patients. Journal of Pathology., 219: 96-102, 2009.

45. Ana Sánchez-de-Abajo, Miguel de la Hoya, Marjo van Puijenbroek, Alicia Tosar, J.A. López-Asenjo, Eduardo díaz-Rubio, Hans Morreau, and Trinidad Calde. Molecular Analyis of Colorectal Cancer Tumors from Patients with Mismatch Repair-Proficient Hereditary Nonpolyposis Colorectal Cancer Suggests Novel Carcinogenic Pathways. Clinical Cancer Research., 13(19): 5729-5735, 2007.

46. Y. Hirai, K. Banno, M. Suzuki, Y. Ichikawa, Y. Udagawa, K. Sugano and Y. Miki. Molecular Epidemiological and Mutational Analysis of DNA Mismatch Repair (*MMR*) Genes in Endometrial Cancer Patients with HNPCC-Associated Familial Predisposition to cancer. Cancer Sciences., 99(9): 1715-1719, 2008.

47. Fay Kastrinos and Spana Syngal. Recently Identified Colon Cancer Predispositions: *MYH* and *MSH6* Mutations. Seminars in Oncology., 34: 418-424, 2007.

48. Takeshi Kambara, Vicki L. J. Whitehall, Kevin J. Spring, Melissa A. Barker, Sven Arnold, Coral V.A. Wynter, Nagahide Matsubara, Noriaki Tanaka, Joanne P. Young, Barbara A

Leggett, and Jeremy R. Jass. Role of Inherited Defects of *MYH* in the Development of Sporadic Colorectal Cancer. Genes, Chromosomes & Cancer., 40: 1-9, 2004.

49. J.J.Y. Sung, J.Y..W. Lau, G..P. Young, Y. Sano, H.M.Chiu, J.S.Byeon, K.G. Yeoh, K.L.Goh, J. Sollano, R. Rerknimitr, T. Matsuda, K.C. Wu, S. Ng, S.Y. Leung, G. Makharia, V.H. Chong, K.Y. Ho, D. Brooks, D.A. Liberman, and F.K.L.Chan. Aisa Pacific Consensus Recommendations for Colorectal Cancer Screening. Gut.,57: 1166-1176, 2008.

50. Michael Pignone, MD, MPH., Somnath Saha, MD, MPH., Tom Hoerger, PhD., and Jeanne Mandeblatt MD, MPH. Cost-Effectiveness Analysis of Colorectal Cancer Screening: A Systematic Review for the U.S. Preventive Services Task Force. Annals of Internal Medicine., 137: 96-104, 2002.

51. V. Johnson, L.R. Lipton, C. Cummings, A. T. Eftekhar Sadat, L. Izatt, S.V. Hodgson, I.C. Talbot, H. J. W. Thomas, A. J. R. Silver, and I. P. M. Tomlinson. Analysis of Somatic Molecular Changes, Clinicopathological Features, Family History and Germline Mutations in Colorectal Cancer Families: Evidence for Efficient Diagnosis of HNPCC and for the Existence of Distinct Groups of Non-HNPCC Families. Journal of Medical Genentics., 42: 756-762, 2005.

52. Noralane M. Lindor, M.D. Hereditary Colorectal Cancer: *MYH*-Associated Polyposis and other Newly Identified Disorders. Best Practice & Research Clinical Gastroenterology., 23: 75-87, 2009.

53. Cappel MS. From Colonic Polyps to Colon Cancer: Pathophysiology Clinical Presentation, Screening and Colonoscopic Therapy. Minerva Gastroenterology Dietol., 53(4): 351-373, 2007.

54. Karam S. Boparai, Evelien Dekker, Susanne van Eeden, Mirjam M. Polak, Joep F. W. M. Bartelsman, Elisbeth M. H. Mathus-Vliegen, Josbert J. Keller, and Carel J. M. Van Noesel. Hyperplastic Polyps and Sessile Serrated Adenomas as a Phenotypic Expression of *MYH*-Associated Polyposis. Gastroenterology., 135: 2014-2018, 2008.

55. Antoni Castells. *MYH*-Associated Polyposis: Adenomas and Hyperplastic Polyps, Partners in Crime? Gastroenterology., 135(6): 1857-1859, 2008.

56. Satoru Yamaguchi, Kazuya Shinmura, Takayuki Saitoh, Seiichi Takenoshita, Hiroyuki Kuwano and Jun Yokota, A single nucleotide polymorphism at the splice donor site of

the human *MYH* base excision repair genes results in reduced translation efficiency of its transcripts. Genes to Cells., 7: 461-474,2002

57. Ted M Kremer, Mikael L Rinne, Yi Xu, Xian Ming Chen and Mark R Kelley. Protection of pulmonary epithelial cells from oxidative stress by *hMYH* adenine glycosylase. Respiratory Research., 5(16): 1-11, 2004

58. Antony Parker, Yesong Gu, William Mahoney, Suk-Hee Lee, Keshav K. Singhi, and A-Lien Lu. Human homolog of the MutY repair protein (h*MYH*) physically interacts with proteins involved in long patch DAN base excision repair. The Journal of Biological Chemistry., 276: 5547-5555, 2001.

59. Karim Bougatef, Raja Marrakchi, Nadia Kourda, Yannick Blondeau Lahely, Sarra Baltagi Ben Jileni, Houssein K. El Khil, Florent Soubrier, and Amel Ben Ammar Elgaaied. Somatic Mutation of MutYH in Tunisian Patients with Sporadic Colorectal Cancer. Journal of Clinical Laboratory Analysis., 21: 372-374, 2007.

60. Marina E Croitoru, MSc., Sean P Cleary, M.D., Terri berk, MSSA., Nando di Nivola, MLT., Ilana Kopolovic, Bharati Bapat, Ph.D., and Steven Gallinger, M.D. Germline *MYH* Mutations in a Clinical-Based Series of Canadian Multiple Colorectal Adenoma Patients. Journal of Surgical Oncology., 95: 499-506, 2007.

61. A Colebatch, M Hirchins, R Williams, A meagher, NJ Hawkins and RL Ward. The Role of *MYH* and Microsatellite Instability in the Development of Sporadic Colorectal Cancer. British Journal of Cancer., 95: 1239-1243, 2006.

62. David Malkin. The Role of p53 in Human Cancer. Journal of Neuro-oncology., 51: 231-243, 2001.

63. Paolo Peterlongo, Nandita Mitra, Ana Sanchez de Abajo, Miguel de la Hoya, Chiara Bassi, Lucio bertario, Paolo Radice, Emily Glogowski, Khedoudja Nafa, Trinidad Caldes, Kenneth Offit and Nathan A. Ellis. Increased Frequency of Disease-Causing *MYH* Mutations in Colon Cancer Family. Carcinogenesis., 27(11): 2243-2249, 2006.

64. Federico Canzian, Reijo Salovaara, Akseli Hemminiki, Paula Kristo, Robert B. Chadwick, LauriA. Aaltonen, and Albert de la Chapelle. Semiantomated Assessment of Loss of Heterozygosity and Replication Error in Tumors. Cancer Research., 56: 3331-3337, 1996.

65. Christina Fleischmann, Julian Peto, Jeremy Cheadle, Bindiya Shah, Julian Sampson and Richard S. Houlston. Comprehensive Analysis of the Contribution of Germline *MYH* Variation to Early-Onset Colorectal Cancer. International Journal of Cancer., 109(4): 554 – 558,2004.

66. Barnetson RA, Devlin L, Miller J, Farrington SM, Slater S, Drake AC, Campbell H, Dunlop MG, and Porteous ME. Germline Mutation Prevalence in the Base Excision Repair Gene, *MYH*, in Patients with Endometrial Cancer. Clinical Genetics., 72: 551-555, 2007.

67. Mohsin Ali, Hyeja Kim, Sean Cleary, Claire Cupples, Steven Gallinger, and Robert Bristow. Characterization of Mutant MUTYH Proteins Associated With Familial Colorectal Cancer. GASTROENTEROLOGY., 135: 449-507,2008.

68. Hong Tao, Kazuya Shinmura, Tomoyuki Hanaoka, Syusuke Natsukawa, Kozo Shaura, Yoichi Koizumi, Yoshio Kasuga, Takachika Ozawa, Toshimasa Tsujinaka, Zhongyou Li, Satoru Yamaguchi, Jun Yokota, Haruhiko Sugimura, and Schoichiro Tsugane. A Novel Spluce-Site Variant of the Base Excision Repair Gene *MYH* is Associated with Production of an Aberrant mRNA Transcript Encoding a Truncated *MYH* Protein not Localized in the Nucleus. Carcinogenesis., 25(10): 1859-1866. 2004.

69. Paul Brennan. Gene–environment interaction and aetiology of cancer: what does it mean and how can we measure it? Carcinogenesis., 23(3): 381-387,2002

70. Ingrid Hedenfalk, M.S., David Duggan, Ph.D., Yidong Chen, Ph.D., Michael Radmacher, Ph.D., Michael Bittner, Ph.D., Richard Simon, D.Sc., Paul Meltzer, M.D., Ph.D., Barry Gusterson, M.D., Ph.D., Manel Esteller, M.D., Ph.D., Mark Raffeld, M.D., Zohar Yakhini, Ph.D., Amir Ben-Dor, Ph.D., Edward Dougherty, Ph.D., Juha Kononen, M.D., Ph.D., Lukas Bubendorf, M.D., Wilfrid Fehrle, M.D., Stefania Pittaluga, M.D., Sofia Gruvberger, M.S., Niklas Loman, M.D., Oskar Johannsson, M.D., Ph.D., Håkan Olsson, M.D., Ph.D., Benjamin Wilfond, M.D., Guido Sauter, M.D., Olli-P. Kallioniemi, M.D., Ph.D., Ake Borg, Ph.D., and Jeffrey Trent, Ph.D. GENE-EXPRESSION PROFILES IN HEREDITARY BREAST CANCER. The New England Journal of Medicine., 344: 539-548, 2002

10.QUESTIONNAIRE

Questionnaire

This questionnaire is for the research program for

prevention and healing of colorectal cancer.

Name: ______________________________

PSESL ______________________________

Date of Investigation: ________________________

Instruction:

Please answer “Yes” or “No” by using check sign or cross sign

A. GENERAL INFORMATION

1. Sex: Female________ Male_____

2. Height__________________

3. Weight__________________

4. Weight Changes in Last 3 Months Yes_____ No____

   If Yes, how many Kilograms increases__________ or decreases__________

5. Previous Diseases and Surgery

Diseases, Surgery	Age

6. Medical History

Regular Pill Taking? Name of the Medicine?

____

____

_____

Vitamins and Microelement supplement? Name of it?

____

____

_____

7. Cancer in the Family Members

Cancer	Relative	Age of Diagnosis, 50,60,70,80

B. DIET

	Everyday	3 X/week	1 X/week	1 X/month
1. Lipid from Animal Products.				
2. Lipid from Plants Products				
3. Majority for Meat Dishes				
4. Sweat Foods				
5. Red Meat				
6. Fish				
7. Eggs				
8. Milk Products				
9. Fermented Milk				
10. Cereal Product				
11. Heavy Spicy Food				
12. Salty Food				
13. Bread				
14. Vegetable				
15. Fruits				
16. Beer				
17. Red Wine				
18. White Wine				
19. Strong Alcohol				

C. FOOD PREPARATION

	YES	NO
1. Fats, Backing, Roasting, and Fires		
2. Steam, Stews, and Short Backing		
3. Microwaves Foods		
4. Re-Cook		
5. Instant Foods		

D. DRINKS

1. Amounts of Liquid per Day ______________________

	Yes	No
2. Non-Gas Mineral Water		
3. Instant Coffee		
4. Coffee from Coffee Bean		
5. Tea		
6. Juice		
7. Soft Drink (Coca Cola, Pepsi, Fanta)		

E. SMOKING Yes__________ No _________

If quit, when do you stop? ______________________

F. PHYSICAL ACTIVITY

1. Type Of Jobs (Sitting or Active) ______________________

2. Free Time Activity (Active or Passive) ________________

3. Sports Training ________________________________

4. Sleeping Hours(Average Hours/day) __________________

G. BOWEL MOVEMENT

	Yes	No
1. Regular Defecation 1 time/Day	Yes	No
2. Regular Defecation 2 times/Day	Yes	No
3. No Regular Defecation W/O Medical Help (soft stool)	Yes	No
4. No Regular Defecation W/O Medical Help (dry and hard stool)	Yes	No
5. No Regular Defecation Medical Help is Needed	Yes	No
6. More than 1 time/ Day	Yes	No
7. Types of Stools		
a. normal stool	Yes	No
b. soft stool	Yes	No
c. watery stool	Yes	No
d. small caliber in diameter	Yes	No
e. bloody stool	Yes	No
f. mucus stool	Yes	No
g. undigestive food in stool	Yes	No
h. light color in stool	Yes	No
i. stone stool	Yes	No
j. hesitation defecation	Yes	No
	Yes	No

8. Constant Stomach Aching with or without Defecation.

k. pain in general

l. specific location? Left side or right side

m. pain after eating

n. pain during defecation

o. pain due to stress

Yes	No
Yes	No
Yes	No
Yes	No
Yes	No