GASTROINTESTINAL POLYPOSIS SYNDROMES

Clinical and molecular aspects of Familial Adenomatous Polyposis and Juvenile Polyposis

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ISBN: 978-90-393-4862-8

Printed by: Gildeprint Drukkerijen B.V.

Cover: Baltimore is renowned for its crab from the Chesapeake Bay. The research described in this thesis was initiated and partly conducted at the Johns Hopkins Hospital in Baltimore, USA. Karkinos (carcinoma) and cancer are the ancient Greek and Latin words for crab, respectively, and the crab is the international symbol for cancer.

Back cover: epithelial loss of SMAD4 expression in a juvenile polyp from a patient with a *SMAD4* germline mutation.

The research described in this thesis was performed at the Department of Pathology of the University Medical Center, Utrecht; the Departments of Pathology and Gastroenterology of the Johns Hopkins Hospital, Baltimore, USA; and the Department of Pathology of the Academic Medical Center, Amsterdam.

Financial support for the publication of this thesis was kindly provided by: Department of Pathology UMC Utrecht, Universiteit Utrecht, J.E. Jurriaanse Stichting, MRC-Holland B.V., Janssen-Cilag B.V., Pfizer B.V., EuroTec B.V., PENTAX Nederland B.V., Sakura Finetek Holland B.V. and QIAGEN Benelux B.V.

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Clinical and molecular aspects of Familial Adenomatous Polyposis and Juvenile Polyposis

GASTROINTESTINALE POLYPOSIS SYNDROMEN

Klinische en moleculaire aspecten van Familiare Adenomateuze Polyposis en Juveniele Polyposis
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
op donderdag 16 oktober 2008 des middags te 12.45 uur.

door

Lodewijk Adriaan Anton Brosens

geboren op 2 juni 1979, te Amsterdam

Promotoren: Prof. dr. G.J.A. Offerhaus
Prof. dr. F.M. Giardiello

Co-promotor: Dr. J.J. Keller

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INTRODUCTION

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HEREDITARY CANCER PRONE DISORDERS OF THE GASTROINTESTINAL TRACT AND COLORECTAL CARCINOGENESIS

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HEREDITARY CANCER PRONE DISORDERS OF THE GASTROINTESTINAL TRACT

Colorectal cancer (CRC) ranks as the third most common cause of cancer related death. In the United States, approximately 150,000 patients are diagnosed with colorectal cancer each year, and one third will die from this disease.[1]

CRC is generally divided into sporadic and familial (hereditary) cases. Most patients (~80%) with CRC have it as a sporadic disease without evidence of an underlying inherited disorder. In approximately 20% of patients with CRC there seems to be a potentially definable genetic component. In the majority of these patients (~15%) there is so called familial predisposition but no known underlying genetic defect. Only in about 5% of patients with CRC a germline genetic defect can be identified. In these cases patients may have hereditary non-polyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP) or another rare colorectal cancer syndrome such as Peutz-Jeghers (PJS), juvenile polyposis (JPS), or missense mutation in the *APC* gene (APC I1307K) as a cause of familial colorectal cancer in patients of Ashkenazi Jewish descent.[2, 3] (Figure 1)

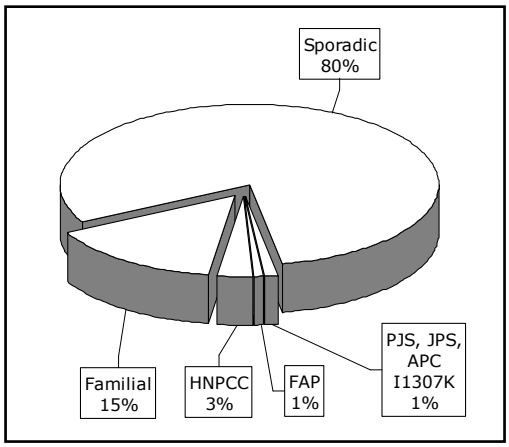

Figure 1. *Causes of colorectal cancer. HNPCC: hereditary non-polyposis colorectal cancer; FAP: familial adenomatous polyposis; PJS: Peutz-Jeghers syndrome; JPS: juvenile polyposis.*

Hereditary non-polyposis colorectal cancer, or Lynch syndrome, is caused by an inherited defect in one of the mismatch repair (MMR) genes, *MLH1* (~50%), *MSH2* (~40%), *MSH6* (~10%), *or PMS2*, causing microsatellite instability (MSI).[4] It is the most frequent form of hereditary CRC, accounting for about 3% of all cases of CRC. The life-time risk of CRC for patients with HNPCC is approximately 70-80% and the average age of CRC diagnosis is 44 years. Multiple synchronous or metachronous colorectal tumors are found in 45% of patients. 60-80% of colorectal tumors in HNPCC are found in the right side of the colon and these tumors often have a mucinous/signet-ring cell differentiation. In addition, patients with HNPCC are at increased risk for a wide variety of extra-colonic malignancies, including endometrial, ovarian, stomach, small bowel, biliary, urinary tract, renal, and brain tumors.[2, 3]

Diagnosis of HNPCC is based on clinical criteria (Amsterdam Criteria I and II) that have been defined for HNPCC.[5, 6] (Table 1) Genetic testing of the MMR genes is indicated when these criteria are fulfilled. However, a substantial fraction of HNPCC patients will be excluded from genetic testing since the sensitivity of the Amsterdam II criteria is 78%.

Therefore, the less stringent Bethesda guidelines were developed to define patients that should undergo MSI testing as a first screening test.[7 8] (Table 2)

Table 1. Amsterdam criteria II [6]

There should be at least 3 relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis)
One should be a first-degree relative of the other 2
At least 2 successive generations should be affected
At least 1 should be diagnosed before age 50
Familial adenomatous polyposis should be excluded in the CRC case(s) if any
Tumors should be verified by pathological examination

Table 2. Revised Bethesda guidelines [7]

Tumors from individuals should be tested for MSI in the following situations:
Colorectal cancer diagnosed in a patient who is less than 50 years of age.
Presence of synchronous, metachronous colorectal, or other HNPCC associated tumors,* regardless of age.
Colorectal cancer with the MSI-H† histology‡ diagnosed in a patient who is less than 60 years of age. §
Colorectal cancer diagnosed in one or more first-degree relatives with an HNPCC-related tumor, with one of the cancers being diagnosed under age 50 years.
Colorectal cancer diagnosed in two or more first- or second-degree relatives with HNPCC-related tumors, regardless of age.

* HNPCC-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel.
† MSI-H: microsatellite instability–high in tumors refers to changes in two or more of the five National Cancer Institute-recommended panels of microsatellite markers.
‡ Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring cell differentiation, or medullary growth pattern.
§ There was no consensus among the Workshop participants on whether to include the age criteria in guideline 3 above; participants voted to keep less than 60 years of age in the guidelines.

In addition, a group of autosomal dominantly inheriting disorders can be distinguished that are characterized by the development of multiple polyps in the gastrointestinal tract. Each of these syndromes is caused by germline mutation of a specific gene (Table 3). Familial adenomatous polposis is the best known and the most frequently occurring. FAP patients typically have innumerable adenomatous polyps throughout the colorectum. Without colectomy, these patients almost inevitably develop CRC at a young age. In addition, there are rare hamartomatous polyposis syndromes, including Peutz-Jeghers syndrome, juvenile polyposis, Cowden syndrome (CS), and Bannayan-Riley-Ruvalcaba syndrome (BRRS).

Each syndrome is characterized by the presence of gastro-intestinal polyps with distinctive histology and may have specific extra-intestinal features. Clinical manifestations, molecular genetics and management of these polyposis syndromes are reviewed in more detail in chapter 3 of this thesis.[9]

Table 3. Gastrointestinal polyposis syndromes and their associated genes

Syndrome	Gene
Familial adenomatous polyposis	*APC*, *MYH* (rare)
Peutz-Jeghers syndrome	*LKB1*
Juvenile polyposis	*SMAD4*, *BMPR1A*
Cowden and Bannayan-Riley-Ruvalcaba syndrome*	*PTEN*

* Likely two variable expressions of the same genetic alteration

COLORECTAL CARCINOGENESIS

CRC develops trough stepwise progression from normal epithelium into dysplastic epithelium and subsequent invasive carcinoma.[10] Additional insights provided a genetic basis for this stepwise progression model, which was described by Fearon and Vogelstein and known as the adenoma-carcinoma sequence. The adenoma-carcinoma sequence is driven by accumulation of molecular alterations, such as inactivation of tumor-suppressor genes (*p53*, *SMAD4*) and activation of oncogenes (e.g. *k-ras*).[11, 12] In addition, expression of cell regulatory proteins, such as cyclooxygenase-2, is altered.[13, 14]

In the great majority of colorectal cancers, aberrant Wnt activation, usually caused by an inactivating mutation of *APC* but sometimes by mutation of *axin-2* or activating mutation of *β-catenin*, initiates the adenoma-carcinoma sequence. The *APC* gene is a tumor-suppressor gene with a central role in the Wnt signaling pathway, which is involved in repression of apoptosis, induction of proliferation, and cell cycle progression. In the absence of Wnt signaling, APC functions in a multiprotein complex with axin and glycogen synthase kinase 3β (GSK-3β) that targets β-catenin for proteasomal degradation. Inactivation of APC or axin, or an activating mutation in *β-catenin* results in disturbed regulation of intracellular β-catenin levels, nuclear translocation of β-catenin and subsequent Wnt target gene transcription.[15]

The adenoma-carcinoma sequence can be regarded as the common denominator of CRC pathogenesis. However, three different pathways leading to the initiation of the adenoma-carcinoma sequence and hence to colorectal cancer have been proposed: "gatekeeper", "caretaker" and "landscaper" defects.[16] Each of these pathways is represented by a cancer prone disorder discussed above and the study of these inherited disorders has given us invaluable insight in colorectal cancer pathogenesis in general.

In patients with FAP and about 80-85% of sporadic colorectal cancers, mutation of the tumor-suppressor gene *APC* is the first step that initiates the adenoma-carcinoma sequence. In this way, APC acts as the "gatekeeper" of colorectal carcinogenesis. Inactivation of APC results in nuclear accumulation of β-catenin and subsequent transcription of Wnt target genes. In addition chromosomal instability is characteristic for these tumors and leads to losses of large parts of chromosomes and additional mutations

in tumor-suppressor genes and oncogenes needed for progression of the adenoma-carcinoma sequence. As a consequence of the inherited defect in *APC*, initiation of the adenoma-carcinoma sequence is accelerated in FAP patients, resulting in the development of innumerable colonic adenomatous polyps, whereas progression of the adenoma-carcinoma sequence follows the normal route and at a normal pace; i.e. similar as in sporadic conventional CRC.[17]

In HNPCC and about 15% of sporadic colorectal cancers, a defective mismatch repair gene is the first step in colorectal carcinogenesis. In sporadic cases, MMR deficiency is usually caused by hypermethylation of CpG-islands in the promotor region of the *MLH1* gene or sometimes by somatic mutation. HNPCC is caused by an inherited defect of one of the MMR genes. MMR deficiency leads to greatly increased mutation rates, particularly in repetitive DNA sequences, which is reflected by microsatellite instability (MSI) and frameshift mutations in growth regulatory genes.[2] Interestingly, somatic mutations in the Wnt-pathway can be found in most MSI tumors, underscoring the importance of this pathway in colorectal carcinogenesis.[18] Because of their role in DNA repair, MMR genes have been addressed as "caretaker" genes. In HNPCC patients, not the initiation, but the progression of the adenoma-carcinoma sequence is accelerated, in contrast to FAP.[17]

A third pathway to colorectal cancer, called the "landscaper" defect, was proposed based on observations in juvenile polyposis syndrome. In this model it is hypothesized that cancer develops as a result of an abnormal stromal environment that can induce carcinogenesis of the adjacent epithelium.[16] The observation that the genetic alterations at chromosome 10q22 (*BMPR1A* locus) occurred predominantly in the stroma of juvenile polyps,[19] gave rise to this model in JPS.[16] More recently, additional evidence in support of the "landscaper" defect in JPS came from a study where BMP-4 was localized exclusively to the mesenchymal compartment of the intestine in mice and disrupted BMP signaling resulted in development a juvenile polyposis-like phenotype.[20] In contrast, homozygous *SMAD4* deletions have been found primarily in the epithelium of juvenile polyps from JPS patients with germline *SMAD4* mutations and *SMAD4* knockout mice.[21, 22] Although further studies are needed, this suggest that SMAD4 may acts as a "gatekeeper", instead of a "landscaper" in JPS pathogensis, which would be in line with the role of SMAD4 in other cancer types.[23]

To conclude, hereditary disorders associated with an increased risk of cancer of the gastrointestinal tract have proven to be valuable models to study colorectal carcinogenesis in general. Based on these cancer prone disorders, several pathways leading to colorectal cancer have been postulated, but there is still much to learn. In addition, quality of life and life-expectancy of patients with these syndromes has dramatically improved in the last decades due to intensive research.

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2

OUTLINE OF THE THESIS

OUTLINE OF THE THESIS

This thesis describes studies on the two gastrointestinal polyposis syndromes familial adenomatous polyposis (FAP) and juvenile polyposis (JPS). FAP is caused by germline mutation of *APC* and is considered the classical model for colorectal cancer development caused by a "gatekeeper" defect that initiates the adenoma-carcinoma sequence. JPS is caused by germline mutation of *SMAD4* or *BMPR1A* and is the model for a different pathway of cancer development, introduced by Kinzler and Vogelstein as the "landscaper" defect. In their model they hypothesize that the primary defect causing neoplastic change in JPS lies within the stroma instead of the epithelium and that stromal changes provide an abnormal environment influencing behavior of adjacent epithelium and ultimately leading to an increased risk of neoplastic transformation.

Chapter 3 gives an overview of the three best known gastrointestinal polyposis syndromes: familial adenomatous polyposis, juvenile polyposis and Peutz-Jeghers syndrome. Clinical manifestations, molecular genetics, pathogenesis, and management of these syndromes are reviewed.

Part I

The first part of this thesis is about familial adenomatous polyposis syndrome. FAP patients are mainly troubled by the development of colorectal adenomatous polyps and the primary cause of death of these patients is colorectal cancer. However, with improved management of colorectal disease and increased life expectancy of FAP patients, duodenal polyps and adenocarcinoma have emerged as major health problems in these patients. Duodenal adenocarcinoma is now the second leading cause of death in FAP. **Chapter 4** discusses the clinicopathological features, management and prevention of duodenal neoplasia in FAP. The literature about surveillance, endoscopic and surgical treatment and the value of NSAID chemoprevention is reviewed. Literature review revealed that results of NSAIDs on regression or prevention of duodenal adenomas in FAP are disappointing.

The lack of a significant effect of NSAID chemoprevention on duodenal polyps prompted us to investigate and compare the expression of the main target of these drugs, cyclooxygenase-2 (COX-2), between colorectal and duodenal adenomas from patients with FAP (**chapter 5**). In **chapter 6**, expression of the mRNA stability protein HuR and its role in progression to malignancy by stabilizing COX-2 mRNA was investigated in the colorectum of patients with FAP.

Part II

The second part of this thesis focuses on clinical and molecular aspects of juvenile polyposis syndrome. JPS is characterized by the presence of multiple histological distinctive hamartomatous polyps and it is associated with an increased risk of cancer, notably colorectal, gastric, small bowel and pancreatic cancer. However, no formal risk assessment of cancer in JPS patients existed. **Chapter 7** describes person-year analysis that was performed to define the magnitude of risk for gastrointestinal cancer in these patients.

JPS is caused by germline mutation of *SMAD4* or *BMPR1A*, and possibly *ENG*. However, a germline mutation in *SMAD4* or *BMPR1A* is found in only 30-40% of JPS patients. These numbers are based on techniques that only detect point mutations or small deletions. In **chapter 8**, a comprehensive genetic analysis was performed in a group of well

documented JPS patients to investigate the role of deletion of one or more exons of *SMAD4*, *BMPR1A*, *ENG* and *PTEN* as the cause of JPS. Using both direct sequencing and multiplex ligation-dependent probe amplification (MLPA) we were able to identify a germline defect in almost 50% of JPS patients. Because this leaves 50% of JPS patients without a known germline defect, it seems likely that other genes may exist that predispose to JPS. In **chapter 9** the potential role of the *TGFBRII* gene in JPS pathogenesis was investigated by sequence analysis and MLPA of this gene in JPS patients without *SMAD4*, *BMPR1A*, *ENG* or *PTEN* germline mutation.
Prevention of polyp and neoplastic development in JPS using chemoprevention, similar as described in FAP (first part of this thesis), may be useful in the treatment of these patients. To explore the potential value of chemoprevention using (selective) COX-2 inhibition, expression of COX-2 in juvenile polyps was investigated (**chapter 10**).
Lastly, in **chapter 11** molecular aspects of juvenile polyps from patients with a germline defect in *SMAD4* were studied. Correlation between SMAD4 immunohostochemistry and underlying genetic defect and the timing of loss of the second allele of *SMAD4* in relation to neoplastic progression were investigated.

3

GASTROINTESTINAL POLYPOSIS SYNDROMES

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Current Molecular Medicine 2007 Feb;7(1):29-46

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ABSTRACT

Colorectal cancer is one of the leading causes of cancer-related death in the Western society, and the incidence is rising. Rare hereditary gastrointestinal polyposis syndromes that predispose to colorectal cancer have provided a model for the investigation of cancer initiation and progression in the general population. Many insights in the molecular genetic basis of cancer have emerged from the study of these syndromes. This review discusses the genetics and clinical manifestations of the three most common syndromes with gastrointestinal polyposis and an increased risk of colorectal cancer: familial adenomatous polyposis (FAP), juvenile polyposis (JPS) and Peutz-Jeghers syndrome (PJS).

FAMILIAL ADENOMATOUS POLYPOSIS

Introduction

Familial adenomatous polyposis is a syndrome characterized by multiple adenomatous polyps in the large bowel and a virtually 100% life-time risk of colorectal cancer. It accounts for approximately 1% of all colorectal cancer cases and occurs in about 1/10,000 live births.[1] FAP is inherited in an autosomal dominant fashion and is caused by a germline mutation in one of the *APC* (*adenomatous polyposis coli*) alleles on chromosome 5q21.[2-5] In about 22-30% of FAP patients no family history of polyposis is noted, indicating that these patients acquired a new spontaneous mutation.[6] In classic FAP, patients have innumerable (>100 to thousands) adenomatous polyps throughout the colorectum. Without prophylactic proctocolectomy, invasive carcinoma usually develops before the 5th decade of life.[1] In addition, a milder variant, termed attenuated FAP (AFAP), has been identified. AFAP is characterized by the presence of less than 100 polyps (oligopolyposis) at presentation and later onset of colorectal cancer (on average 12 years later than in classic FAP). Some of these patients have severe upper gastrointestinal manifestations.[1]

Patients with FAP can also develop duodenal and gastric polyps, extra-intestinal malignancies (desmoid tumors, thyroid, pancreatic and biliary tree carcinoma, brain tumors and hepatoblastoma) and benign extra-intestinal lesions (lipomas, fibromas, sebaceous and epidermoid cysts, osteomas, occult radio-opaque jaw lesions, dental abnormalities, congenital hypertrophy of the retinal pigment epithelium and nasopharyngeal angiofibroma). The combination of colorectal polyposis and a primary central nervous system malignancy (medulloblastoma) is called Crails syndrome.[1]

Adenomatous polyps in FAP are mostly sessile and spherical or lobulated and range from barely visible to pedunculated lesions up to 1 cm or more. Dysplasia starts in single crypts, called a dysplastic aberrant crypt focus (ACF), single crypt adenoma or microadenoma (Figure 1A). Multiple aberrant crypt foci in a colon is unique to FAP. Subsequently, dysplasia progresses following the multistep ACF-adenoma-carcinoma sequence as proposed by Kinzler and Vogelstein in 1996.[7] Histologically, adenomas in FAP resemble sporadic adenomas.

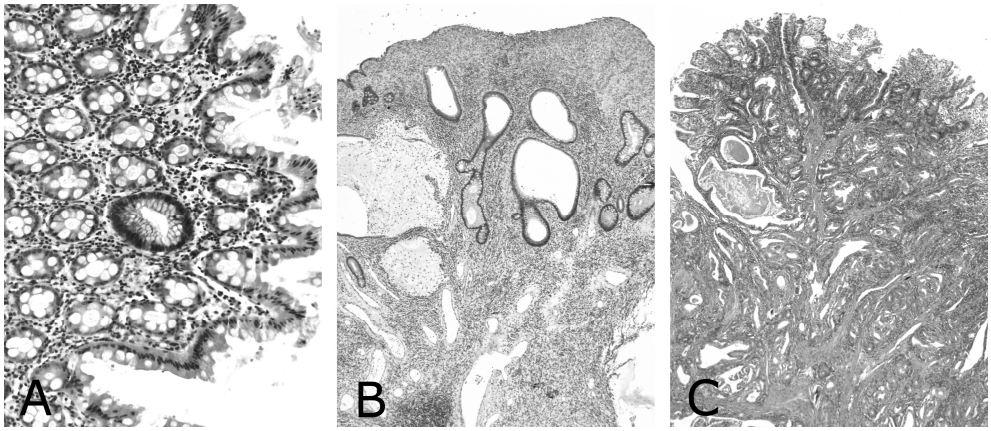

Figure 1. *A) Single crypt adenoma in FAP. B) Typical juvenile polyp with erosion of surface epithelium, expanded mesenchymal stroma and cystically dilated crypts with reactive change. C) Typical microscopic picture of a Peutz-Jeghers polyp with arborizing smooth muscle proliferation and elongated crypts with reactive epithelial cells.*

Clinical manifestations

Colorectum

The presence of colorectal adenomatous polyposis is the hallmark feature of FAP. Adenomatous polyps develop throughout the colorectum starting in childhood and adolescence. By age 15, about 50% of FAP patients have colorectal adenomas, and by age 35, 95% are affected. If left untreated, invasive carcinoma develops at an average age of 34.5 to 43 years and the lifetime risk of colorectal carcinoma is virtually 100%.[1]

Duodenum

The duodenum is the second most common site of adenoma development in FAP patients with a predilection for the second and third parts and the periampullary region.[8] Duodenal adenomas can be found in 30-70% of FAP patients and the lifetime risk of duodenal adenoma development is nearly 100%.[9]

Severity of duodenal polyposis is classified using the Spigelman staging system which describes five (0-IV) stages (Table 1). Points are assigned for size (1-4, 5-10, >10 mm), number (1-4, 5-20, >20), histology (tubular, tubulo-villous, villous) and severity of dysplasia (low-, high-grade). Stage I reflects mild disease, whereas stage III to IV represents severe duodenal polyposis and high risk of developing malignancy.[8]

Stage II or stage III duodenal disease is found in 70 to 80% of patients with FAP, and stage I or stage IV disease in 10 to 20%. However, by 70 years of age 52% of FAP patients have stage IV duodenal polyposis.[8-10]

The stage of duodenal polyposis progresses over time. In approximately 10 years, progression of duodenal polyposis occurs in 42 to 73% of FAP patients, and the time needed for progression by one stage ranges between 4 to 11 years. Moreover, severity of duodenal polyposis increases with age and the risk of developing stage III or IV disease is exponentially increased after age 40.[9]

Table 1. Spigelman classification for duodenal polyposis in FAP

Criteria	Points		
	1	2	3
Polyp number	1-4	5-20	>20
Polyp size (mm)	1-4	5-10	>10
Histology	Tubular	Tubulo-villous	Villous
Dysplasia	Low-grade		High-grade

Stage 0: 0 points. Stage I: 1-4 points. Stage II: 5-6 points. Stage III: 7-8 points. Stage IV: 9-12 points

Duodenal/periampullary cancer is the leading cause of death in FAP patients after colorectal cancer. Patients have an 100 to 330 fold higher chance to develop duodenal cancer compared to unaffected individuals and estimates of the cumulative risk of duodenal cancer ranges from 4 to 10% at age 60-70.[9, 11, 12] The risk of developing duodenal malignancy increases with higher Spigelman stages. Stage II and III disease are associated with 2.3% and 2.4% risk, respectively, while stage IV duodenal polyposis carries a 36% risk of developing duodenal cancer.[10] Prophylactic duodenectomy should be considered in patients with stage IV disease.

Stomach

In contrast to duodenal polyps, gastric polyps in FAP patients are usually benign fundic gland polyps. These lesions occur in the fundus and the body of the stomach in about 50% of FAP patients. Histologically, these polyps are characterized by dilatation and cystic change of the fundic glands. Although dysplasia has been described, fundic gland polyps rarely show malignant transformation.[13] Approximately 10% of the gastric polyps are adenomas, which can be found throughout the stomach.[8, 9] Interestingly, in Japan, a high-risk country for gastric cancer, adenomatous stomach polyps in FAP patients occur more frequently than in Western countries. In Japanese and Korean FAP patients a 3 to 4 times higher risk of gastric cancer was found compared to the general population.[14, 15] In contrast, person-year analysis revealed that western FAP patients have no increased risk of gastric cancer.[12]

Desmoid tumors

Desmoid tumors (or fibromatosis) are slow growing tumors originating from the mesenchymal primordial germ-cell layer. They are composed of sheets of elongated myofibroblasts, arranged in fascicles and whorls with abundant collagen matrix. Desmoids occur in approximately 10% of FAP patients most frequently within the abdomen and small intestinal mesentery, but also in the abdominal wall or in the extremities. FAP patients have an 852 times higher risk of developing desmoids compared to the general population.[16, 17]

Although desmoids have no metastatic potential, they can cause obstructive complications as a result of local growth. Desmoid tumors are the cause of death in a significant proportion of patients with FAP treated by colectomy. In particular, intra-abdominal desmoid tumors have a poor prognosis compared to those of the abdominal wall. Death can result from local expansion and invasive growth with resulting damage to intra-

abdominal structures, such as intestines, ureters and blood vessels. In addition, peri-operative complications in patients undergoing surgery for intra-abdominal desmoids are an important cause of death.[17]
The exact etiology of desmoid tumors is unclear. However, surgical trauma is considered as a major risk factor since these lesions frequently develop after a patient has had surgery. Also, recurrence rates after incomplete resection are high. In addition, sex hormones, in particular estrogens, may play a role in the development of these tumors.[16,17]

Extra-intestinal malignancies

Extra-intestinal malignancies that have been associated with FAP, include thyroid, pancreatic,[18] biliary tree,[19, 20] hepatoblastoma,[21] and medulloblastoma.[22] Thyroid cancer, predominantly papillary carcinoma, can be found in 1-2% of FAP patients. The relative risk of developing this malignancy ranges from 7.6 to 20.9, although the absolute life time risk is low (2.1%).[15, 18, 23] Thyroid cancer in FAP is typically diagnosed in the third decade of life and female patients are at a higher risk than males.[23, 24] Annual physical examination of the thyroid is recommended and ultrasonography should be considered.[18] The relative risk of pancreatic adenocarcinoma is 4.5. The absolute life time risk, however, is low (1.7%).[18]
Hepatoblastoma occurs during the first seven years of life in about 0.3% of patients with FAP or at-risk for FAP, with an 800-fold higher risk of this rare tumor compared to the general population.[21]
Finally, the presence of a brain tumor and multiple colorectal polyps is called Crails syndrome. FAP patients usually present at young age with medulloblastoma and colorectal polyposis. Central nervous system malignancies have also been associated with hereditary non-polyposis coli cancer (HNPCC), but tumors in these patients are usually astrocytomas or glioblastomas and present later in life. The association of glioblastoma with HNPCC is known as Turcot's syndrome.[22, 25]

Benign extra-intestinal manifestations

A variety of benign extra-intestinal lesions have been described in association with FAP. Some of these phenotypic markers can be used as diagnostic tools in the examination of first degree relatives of FAP patients.[26]
Congenital hypertrophy of the retinal pigment epithelium (CHRPE) can be found in more than 90% of patients with FAP. CHRPE are discrete round to oval darkly pigmented areas in the ocular fundus ranging in size from 0.1 to 1 optic-disc diameter. They consist of multiple hyperplastic layers of retinal pigment epithelium with hypertrophied cells filled with large spherical melanosomes. Although these lesions are asymptomatic, the presence of bilateral and/or multiple (>4) CHRPE can be used as a specific clinical marker for the identification of asymptomatic carriers in FAP families.[26, 27]
Osteomas of the maxilla and mandibula are noted in approximately 80% of FAP patients.[28] Occult radio-opaque jaw lesions are osteosclerotic bone lesions that can be demonstrated by panoramic radiographs of the jaw. These lesions can be used as predictors for polyp development in families with FAP and jaw lesions.[29]
In addition, a variety of dental abnormalities, including impacted teeth, supernumerary or congenitally missing teeth, and fused roots of molars, can occur in 17 to 75% FAP patients.[28] FAP patients can also develop cutaneous lesions including lipomas, fibromas,

sebaceous and epidermoid cysts.[30, 31] Finally, nasopharyngeal angiofibroma is a highly vascular locally invasive tumor most often occurring in the nares or nasopharynx of adolescent boys. It is 25 times more common in FAP patients than in the general population.[32]

Genetic defect

In 1987 the genetic defect causing FAP was linked to chromosome 5q21[2] and in 1991 the *APC* gene was identified.[3-5] The *APC* gene is a tumor-suppressor gene with 15 exons, encoding a 2843 amino acid protein with a key function in the Wnt signaling pathway. Wnt signaling is involved in repression of apoptosis, induction of proliferation and cell cycle progression.[33]

More than 300 different *APC* gene mutations have been reported in FAP. Germline mutations are mainly found in the 5' half of the *APC* gene, particularly in codons 1061 and 1309. Most mutations are frameshifts due to insertions or deletions, or nonsense mutations, leading to truncated APC proteins.[1, 34] A high frequency of somatic *APC* mutations is found in the so-called mutation cluster region in the 5' part of exon 15, between codons 1286 and 1513 (Figure 2).[35]

Germline mutations in the *APC* gene are found in about 80-90% of patients with classic FAP and in about 10-30% of patients with AFAP.[34, 36] Until recently, no other genetic cause for the remainder of patients with classic or attenuated FAP was known. However, in 2002 defects in the base excision repair gene *MYH* were identified in patients with both classic and attenuated FAP in which no germline *APC* mutation could be found. Adenomatous polyposis in these patients is inherited in an autosomal recessive way with biallelic inactivation needed to develop the phenotype. MYH has a repair function critical for *APC*, and the *APC* gene is particularly vulnerable to loss of function.[37, 38]

Genotype-phenotype correlations

Several genotype-phenotype correlations have been established in FAP. Classic FAP is caused by *APC* mutations between codons 169 and 1393, and mutations between codon 1250 and codon 1464 are associated with severe polyposis (>1000 colorectal polyps).[1, 39] Moreover, mutations at the 5' and 3' extremes and in exon 9 of the *APC* gene tend to present as attenuated FAP, characterized by less than 100 colorectal polyps and malignant transformation occurring 10-20 years later than in patients with classic FAP (Figure 2).[1, 40, 41]

Genotype-phenotype correlations for duodenal polyposis in FAP are less clear. However, a severe duodenal phenotype appears to be associated with mutations in exon 15 of the *APC* gene, particularly distal to codon 1400.[9] Desmoid tumors have been associated with mutations 3' of codon 1444 of the *APC* gene,[42, 43] although other investigators have not found this relationship.[16, 44] Thyroid cancer appears associated with germline mutations in the 5' part of exon 15, outside codons 1286-1513, and with an increased frequency at codon 1061.[23] A multiplicity of extra-intestinal lesions has been associated with mutations in codons 1465, 1546 and 2621 and ocular fundus lesions (CHRPE) are associated with mutations between codons 463 and 1444 of the *APC* gene.[27, 44, 45]

To conclude, understanding genotype-phenotype correlations can be helpful in predictive testing of at-risk subjects. However, caution should be taken in genetic counseling of patients with FAP, since considerable phenotypic variability occurs among individuals and

families with identical *APC* mutations.[46] Therapeutic decisions should, therefore, be based on the clinical findings in individual patients, not site of gene mutation.[34]

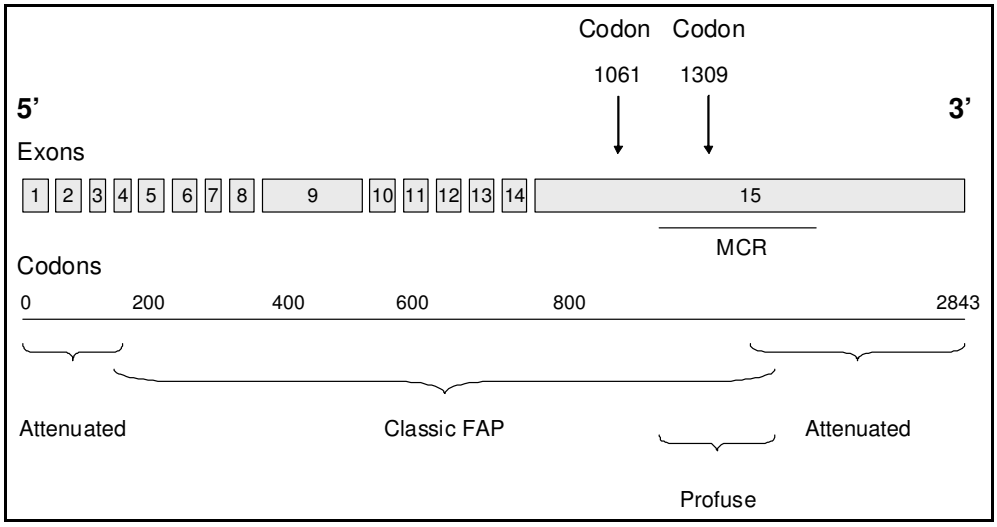

Figure 2. *Schematic representation of the APC gene, consisting of 15 exons and 2843 codons. Most germline mutations are found in the 5' half the APC gene, particularly in codons 1061 and 1309. A high frequency of somatic APC mutations is found in the so-called mutation cluster region between codons 1286 and 1513. Germline mutations in the central part of the gene represent classic FAP. Germline mutations between codons 1250 and 1464 associate with profuse polyposis, whereas mutations in the extremities of the APC gene cause attenuated FAP.*

Cancer pathogenesis

The *APC* gene is a key tumor-suppressor gene in the Wnt signaling pathway. In the absence of Wnt signaling, APC functions in a multiprotein complex with axin and glycogen synthase kinase 3β (GSK-3β) that targets β-catenin for proteasomal degradation. Inactivation of APC leads to disturbed regulation of intracellular β-catenin levels, nuclear translocation of β-catenin and Wnt target gene transcription. Wnt target genes are involved in repression of apoptosis, induction of proliferation, and cell cycle progression.[7, 33]

In 1996, Kinzler and Vogelstein proposed a paradigm for carcinoma development in FAP and sporadic colorectal cancer. In this model, intestinal carcinogenesis follows a stepwise progression through the so-called ACF-adenoma-carcinoma sequence. APC acts as the "gatekeeper" in the initiation of this oncogenic sequence. Once the *APC* gene is mutated, additional mutations in tumor-suppressor genes (e.g. *p53* and *SMAD4*) and proto-oncogenes (e.g. *K-Ras*) drive the progression of the adenoma-carcinoma sequence (Figure 3).[7] Also, expression of cell regulatory proteins is changed, including cyclooxygenase-2 (COX-2), which is increasingly expressed in consecutive stages of the adenoma-carcinoma sequence.[47] COX-2 is a key enzyme in the conversion of arachidonic acid to prostaglandin, which regulates cellular functions such as cell proliferation, apoptosis and angiogenesis. Recently, a direct link between COX-2 upregulation and Wnt signaling was shown. In the absence of functional APC, binding of prostaglandin E2 to its receptor EP2 promotes the

release of GSK-3β from its complex with axin, leading to increased intracellular β-catenin levels, Wnt target gene transcription and colon cancer cell proliferation.[48] Selective and non-selective inhibition of COX-2 has been studied in chemoprevention trials and causes regression of adenomas in FAP.[49, 50]

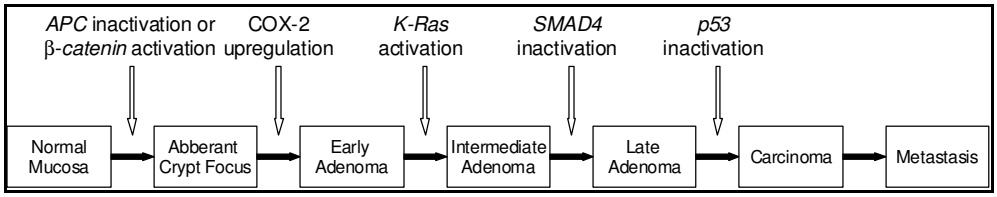

Figure 3. *The adenoma-carcinoma sequence. Activation of the Wnt signaling pathway, by an inactivating APC mutation or an activating β-catenin mutation, is regarded as the first step in the adenoma-carcinoma sequence. Then, additional mutations in oncogenes (e.g. K-Ras) and tumor-suppressor genes (e.g. p53 and SMAD4) drive further progression of the adenoma-carcinoma sequence.*

Management

Colorectum

Screening and surveillance. First degree relatives of patients with FAP should be screened for FAP between age 10-12. For these individuals, *APC* gene testing is the test of choice (Table 2). However, at-risk individuals in which no informative genetic test can be obtained should be enrolled in an endoscopic screening program. These patients should have a yearly sigmoidoscopy starting at 12 years of age, with reduced screening frequency each subsequent decade up to age 50. After 50 years of age, patients should follow guidelines for colorectal cancer screening in average-risk patients.[1, 51] For individuals suspected of AFAP, gene testing is recommended if 20 or more cumulative colorectal adenomas are found.[1] Endoscopic screening with colonoscopy in patients at risk for AFAP should occur at age 12, 15, 18 and 21, and then every 2 years.[51]

Table 2. Indications for *APC* gene testing

≥100 colorectal adenomas
First-degree relatives (>10 years old) of FAP patients
≥20 cumulative colorectal adenomas (suspected for AFAP)
First-degree relatives (>10 years old) of AFAP patients

Treatment. To prevent colorectal carcinoma in FAP patients, prophylactic colectomy should be performed shortly after diagnosis of adenomatous polyposis. Surgical options include subtotal colectomy with ileorectal anastomosis, total proctocolectomy with Brooke ileostomy (or with continent ileostomy) and total proctocolectomy with mucosal proctectomy and ileo-anal pull-trough (with pouch formation). Patients who have subtotal colectomy and ileorectal anastomosis should have life-long endoscopic surveillance of the remaining rectal segment every six months, since cancer in the retained rectum develops in approximately 25% of these patients. In about 16% of these individuals, proctocolectomy is eventually needed. Patients with dense polyposis and carcinoma at the time of subtotal colectomy have a particularly high risk of developing rectal cancer.

Therefore, these patients should have total proctocolectomy with either ileostomy or restorative proctocolectomy.[51-53]

Chemoprevention. Non-steroidal anti-inflammatory drugs (NSAIDs) have been used in chemoprevention studies of colorectal adenoma development. Chemoprevention of polyps in the retained rectum of patients with FAP with selective and non-selective COX-2 inhibitors has been shown to reduce the number and size of polyps in short-term,[49, 50] and long-term studies with sulindac.[54] However, the effects are variable and endoscopic surveillance should still be performed stringently. The main benefit obtained by the use of NSAIDs is that endoscopic surveillance is more straightforward due to decreased numbers and smaller polyps.[54] Primary chemoprevention of adenomas in phenotypically unaffected *APC* gene mutation positive patients did not prevent development of polyposis.[55]

Upper gastrointestinal tract

Screening and surveillance. Baseline upper gastrointestinal endoscopy is recommended between age 25 and 30.[51] Thereafter, endoscopic surveillance of duodenal polyposis should be adjusted to the Spigelman-stage of duodenal polyposis. In general, recommendations include: stage 0 every four years, stage I and II every 2 to 3 years, stage III and IV every 6 to 12 months and for stage IV surgery should be considered (Table 3).[10, 51]

Table 3. Recommendations for management of duodenal polyposis in FAP, adjusted to the Spigelman stage of duodenal polyposis

Spigelman stage	Endoscopic frequency	Chemoprevention	Surgery
Stage 0	4 years	No	No
Stage I	2-3 years	No	No
Stage II	2-3 years	+/-	No
Stage III	6-12 months	+/-	+/-
Stage IV	6-12 months	+/-	Yes

Treatment. Treatment options for duodenal polyposis are pharmacologic therapy, endoscopic treatment (including snare-excision, laser therapy, photodynamic therapy and argon-plasma therapy), and surgery.[9]

Endoscopic treatment of duodenal polyposis is fraught with high recurrence rates, varying from 50 to 100%. Therefore, the benefit of endoscopic therapy is controversial, but it may be useful in individual cases and to postpone surgery.[9]

Surgery for duodenal polyposis is generally offered to patients with stage IV duodenal polyposis. Surgical options include local surgical treatment (duodenotomy with polypectomy and/or ampullectomy), pancreas and pylorus preserving duodenectomy or classical pancreaticoduodenectomy. High recurrence rates have been reported in patients treated with local surgery. Therefore, pancreas and pylorus preserving duodenectomy or classical pancreaticoduodenectomy is indicated in patients with severe duodenal disease, failed endoscopic or local surgical management, and carcinoma development. However, morbidity and mortality of these surgical procedures must be weighed against the risk of developing duodenal malignancy.[9, 51]

Chemoprevention. Chemoprevention trials of duodenal polyps with NSAIDs show conflicting result. Some groups find modest regression of small duodenal adenomas in patients treated with 400 mg sulindac or 800 mg celecoxib, however, most reports find no significant effect on duodenal polyposis.[9]

Desmoid tumors

Desmoids in the abdominal wall and extremities can usually be excised with few complications, although recurrence rates are high (10-70%). In case of recurrence, re-resection, or treatment with sulindac and anti-estrogens can be considered. In addition, radiotherapy can be used for recurrence of desmoids on the extremities.[17]

The usual first line treatment of symptomatic mesenteric or retroperitoneal desmoids is medical treatment with regiments such as combination of sulindac (200 mg/day) and an anti-estrogen (e.g. tamoxifen 20-40 mg/day). The effect of treatment should be evaluated every three to six months by CT scan. In addition, kidney function should be controlled and stenting should be considered in case of ureteric obstruction from desmoid disease. Radical resection of mesenteric or retroperitoneal desmoids is often impossible. Also, major complications occur in about half of the patients and post-operative mortality rates vary from 10 to 60%. In almost 80% of these patients, desmoids will recur within 5 years. However, surgery can be considered if considerable symptoms result from small, well-defined desmoids. Intestinal by-pass surgery is indicated in patients with signs of intestinal ischemia or bowel obstruction. Larger desmoids, that involve vital structures, should be attacked with cytotoxic agents.[17]

Life-expectancy and causes of death

As a result of screening and prophylactic surgery, life expectancy of patients with FAP has significantly improved in the last several decades. However, FAP patients still have a 3.35 fold elevated risk of dying compared to the general population.[56] The main causes of dead are upper gastrointestinal malignancy, peri-operative complications and desmoid disease.[56, 57]

JUVENILE POLYPOSIS

Introduction

Juvenile polyposis is an autosomal dominant syndrome characterized by multiple juvenile polyps primarily in the colorectum but also elsewhere in the gastrointestinal tract. The incidence is approximately 1/100,000 live births. The first histological description of a juvenile polyp was by Diamond in 1939,[58] and McColl et al. introduced the term juvenile polyposis in 1964.[59]

Solitary juvenile polyps occur in approximately 2% of the pediatric population and are not associated with an increased risk of colorectal cancer.[60] In contrast, in the setting of juvenile polyposis, there is an increased risk of gastrointestinal malignancy. JPS is generally defined as: A) more than 3-5 juvenile polyps in the colorectum, and/or B) juvenile polyps throughout the gastrointestinal tract, and/or C) any number of juvenile polyps with a positive family history of juvenile polyposis.[61, 62]

Other syndromes to be excluded and associated with colorectal juvenile polyps include Bannayan-Riley-Ruvalcaba, Cowden, and Gorlin syndrome. Each of these disorders is

characterized by specific extra-intestinal features in addition to intestinal polyps. In some patients, however, exclusion of one these syndromes can be difficult since extra-intestinal characteristics may only appear at later age.[63]
Macroscopically, juvenile polyps typically have a spherical, lobulated and pedunculated appearance and vary in size from 5 to 50 mm, often with surface erosion. On histology, solitary juvenile polyps have abundant stroma composed of inflamed and edematous granulation tissue surrounding cystically dilated glands containing mucin. The glands are lined by cuboidal to columnar epithelium with reactive changes. Juvenile polyps in juvenile polyposis may have similar appearances as sporadic juvenile polyps, but often have a frond-like growth pattern with relatively less stroma, fewer dilated glands and more proliferative smaller glands (Figure 1B).[64]

Clinical manifestations

Presentation

Juvenile polyposis typically presents in the first or second decade of life with rectal bleeding, a prolapsed rectal polyp, abdominal pain, diarrhea or anemia. In 20-50% of these patients a family history of juvenile polyposis is present.[62, 65, 66] JPS can also present in infancy with severe gastrointestinal bleeding, diarrhea, protein-losing enteropathy and failure to thrive. Death may occur at a young age in these patients if supportive care is not provided. Family history is usually negative. This latter form is also called JPS of infancy.[62,66]
Polyps most commonly occur in the colorectum and vary in number from three to several hundreds. Polyps can also be found in the upper gastrointestinal tract, particularly in the stomach.[67-69] Howe et al. report an incidence of upper gastrointestinal polyps in about 40 to 65%.[70] Rarely, profuse gastric juvenile polyposis is found in the absence colonic polyps.[71]

Cancer risk

Previously, juvenile polyps were thought to harbor no malignant potential. This appears true for sporadic solitary juvenile polyps,[60] but not in juvenile polyposis. Reports describing adenomatous change in colonic juvenile polyps,[61, 67, 72-74] adenomas,[61, 67, 74] and colorectal carcinoma in patients with JPS,[61, 67, 72-74] suggest an increased risk of colorectal cancer. In addition, gastric,[70, 75, 76] duodenal,[70] and pancreatic cancer[70, 77] have been described in these patients.
Few studies estimate the colorectal cancer risk in JPS, and these calculations vary widely. Jass et al. found colorectal cancer in 18 of 87 (20.7%) JPS patients at a mean age of 34 years (range 15-59).[62] However, since most of these patients had undergone colectomy, the cumulative risk of colorectal cancer was estimated as high as 68% at age 60.[78]
A review of medical records of the Iowa JPS kindred of 29 patients with juvenile polyposis revealed 11 patients with colorectal cancer (38%), 4 with gastric (13.7%), 1 with duodenal (3.4%), and 1 with pancreatic cancer (3.4%). The cumulative risk of colorectal and upper gastrointestinal malignancy was 55% with a median age of colorectal and upper gastrointestinal cancer of 42 (range 17.4-68.2) and 57.6 (range 20.5-72.8) years, respectively.[70] In the same report, a literature review revealed 42 cases of colorectal cancer (31.5%), 15 cases of stomach cancer (11.3%), one case of duodenal (0.75%), and

one pancreatic carcinoma (0.75%) in 133 patients with familial juvenile polyposis from 22 families.[70]
In a review of published reports, Coburn et al. found 34 colorectal cancers in 218 JPS patients (15.5%) and a mean age of cancer diagnosis of 35.5 (range 4-60) years. In addition, they found one gastric and one duodenal carcinoma.[65]
Although considerable variation in reports exists, it seems evident that JPS patients carry an increased risk of colorectal and possibly gastric cancer.

Extra-intestinal manifestations

A variety of extra-intestinal manifestations in JPS have been described, although most are based on case reports. Also, extra-intestinal features in JPS are difficult to interpret due to an unclear distinction between JPS, Cowden, and Bannayan-Ruvalcaba-Riley syndrome.
Extra-intestinal manifestations have been reported in about 10 to 78% of JPS patients.[66, 79] Anomalies described, include hypertelorism, macrocephaly, digital clubbing, polydactily, mental retardation, hydrocephalus, congenital cardiac anomalies, pulmonary arteriovenous malformations, pulmonary stenosis, teleangiectasias, Meckels diverticulum, intestinal malrotation, cryptorchidism, and bifid uterus and vagina.[59, 65, 71, 77, 79]
An association between JPS and hereditary hemorrhagic teleangiectasias (HHT) exists. Hereditary hemorrhagic teleangiectasia (Osler-Weber-Rendu syndrome) is an autosomal dominant disorder of vascular dysplasia affecting many organs. Characteristic features are teleangiectasias of skin and mucosal surfaces, pulmonary, cerebral and hepatic arteriovenous malformations and hemorrhage as a consequence of these lesions. HHT is caused by mutations in two endothelial-specific receptors for TGF-β: *ENG (Endoglin)* and *ACVRL1 (ALK1)* (Figure 4).[80] Case reports of juvenile polyposis patients with symptoms of HHT, including arteriovenous malformations in the lung,[81-84] liver,[83, 84] and skin[84] and gastrointestinal teleangiectasias,[82, 83] raised the suggestion of a common genetic cause for these two syndromes. In 2004, HHT and juvenile polyposis were genetically linked by the discovery of *SMAD4* mutations in patients with both conditions,[85] and germline mutations in *ENG* were recently described in two JPS patients.[86]
In JPS patients with germline *SMAD4* or *ENG* mutations, screening should be considered for arteriovenous malformations using chest radiography, magnetic resonance imaging of the brain and liver sonography.[83, 85] In addition, digital clubbing and pulmonary osteoarthropathy are frequently described in combination with arteriovenous malformations.[79, 81]

Bannayan-Riley-Ruvalcaba syndrome and Cowden syndrome

Bannayan-Riley-Ruvalcaba syndrome (BRRS) and, to a lesser extent, Cowden syndrome (CS) share the intestinal phenotype of juvenile polyposis. Hamartomatous intestinal polyps occur in about 45% of BRRS patients.[87] In CS, intestinal polyps occur less frequently, although the incidence is unclear. In addition, polyps in CS are usually less abundant and asymptomatic, and typical juvenile polyps in CS are rare.[88]
Intestinal polyposis is not pathogmonic for BRRS or CS and both syndromes are marked by specific extra-intestinal features. BRRS is characterized by macrocephaly, developmental retardation, genital pigmentation, hemangiomas, lipomas, intestinal polyps and lipid myopathy.[87] Clinical features of CS include mucocutaneous lesions (facial trichilemmoma, acral keratoses, papillomatous papules and mucosal lesions are pathogmonic), increased

risk of breast and thyroid carcinoma, macrocephaly and a range of minor features, including gastrointestinal hamartomas. About 75% of CS patients have thyroid disease, usually goiter and/or adenoma. CS patients are at increased risk of breast cancer (25-50%), non-medullary thyroid malignancy (10%), and possibly endometrial carcinoma.[88]
CS and BRRS are autosomal dominant diseases caused by a germline defect in the *PTEN* gene, which is found in approximately 80% of CS and 60% of BRRS patients.[89, 90] Since patients fulfilling criteria for both CS and BRRS have been described, and CS and BRRS are caused by mutations in the same gene, these two syndromes are likely two variable expressions of the same genetic alteration.[90]

Genetic defect

Currently alterations in two genes, *SMAD4* and *BMPR1A*, are identified causes of JPS. Both encode proteins involved in TGF-β/BMP signaling (Figure 4). In 1998, Howe et al. discovered *SMAD4*, located on chromosome 18q21.1, as a gene responsible for JPS.[91] Germline mutations in *SMAD4* are found in 16-24% of JPS patients, most in the 3' half of the gene, encoding the highly conserved MH2 domain, which is involved in SMAD oligomerization and transcriptional activation.[91-94] In 2001, *bone morphogenetic protein receptor 1A* (*BMPR1A*), located on chromosome 10q22.3, was identified as a second JPS gene.[95] Germline *BMPR1A* mutations are noted in 17-24% of JPS patients.[92-94] Since germline mutations in *SMAD4* or *BMPR1A* are identified in a minority of patients with clinically defined juvenile polyposis,[92] other components of the TGF-β signaling pathway have been studied. Other *SMAD* genes, including *SMAD1*, *SMAD2*, *SMAD3*, *SMAD5* and *SMAD7* were analyzed, but no germline mutations in these genes were found in JPS patients.[96, 97] In addition, germline *BMPR2*, *BMPR1B* and *ACVRL1* mutations were excluded as a cause for JPS.[92] Recently, germline mutations in the HHT gene *ENG*, encoding the TGF-β co-receptor endoglin, were reported in two patients with JPS.[86]
The role for germline *PTEN* (chromosome 10q23.3) mutations in juvenile polyposis is unclear. Some investigators suggested that *PTEN* could be involved in JPS,[98] while others disagree.[99] Discriminating between JPS and CS can be difficult since the penetrance of CS is less than 10% below age 15.[63] Currently, *PTEN* mutations in patients with JPS likely represent CS or BRRS patients that have not yet expressed the clinical features of these conditions.[100]

Genotype-phenotype correlation

Since juvenile polyposis is a rare disorder, only a few genotype-phenotype studies exist. Patients with either a *SMAD4* or *BMPR1A* germline mutation express a more prominent juvenile polyposis phenotype (i.e. more family history of JPS, >10 polyps, and higher frequency of family history of gastrointestinal cancer) compared to those without an identified germline mutation.[93] Moreover, *SMAD4* mutations have been associated with a more aggressive gastrointestinal phenotype compared to *BMPR1A* mutation carriers. Handra-Luca and colleagues, found a higher incidence of colonic adenomas in carriers of *SMAD4* mutations compared to those with *BMPR1A* or *PTEN* mutations, and carcinoma was only found in patients with *SMAD4* mutations.[101]
In addition, carriers of germline *SMAD4* mutations have more severe gastric polyposis than patients with a *BMPR1A* mutation or those in whom no germline mutation could be

identified.[93, 94, 101] Finally, the combined syndrome of JPS and hereditary hemorrhagic teleangiectasia has been associated with germline mutations in *SMAD4*.[85]

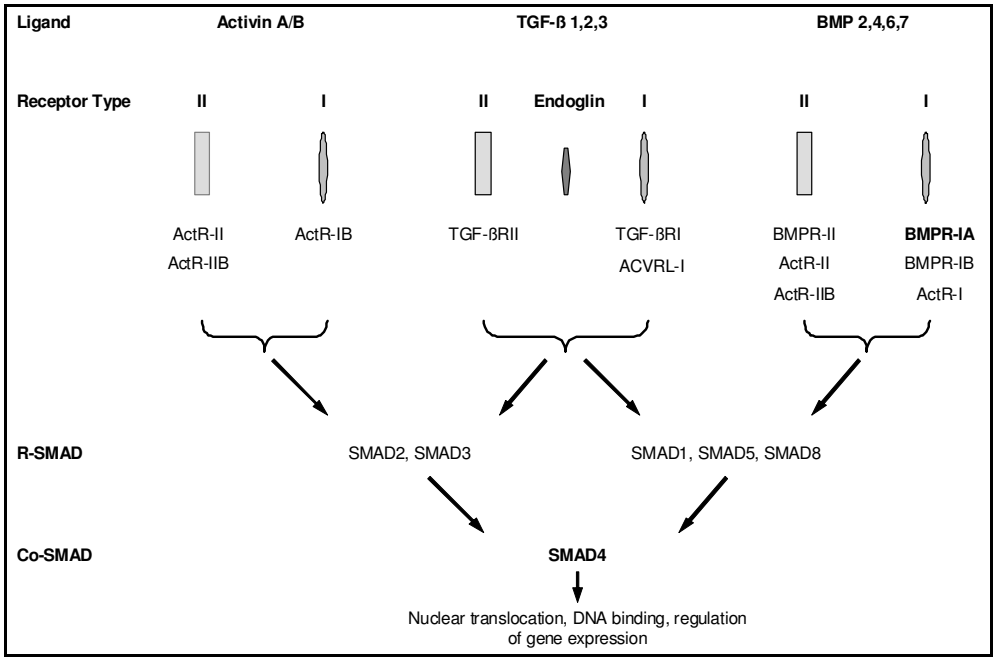

Figure 4. *The TGF-β signaling pathway. Binding of a TGF- β ligand to a type II receptor results in recruitment of a type I receptor and subsequent phosphorylation of the type I receptor by the type II receptor. The type I receptor then phosphorylates and activates receptor-regulated SMADs (R-SMADs) which subsequently form complexes with the common SMAD (Co-SMAD) SMAD4. The activated SMAD complexes translocate to the nucleus where they regulate target gene transcription. Inhibitory SMADs (I-SMADs) SMAD6 and SMAD 7 inhibit TGF-β signaling by competing with R-SMADs for receptor or Co-SMAD interaction and by targeting the receptors for degradation. Endoglin is a co-receptor for TGF-β1 and TGF-β3. Abbreviations: Act: activin, ActR: Activin receptor, ALK: Activin-like kinase, BMP: Bone morphogenetic protein, BMPR: BMP receptor, TGF-βR: TGF-β receptor, ACVRL-1: Activin A receptor type-II like 1. Commonly used synonyms are: ActR-1/ALK2, ActR-IB/ALK4, TGF-βR-I/ALK5, ACVRL1/ALK1, BMPR-IA/ALK3, BMPR-IB/ALK6.*

Cancer pathogenesis

Malignant transformation in JPS likely occurs in adenomatous foci within juvenile polyps, and/or in adenomas that arise as separate lesions. Reports describing adenomatous foci in juvenile polyps suggest that these polyps can undergo neoplastic change.[61, 67, 72-74] In addition, carcinoma *in situ* and adenocarcinoma have been described within juvenile polyps.[68, 102, 103]

Goodman and colleagues first proposed a model for polyp development and neoplastic transformation in juvenile polyps. They suggested a pathogenic sequence from epithelial hyperplasia, leading to hyperplastic polyps which become inflamed and enlarge, forming juvenile polyps. Subsequently, focal adenomatous areas develop in some juvenile polyps giving rise to adenoma and eventually adenocarcinoma.[67] Moreover, Jass et al. showed

that dysplasia could be found frequently in juvenile polyps, particularly in atypical juvenile polyps. Adenomatous epithelium was found in 9% of typical juvenile polyps, but 47% of atypical juvenile polyps (i.e. multilobulated, relatively less lamina propria and more epithelium and villous or papillary configuration).[62]

Although alterations in genes with a function in the TGF-β/BMP signaling pathway cause JPS, molecular mechanisms of polyp development are still poorly understood. The TGF-β signaling pathway is a key tumor-suppressor pathway, regulating cellular functions such as cell proliferation, adhesion and differentiation. The TGF-β family comprises several structurally related secreted cytokines, including TGF-β isoforms, activins, and BMPs. Signal transduction is mediated through binding of these cytokines to membrane bound receptors and subsequent intracellular signal transduction mediated by SMAD proteins. Of these proteins, SMAD4 has a central role in TGF-β, activin, and BMP signaling (Figure 4).[104]

In 1998, Kinzler and Vogelstein introduced the "landscaper" hypothesis. Based on the observation that the genetic alterations at chromosome 10q22 (*BMPR1A* locus) occur predominantly in the stroma,[105] they hypothesized that stromal changes provide an abnormal environment influencing behavior of adjacent epithelium and ultimately leading to an increased risk of neoplastic transformation.[106] Recently, BMP-4 has been localized exclusively to the mesenchymal compartment of the intestine in mice. Disrupted BMP signaling resulted in development of ectopic crypts perpendicular to the crypt-villus axis, leading to polyps resembling juvenile polyps.[107, 108] Consequently, disrupted mesenchymal-epithelial communication, by defective BMP signaling, is hypothesized to cause the "landscaper" defect.[107] Interestingly, in long term observation, neoplastic change was observed in the polyps of these mice. Nuclear translocation of β-catenin and overexpression of Wnt targets in these dysplastic foci suggested Wnt activation. This was interpreted as compelling evidence that stromal-epithelial cross-talk in these polyps underlies conventional adenoma-carcinoma sequence initiation. Furthermore, BMP signaling appeared to restrict ectopic crypt formation by antagonizing Wnt signaling. He et al. proposed that BMP-4 promotes PTEN activation in intestinal stem cells, which in turn represses β-catenin/TCF4 through the PI3 kinase-AKT pathway.[108] Lastly, disturbed stromal TGF-β signaling ultimately leads to epithelial neoplasia of the prostate in mice.[109]

Whether germline *SMAD4* mutations in JPS also act via a "landscaper" mechanism is unclear. Historically, *SMAD4* is known as a tumor-suppressor gene in several cancer types.[110] One study found homozygous *SMAD4* deletions primarily in the epithelium, but also in stromal fibroblasts and pericryptal myofibroblasts, of juvenile polyps of JPS patients with germline *SMAD4* mutations. These findings suggest that SMAD4 acts as a "gatekeeper", instead of a "landscaper" and that juvenile polyps are not just stromal lesions, but epithelium is also involved in hamartoma development.[111]

LOH of *SMAD4* was found in 1 out of 11 polyps from five JPS patients.[91] Also heterozygous *SMAD4* knockout mice showed LOH in epithelium of polyps resembling juvenile polyps.[112] Finally, SMAD4 protein expression was absent in almost all polyps from *SMAD4* mutation carriers, suggesting a second-hit mechanism in polyp formation in JPS.[113] In contrast, no LOH of *BMPR1A* was found in either epithelial or stromal cells of JPS polyps, arguing against a typical two-hit tumor-suppressor function for BMPR1A.[95]

Management

Screening. Patients at risk or with a high suspicion of JPS should have endoscopic screening of the colon and upper gastrointestinal tract at age 15 or at the time of first symptoms.[6, 114, 115] At the time of diagnosis of JPS, the entire gastrointestinal tract should be examined for the presence of polyps.[68] Genetic testing can be useful for at-risk members from families where germline mutations have been identified. If no germline mutation is found in such an at-risk person, they do not have JPS and can be enrolled in screening programs for the general population.[114]

Surveillance and treatment. Endoscopic examination of the colon and upper gastrointestinal tract is recommended every two to three years in patients with JPS. In patients with polyps, endoscopic screening should be performed yearly, until the patient is polyp free.[6, 114-116] Patients with mild polyposis can be managed by frequent endoscopic examinations and polypectomy.[116]

Prophylactic surgery is indicated in patients with polyposis (>50-100) unable to be managed endoscopically, severe gastrointestinal bleeding or diarrhea, juvenile polyps with adenomatous changes, and patients with a strong family history of colorectal cancer.[115-117] Surgical options include subtotal colectomy with ileorectal anastomosis (IRA) or total proctocolectomy with pouch.[116, 117] It is unclear which type of surgery is preferable but, in analogy with FAP, may depend on the extent of rectal polyposis.[118] However, no relationship was found between rectal polyp burden and the type of surgery.[117] Moreover, recurrence of rectal polyps in patients with subtotal colectomy is frequent[116] and recent data show that about half of these individuals require subsequent proctectomy.[117] Therefore, total proctocolectomy has been advocated as the initial surgery for patients with massive juvenile polyposis, unable to be managed endoscopically.[116] Although the surgery of choice in JPS remains debatable, patients need frequent post-operative endoscopic surveillance because of high recurrence rates of polyps in the remnant rectum and the pouch.[117]

Endoscopic treatment of gastric polyps is often difficult, and patients with symptomatic gastric polyposis (e.g. severe anemia) eventually need subtotal or total gastrectomy.

Chemoprevention. Recently noted, COX-2 expression is higher in JPS polyps than in sporadic juvenile polyps and correlates with polyp size and dysplasia.[119] This observation suggests that chemoprevention using selective or non-selective COX-2 inhibitors could be beneficial in JPS. Currently, NSAID chemoprevention in JPS has not been studied systematically. Anecdotally, Oncel et al. described the use of sulindac 300 mg per day in two JPS patients who had proctocolectomy with a pouch and subsequent polypectomy from the pouch. These patients were followed for 2 and 9 years with no further polyp development in the pouch.[117] The value of NSAID chemoprevention in JPS requires further investigation.

PEUTZ-JEGHERS SYNDROME

Introduction

Peutz-Jeghers syndrome is an autosomal dominant disorder, characterized by hamartomatous intestinal polyposis and mucocutaneous skin pigmentation. After juvenile polyposis, it is the most common hamartomatous syndrome with an incidence of one per 120,000-200,000 live births.[6, 120, 121] First described as an inherited condition by Dr Peutz

in 1921, followed by a comprehensive report by Dr Jeghers in 1949, the eponym Peutz-Jeghers was assigned to this disorder in 1954.[120, 122]
Diagnostic criteria for PJS are: A) three or more histologically confirmed Peutz-Jeghers polyps, or B) any number of Peutz-Jeghers polyps with a family history of PJS, or C) characteristic, prominent, mucocutaneous pigmentation with a family history of PJS, or D) any number of Peutz-Jeghers polyps and characteristic, prominent, mucocutaneous pigmentation.[120, 123]
Peutz-Jeghers polyps typically occur in the small intestine, although these lesions can also be found in the stomach, large intestine, and rarely in the gallbladder, respiratory and urinary tract.[124] Macroscopically, the polyps are 5 to 50 mm in size and can be pedunculated or sessile. On histology, the center of the polyp is composed of smooth muscle with a tree-like branching pattern. Overlying the smooth muscle core, is mucosa native to the region, heaped into folds producing a villous pattern (Figure 1C). Pseudo-invasion (epithelial misplacement involving all layers of the bowel wall) has been described in 10% of the small intestinal PJS polyps, and may, thereby, mimic a well differentiated adenocarcinoma.[123, 125]

Clinical manifestations

Presentation

Presenting symptoms of Peutz-Jeghers syndrome include bowel obstruction, polyp intussusception, abdominal pain, rectal bleeding and anemia. About 50% will present with obstruction or intussusception, 25% presents with abdominal pain, and rectal bleeding and polyp extrusion are found in 13 and 7%, respectively.[121, 126] Symptoms usually occur during the first decade of life, and 50-60% of patients will have complaints before the age of 20.[127] In addition, PJS patients can present with gastrointestinal malignancy.[121]
Mucocutaneous skin pigmentation, characterized by increased melanocytes at the epidermal-dermal junction and increased melanin in the basal cells, is the hallmark feature of Peutz-Jeghers syndrome. These pigmented macules are usually between 1 and 5 mm in size and cluster around the mouth, eyes, nostrils, and the peri-anal area. Pigmented spots can also be found on the fingers and toes and rarely on the dorsal and volar aspects of the hands and feet. Pigmentation can be present from birth, but may develop in early infancy, and is usually present before gastrointestinal manifestations arise. Although buccal pigmentation tends to persist, skin pigmentation can fade with age.[121]
The presence of multiple hamartomatous polyps with typical Peutz-Jeghers histology in the large and/or small intestine is diagnostic for PJS. Polyps in PJS are less numerous than in FAP, ranging from zero to several dozens per intestinal segment. Polyps are most prevalent in the small intestine (64-96% of patients), but can also be found in the colon (27-53% of patients) and stomach (24-49% of patients).[121, 126] Solitary Peutz-Jeghers polyps can occur as sporadic lesions in patients without a family history or any other features of PJS. In addition to intestinal polyps, hamartomas have been described in the gallbladder, nasopharynx, trachea, bladder, and ureter.[124]

Cancer risk

Peutz-Jeghers patients have an increased risk for several malignancies including small intestinal, stomach, pancreas, colon, esophagus, ovary, uterus, lung, and breast cancer.[128-131] Several investigators have estimated the cancer risk in PJS.

Giardiello et al. reported 15 malignancies in 31 PJS patients (48%). Most cancers were extra-intestinal and only 4 gastrointestinal cancers were found. They reported a 18 times greater risk of cancer development in PJS patients compared to the general population.[128]
Spigelman et al. studied 72 PJS patients and found 16 cancers (22%), of which 9 were gastrointestinal and 7 were extra-intestinal malignancies. The estimated relative risk of death was 13 due to gastrointestinal malignancy, and 9 due to any malignancy. The chance of dying of cancer was 48% at age 57.[130]
Boardman et al. found a relative risk of cancer of 18.5 in women and 6.2 in men with PJS (overall relative risk of 9.9). Men had a relative risk of gastrointestinal cancer of 30.3 and women of 150.9, the total relative risk was 50.5 for men and women together. The relative risk of breast and gynecologic cancer in women was 20.3.[131]
In 2000 Giardiello and colleagues reviewed the literature and estimated the risk of cancer in PJS based on 210 PJS patients described in 6 publications. They found a relative risk of all cancers of 15.2 and a cumulative risk of developing cancer of 93% from age 15 to 64. Moreover, PJS patients were at a significantly greater relative risk of cancer of the small intestine (RR 520), stomach (RR 213), pancreas (RR 132), colon (RR 84), esophagus (RR 57), ovary (RR 27), uterus (RR 16), lung (RR 17), and breast (RR 15.2).[129]
Recently, a large study among 240 patients with Peutz-Jeghers syndrome carrying germline *LKB1/STK11* mutations found an overall risk of developing cancer at ages 20, 30, 40, 50, 60 and 70 years of 1%, 3%, 19%, 32%, 63% and 81%, respectively. The most common cancers found were gastroesophageal, small bowel, colorectal and pancreatic cancer. Women had an increased risk of breast cancer of 32% at age 60.[132]
In summary, these studies show that PJS patients are at high risk for cancer, although exact figures remain unclear. The relative risk of developing cancer ranges between 9.9 and 18 fold [128, 129, 131] and the cumulative risk between 80-90% at age 70.[129, 132]

Genital tract tumors

Several gonadal malignancies occur in PJS patients.[133, 134] In female patients, sex cord tumors with annular tubes (SCTAT) are found in almost all individuals in whom the ovaries are examined. Patients with these tumors can present with menstrual irregularity, hyperestrogenism or sexual precocity. These lesions occur bilaterally and usually have a benign behavior, although a clinically malignant course has been reported.[133]
In male PJS patients calcifying Sertoli cell tumors, also referred to as testicular tumors resembling SCTAT, have been described. Presenting symptoms are gynecomastia, rapid growth and advanced bone age due to hyperestrogenism. Patients often present at a young age, between age 2-6 years old. Although a benign tumor, it does have malignant potential. Orchidectomy is the curative treatment.[135, 136]
Finally, adenoma malignum, or minimal deviation adenocarcinoma of the cervix, has been reported in PJS patients. Presenting symptoms include abnormal vaginal bleeding or a mucoid vaginal discharge. It is an extremely well differentiated adenocarcinoma of the cervix, usually of mucinous type, with malignant behavior and a poor prognosis.[133, 134]

Genetic defect

In 1998 two groups independently identified the serine-threonine kinase gene (*STK11/LKB1*), located on chromosome 19p13.3, as responsible for the Peutz-Jeghers syndrome.[137, 138] *LKB1* is a tumor-suppressor involved in intracellular signal transduction

and cellular polarity.[139] Mutations in *LKB1* result in inactivation of the kinase activity,[140] although germline mutations that allow retention of kinase activity have recently been reported.[141]

LKB1 is composed of nine exons, coding a 433 amino acid protein. Although mutations can occur throughout exons 1 to 9, about 40% are found in exon 1 to 6.[132, 142, 143] More than 75% of the mutations in *LKB1* are frameshift or nonsense mutations, resulting in a truncated protein. In-frame deletions or missense mutations occur less commonly at conserved amino acids within the kinase core of the protein.[137, 138, 140, 141, 143-145]

Germline mutation in *LKB1* can be identified in about 50 to 70% of PJS patients. Although shortcomings in mutational analyses and patient selection have been suggested to account for the large proportion of germline mutation-negative PJS patients, genetic heterogeneity has also been considered.[141-144, 146] A possible second locus was proposed at chromosome 19q13.4. However, no germline mutations in this region have been identified.[147, 148] In addition, several LKB1/STK11-interacting proteins, including STRAD on chromosome 17q23.3,[149, 150], BRG1, MO25,[150] and LIP1 (LKB1-interacting protein) on chromosome 2q36,[148] were excluded as PJS genes. A recent study found germline *LKB1* defects in 94% of patients who clinically met the criteria of Peutz-Jeghers, arguing against the existence of a second PJS locus.[151]

Genotype-phenotype correlations

Considerable inter- and intra-familial phenotypic variation of expression exists in PJS kindreds and little is known about the natural course of PJS in relationship to site and type of *LKB1* germline mutation. Several studies have evaluated genotype-phenotype correlations in PJS.

Some studies found that cancer risk differs between PJS patients with and without detectable *LKB1* mutations.[141, 144, 146] However, a recent large multi center study did not find such correlation.[152]

Also the site and type of mutation have been associated with differences in cancer risk for patients with PJS in some small studies. One study found that PJS patients with missense mutations had a later age of onset of gastrointestinal symptoms, gastric polyposis, and first polypectomy compared to patients with truncating mutations or with no detectable mutation.[146] Another group reported that in-frame deletions, splice site mutations, and missense mutations in the part of the gene encoding protein domains important for ATP binding (codon 49-106) and the site of catalysis (codon 123-171) were rarely associated with cancer, whereas mutations in the C-terminus and in the part of the gene encoding protein domains important for substrate recognition (codon 171-225) were more frequently associated with malignancies.[141] A recent large collaborative study, however, did not find a correlation between type or site of *LKB1* mutation and cancer risk.[152]

Cancer pathogenesis

The pathogenic mechanisms involved in gastrointestinal polyp development and carcinogenesis in PJS are largely unknown. Reports describing adenomatous and carcinomatous change within hamartomas, suggest that malignancy develops within hamartomas, following a hamartoma-adenoma-carcinoma sequence, comparable to that in FAP.[153-156] In addition, several studies provided molecular evidence of a hamartoma-adenoma-carcinoma sequence in PJS. A second hit in *LKB1* causing loss of heterozygosity

(LOH) in adenomatous and carcinomatous lesions in PJS polyps was noted by several investigators.[145, 157, 158] Consequently, *LKB1* was thought to act as a typical tumor-suppressor gene.[159] In addition, LOH of *p53*, and *K-Ras* and *β-catenin* mutations were found in adenomas developing in hamartomatous polyps, indicating that molecular alterations in these genes drive carcinogenesis in PJS as well.[145, 158]
However, the precise frequency of LOH of *LKB1* in PJS polyps in humans remains unclear, and studies in mice showed that loss of the wild-type *LKB1* allele is not a prerequisite for the formation of hamartomatous polyps.[160] Therefore, the need for a second-hit in *LKB1* during polyp development in PJS and, hence, the role of *LKB1* as a typical 'Knudson' two-hit tumor-suppressor gene, is questioned.
The identification of LKB1 as important in cellular polarity may provide new insights in the molecular mechanism of polyp and carcinoma development in PJS.[139] One theory suggests mucosal prolapse as a pathogenic mechanism underlying the development of typical hamartomatous polyps in PJS. In this hypothesis PJS hamartomatous polyps represent an epiphenomenon to the cancer prone condition and the hamartoma-adenoma-carcinoma sequence as such does not exist.[161] Loss of polarity function may also affect asymmetric stem cell division in PJS and lead to expansion of the stem cell pool.[162] This could contribute to polyp formation and explain the increased cancer risk as well.

Management

Screening and surveillance of PJS patients is essential since complications of polyposis resulting in repeated acute laparotomy with the risk of short-bowel syndrome can be prevented.[163] In addition, PJS patients are at increased risk for numerous malignancies. Management of small bowel polyps is problematic, since most endoscopic techniques fail to visualize and treat polyps in this region. However, modern endoscopic techniques have improved surveillance and treatment of small bowel polyposis.
Screening. Genetic testing of at-risk offspring of PJS patients is indicated at the time symptoms occur or in the late teens if symptoms do not occur. In addition, in patients with a negative family history, genetic testing is indicated in patients with Peutz-Jeghers polyps or typical pigmentation.[6, 146]
Surveillance and treatment. Most authors recommend endoscopic surveillance of the upper gastrointestinal tract at a two year interval starting at age 10.[6, 120, 121] However, others recommend upper gastrointestinal endoscopic surveillance every three years, starting at age 25.[115] In addition, a barium study is recommended every 2 years to evaluate small intestinal polyposis. Polyps larger than 1.5 cm should be removed by push enteroscopy and/or laparotomy with intra-operative enteroscopy. Small polyps can be removed by snare polypectomy, larger polyps may require enterotomy.[120, 121, 163] In the future, wireless capsule endoscopy may prove to be an effective method for evaluating small bowel polyposis in PJS.
Colonoscopic examination should occur every three years starting at the time of first symptoms or in the late teens in patients that did not develop symptoms.[6, 115]
Multiple bowel resections due to gastrointestinal complications of polyps may eventually result in short-bowel syndrome. Therefore, prevention of surgery is key in the treatment of PJS. However, surgery may be inevitable in acute situations, or in case of malignancy.
To evaluate presence of pancreatic tumors, endoscopic or abdominal ultrasound is indicated every one or two years, starting at age 30.[120, 121] Female patients should perform

regular breast self-examination, and undergo breast radiology every 5 years from 25 to 45 years. Thereafter, breast radiology should occur every two years between age 45 and 50, and yearly after the age of 50.[120] In addition, pelvic ultrasound, and cervical smears should be performed yearly.[120, 121] Finally, affected or at-risk males should perform regular self examination of the testes and have scrotal ultrasound until puberty or in the presence of feminizing symptoms.[120]

Chemoprevention: Several investigators showed increased levels of COX-2 in hamartomas[164-166] and carcinomas[164, 166] of PJS patients. Udd et al. studied the effect of COX-2 inhibition in *LKB*$^{+/-}$ mice and PJS patients. They observed decreased numbers of polyps larger than 2 mm in *LKB1*$^{+/-}$ mice treated with celecoxib or *LKB*$^{+/-}$ mice in which one or two *COX-2* alleles were knocked out. Interestingly, the effect of celecoxib treatment on polyp burden was greater than the effect that was observed in COX-2 deficient mice, indicating an effect of a COX-2 independent mechanism. Moreover, no effect on polyps smaller than 2 mm was observed, which points to a role for COX-2 in polyp progression rather than polyp initiation.[167] In addition, they performed a pilot clinical trial in which eight PJS patients were treated with 400 mg celecoxib per day for 6 months. Gastroscopy was performed before and after 6 months of treatment. Clinical data from 6 patients were used in the final analysis. In 2 of 6 patients a significant reduction of gastric polyp burden was observed.[167] These results indicate that COX-2 inhibition may be beneficial in at least a subset of PJS patients. However, a randomized study is necessary to further evaluate the potential of long-term COX-2 treatment in PJS.

SUMMARY AND CONCLUDING REMARKS

FAP, JPS and PJS are the most well-known and clinically relevant gastrointestinal polyposis syndromes. Although rare, recognition of these conditions is important in view of the consequences for the patients as well as family members. These hereditary gastrointestinal polyposis syndromes also serve as paradigms for understanding gastrointestinal carcinogenesis. FAP was the first polyposis syndrome molecularly characterized by the discovery of the *APC* gene. Tumorigenesis in FAP is considered the prototype of the adenoma-carcinoma sequence in the large bowel due to disrupted 'gatekeeper' function of APC and subsequent Wnt activation accompanied by an accumulation of genetic changes and resultant clonal expansion. The molecular genetics of juvenile polyposis and Peutz-Jeghers syndrome are less well understood. In JPS the primary defect may be stromal rather than epithelial and this so-called "landscaper" defect may ultimately lead to neoplastic transformation in the overlying epithelium, though the polyps are not neoplastic per se. On the contrary, they may be considered true hamartomas, i.e. anomalies in the developmental patterning of the gut. In PJS loss of polarity function may be a critical pathogenic mechanism underlying polyp formation and tumorigenesis. Loss of proper polarity regulation may affect asymmetric stem cell division, leading to an expanded stem cell compartment. Secondary changes due to mucosal prolapse of the bowel mucosa may then contribute to the typical appearance of the polyps. Whether or not the polyps are preneoplastic remains to be determined.

Future studies on the molecular and clinical aspects of these syndromes may eventually result in a better understanding of gastrointestinal polypogenesis and carcinogenesis, and improved management of patients afflicted by these disorders.

AKNOWLEDGEMENTS

This work was supported by The Netherlands Digestive Disease Foundation (MLDS) grant WS 04-06. We thank Wilfried Meun for his help in preparing the figures.

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PART I

FAMILIAL ADENOMATOUS POLYPOSIS

4

PREVENTION AND MANAGEMENT OF DUODENAL POLYPS IN FAMILIAL ADENOMATOUS POLYPOSIS

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Gut 2005 Jul;54(7):1034-43

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ABSTRACT

Familial adenomatous polyposis (FAP) is one of two well described forms of hereditary colorectal cancer. The primary cause of death from this syndrome is colorectal cancer which inevitably develops usually by the fifth decade of life. Screening by genetic testing and endoscopy in concert with prophylactic surgery has significantly improved the overall survival of FAP patients. However, less well appreciated by medical providers is the second leading cause of death in FAP, duodenal adenocarcinoma. This review will discuss the clinicopathological features, management, and prevention of duodenal neoplasia in patients with familial adenomatous polyposis.

FAMILIAL ADENOMATOUS POLYPOSIS

FAP is an autosomal dominant disorder caused by a germline mutation in the *adenomatous polyposis coli* (*APC*) gene. FAP is characterized by the development of multiple (≥100) adenomas in the colorectum. Colorectal polyposis develops by age 15 years in 50% and age 35 years in 95% of patients. The lifetime risk of colorectal carcinoma is virtually 100% if patients are not treated by colectomy.[1]

Patients with FAP can also develop a wide variety of extraintestinal findings. These include cutaneous lesions (lipomas, fibromas, and sebaceous and epidermoid cysts), desmoid tumors, osteomas, occult radio-opaque jaw lesions, dental abnormalities, congenital hypertrophy of the retinal pigment epithelium, and nasopharyngeal angiofibroma. In addition, FAP patients are at increased risk for several malignancies, such as hepatoblastoma, pancreatic, thyroid, biliary-tree, and brain tumors.[1]

Other gastrointestinal manifestations commonly found in FAP patients are duodenal adenomas, and gastric fundic gland and adenomatous polyps. Of concern, duodenal cancer is the second leading cause of death after colorectal cancer in these individuals.

EPIDEMIOLOGY OF DUODENAL POLYPS AND CANCER

After the colorectum, the duodenum is the second most commonly affected site of polyp development in FAP (Figure 1).[2, 3] Duodenal adenomas can be found in 30–70% of FAP patients[2-4] and the lifetime risk of these lesions approaches 100%.[4, 5]

Duodenal/periampullary adenocarcinoma is the leading cause of death in FAP after colorectal cancer.[6] These patients have a 100–330-fold higher risk of duodenal cancer compared with the general population.[7, 8] Of note, duodenal cancer is rare in the population, with an incidence of 0.01–0.04%.[9] Estimates of the cumulative risk of developing duodenal cancer in FAP range from 4% at age 70 years to 10% at age 60 years.[10, 11] Recently, a large prospective five nation study set the cumulative incidence rate of duodenal cancer at 4.5% by age 57 years. The median age of duodenal cancer development was 52 years (range 26–58).[4]

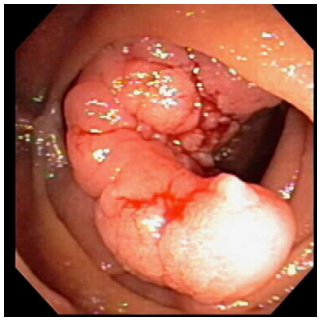

Figure 1. *Polyps in the second part of the duodenum in a patient with familial adenomatous polyposis.*

UPPER GASTROINTESTINAL POLYP DISTRIBUTION AND TYPE

Polyps can be found throughout the duodenum, but the second and third portion and the periampullary region are the most commonly affected sites. This pattern probably reflects exposure of duodenal mucosa to bile acids,[12] suggesting a role for these compounds in duodenal carcinogenesis.[13] Most polyps in the duodenum are adenomas whereas polyps in the stomach are usually benign nonadenomatous fundic gland lesions. However, approximately 10% of gastric polyps are adenomas.[3, 12] Interestingly, Japanese and Korean FAP patients have a 3–4 times higher risk of gastric cancer compared with the general population[14, 15] whereas no increased risk has been found in Western countries.[7] Besides polypoid neoplasia, flat adenomas can be found in the duodenum of approximately 30% of FAP patients and careful follow up of these lesions is recommended.[16]

GENOTYPE-PHENOTYPE CORRELATION IN DUODENAL POLYPOSIS

The cause of FAP is germline mutation of the *APC* gene. The *APC* gene is a tumor suppressor gene with 15 exons that encodes a 2843 amino acid protein with a molecular weight of 309 kDa. One third of all germline mutations occur in codons 1061 and 1309 (Figure 2).[1]

Several genotype-phenotype correlations for colonic polyposis in FAP have been established. Mutations between codon 1250 and codon 1464 are associated with profuse polyposis (>5000 colorectal polyps) and those in codon 1309 with early onset of adenoma development (10 years earlier) and colorectal cancer (age <35 years).[17, 18] Mutations at the 5′ and 3′ extremes of the *APC* gene cause attenuated FAP, characterized by oligopolyposis (less than 100 colorectal polyps) at presentation and later onset of colorectal cancer development (age >50 years).[1]

The relationship between severity of duodenal polyposis and mutations in the *APC* gene is less well understood. Taken together, published reports are inconsistent (Table 1). One study failed to detect a correlation between the site of mutation and the severity of duodenal polyposis.[18] In another, severe duodenal polyposis was found in patients with 5′ mutations.[19] Still others correlate severe duodenal disease with mutations in the central part of the gene.[20] However, most reports indicate that mutations in exon 15 of the *APC* gene, particularly distal to codon 1400, give rise to a severe duodenal phenotype.[11, 17, 21-27]

Table 1. Genotype-phenotype correlations for upper gastrointestinal polyposis in FAP

Author	No. of FAP patients	Findings	Conclusion
Groves[22]	129 patients with known *APC* mutation	245 patients underwent upper GI endoscopy, 129 had known germ-line mutation. Mutations after codon 1400 tend to give rise to more severe duodenal polyposis	Exon 15 (distal)
Attard[21]	15 patients with known *APC* mutation	24 pediatric patients from 21 families underwent upper GI endoscopy. 15 patients had known *APC* mutation. Patients with upper GI adenomas were more likely to have mutation between codon 1225 and 1694	Exon 15 (distal)
Matsumoto[24]	4 members of 1 family	4 patients from 1 family with severe duodenal adenomatosis and a frame shift mutation in codon 1556	Exon 15 (distal)
Legget[23]	2 members of 1 family	2 members of 1 family with sparse colonic but severe upper GI adenomatosis and a two bp. deletion in codon 1520	Exon 15 (distal)
Trimbath[27]	1 (AFAP)	AFAP patient presenting with ampullary adenocarcinoma and distal 3' (exon 15) *APC* mutation	Exon 15
Bjork[11]	15 patients with known *APC* mutation	19 patients with stage IV duodenal adenomatosis or carcinoma.15 *APC* mutations were detected, 12 were downstream of codon 1051 in exon 15	Exon 15
Bertario[17]	399 patients from 78 families with known *APC* mutation	Mutations between codon 976 and 1067 are associated with 3-4 fold increased risk of duodenal adenomas	Exon 15 (proximal)
Enomoto[26]	62 patients from 30 families with known *APC* mutation	Patients with germ-line mutations between codon 564 and 1465 have higher frequencies of upper GI adenomas than patients with a mutation between codon 157 and 416	Exon 10-15
Matsumoto[25]	34 patients from 25 families with known *APC* mutation	Patients with distal (exon 10-15) *APC* mutations have higher prevalence of duodenal adenomas than patients with proximal (exon 1-9) mutations	Exon 10-15
Saurin[20]	33 patients from 17 families with known *APC* mutation	Mutation in central part (279-1309) risk factor for development of severe duodenal adenomatosis	Codon 279-1309
Soravia[19]	7 AFAP kindreds	Kindreds with 5' end mutations (exon 4 and 5) have more duodenal adenomas than kindreds with mutations in exon 9 and 3' distal end	Exon 4 and 5
Friedl[18]	86 patients from 77 families with known *APC* mutation	134 patients from 125 families had duodenal adenomas. From 86 patients the germ-line mutation was known No correlation between site of mutation and duodenal adenomatosis	No correlation

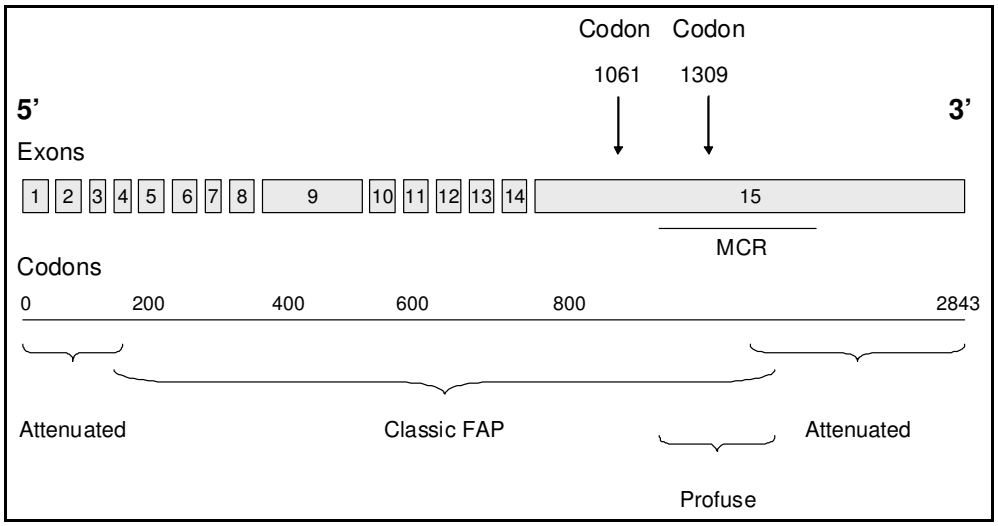

Figure 2. *Schematic representation of the adenomatous polyposis coli (APC) gene, consisting of 15 exons and 2843 codons. One third of all germline mutations occur in codons 1061 and 1309. Mutations at the extremes of the APC gene present as attenuated familial adenomatous polyposis.*

UPPER GASTROINTESTINAL ADENOMACARCINOMA SEQUENCE

The adenoma-carcinoma sequence describes colorectal carcinogenesis as a stepwise progression of normal intestinal mucosa to aberrant crypt foci, adenoma, and finally invasive carcinoma (Figure 3). Activation of the Wnt signaling pathway, by biallelic inactivating *APC* mutation or an activating *β-catenin* mutation, can be regarded as the initiating step. Subsequent mutations in tumor suppressor genes (for example, *p53* and *SMAD4*) and oncogenes (for example, *K-Ras*) lead to neoplastic progression of the adenomacarcinoma sequence.[28] Also, expression of important cell regulatory proteins is changed. One of these is cyclooxygenase 2 (COX-2), which is increasingly expressed in consecutive stages of the adenoma-carcinoma sequence.[29, 30]

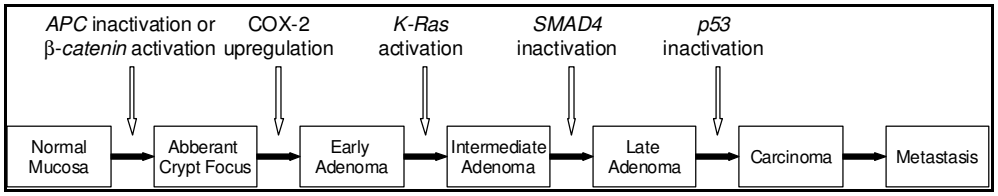

Figure 3. *The adenoma-carcinoma sequence. Activation of the Wnt signaling pathway, by an inactivating adenomatous polyposis coli (APC) mutation or an activating β-catenin mutation, is regarded as the first step in the adenoma-carcinoma sequence. Then, additional mutations in oncogenes (for example, K-Ras) and tumor suppressor genes (for example, p53 and SMAD4) drive further progression of the adenoma-carcinoma sequence. COX-2, cyclooxygenase 2.*

The adenoma-carcinoma sequence, first identified for colorectal tumorigenesis, has been observed in the setting of duodenal carcinogenesis in patients with both FAP and sporadic

disease. Spigelman and colleagues[31] found a strong association between duodenal adenomas and duodenal cancer, showing that villous histology, moderate or severe dysplasia, and the presence of stage IV duodenal polyps were associated with malignant change. Also, case reports of duodenal carcinoma development in or near adenomas have been described.[32, 33] Moreover, Kashiwagi et al. noted p53 overexpression in 25% of tubular, 72% of tubulo-villous/villous adenomas, and 100% of duodenal carcinomas,[34] and *K-Ras* codon 12 mutations have been detected in duodenal adenomas and carcinomas.[35] In addition, *SMAD4* mutations play a role in polyp development in the upper intestine in mice.[36] Lastly, Resnick and colleagues[37] demonstrated that transforming growth factor-α (TGF-α) expression was greater in duodenal carcinomas than in adenomas, and that epidermal growth factor receptor (EGF-R) expression correlated with the degree of dysplasia in duodenal adenomas. These studies reveal that additional molecular alterations drive the transition of adenoma into carcinoma.

COX-2 is known to be an important mediator of colorectal neoplasia progression but expression of COX-2 has not been extensively studied in duodenal or upper gastrointestinal adenomas. Shirvani and colleagues[38] found constitutive COX-2 expression in normal duodenum and esophagus and significantly higher levels in esophageal dysplastic tissues. Furthermore, these investigators showed that COX-2 expression in Barrett's esophagus increased in response to pulses of acid or bile salts. COX-2 expression is also elevated in gastric cancers.[39]

CLASSIFICATION OF DUODENAL POLYPOSIS

The most useful system for rating the severity of duodenal polyposis was developed by Spigelman and colleagues. This classification describes five (0–IV) stages. Points are accumulated for number, size, histology, and severity of dysplasia of polyps (Table 2). Stage I indicates mild disease whereas stages III–IV imply severe duodenal polyposis.[12] Approximately 70–80% of FAP patients have stage II or stage III duodenal disease, and 20–30% have stage I or stage IV disease.[12, 40] The estimated cumulative incidence of stage IV duodenal disease, however, is 50% at age 70 years.[4, 41]

Table 2. Spigelman classification for duodenal polyposis in FAP[12]

Criteria	Points		
	1	2	3
Polyp number	1-4	5-20	>20
Polyp size (mm)	1-4	5-10	>10
Histology	Tubular	Tubulo-villous	Villous
Dysplasia	Low-grade		High-grade

Stage 0: 0 points. Stage I: 1-4 points. Stage II: 5-6 points. Stage III: 7-8 points. Stage IV: 9-12 points

Several investigators have shown that duodenal polyposis slowly progresses. One study followed 114 FAP patients for 51 months and found progression of polyps in size (26%), number (32%), and histology (11%).[42] When individuals are followed for longer, duodenal polyps advance in Spigelman stage. Heiskanen and colleagues[5] reported worsening

polyposis in 73% of 71 FAP patients followed for 11 years. The median interval for progression by one stage was 4–11 years. Another group reported a stage change in 42% of patients with an average time of evolution by one stage of 3.9 years. Also, the risk of developing stage III or IV disease exponentially increases after age 40 years.[43] The Spigelman classification also correlates with risk of duodenal malignancy. Stages II, III, and IV disease are associated with a 2.3%, 2.4%, and 36% risk of duodenal cancer, respectively.[40]

MANAGEMENT

Surveillance

As noted, duodenal polyposis is ingravescent over time. Consequently, surveillance of the upper gastrointestinal tract for the development of neoplasia by end and side viewing scopes is recommended by most authorities. One long term upper tract surveillance study of 114 FAP patients failed to prevent the development of duodenal adenocarcinoma in six patients.[40] These findings emphasize the need to adjust the frequency of surveillance and to entertain surgical treatment with increasing severity of disease. Recommendations concerning the age of initiation of upper tract surveillance are not uniform. Some propose that screening for upper gastrointestinal disease should start at the time of FAP diagnosis.[44] The NCCN (National Comprehensive Cancer Network), after review of all case reports of duodenal cancer in FAP patients, recommended a baseline upper gastrointestinal endoscopic examination at 25–30 years of age.[45] Guidelines for continued endoscopic surveillance after baseline examination have been developed according to Spigelman stage by several authorities.[40, 45] In general, recommendations include stage 0 every 4 years; stage I every 2–3 years; stage II every 2–3 years; stage III every 6–12 months with consideration for surgery; and stage IV strongly consider surgery (Table 3).

Table 3. Recommendations for management of duodenal polyposis in FAP, adjusted to the Spigelman stage of duodenal polyposis

Spigelman stage	Endoscopic frequency	Chemoprevention	Surgery
Stage 0	4 years	No	No
Stage I	2-3 years	No	No
Stage II	2-3 years	+/-	No
Stage III	6-12 months	+/-	+/-
Stage IV	6-12 months	+/-	Yes

Endoscopic treatment

Endoscopic treatment options for duodenal lesions include snare excision, thermal ablation, argon plasma coagulation, and photodynamic therapy (PDT). Most reports of endoscopic therapy use snare excision. However, duodenal adenomas are often flat non-polypoid structures and, therefore, difficult to remove using conventional snare excision. For these cases, prior submucosal saline/adrenaline infusion may facilitate removal and

reduce the risk of hemorrhage and perforation.[40] In addition, thermal ablation,[5, 46] argon plasma coagulation,[47] or PDT[48-51] may be suitable.
PDT is a non-thermal technique relying on the combined effect of a low power activating light and a photosensitizing drug that is selectively retained within neoplastic tissue with minimal retention in surrounding normal tissue. Few reports of PDT for adenomas in the gastrointestinal tract exist. Loh and colleagues[50] successfully applied PDT for resection of colorectal adenomas: 7/9 treated adenomas were eradicated. Others have used PDT for resection of neoplastic lesions in the upper gastrointestinal tract but results are disappointing (Table 4).[48, 49, 51]
Endoscopic treatment of duodenal neoplasia for Spigelman stage II and III polyposis has been pursued by some investigators. However, the benefit of this approach in eradicating duodenal polyposis is difficult to justify but may be useful in individual cases. Literature reports of endoscopic treatment for FAP patients with duodenal/ampullary polyps are summarized in table 4. These publications reveal that endoscopic treatment is usually insufficient to guarantee a polyp-free duodenum and fraught with complications. Recurrence rates of adenomatous tissue in duodenum of FAP patients treated endoscopically range from 50% to 100%.[44, 46, 52, 53] Lower recurrence was reported by Norton and colleagues[54, 55] but their study population also included patients with sporadic duodenal lesions. In summary, endoscopic treatment appears useful in individual cases but follow up remains necessary and surgical intervention is often indicated in patients with more severe polyposis.

Surgery

Surgical options utilized to treat duodenal polyposis include local surgical treatment (duodenotomy with polypectomy and/or ampullectomy), pancreas and pylorus sparing duodenectomy, and pancreaticoduodenectomy. There are no randomized studies published to help guide surgical selection.
Publications of local surgical treatment with duodenotomy for duodenal polyposis in FAP patients are summarized in table 5. This surgery has proven insufficient to guarantee a polyp-free duodenum, with most studies reporting high recurrence rates in FAP patients with severe duodenal adenomatosis.[5, 44, 46, 53, 56-59] Farnell and colleagues[60] found a lower recurrence of duodenal polyps of 32% and 43% at five and 10 years of follow up, respectively. But this investigation also included sporadic duodenal polyposis cases and concludes that recurrence was higher in patients with a polyposis syndrome. Nevertheless, duodenotomy may be indicated in patients with one or two dominant worrisome duodenal lesions in otherwise uninvolved or minimally involved intestine. In the future, the postoperative use of chemopreventive medication may be a useful strategy.
More radical surgery, in the form of classical pancreaticoduodenectomy, or pylorus or pancreas preserving duodenectomy, has been indicated for patients with severe polyposis (stage IV), failed endoscopic or local surgical treatment, and carcinoma development. Others recommend consideration of surgery in patients with stage III polyposis.[44, 46, 53, 56, 58-63] Low recurrence rates of polyposis have been reported with these procedures (Table 6). The specific choice of procedure appears related to local expertise and the site of polyp involvement. Use of endoscopic retrograde cholangiopancreatograpy to evaluate biliary duct involvement in patients with ampullary lesions or those with laboratory test perturbations has been suggested to direct appropriate surgery. In the final analysis, the

morbidity and mortality of these surgeries must be weighed against the risk of developing duodenal adenocarcinoma.

Table 4. Endoscopic treatment for duodenal neoplastic lesions

Author	Treatment	Follow-up	Patients	Outcome	Postoperative
Soravia[53]	Endoscopic resection n.o.s.	4-34 months (mean 18)	6 FAP	Recurrence of duodenal adenomas in all 5 surviving patients	1 patient died of acute pancreatitis after endoscopic ampullectomy
Bertoni[52]	Snare papillectomy	18 months	2 FAP	Recurrence in 1 patient, successfully retreated	1 oozing-type hemorrhage and 1 mild pancreatitis, controlled by conservative measures
Morpurgo[44]	3 snare polypectomy, 2 argon plasma therapy	6-24 months (mean 19)	5 FAP	Recurrence in 3 patients	No postoperative complications
Alarcon[46]	Snare polypectomy and thermal contact ablation	14-83 months (mean 43.5)	3 FAP	Recurrence in 3 patients	NS
Heiskanen[5]	5 snare excision, 1 YAG laser coagulation	0.4-15.1 years (median 6.8)	6 FAP	No significant difference in Spigelman stage preoperative and at latest endoscopy	Patient treated with YAG laser developed mild pancreatitis
Norton[54]	Ampullary ablative therapy	1-134 months (median 24)	59 FAP, 32 sporadic	Return to normal histology in 44 % of sporadic and 34% of FAP lesions	12 patients had mild complications, 3 severe complications: 1 duodenal stenosis, 1 postcoagulation syndrome, 1 necrotizing pancreatitis
Norton[55]	Snare excision of papilla	2-32 months (median 9)	15 FAP, 11 sporadic	Recurrence rate of adenomatous tissue of 10%	2 minor bleedings, 4 mild pancreatitis, 1 duodenal perforation
Mlkvy[49]	PDT with ALA or Photofrin		4 FAP patients with duodenal polyps	Superficial necrosis and no polyp reduction after PTD with ALA. Deep necrosis and moderate polyp reduction after PDT using Photofrin.	Mild skin photosensitivity using Photofrin
Regula[48]	PDT with ALA		2 duodenal adenomas, 3 ampullary carcinomas	Superficial necrosis of adenomas and in 2 adenocarcinomas. 1 adenocarcinoma unaffected.	Side effects included mild skin photosensitivity, nausea/vomiting and transiently increases ASAT
Loh[50]	PDT with HpD or Photofrin	3-50 months (median 5.5)	8 patients with 9 colorectal adenomas	7 adenomas successfully eradicated	No local complications
Abulafi[51]	PDT with HpD		10 patients with ampullary carcinoma unsuitable for surgery	Remission for 8 to 12 months in 3 patients with small tumors. In 4 patients with small tumors bulk was reduced. No improvement in patients with extensive disease	3 patients with moderate skin sensitization

NS: not stated; PDT: photodynamic therapy; ALA: 5-aminolaevulinic acid;
HpD: hematoporphyrin derivate or Photofrin

Table 5. Local surgical treatment (duodenotomy with polypectomy and/or ampullectomy) for duodenal neoplastic lesions

Author	Treatment	Follow-up	Patients	Outcome	Postoperative
Soravia[53]	Duodenotomy with polypectomy (1) or ampullectomy (4)	4-34 months (mean 18)	5 FAP	Recurrence in 4 patients. 1 patient died of cancer	1 transient duodenal fistula
Morpurgo[44]	Transduodenal ampullectomy (1) or polyp excision (1)	6-24 months (mean 19)	2 FAP	Recurrence in 1 patient	1 severe pancreatitis
Alarcon[46]	Local resection	8-33 months (mean 20.2)	5 FAP	Recurrence in 4 patients. 1 had progressive metastatic adenocarcinoma	NS
Heiskanen[5]	Duodenotomy	0.4-15.1 years (median 6.8)	15 FAP	No significant difference in Spigelman stage preoperative and at latest endoscopy	No postoperative complications
Penna[57]	Duodenotomy with polypectomy	5-36 months (mean 13.3)	12 FAP	Recurrence in 12 patients	NS
Penna[56]	Duodenotomy with polypectomy	36-72 months (mean 53)	6 FAP	Recurrence in 6 patients	1 cholecystectomy for cholecystitis, 2 duodenal fistulas
de Vos tot Nederveen[58]	Duodenotomy with ampullectomy	4-13 months (mean 11)	8 FAP	Recurrence in 6 patients	1 minor morbidity*
de Vos tot Nederveen[58]	Duodenotomy with polypectomy	5-103 months (mean 29)	22 FAP	Recurrence in 17 patients. 1 death from metastatic disease	1 minor morbidity*
Ruo[59]	Duodenotomy with ampullectomy	35 months	1 FAP	Gastric cancer arising from a polyp at 35 months	No postoperative complications
Farnell[60]	Transduodenal local excision	10 years	53 sporadic and FAP patients	Recurrence rate of 32 % at 5 years and 43 % at 10 years of follow-up	3 pancreatitis, 3 leaks, 2 delayed gastric emptying, 2 ileus, 1 fluid overload

* i.e. wound infection, atelectasis, or urinary tract infection.
NS: not stated.

Table 6. Pancreaticoduodenectomy and pylorus or pancreas preserving duodenectomy for duodenal neoplastic lesions

Author	Treatment	Follow-up	Patients	Outcome	Postoperative
Soravia[53]	Pancreatico-duodenectomy	NS	1 FAP	Unknown	NS
Morpurgo[44]	Pancreatico-duodenectomy	NS	4 FAP	No recurrence reported	Increased number of bowel movements. One patient required pouch excision and end ileostomy to control diarrhea. 3 patients experienced weight loss, 1 patient had episodes of pancreatitis
Alarcon[46]	Pancreas sparing duodenectomy	40-50 months (mean 45.7)	3 FAP	No recurrence. Two of three patients had a small tubular adenoma in the duodenal bulb.	NS
Penna[56]	Pancreatico-duodenectomy	9-108 months (mean 42)	7 FAP severe duodenal polyposis	No recurrence in patients treated for severe duodenal polyposis.	1 pancreatic fistula, 1 upper GI hemorrhage
		1-9 years	5 FAP duodenal cancer	Only 1 patients with duodenal cancer survived >4 years	Resection not possible in 2 because of peritoneal carcinomatosis or distal lymph node involvement
de Vos tot Nederveen[58]	Pancreatico-duodenectomy	7-96 months (mean 47)	23 FAP	Recurrence in 3, 6 died of metastatic disease	5 minor morbidity*, 12 major morbidity†, 1 patient died of postoperative complications
de Vos tot Nederveen[58]	Pancreas sparing duodenectomy	2-15 months (mean 11)	6 FAP	No recurrence	1 minor morbidity*, 3 major morbidity†
de Vos tot Nederveen[58]	Pylorus preserving duodenectomy	7-93 months (mean 45)	12 FAP	Recurrence in 3 of 9, 3 died of metastatic disease	1 minor morbidity*, 4 major morbidity†
Ruo[59]	(Pylorus preserving) pancreatico-duodenectomy	37-162 months (mean 70.5)	7 FAP	1 patient developed jejunal adenomas 12 years after operation	1 patient developed pancreatic ascites
Chung[62]	Pancreas sparing duodenectomy	0.5-3 years (mean 2.1)	4 FAP	No recurrence	1 gastric retention, 1 pancreatic fistula
Kalady[63]	Pancreas sparing duodenectomy	10 years	3 FAP	1 had polyp recurrence in jejunum at 5 years of follow-up	1 postoperative wound infection, 1 biliary leak
Balladur[61]	(Pylorus preserving) pancreatico-duodenectomy	24 and 28 months	2 FAP	No recurrence	NS
Farnell[60]	(Pancreas sparing) pancreatico-duodenectomy	0.3-16 years (mean 5.6)	25 FAP and sporadic	No recurrences	10 leaks, 4 delayed gastric emptying, 1 delirium tremens, 3 abscesses. 1 patient died from bleeding and sepsis related to hepaticojejunostomy leak. Morbidity was higher after pancreas sparing duodenotomy.

* i.e. wound infection, atelectasis, or urinary tract infection.
† i.e. anastomotic leakage, fistula formation, wound abscess, sepsis, pancreatitis.
NS: not stated.

Pharmacological treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) regress colorectal adenomas in FAP patients. The value of these agents for duodenal polyposis regression is unclear. Studies of duodenal adenoma regression have primarily utilized sulindac (NSAID) and selective COX-2 inhibitors (Table 7).

Nugent and colleagues[64] compared the effect of sulindac (n=12) and placebo (n=12) on the number of duodenal polyps. Polyp number decreased in five patients, increased in one, and was unchanged in five after six months of treatment with sulindac 400 mg/day.

The difference between sulindac and placebo treated patients was not significant, possibly due to lack of statistical power. However, a second evaluation of endoscopic videotapes from this cohort revealed a statistically significant effect on small (<2 mm) duodenal

Table 7. FAP patients treated with sulindac, celecoxib or refecoxib for duodenal adenomas

Author	Treatment dose/day	Type of study	Duration	Pts	Outcome	Side effects
Nugent[64] Debinski[65]	Sulindac 400 mg	Randomized controlled clinical trial	6 months	11	Number of polyps ↓ in 5 patients (p=0.12 vs. placebo). Second evaluation: effect on small polyps (≤2mm) (p=0.02)	1 patient with indigestion
Seow-Choen[66]*	Sulindac 300 mg	Randomized controlled clinical trial	6 months	15	No effect	No adverse events reported
Richard[67]	Sulindac 300 mg	Clinical trial	10-24 months	5	No regression of small residual polyps. 3 patients developed large polyps; 1 breakthrough carcinoma	2 patients with abdominal cramp. 1 patient with upper GI bleeding
Phillips[68]	Celecoxib 800 mg	Randomized controlled clinical trial	6 months	30	Number of polyps ↓ compared to placebo (p=0.03)	1 patient with allergic reaction. 1 patient with symptoms of dyspepsia
Winde[69]	Sulindac 50-300 supp dose reduction	Prospective, controlled, nonrandomized phase II dose-finding study	Up to 4 years	xx	No effect on upper GI polyps	2 patients with mild gastritis due to NSAID
Maclean[70†]	Refecoxib 25 mg	Randomized controlled clinical trial	6 months	6	Improvement in 2 patients with stage III polyposis; no effect in 4 patients; no effect in ursodeoxycholic acid group	
Parker[71]	Sulindac 300 mg	Case report		1	No recurrence of duodenal polyps	
Theodore[72]	Sulindac 300-400 mg	Case reports	5 and 14 years	2	Sulindac normalized adenomatous ampulla and induced elimination of moderate dysplasia	
Waddell[73]	Sulindac 300-400 mg	Case reports	4.5-5 years	2	No effect on gastric and small intestinal polyps	

* The control group was treated with calcium and calciferol.
† The control group was treated with ursodeoxycholic acid

polyps whereas larger (>3 mm) duodenal polyps were unaffected.[65] Another randomized crossover trial that compared sulindac 300 mg/day with calcium and calciferol revealed no effect on duodenal polyps in 15 patients who completed six months of treatment with sulindac.[66]

Richard and colleagues[67] treated eight FAP patients with residual small periampullary polyps with sulindac 300 mg/day for at least 10 months. Sulindac was discontinued in three patients due to side effects. Follow up endoscopy was performed every six months or at discontinuation of treatment. None of the patients showed regression of polyps; three patients developed large polyps and one an infiltrating carcinoma while on this drug.

A large randomized trial by Phillips and colleagues,[68] with statistical power to detect small differences, investigated the effect of the specific COX-2 inhibitor celecoxib on duodenal polyp number and total polyp area. A 14% decrease in polyp number was found after six months of celecoxib 800 mg/day (n=32) compared with placebo (n=17) which was not statistically significant. Paired assessment of endoscopic videotapes, however, revealed a significant difference (p=0.033), although no effect on polyp area was noted.

Winde and colleagues[69] preformed a prospective, controlled, non-randomized phase II dose finding study for sulindac. These investigators compared effects of sulindac suppositories (n=28) with placebo (n=10) on rectal and upper gastrointestinal adenomas in patients that underwent colectomy. They found complete or partial reversion of rectal polyps but no effects on duodenal and papillary adenomas.

Preliminary data from a trial comparing another specific COX-2 inhibitor, rofecoxib 25 mg/day, with ursodeoxycholic acid (controls) for duodenal polyps showed a response in two of six patients on rofecoxib and in none of the controls (n=6). Of note, both responsive patients had stage III disease whereas none of the patients with stage IV disease improved.[70]

A case report described that sulindac 300 mg/day prevented the recurrence of severe duodenal polyposis in a patient with FAP.[71] Another described two patients in whom treatment with sulindac 300–400 mg/day normalized an adenomatous ampulla and eliminated moderate dysplasia.[72] In contrast, Waddell and colleagues[73] observed no effect of sulindac 300–400 mg/day on gastric and small intestinal polyps in two patients with FAP. In addition to chemoprevention with NSAIDs, H2 blockers have been studied. No significant difference was found in duodenal polyp number or adduct formation between the ranitidine and placebo groups.[74]

In conclusion, the results of NSAID and other compounds on regression or prevention of duodenal adenomas in FAP appear disappointing, although regression of small adenomas may occur.[65]

MOLECULAR MECHANISMS OF CHEMOPREVENTION WITH NSAIDS

Studies of chemoprevention/regression of duodenal polyps in FAP have primarily utilized NSAIDs. The action of these agents has been divided into COX dependent, mediated through inhibition of the COX enzymes, and COX independent, caused by direct actions of NSAIDs on different molecular mechanisms.

COX dependent mechanisms

NSAIDs are best known for inhibitory effects on COX-1 and COX-2, key enzymes in the conversion of arachidonic acid to prostaglandins (PGs) (Figure 4). COX-1 expression occurs in most tissues whereas COX-2 is expressed in response to growth factors, lipopolysaccharide, cytokines, mitogens, and tumor promoters.[75] PGs are involved in cellular functions such as angiogenesis and cell proliferation. Therefore, inhibition of PG synthesis could explain part of the antineoplastic effects of NSAIDs. Also, COX-2 inhibition has antiangiogenic effects, as confirmed by several different studies.[76-78] COX-2 inhibition may also induce apoptosis, mainly via inhibition of PGE2,[79] and inhibit invasive properties of cancer cells. COX-2 was induced by co-culture and promoted invasion in vitro that was inhibited by NSAIDs or RNAi against COX-2.[80]

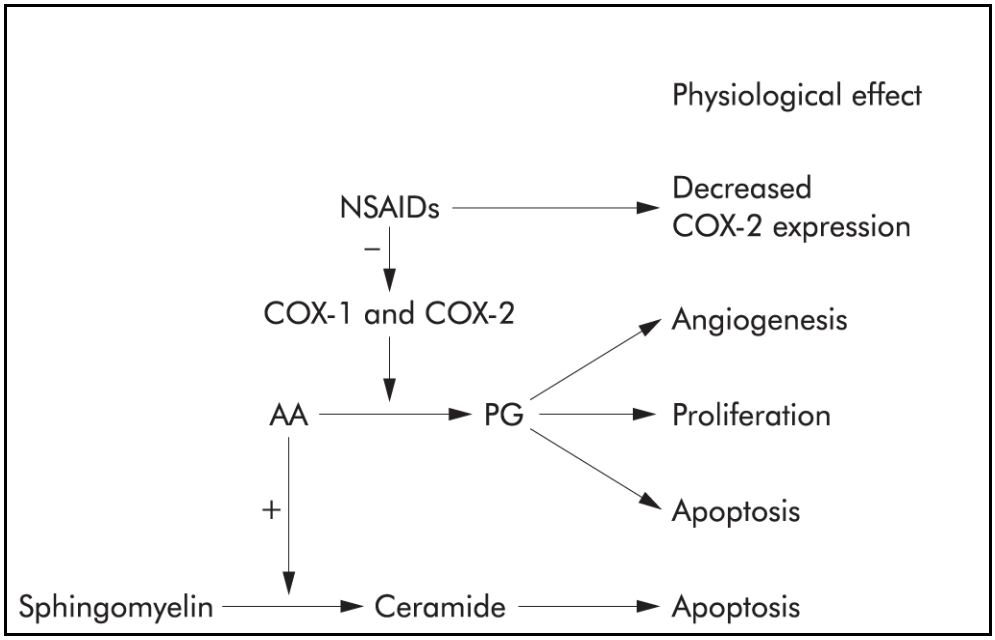

Figure 4. *Cyclooxygenase (COX) dependent chemopreventive mechanisms of non-steroidal anti-inflammatory drugs (NSAIDs). PG, prostaglandins; AA, arachidonic acid.*

COX independent mechanisms

Several lines of evidence support the importance of COX independent means of action of NSAIDs. Firstly, high doses of NSAIDs induce apoptosis in COX-1 or COX-2 deficient cell lines[81] and, secondly, PGs do not rescue these cells from apoptosis.[82]

Various COX-2 independent targets for NSAIDs have been proposed (Figure 5). β-catenin appears to be an important target as both indomethacin and exisulind reduce β-catenin expression in colorectal cancer cells.[83, 84] Also, NSAIDs induce apoptosis via both the membrane bound and mitochondrial pathway. High doses of aspirin antagonize the transcription factor nuclear factor-κβ,[85] which regulates expression of antiapoptotic genes encoding proteins such as TRAF, c-IAP, c-FLIP, Bcl-X_L, and A1. Several studies indicate a

role for proteins of the Bcl-2 family in the apoptotic response to NSAIDs, and the membrane death receptor apoptotic pathway may also be involved.[86] Furthermore, TGF-β signaling is implicated in NSAID chemoprevention.[87] NSAIDs affect cell adhesion[88] and lipoxygenase metabolism,[89] which reduce colorectal cancer cell invasion and could explain part of the apoptotic response to NSAIDs in colorectal cancer cells. Finally, it appears that members of the peroxisome proliferator activated receptor (PPAR) family, PPARδ and PPARγ, are directly targeted by NSAIDs and PGs.[90-93]

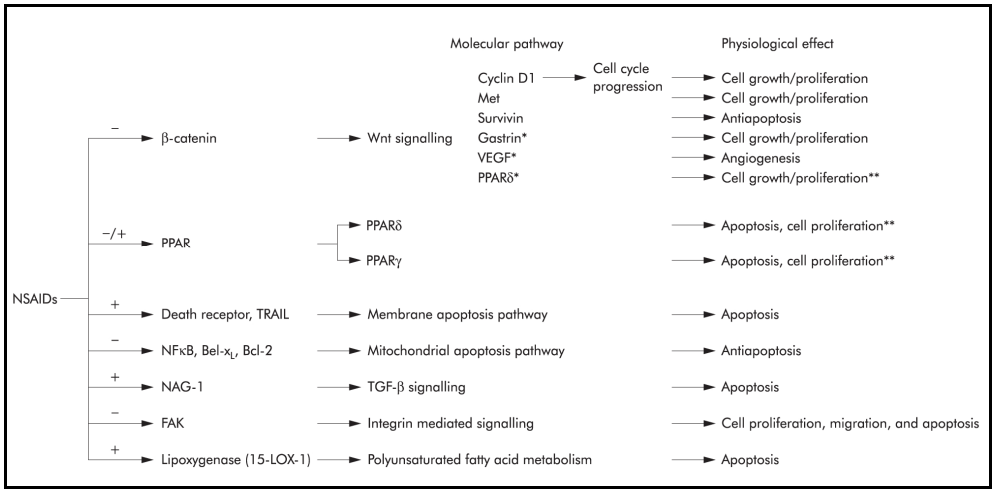

Figure 5. *Cyclooxygenase (COX) independent chemopreventive mechanisms of non-steroidal anti-inflammatory drugs (NSAIDs). *Genes with a T cell factor 4 responsive element in their promoter, but no reports of downregulation in response to NSAIDs. **Contradictory reports. PPAR, peroxisome proliferator activated receptor; TGF-β, transforming growth factor β; NF-κβ, nuclear factor-κβ; VEGF, vascular endothelial growth factor; TRAIL, tumor necrosis factor related apoptosis inducing ligand.*

CONCLUSIONS AND FUTURE DIRECTIONS

With improvement in the management of colorectal disease and increased life expectancy, duodenal polyposis and malignancy have emerged as major health problems in patients with FAP. Although most patients eventually develop duodenal polyps, these lesions occur at later age and have lower potential for malignant change compared with colonic polyps. Moreover, duodenal adenomas seem less responsive to chemoprevention with NSAIDs than colonic counterparts.

Currently, the main treatment options for duodenal polyposis are frequent surveillance and targeted endoscopic treatment, adjusted by severity of duodenal lesions. However, these modalities alone cannot guarantee a polyp-free duodenum.[40] In patients with severe disease, duodenotomy or duodenectomy may be necessary. Drug therapy of duodenal adenomas would be appropriate treatment but most published reports find no significant effect of NSAIDs or COX-2 inhibitors on duodenal adenoma regression.

Increasing insights into the molecular changes during the adenoma-carcinoma sequence in the duodenum may point to future treatment strategies. Duodenal mucosa is exposed to different environmental factors than that in the colon. Low pH and bile acids may affect

control of growth and malignant potential of duodenal tumors.[12, 13, 38] Little is known about the role of potential molecular targets for chemoprevention, including COX-2, PPARδ, PPARγ, TGF-β receptor type II, EGF-R, and inducible nitric oxide synthase. More powerful chemopreventive/regressive regimens could result from combinations of NSAIDs or COX-2 inhibitors with other drugs, such as selective inhibitors of receptor tyrosine kinases or EGF-R. Further study is needed to understand the molecular changes in duodenal adenoma development and identify molecular targets for chemoprevention and regression of duodenal polyposis.

ACKNOWLEDGEMENTS

Supported by The Queen Wilhelmina Fund/Dutch Cancer Society, The John G Rangos, Sr. Charitable Foundation, The Clayton Fund, and NIH grants 53801, 63721, 51085 and P50 CA 93–16.

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5

INCREASED CYCLOOXYGENASE-2 EXPRESSION IN DUODENAL COMPARED WITH COLONIC TISSUES IN FAMILIAL ADENOMATOUS POLYPOSIS AND RELATIONSHIP TO THE -765G -> C *COX-2* POLYMORPHISM

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Clinical Cancer Research 2005 Jun;11(11):4090-6

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ABSTRACT

Background: Colorectal cancers arising in patients with familial adenomatous polyposis (FAP) can be largely prevented by polyp surveillance and prophylactic colectomy. As a result, duodenal adenocarcinoma has become a leading cause of death in patients with FAP. Cyclooxygenase-2 (COX-2) inhibition is effective against colorectal polyposis in FAP, but is less effective in treating duodenal polyps. We compared the expression of COX-2 in duodenal and colorectal adenomas from patients with FAP and from patients with sporadic neoplasms and correlated expression to a *COX-2* promoter polymorphism (-765G>C) that is reported to influence COX-2 expression.

Methods: The study population included 36 FAP patients with colonic adenomas, 22 FAP patients with duodenal adenomas, 22 patients with sporadic duodenal adenomas, and 17 patients with sporadic duodenal adenocarcinoma. Neoplastic and corresponding normal tissue COX-2 expressions were determined using immunohistochemistry on tissue microarrays. The prevalence and ethnic distribution of a polymorphism in the *COX-2* promoter that influences COX-2 expression (-765G>C) were determined in DNA from 274 individuals by real-time quantitative PCR.

Results: Among patients with FAP, histologically normal duodenal mucosa showed higher COX-2 expression than normal colonic mucosa ($P<0.02$), and duodenal adenomas had higher COX-2 expression than colonic adenomas ($P<0.01$). In addition, the normal duodenum of patients with FAP showed higher COX-2 expression than the normal duodenal mucosa of patients with sporadic adenomas ($P<0.05$). COX-2 expression was significantly higher in the normal-appearing mucosa of patients with FAP carrying the -765GG genotype compared with those carrying the -765GC or -765CC genotypes ($P<0.01$). The -765C genotype was more common in African Americans than in Caucasians (52% versus 33%, $P<0.01$).

Conclusion: High COX-2 expression in the normal and adenomatous duodenal mucosa of patients with FAP may explain the poorer response of these neoplasms to chemoprevention with COX-2 inhibitors.

INTRODUCTION

Familial adenomatous polyposis is caused by germline mutations in the *adenomatous polyposis coli* (*APC*) gene, which leads to the development of innumerable adenomatous polyps throughout the colorectum. Without colectomy, colorectal carcinoma is almost inevitable usually by the fifth decade of life. In recent decades, colorectal cancer screening and prophylactic surgery have significantly improved the survival of patients with FAP. However, the life expectancy of patients with FAP is still below that of the general population, largely due to the risk of developing upper gastrointestinal tract malignancy.

The duodenum is the second commonest site of adenoma development in patients with FAP, and ~5% of patients with FAP will develop duodenal cancer during their lifetime.[1-3] Currently, the main management options for patients with duodenal adenomatosis are endoscopic surveillance and selective surgical resection. Duodenal surgery, either a pancreas preserving duodenectomy or pancreaticoduodenectomy, is indicated for patients with either severe duodenal polyposis or duodenal carcinoma. However, these therapeutic options do not adequately manage the duodenal neoplasia that arises in the setting of FAP.[4] Colorectal adenomas occurring in patients with FAP have been shown to regress with

sulindac, a nonsteroidal anti-inflammatory drug, and with cyclooxygenase-2 inhibitors.[5, 6] These drugs have been targeted towards treating duodenal adenomas but results have been modest and conflicting. Some investigators have shown that sulindac and COX-2 inhibitors can reduce small duodenal adenomas,[7, 8] but not large adenomas,[9] whereas others have not.[9-11]

The rationale behind using nonsteroidal anti-inflammatory drugs as chemopreventive agents is their inhibitory effect on the COX enzymes. Two isoforms of COX exist, COX-1 and COX-2, which are involved in the conversion of arachidonic acid into prostaglandins. Prostaglandins, in turn, regulate cellular functions such as angiogenesis and cell proliferation and have been associated with progression of tumor development in a size-dependent manner.[12] In the colorectum, COX-2 is increasingly up-regulated with progression from adenoma to carcinoma.[12-15] The importance of COX-2 expression in the pathogenesis of colorectal neoplasia is dramatically illustrated by the marked reduction of polyps seen in *Apc^{MIN}* mice crossed with *Cox-2* knockout mice.[16] The mechanisms by which COX-2 overexpression may contribute to tumorigenesis have been extensively studied and include antiapoptotic, proangiogenic, and proinvasive effects.[17] Other gastrointestinal tract neoplasms are associated with increases in COX-2 expression including esophageal and gastric neoplasia.[18, 19] However, duodenal neoplasms in the setting of FAP have not, to our knowledge, been investigated for COX-2 expression.

Therefore, to provide information concerning the potential to prevent the progression of duodenal neoplasms in patients with FAP using COX-2 inhibitors, we evaluated the expression of COX-2 in duodenal adenomas of patients with FAP compared with that in duodenal tissues from patients with sporadic duodenal adenomas and duodenal carcinomas as well as with that in colonic mucosa from patients with FAP. In addition, we examined the association between intestinal COX-2 expression and the presence of a common *COX-2* promoter polymorphism (-765C>G).[20, 21]

METHODS

Patients

Patients were selected by searching the Johns Hopkins Surgical Pathology archives for patients with FAP and colonic and duodenal polyps and for patients with sporadic duodenal adenoma and/or duodenal carcinoma. Thirty-six patients with FAP had colon polyps (19 male, mean age: 34.5±18.1 (SD) years; 17 female, mean age 33.8±13.9 years) and 22 patients with FAP had duodenal polyps (7 male, mean age 48.4±11.5 years; 15 female, mean age 44.8±11.7 years). Two patients with FAP had colorectal adenocarcinoma and two had duodenal adenocarcinoma in association with colonic and duodenal polyposis, respectively. In addition, 22 patients with sporadic duodenal adenomas (11 male, mean age: 66.1±12.5 years; 11 female, mean age: 61.2±17.1 years) and 17 patients with sporadic duodenal carcinomas (11 male, mean age: 66.2±9.7 years; 7 female, mean age: 54.1±14.9 years) were included in the analysis (Table 1).

To investigate the correlation between the -765G/C *COX-2* promoter genotype and COX-2 expression, normal duodenal mucosa was obtained from 93 patients who underwent pancreaticoduodenectomy for pancreatic adenocarcinoma. To determine the prevalence of the *COX-2* promoter polymorphism by ethnicity, 219 unselected Caucasian patients and 50 African American patients were genotyped (206 with pancreatic cancer, 46 with familial

adenomatous polyposis, 17 with benign gallbladder disease, and 5 with chronic pancreatitis). The study was approved by the Institutional Review Board of the Johns Hopkins University.

Table 1. Tissue microarrays (TMA) and patient characteristics

TMA	No	Adenoma	Normal	Carcinoma	Mean age ± SD	
					Male	Female
FAP Colon*	5	133 different polyps from 36 different patients	Matched normal mucosa from 30 patients	Matched carcinoma from 2 patients	34.5±18.1 (N=19)	33.8±13.9 (N=17)
FAP Duodenum*	1	49 different polyps from 22 FAP patients	Matched normal mucosa from 13 patients	Matched carcinoma from 2 patients	48.4±11.5 (N=7)	44.8±11.7 (N=15)
Sporadic Duodenal Adenoma	1	36 different sporadic polyps from 22 patients	Matched normal mucosa from 15 patients		66.1±12.5 (N=11)	61.2±17.1 (N=11)
Sporadic Duodenal Carcinoma	2	Matched adenoma from 7 patients	Matched normal mucosa from 15 patients	17 sporadic duodenal carcinomas from 17 patients	66.2±9.7 (N=11)	54.1±14.9 (N=7)
Normal Duodenal mucosa from patients with pancreatic adenocarcinoma	3		Normal duodenum mucosa from 93 patients		67.1±10.8 (N=51)	65.8±11.2 (N=42)

*COX-2 expression was also determined in colonic mucosa of 9 and duodenal mucosa of 2 FAP patients using individual paraffin sections of their tissues.

Cyclooxygenase-2 measurement

Tissue microarrays

Formalin-fixed paraffin embedded tissues were collected to generate 13 tissue microarrays (Table 1). For tissue microarray construction, representative areas containing morphologically defined normal mucosa, adenoma, or carcinoma were identified on an H&E-stained reference slide by an experienced pathologist (CAID) and encircled on the paraffin block. Tissue microarrays were constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD). The diameter of the punched core was 1.4 mm. Serial sections were cut from these tissue microarrays, one of which was stained with H&E as a reference.

Immunohistochemistry

Immunohistochemistry for COX-2 was done on unstained 4 µm sections as previously described.[22] An anti–COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI) was used at a dilution of 1:100. Two independent observers (CAID and LAAB) scored the

intensity of epithelial COX-2 staining in a semiquantitative manner on a five-grade scale: absent, weak, moderate, strong, or very strong COX-2 labeling.[23, 24] Stromal staining was scored separately. In assigning scores, the observers assessed all of the tissue cores on the tissue microarrays. In most cases, multiple cores from multiple polyps and multiple cores from normal mucosa or carcinoma were present. The highest score was used in the statistical analysis.

-765G/C genotyping

DNA isolation

Genomic DNA was obtained from deparaffinized formalin-fixed paraffin-embedded tissue of 46 patients with FAP using TK buffer [200 µg/mL of proteinase K and 0.5% Tween 20, 50 mmol/L Tris (pH=9), 1 mmol/L NaCl, 2 mmol/L EDTA]. After overnight incubation in 50 µL TK buffer at 56°C, tubes were incubated at 95°C for 10 minutes to inactivate the proteinase K.[25] In addition, DNA was isolated using Qiagen Tissue Kits (Qiagen, Valencia, CA) from freshfrozen normal duodenum or normal pancreas tissue of 206 patients with pancreatic adenocarcinoma who had undergone Whipple resection, as well as formalin-fixed paraffin-embedded gall bladder tissue from 17 patients with benign gall bladder disease who had undergone cholecystectomy and 5 patients with chronic pancreatitis who had undergone Whipple resection.

Real-time PCR

The -765G>C promoter polymorphism was detected in the SmartCycler1 (Cepheid, Sunnyvale, CA) using the following primers: COX2RealTimeFor: 5′-cattaactatttacagggtaactgcttagg-3′ and COX2RealTimeRev: 5′-ccccctccttgtttcttgga-3′. Fluorescent MGB probes (Applied Biosystems) were used to detect the G allele (probe 1, 765G: 6-FAM-5′-ctttcccgcctctct-3′) and the C allele (probe 2, 765C: TET-5′-ctttccccccctctct-3′).[26] Samples were assayed in a 26 µL reaction mixture containing 12.5 µL Quantitect Buffer (Qiagen), 1.25 µL of each primer (final concentration 0.5 µmol/L), 0.25 µL of each probe (final concentration 0.1 µmol/L), 9.5 µL diethyl pyrocarbonate–treated H_2O, and 50 ng of sample genomic DNA. PCR reactions were done starting with 94°C for 15 minutes to activate HotStarTaq DNA polymerase, followed by 45 cycles of 94°C for 15 seconds and 60°C for 30 seconds. Two samples with known genotype and a water control were simultaneously assayed in each run.

Sequencing

Fourteen samples were sequenced to validate the single nucleotide polymorphism real-time PCR assay. After initial amplification of the promoter region containing the single nucleotide polymorphism of interest (primers, COX2For: 5′-gcatacgttttggacatttag; COX2Rev: 5′-ctaccttcagtgtacatagc), the PCR product was purified using the QIAquick PCR Purification Kit (Qiagen). Subsequently, samples were sequenced using an internal forward primer (COX2IntFor: 5′-gttttggacatttagcgtcc) and the Applied Biosystems 3730 DNA Analyzer.

Statistics

Nonparametric χ^2 tests were used to assess differences between groups and to assess the correlation between COX-2 expression and *COX-2* genotype. In addition, χ^2 tests were used to compare the observed genotype prevalence with the expected prevalence of each genotype for a population in Hardy-Weinberg equilibrium.

RESULTS

Cyclooxygenase-2 expression in the colon of patients with familial adenomatous polyposis.

COX-2 was expressed in the normal-appearing and adenomatous colonic epithelium of all patients. The level of COX-2 expression in normal colonic mucosa was similar to levels found in colorectal adenomas from patients with FAP. The colon carcinomas from patients with FAP had significantly higher COX-2 expression than normal-appearing (χ^2=14.9, P=0.0019) and adenomatous mucosa (χ^2=17.9, P=0.00045; Table 2; Figure 1A, C, and E).

Table 2. Immunohistochemical scoring of COX-2 expression in familial adenomas of the duodenum and colon and sporadic duodenal adenomas and carcinomas

	COX-2 Expression*					
	Absent (%)	Weak (%)	Moderate (%)	Strong (%)	Very strong (%)	P
FAP Colon						
Normal (n=30)	0	3(10)	22(73)	3(10)	2(7)	0.0019[£]
Adenoma (n=36)[†]	0	4(11)	22(61)	8(22)	2(6)	0.00045[¥]
Carcinoma (n=2)	0	0	0	0	2(100)	
FAP Duodenum						
Normal (n=13)	1(8)	1(8)	3(23)	2(15)	6(46)	
Adenoma (n=22)[‡]	1(5)	0	11(50)	2(9)	8(36)	
Carcinoma (n=2)	0	0	0	1(50)	1(50)	
Sporadic duodenum adenoma						
Normal (n=15)	1(7)	0	12(80)	1(7)	1(7)	0.0224[§]
Adenoma (n=22)[€]	0	1(4)	7(32)	7(32)	7(32)	
Sporadic duodenum carcinoma						
Normal (n=17)	0	0	12(71)	4(23)	1(6)	0.024[£]
Adenoma (n=9)[$]	0	0	6(67)	2(22)	1(11)	
Carcinoma (n=17)	0	0	5(29)	5(30)	7(41)	
All sporadic duodenum adenomas[#]						
Normal (n=32)	1(3)	0	24(75)	5(16)	2(6)	
Adenoma (n=31)	0	1(3)	13(42)	9(29)	8(26)	

* The highest score was used in statistical analysis if multiple cores from multiple polyps, normal mucosa or carcinoma from the same patient were present; [†]123 different polyps from 27 different FAP patients; [‡] 49 different polyps from 22 different FAP patients; [€] 36 different polyps from 22 different patients; [$] 13 different polyps from 9 different patients; [#] Pooled adenomas from patients with sporadic duodenal adenomas and adenomas that were found in patients with sporadic duodenal carcinoma; [£] carcinoma vs. normal; [§] normal vs. adenoma

Cyclooxygenase-2 expression in the duodenum of patients with familial adenomatous polyposis.

COX-2 was similarly expressed in the normal duodenal mucosa, duodenal adenomas, and duodenal carcinomas (Table 2; Figure 1B, D, and F). Normal appearing duodenal mucosa from FAP patients showed significantly higher levels of COX-2 than normal-appearing FAP colonic mucosa (Figure 1B versus A; χ^2=12.5, P=0.014). Similarly, duodenal adenomas from patients with FAP showed higher levels of COX-2 than colonic adenomas from patients with FAP (χ^2=13.3, P=0.01; Figure 1C and D).

Cyclooxygenase-2 expression in sporadic and familial adenomatous polyposis duodenal adenomas.

Normal duodenal mucosa was available from 15 of 22 patients that had sporadic duodenal adenomas. The majority (80%) showed moderate COX-2 expression in normal duodenal mucosa whereas COX-2 was expressed in all duodenal adenomas. Furthermore, sporadic duodenal adenomas had significantly higher COX-2 expression than normal duodenal mucosa (χ^2=11.4, P=0.0224) with most adenomas exhibiting strong COX-2 expression (Table 2). Moreover, normal duodenal mucosa from FAP patients showed statistically significantly higher levels of COX-2 than normal duodenal mucosa from patients with sporadic duodenal adenomas (χ^2=10.2, P=0.037). This observation is further supported by the finding that 69.2% of matched normal and adenomatous duodenal mucosa of FAP patients showed the same COX-2 intensity, whereas only 33.3% of sporadic duodenal adenomas showed the same level of COX-2 expression in their normal duodenal mucosa (χ^2=8.4, P=0.015; Table 3). These results could not be explained by differences in the grade of adenoma or how the adenoma was obtained (by resection, polypectomy, or biopsy) between patients with FAP and those with sporadic duodenal adenomas.

Cyclooxygenase-2 expression in sporadic duodenal carcinomas.

All 17 patients with sporadic duodenal carcinoma expressed COX-2 in normal duodenal mucosa, mostly with moderate labeling intensity. Most of the 17 sporadic duodenal carcinomas exhibited strong or very strong COX-2 immunoreactivity. In addition, all nine adenomas associated with duodenal carcinoma displayed COX-2 immunostaining (Table 2). Duodenal carcinomas had statistically significantly higher levels of COX-2 expression than normal duodenal mucosa (χ^2=7.5, P=0.024).

Stromal cyclooxygenase-2 immunoreactivity.

Stromal COX-2 labeling was focal. It was observed mainly in macrophages underlying the epithelium (Figure 1A, arrow). COX-2 labeling was strong in eroded areas and seen in macrophages underlying the erosion.

Distribution of the -765G>C polymorphism.

In patients with FAP, the GG, GC, and CC genotype frequencies were 65.2%, 28.3%, and 6.5%, respectively (Table 4). The GG, GC, and CC genotypes occurred at 63.2%, 32.4%, and 4.4% in the control group, respectively. African Americans were more likely to carry a -765C polymorphism than Caucasians (χ^2=10.01, P=0.0067; Table 4). All genotypic distributions are in Hardy-Weinberg equilibrium (P≥0.05).

Table 3. COX-2 expression in matched normal and adenomatous duodenal mucosa of patients with FAP and with sporadic duodenal adenomas

Patient group	N=P	P>N	N>P
FAP (n=13)	9 of 13 (69.2%)	2 of 13 (15.4%)	2 of 13 (15.4%)
Patients with sporadic duodenum adenomas (n=15)	5 of 15 (33%)	10 of 15 (66.7%)	0
		χ^2=8.4, P=0.015	

NOTE: N=P, normal mucosa has the same COX-2 intensity as adenomatous mucosa; P>N, adenoma has higher COX-2 intensity than normal mucosa; N>P, normal mucosa has higher COX-2 intensity than adenoma.

Table 4. Prevalence of –765G/C COX-2 genotype in patients with FAP and disease controls and ethnic distribution of the –765G/C COX-2 polymorphism

	Prevalence				
	FAP [n (%)]	**Controls* [n (%)]**	**Caucasian [n (%)]**	**African American [n (%)]**	**Other/unknown (n)**
-765 GG	30 (65.2)	144 (63.2)	146 (66.7)	24 (48)	4
-765 GC	13 (28.3)	74 (32.4)	66 (30.1)	20 (40)	1
-765 CC	3 (6.5)	10 (4.4)	7 (3.2)	6 (12)	0
	46 (100)	228 (100)	219 (100	50 (100)	5
	FAP vs. Controls: χ^2=0.606, P=0.74		Caucasian vs. African American: χ^2=10.011, P=0.0067		

* 206 with pancreatic cancer, 17 with benign gallbladder disease, and 5 with chronic pancreatitis.

Correlation between -765 genotype and cyclooxygenase-2 expression.

Carriers of the -765GG genotype with FAP had higher COX-2 expression in their normal colonic and duodenal mucosa than the -765GC/CC carriers (χ^2=9.4, P=0.009) (Table 5). In contrast, there was no significant difference in COX-2 expression in the adenomatous mucosa of GG carriers (χ^2=1.4. P=0.495). There was also no correlation between the level of COX-2 expression in the normal duodenal mucosa of 93 patients with pancreatic cancer and their -765G/C *COX-2* genotype (data not shown). Recent studies indicate that COX-2 activity may be influenced in certain tissues by estrogen.[27] However, we found no evidence of any difference in COX-2 expression in normal appearing mucosa or adenomas by gender.

Table 5. Correlation between intestinal immunohistochemical COX-2 staining and -765 COX-2 genotype

	Normal				Adenoma			
	Immunohistochemical score				Immunohistochemical score			
	Absent/weak	Moderate	(Very) Strong		Absent/weak	Moderate	(Very) Strong	
GG	1	15	10	26	1	17	13	31
GC/CC	5	8	1	14	1	11	4	16
				40				47
	χ^2=9.4, P=0.009				χ^2=1.4, P=0.495			

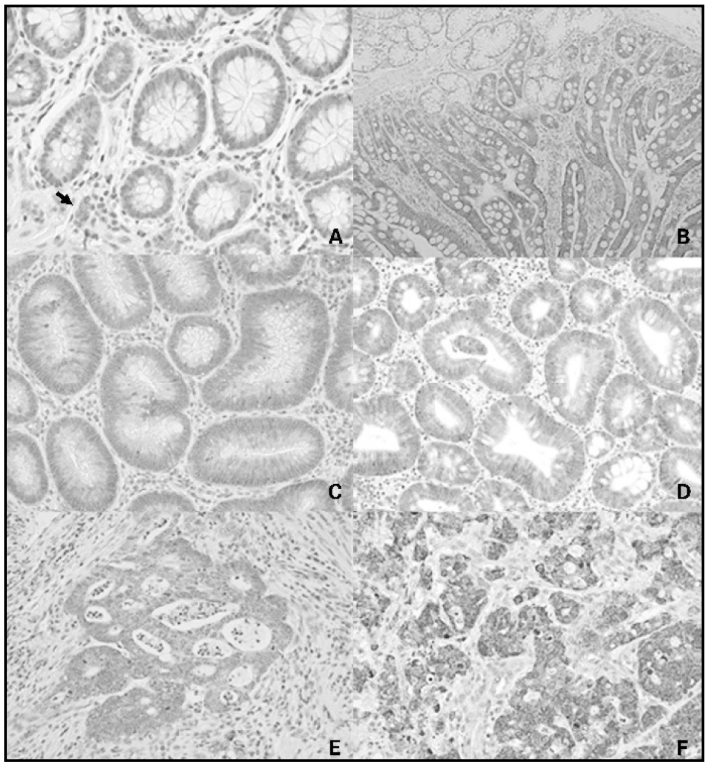

Figure 1. *COX-2 immunoreactivity in duodenum and colon. A, normal colon mucosa in a patient with FAP exhibiting weak COX-2 immunoreactivity (x100). Arrow, macrophage labeling. B, normal duodenal mucosa from a patient with FAP demonstrating strong COX-2 immunoreactivity. C, colon adenoma in FAP showing moderate COX-2 staining (x64). D, duodenum adenoma in FAP showing strong COX-2 staining (x64). Colon carcinoma (E; x64) and duodenum carcinoma (F; x64) showing very strong COX-2 immunoreactivity.*

DISCUSSION

In this study, we find that COX-2 levels increase with increasing stage of duodenal neoplasia among patients with sporadic disease. This pattern of COX-2 expression has been described for colorectal and other neoplasms and is consistent with a similar adenoma-carcinoma progression sequence for duodenal neoplasia as has been observed for colorectal and other neoplasms.[28]

Second, we also find greater COX-2 expression in the normal duodenal mucosa and duodenal adenomas of patients with FAP than in patients with sporadic duodenal neoplasias. Other investigators have noted that normal duodenal mucosa from patients with FAP exhibits increased cell proliferation and ultrastructural changes in cell adhesion function compared with non-FAP controls.[28-30] In addition, several genes, including COX-2, are up-regulated in the normal-appearing colon mucosa of *Apc*^{MIN} mice and in patients with colorectal cancer.[31] Increased COX-2 expression in histologically normal mucosa from patients with FAP may result from impaired Wnt signaling through a possible transcription factor 4 binding element in the *COX-2* promoter.[32] In addition, gastrin expression is increased by APC inactivation, and in certain tissues gastrin expression can increase COX-2 expression.[33, 34]

In addition, we also find that among patients with FAP, COX-2 expression is higher in normal duodenal mucosa than in normal colonic mucosa. Whereas previous studies have compared COX-2 expression in small intestinal cancers and colorectal cancers, where expression patterns were found to be similar,[35] COX-2 expression has not been studied in the duodenal tissues of patients with FAP. The higher expression of COX-2 in duodenal mucosa than in colonic mucosa could explain the lower response of duodenal adenomas compared with colonic adenomas to chemoprevention with nonsteroidal anti-inflammatory drugs and COX-2 inhibitors. Other factors that could contribute to differences between colonic and duodenal polyp responses to COX-2 inhibitors, such as greater resistance of duodenal adenomas to apoptosis or differences in the bioavailability of COX-2 inhibitor drugs in the colon versus the duodenum, require investigation. Interestingly, although it is plausible that treatment responses to standard doses of COX-2 inhibitors would be influenced by the amount of COX-2 protein in target tissues, such a relationship has not been clearly shown. Nonsteroidal anti-inflammatory drugs have been shown to reduce COX-2 expression in vitro[36] and in *Apc*$^{MIN/+}$ mouse.[37] Indeed, in a previous study examining molecular correlation of adenoma responses and resistance to sulindac, COX-2 expression was lower (although still present) in sulindac-resistant colonic adenomas than in pretreatment adenomas that subsequently regressed with sulindac.[22] Our results raise the possibility that higher dosages of COX-2 inhibitors could be more effective against duodenal adenomas as has been suggested for sulindac-resistant adenomas. Future studies should consider measuring duodenal COX-2 expression in patients with FAP undergoing treatment to determine if expression levels predict response to COX-2 inhibitors.

The mechanism(s) responsible for higher COX-2 expression in duodenal mucosa is not known. Several studies have suggested a role for bile acids, such as the unconjugated bile acid chenodeoxycholate, in the development of duodenal neoplasia in *Apc*$^{MIN/+}$ mice.[38] Also, a correlation has been observed between the site of a patients' duodenal adenoma development and the site of exposure of their mucosa to bile.[39] Ex vivo experiments have shown that COX-2 expression increases in response to exposure to pulses of bile acids and stomach acid.[19, 40] Other studies have indicated a chemopreventive effect for ursodeoxycholic acid [41] and combined sulindac and ursodeoxycholic acid in mouse and rat intestine.[42]

Finally, we investigated whether a recently reported single nucleotide polymorphism in the *COX-2* promoter affected the level of COX-2 expression in intestinal mucosa. Papafili et al.[21] showed that the -765C allele had lower *COX-2* promoter activity than the -765G

allele. In addition, the -765GC and -765CC genotypes correlate with decreased risk of myocardial infarct and stroke and decreased COX-2 expression in atherosclerotic plaques compared with -765GG.[20] We found higher COX-2 expression in the normal, but not in the adenomatous, mucosa of patients with FAP who carried -765GG alleles than in those with the -765GC or -765CC genotype. Because the -765 *COX-2* polymorphism influences COX-2 expression in the normal-appearing gastrointestinal mucosa of patients with FAP, it is possible that this polymorphism could influence the number of adenomas that develop in these patients, similar to the effect observed when *COX-2* is knocked out in animal models of FAP.[16] However, our results indicate that once polyps have formed, *COX-2* genotype does not influence COX-2 expression, suggesting that *COX-2* genotype may not influence the progression of these neoplasias. Interestingly, we also found that African Americans are significantly more likely to be carriers of -765C alleles, raising the possibility that African Americans could be more prone to the beneficial and adverse effects (such as toxicity from nonsteroidal anti-inflammatory drugs) of having lower COX-2 expression. Further study is needed to assess whether this polymorphism acts as a modifier of FAP phenotype or affects COX-2 expression elsewhere in the gastrointestinal tract, and whether genetic differences in the level of COX-2 expression influence patients' response to COX-2 inhibitors.

There is a need to improve the chemopreventive strategies for duodenal neoplasia occurring in the setting of FAP. In addition to COX-2, several other molecular targets merit consideration in chemoprevention studies including peroxisome proliferator activated receptors δ and γ,[43] epidermal growth factor receptor,[44] ornithine decarboxylase,[45] and nuclear factor κB pathway.[46]

In conclusion, we have found that COX-2 is more highly expressed in the duodenal adenomas and normal duodenal mucosa of patients with FAP and this increased COX-2 expression may contribute to the poorer response of these neoplasms to chemoprevention with COX-2 inhibitors. Further investigation is needed to determine the role of the -765G/C *COX-2* promoter polymorphism on *COX-2* gene expression in the gastrointestinal tract and its effect on the response to COX-2 inhibitors.

ACKNOWLEDGMENTS

Supported by The Queen Wilhelmina Fund/Dutch Cancer Society, The John G Rangos, Sr. Charitable Foundation, The Clayton Fund, and NIH grants CA 53801, 63721, 51085, P50 CA62924 and P50 CA93–16. We thank Kieran Brune, Mike Mullendore and Katharine Romans for technical support.

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6

INCREASED EXPRESSION OF CYTOPLASMIC HuR IN FAMILIAL ADENOMATOUS POLYPOSIS

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Cancer Biology and Therapy 2008 Mar;7(3): 424-7

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ABSTRACT

Background: HuR is an mRNA stability factor that binds to the AU-rich element-containing 3' untranslated region of the transcript. HuR overexpression is associated with increased tumor growth. Increased cytoplasmic HuR expression occurs in several cancer types, including colorectal cancer where it may contribute to the increased cyclooxygenase-2 (COX-2) expression observed during tumorigenesis. To investigate expression of HuR in the colorectal adenoma-carcinoma sequence, we examined expression of HuR in colorectal mucosa of patients with familial adenomatous polyposis (FAP) and sporadic colorectal cancer with correlation to COX-2 expression.

Methods: HuR and COX-2 protein expression were studied by immunohistochemistry of normal colon mucosa (N=20), adenomas (N=112) and carcinomas (N=9) from patients with FAP, and 141 sporadic colorectal adenocarcinomas (Dukes B and C).

Results: Cytoplasmic HuR staining was found in the epithelium of 10% of normal mucosa, 14.3% of adenomas and 88.9% of adenocarcinomas from FAP patients ($P<0.01$) and in 68.8% of sporadic colorectal carcinomas. High epithelial COX-2 immunostaining was observed in 10% of normal, 8% of adenomas and all adenocarcinomas from FAP patients ($P<0.01$) and in 69.5% of sporadic colorectal carcinomas. Positive cytoplasmic HuR immunostaining correlated with high COX-2 immunoreactivity in colon mucosa of FAP patients ($P<0.01$) and in sporadic colorectal carcinomas. ($P=0.016$)

Conclusion: HuR is increasingly expressed in the cytoplasmic epithelial compartment in consecutive stages of the adenoma-carcinoma sequence in FAP. Also, COX-2 levels correlate with cytoplasmic expression of HuR in colonic epithelium of FAP patients and in sporadic colorectal cancer specimens. The role of cytoplasmic expression of HuR as a biomarker for progression of adenomas in FAP needs further study.

INTRODUCTION

Aberrant expression of gene products is a general feature of tumorigenesis. Gene expression can be regulated at the level of DNA, RNA or the protein itself. RNA regulation occurs through alterations in translational efficiency and in mRNA stability. mRNA stability depends on cis-elements in the RNA and trans-acting factors. A well studied mRNA instability element is the AU-rich element (ARE) in the 3' untranslated region (3'UTR). HuR, a member of the Hu-family of proteins, can bind ARE-containing mRNAs through its RNA recognition motif. It is postulated that HuR binds these mRNAs in the nucleus and accompanies them into the cytoplasm (i.e. nucleocytoplasmic translocation), thereby protecting the mRNA from degradation, and affecting the protein expression levels of target genes.[1]

HuR overexpression is associated with increased tumor growth and increased cytoplasmic HuR expression is described in malignancies of various organs.[2-4] In colorectal carcinogenesis a gradual increase in cytoplasmic HuR levels was observed in a small series of adenomas and carcinomas[5] and overexpression of HuR increased the expression of the cyclooxygenase-2 in human colon cancer cells.[6, 7] It has been hypothesized that HuR contributes to neoplastic growth by regulating expression of genes involved in carcinogenesis that harbor an ARE in the 3'UTR, such as COX-2, β-catenin and vascular endothelial growth factor (VEGF).[5, 6, 8, 9]

To further investigate HuR expression in colorectal neoplasia, we explored colorectal mucosa from patients with familial adenomatous polyposis and sporadic CRC. FAP is a rare inherited disorder characterized by the presence of hundreds of colorectal adenomas and inevitably CRC at a young age and serves as a model for sporadic carcinogenesis.[10] We assessed HuR expression in normal, adenomatous and carcinomatous colorectal mucosa from patients with FAP and in sporadic CRC. In addition, we examined expression of COX-2 with correlation to HuR expression.

METHODS

Patients

HuR and COX-2 expression were studied in normal flat colorectal mucosa of 20 FAP patients, 112 colorectal adenomas of 26 FAP patients and in 9 colorectal adenocarcinomas of FAP patients. Twenty-four patients were from The Johns Hopkins Hospital, and 7 were from The Helsinki University Central Hospital. In addition, HuR and COX-2 expression were studied in 141 sporadic colorectal adenocarcinomas (Dukes B (n=84) and C (n=57)) from The Helsinki University Central Hospital (81 male, mean age 65.6±12.1 (SD); 60 female, mean age 68.2±12.0). The study was approved by the Institutional Review Board of The Johns Hopkins University and Helsinki University Central Hospital.

Tissue microarrays

Formalin-fixed paraffin embedded tissue of FAP patients was collected to prepare tissue microarrays (TMAs). For TMA construction, representative areas containing morphologically defined normal mucosa, adenoma or carcinoma were identified on an H&E stained reference slide by an experienced pathologist (CAID) and encircled on the paraffin block. TMAs were constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD). The diameter of the punched core was 1.4 mm. Serial sections were cut from these TMAs, one of which was stained with H&E as a reference. Regular pathological slides were used for IHC on sporadic colorectal carcinomas.

Immunohistochemistry

Immunohistochemistry for HuR was performed on unstained 4-µm sections as described previously.[4] In brief, specimens were deparaffinized and antigen was retrieved using a microwave oven (4x5 min in 700W in 0.01 M Na-citrate buffer, pH=6). The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and in blocking solution (1.5:100 normal horse serum in PBS) for 15 min to block unspecific binding sites. Immunostaining was performed with a monoclonal HuR antibody (1:10.000 dilution; 19F12, a kind gift of Clonegene, LLC, Hartford, CT) in PBS containing 0.1% sodium azide and 0.5% BSA. The specimens were incubated with the antibodies at room temperature overnight. The sections were then treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and avidin-biotin peroxidase complex (Vectastain ABComplex, Vector Laboratories). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO, USA), and the sections were counterstained with Mayer's hematoxylin. COX-2 immunostaining was performed as

described previously with a COX-2 specific anti-human monoclonal antibody (1:100 dilution; #160112, Cayman Chemical Co., Ann Arbor, MI, USA).[11, 12]

Scoring

Nuclear and cytoplasmic HuR immunostaining were evaluated separately by two independent observers (AR and LAAB) and scored as either positive or negative. Epithelial COX-2 immunoreactivity was scored by two independent observers (CAID and LAAB) in a semi-quantitative manner on a 5-grade scale: absent, weak, moderate, strong or very strong COX-2 labeling. Stromal expression of COX-2 was assessed in our previous study.[12] In this study it was occasionally observed particularly in macrophages in areas with surface erosion. However, since stromal expression did not play a role, it was discarded in the current study and only epithelial expression was taken into account. In assigning the scores, all of the tissue cores on the TMA's were assessed. The highest score was considered representative.

Statistical analysis

The intensity of COX-2 immunostaining was categorized into COX-2 low (absent, weak and moderate) and COX-2 high (strong and very strong). The highest scoring tissue core was used in the final analysis in cases where multiple cores from the same polyp and multiple cores from normal mucosa or carcinoma were present on the TMA's. COX-2 expression was correlated with cytoplasmic HuR in each individual core. Samples negative for both nuclear and cytoplasmic HuR were excluded from the analysis. Non-parametric χ^2-test was used to assess differences in COX-2 and HuR expression between groups and to assess the correlation between COX-2 and HuR.

RESULTS

HuR expression

FAP colon mucosa

Nuclear HuR staining was positive in the epithelium of 95% (19/20) of normal tissue samples, 92.9% (104/112) of adenomas, and 88.9% (8/9) of carcinomas from patients with FAP (χ^2=0.354, P=0.84). Cytoplasmic HuR immunoreactivity was positive in the epithelium of 10% (2/20) of normal tissues, 14.3% (16/112) of adenomas and 88.9% (8/9) of carcinomas of patients with FAP (χ^2=13.785, P=0.0002). (Table 1) A gradual increase in the frequency and the intensity of cytoplasmic relative to nuclear HuR staining, consistent with nucleocytoplasmic translocation of HuR, was observed during the adenoma-carcinoma sequence in FAP.

Sporadic Colorectal Cancer

The epithelium of all sporadic CRC specimens was positive for HuR, but the localization of the immunoreactivity varied. Nuclear HuR expression was found in 70.2% (99/141) and cytoplasmic immunostaining in 68.8% (97/141) of carcinomas. (Table 1)

Stromal HuR expression

Stromal HuR staining was always present in the colorectal samples and exclusively nuclear, mostly located in lymphocytes, plasma cells and myofibroblasts.

Correlation between cytoplasmic HuR and COX-2

High epithelial COX-2 expression was found in 10% (2/20) of normal samples, 8% (9/112) of adenomas and 100% (9/9) of colorectal carcinomas from FAP patients (χ^2=17.7, P=0.000026) (Table 1). Although cytoplasmic HuR staining correlated with high COX-2 expression in normal FAP epithelium (χ^2=19.39, P=0.000011), no positive correlation was found in adenomas and carcinomas of FAP patients.

In sporadic colorectal carcinomas, high COX-2 expression was present in the epithelium of 69.5% (98/141) of cases. Cytoplasmic HuR immunostaining correlated with COX-2 expression in sporadic CRC. 24 of 44 (54.5%) colorectal cancer samples with negative cytoplasmic HuR immunoreactivity had high COX-2 immunoreactivity, whereas 74 of the 97 (76.3%) colorectal cancer samples with positive cytoplasmic HuR immunostaining showed high COX-2 expression (N=141, χ^2=5.77, P=0.016). No statistically significant differences were observed between FAP associated CRC and sporadic CRC. COX-2 and HuR did not correlate with age, gender, tumor stage or grade (data not shown).

Table 1. Epithelial HuR and COX-2 expression in sporadic colorectal carcinoma and FAP colon mucosa

		COX-2 high			HuR Cytoplasmic positive			HuR Nuclear positive		
	N	N	%	P	N	%	P	N	%	P
FAP colon[a]										
Normal	20	2	10.0		2	10.0		19	95.0	
Adenoma[b]	112	9	8.0		16	14.3		104	92.9	
Carcinoma	9	9	100.0	<0.01[c]	8	88.9	<0.01[c]	8	88.9	0.84
Sporadic colorectal cancer										
	141	98	69.5	0.11[d]	97	68.8	0.37[d]	99	70.2	0.41[d]

[a] When multiple cores from the same normal, polyp or carcinoma mucosa were present on the TMA, the highest score was used in the analysis. [b] 112 adenomas from 26 patients. [c] Normal vs. Carcinoma. [d] FAP vs. Sporadic CRC

DISCUSSION

HuR expression was investigated in normal colorectal mucosa, adenomas and carcinomas of patients with FAP and in sporadic Dukes B and C colorectal carcinomas. We found increasing cytoplasmic HuR immunoreactivity and nucleocytoplasmic translocation of HuR in consecutive stages of the adenoma-carcinoma sequence in FAP colon epithelium. In addition, we noted a correlation between cytoplasmic HuR positivity and COX-2 staining in sporadic carcinomas.

Nuclear HuR expression was almost invariably present in FAP colon epithelium and in 70% of sporadic colorectal carcinomas. However, cytoplasmic immunoreactivity gradually increased in subsequent stages of the adenoma-carcinoma sequence in FAP (10% in normal, 14.3% in adenomas and 88.9% in carcinomas), and was found in almost 70% of sporadic colorectal carcinomas. Moreover, the abundance of cytoplasmic relative to nuclear HuR immunostaining increased during consecutive stages of the adenoma-carcinoma sequence. These results correspond to a study of HuR expression in a limited number of

samples (15 normal and neoplastic colon specimens), which reported increasing cytoplasmic HuR intensity and relative cytoplasmic abundance in consecutive stages of progression to malignancy.[5] Interestingly, Denkert et al. found a gradual increase of cytoplasmic HuR expression in subsequent Dukes stages: 38% of Dukes A carcinomas and 50% of Dukes B carcinomas, 58% of Dukes C carcinomas and 85% of Dukes D carcinomas exhibited cytoplasmic HuR expression.[8] Taken together, cytoplasmic HuR expression appears associated with progression to cancer. Whether HuR expression reflects other changes underlying these processes (an epiphenomenon), or is truly involved in carcinogenesis affecting the expression of major growth regulatory/promoting genes remains to be elucidated. In this regard, the induction of HuR in CRC cells in vitro increased the expression of COX-2,[6] an important enzyme in progression of the adenoma-carcinoma sequence. This observation also corresponds to our finding that the cytoplasmic HuR expression correlated to COX-2 expression in sporadic CRC.

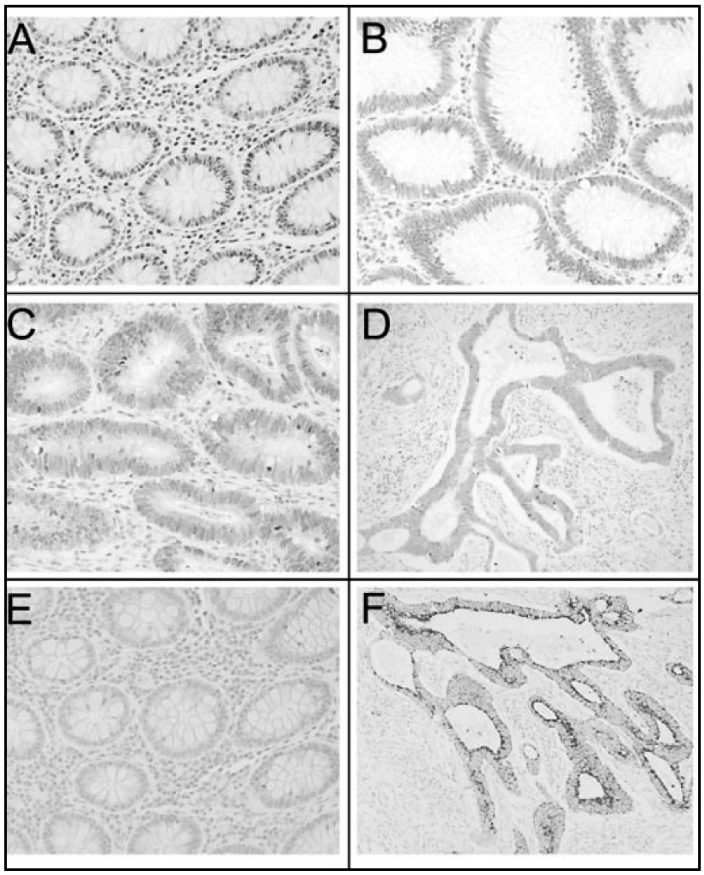

Figure 1. *HuR is localized exclusively to the nucleus of normal colon epithelium (A) and of a colon adenoma (B). Nuclear and cytoplasmic HuR are both positive in a colon adenoma (C). HuR is mainly localized to the cytoplasm of a colorectal carcinoma (D). Low COX-2 immunostaining in normal colon epithelium (E). High COX-2 immunostaining in the same carcinoma as presented in D.*

Few colorectal adenomas of FAP patients showed cytoplasmic HuR expression. Whether cytoplasmic HuR expression reflects an increased potential for progression to malignancy remains to be shown. An increased rate of p53 expression (which is considered a late

event in the adenoma-carcinoma sequence) was not found in these cases (data not shown) but further studies addressing the value of HuR expression as a marker for tumor progression of adenomas are warranted. COX-2 expression did not correlate with cytoplasmic HuR in these adenomas. COX-2 is variably and often focally expressed in adenomas, but consistently expressed and more pronounced in carcinomas. Whether different mechanisms of COX-2 overexpression between adenoma and carcinomas exist is not known.

Although the mechanisms that regulate HuR expression are largely undefined, several signaling pathways influence cytoplasmic HuR expression and activity. For instance, mitogen-activated protein (MAP) kinases p38 and ERK increase cytoplasmic presence of HuR and its mRNA stabilizing function. Also AMP-activated protein kinase (AMPK) has been implicated in regulation of HuR expression. Increased AMPK activity reduces cytoplasmic HuR expression while reduced AMPK activity increases expression of cytoplasmic HuR and HuR targets, including proteins with key functions in malignant development.[13]

In conclusion, in this first report of HuR expression in FAP we found increasing HuR expression in subsequent stages of the adenoma-carcinoma sequence. In addition, this study suggests that HuR is associated with progression to malignancy by stabilizing COX-2 mRNA and, thereby, increasing COX-2 levels in the colon.

ACKNOWLEDGEMENTS

Supported by The Netherlands Digestive Disease Foundation (MLDS) grant WS 04-06, The John G Rangos, Sr. Charitable Foundation, The Clayton Fund, and NIH grants CA 53801, 63721, 51085 and P50 CA 93–16.

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PART II

JUVENILE POLYPOSIS

7

RISK OF COLORECTAL CANCER IN JUVENILE POLYPOSIS

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Gut 2007 Jul;56(7):965-7

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ABSTRACT

Background: Juvenile polyposis (JPS) is an autosomal dominant syndrome characterized by the development of hamartomatous gastrointestinal polyps and is associated with colorectal cancer. However, the relative and absolute risk of colorectal malignancy in these patients is not known.

Methods: The incidence rates of colorectal cancer in patients with JPS were compared with that of the general population through person-year analysis with adjustment for demographics.

Results: In patients with JPS, the RR (95% CI) of colorectal cancer was 34.0 (14.4 to 65.7). Similar risks were noted in both males (30.0, 9.6 to 68.6) and females (43.7, 8.8 to 125). The cumulative life-time risk for colorectal cancer was 38.7%. The mean (SD) age of diagnosis of colorectal cancer was 43.9 (10.4) years. Other gastrointestinal malignancies were not noted in this cohort.

Conclusion: Patients with JPS have a markedly increased RR and absolute risk for colorectal cancer and require vigilant colorectal surveillance starting at young age. A low threshold for recommending surgery with consideration for removal of the entire colorectum seems warranted.

INTRODUCTION

Juvenile polyposis is an autosomal dominant syndrome characterized by the development of histopathological juvenile polyps in the gastrointestinal tract. Polyps usually occur by the third decade of life and primarily affect the colorectum.[1] Recently, investigators have discovered that germline mutations in the *BMPR1A* and *SMAD4/MADH4* gene cause this disorder in a minority (39%) of patients.[2] Also, mutation of the *ENG* gene may or may not be a cause of early-onset JPS.[3, 4] JPS has been associated with increased risk of colorectal cancer. Evidence for this concept comes from a limited number of case series and collections of literature case reports, which provide variable estimates of the risk of colorectal cancer.[5-12] Also, other malignancies, including gastric, small bowel and pancreatic cancer, have been noted in some studies. However, a formal risk assessment of gastrointestinal cancer in patients with JPS has not been reported.

The purpose of this study was to define the magnitude of risk for colorectal cancer in patients with JPS. The occurrence of colorectal cancer in patients with JPS from The Johns Hopkins Polyposis Registry and Clinic, The Johns Hopkins Hospital, Baltimore, Maryland, USA, were compared with the general population of the US through person-year analysis.

METHODS

Patient data were collected from The Johns Hopkins Polyposis Registry and clinic. Patients were defined as having JPS according to the following accepted clinical criteria:[1, 7] (1) at least five juvenile polyps in the colorectum, (2) juvenile polyps throughout the gastrointestinal tract or (3) any number of juvenile polyps in a person with a known family history of juvenile polyps. This study was approved by the Johns Hopkins Joint Committee on Clinical Investigation (institutional review board).

A risk assessment for colorectal adenocarcinoma was performed. Computation of person-years at risk for colorectal cancer started on 1 January 1970 and lasted until 1 July 2005. Patients were considered to be at risk from birth until date of diagnosis of colorectal

cancer, the date of death or the closing date of the study. Patients were censored at age 85 years.

Person-years at risk were calculated for ages 0–84 years according to sex, race and age-specific categories during the subsequent 5-years calendar time period of observation using a computer program for cohort analysis.[13] Expected colorectal cancer cases were calculated by multiplying the number of person-years for each of 5-year age groups and sex by the corresponding race-, age-, sex- and calendar time-specific incidence rate for the general US population. The Surveillance, Epidemiology and End Results data for the US population[14] were used utilizing 5-year calendar time periods as conventionally provided by this dataset. The ratio of observed carcinomas to the expected number was computed with a test of significance and 95% CIs assuming a Poisson distribution. This ratio forms the relative risk (RR) and compares cancer risk of the study population with that of the general population. Using the absolute rates for each 5-year age group, the cumulative risk was calculated using the formula: cumulative risk=1-exp (-cumulative rate).[13]

Table 1. Patients with juvenile polyposis who developed colorectal cancer

Age at diagnosis of CRC (years)	Sex	Race	Prior partial colectomy (age in years)	Death from CRC
30	F	W	No	No
32	F	W	Yes (28)	No
37	M	W	Yes (18)	Yes
41	M	W	No	Yes
48	F	W	No	No
52	M	W	Yes (19)	Yes
53	M	W	No	Yes
58	M	W	No	Yes

F, female; M, male; W, white; CRC, colorectal cancer

RESULTS

The study population for the person-year analysis consisted of 84 patients with JPS from 44 pedigrees contributing 1652.2 person-years of follow-up. This included 35 white and 7 black males (738.9 person-years of follow-up), and 39 white and 3 black females (913.3 person-years of follow-up).

Table 1 lists the patients with JPS having colorectal adenocarcinoma. The mean (SD) age of diagnosis of colorectal cancer was 43.9 (10.4) years. Two patients with prior prophylactic colectomy with ileoanal anastomosis and one with colectomy and Hartman's pouch developed subsequent cancer in the retained rectum. In all, 5 of 8 (63%) patients with colorectal cancer died of this malignancy. No cases of esophageal, gastric, small bowel or pancreatic cancer were noted in this cohort.

Table 2 shows the results of the person-year analysis for colorectal cancer. The RR (95% CI) for colorectal cancer in patients with JPS was 34.0 (14.4 to 65.7). This significantly increased risk was similar in males (30.0, 9.6 to 68.6) and females (43.7, 8.8 to 125). On the basis of an 80-year life span, the absolute risk for colorectal cancer was 38.7 per 100 persons.

Table 2. Risk analysis of colorectal cancer in patients with juvenile polyposis as compared with the general population of the United States (SEER data)

Group	Person-years	Numbers of CRC	Relative Risk (O/E)	95% confidence limits	Rate per 100,000 (person-years)
Males	738.9	5	30.0	9.6 to 68.6	676.7
Females	913.3	3	43.7	8.8 to 125.0	328.5
Combined	1652.2	8	34.0	14.4 to 65.7	484.2

CRC, colorectal cancer

DISCUSSION

In this study performed by person-year analysis, both male and female patients with JPS had a significantly elevated RR (34.0) of colorectal cancer compared with the general population. The life-time risk of colorectal cancer was calculated at 39%. These findings are consistent with several case series,[5, 7-12] and a publication that compiled literature reports,[6] estimating a 13–38% frequency of colorectal cancer in patients with JPS. Jass[15] reported a 68% cumulative risk of colorectal cancer in patients from the St Mark's Registry but details were not provided.

In JPS, as in the other inherited syndromes of colorectal neoplasia, increased risk of colon malignancy seems to be associated with a younger age of diagnosis. In this study, the mean age of colorectal cancer diagnosis was 43.9 years with one case being diagnosed at age 30 years. In two previous reports, the mean age of colorectal cancer diagnosis was 34 years with one individual being diagnosed early at age 15 years.

Extracolonic gastrointestinal cancers (<30 confirmed literature cases) have been reported in other studies of patients with JPS. These have included stomach, small bowel and pancreatic cancers.[8, 9, 11, 12] None of the patients in our cohort had these malignancies, and consequently, formal risk analysis for these tumors could not be performed. Evaluation of literature reports shows gastric and small bowel tumors, occurring together at about one-fifth the frequency of colorectal cancers in this patient group.

A caution raised by comparison of a registry-based population to the general US population is detection bias—that is, surveillance of the population in the registry may lead to higher diagnosis of certain disorders compared with the general population. Although this concern cannot be discounted, none of the patients with malignancy came to the attention of the registry as being secondary to the diagnosis of colorectal cancer. Also, the risks generated for colorectal cancer in patients with JPS in this analysis are consistent with the prevailing literature.

Moreover, our estimates of colorectal cancer risk are probably conservative, as patients with partial colectomy were not censored from the analysis at the time of surgery. In this regard, three of the patients with colorectal cancer developed malignancy in the retained rectum several years after the partial colectomy. Finally, marked risk of colorectal neoplasia is also noted in a transgenic mouse model of JPS produced by inhibition of bone morphogenetic protein signaling. In these animals, intestinal epithelial neoplasia and disruption of the Wnt pathway were noted in a majority of mice.[16]

In summary, this study, taken together with other literature data, argues for a markedly increased risk of colorectal cancer in patients with JPS. These findings are in concert with expert opinion, which recommends the commencement of screening for JPS in at-risk individuals at age 15 years (or earlier if the patient is having symptoms).[17, 18] Screening with genetic testing is preferable, but if not feasible, colonoscopy at an interval of every 3 years is advised. Surveillance of affected individuals is advocated at least biennially by colonoscopy initiated by age 15 years.[18] Also, others advise periodic upper endoscopy and evaluation of the small intestine.[18] Based on colorectal cancer risk estimates, a low threshold for recommending colectomy (i.e., when colorectal dysplasia is present or adequate surveillance is not possible) with consideration for removal of the entire colorectum seems warranted.

ACKNOWLEDGMENTS

Supported by The John G Rangos, Sr. Charitable Foundation, The Clayton Fund, and NIH grants CA 53801, 63721, 51085 and P50 CA 93–16, and The Netherlands Digestive Disease Foundation (MLDS) grant WS 04-06. We thank Ms Linda Welch for technical support.

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8

LARGE GENOMIC DELETIONS OF *SMAD4*, *BMPR1A* AND *PTEN* IN JUVENILE POLYPOSIS

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Gut 2008 May;57(5):623-7

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ABSTRACT

Background: Juvenile polyposis syndrome (JPS [MIM 174900]) is a rare autosomal dominant disorder characterized by multiple gastrointestinal juvenile polyps and an increased risk of colorectal cancer. This syndrome is caused by germline mutation of either *SMAD4* or *BMPR1A*, and possibly *ENG*. *PTEN,* originally linked to Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, has also been associated with JPS. By direct sequencing, germline mutations are found in only 30-40% of patients with a JPS phenotype. Therefore, alternative ways of inactivation of the known JPS genes, or additional genes predisposing to JPS may be involved. In this study, a comprehensive genetic analysis of *SMAD4*, *BMPR1A*, *PTEN* and *ENG* is performed through direct sequencing and multiplex ligation-dependent probe amplification (MLPA) in JPS patients.

Methods: Archival material of 29 patients with JPS from 27 families was collected. Direct sequencing and MLPA analysis were performed to search for germline defects in *SMAD4*, *BMPR1A*, *PTEN* and *ENG*.

Results: A germline defect in *SMAD4*, *BMPR1A* or *PTEN* was found in 13 of 27 (48.1%) unrelated JPS patients. Nine mutations (33.3%) were detected by direct sequencing, including six (22.2%) *SMAD4* mutations and three (11.1%) *BMPR1A* mutations. MLPA identified four additional patients (14.8%) with germline hemizygous large genomic deletions, including one deletion of *SMAD4*, one deletion of exons 10 and 11 of *BMPR1A*, and two unrelated patients with deletion of both *BMPR1A* and *PTEN*. No *ENG* gene mutations were found.

Conclusion: Large genomic deletions of *SMAD4*, *BMPR1A* and *PTEN* are a common cause of JPS. Using direct sequencing and MLPA, a germline defect was detected in 48.1% of JPS patients. MLPA identified 14.8% (4/27) of these mutations. Since a substantial percentage of JP patients carry a germline deletion and MLPA is a reliable and user friendly technique, we conclude that MLPA is a valuable adjunct in JPS diagnosis.

INTRODUCTION

Juvenile polyposis is an autosomal dominant disorder characterized by multiple gastrointestinal juvenile polyps and an increased risk of colorectal cancer.[1] Clinically JPS is defined by the presence of more than 3-5 juvenile polyps, or any number of juvenile polyps and a positive family history of juvenile polyposis.[2, 3] Juvenile polyps are hamartomas with a distinctive histology and most frequently encountered in the colorectum.

In the past decade mutations in the *SMAD4* and *BMPR1A* genes were identified as the cause of JPS.[4, 5] However, a germline defect in these genes is found in a minority of JPS patients. The largest study analyzed 77 JPS patients by direct sequencing of *SMAD4* and *BMPR1A* and found a germline mutation of *SMAD4* in 18.2% and of *BMPR1A* in 20.8% of patients.[6] Others have reported similar results.[7-9] Therefore, alternative ways of inactivation of the known JPS genes, or additional, yet unidentified, genes predisposing to JPS may be involved.

Other components of the TGF-β/BMP pathway, including *SMAD1*, *SMAD2*, *SMAD3*, *SMAD5*, *SMAD7*, *BMPR2*, *BMPR1B* and *ACVRL1*, were studied but no mutations have been found in these genes.[6, 10, 11] Recently, two patients with JPS were reported to have a germline mutation in the gene encoding the TGB-β co-receptor Endoglin (*ENG*).[12] Therefore, *ENG*

was proposed as a potential novel susceptibility gene of JPS,[12] but this has not been confirmed.[13] Also *PTEN*, originally linked to Cowden syndrome (CS [MIM 158350]) and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]), has been associated with JPS.[14, 15] However, others have not found *PTEN* germline mutations in JPS.[16, 17] Consequently, *PTEN* mutations in patients with juvenile polyposis likely represent CS or BRRS patients that have not (yet) expressed the extraintestinal clinical features of these conditions.[18, 19]

Interestingly, germline contiguous deletion of *BMPR1A* and *PTEN* is reported in patients with multiple juvenile polyps. However, it is unclear whether these patients are true JPS patients or BRRS/CS patients that have not yet displayed the clinical features of these conditions.[20-23]

Multiplex ligation-dependent probe amplification (MLPA) is a novel technique that can detect copy number changes in genomic DNA sequences.[24] In the current study, direct sequencing and MLPA were combined to perform a comprehensive genetic analysis in a group of well documented JPS patients and to address the question whether large genomic deletions of any of the known JPS genes may cause JPS.

METHODS

Patients and patient selection

Archival material from 29 JPS patients from 27 families was collected from The Johns Hopkins Polyposis Registry and clinic (Baltimore, MD, USA) and two academic hospitals in the Netherlands (Academic Medical Center, Amsterdam, and University Medical Center, Utrecht). Patients were defined as having JPS according to the accepted clinical criteria[2, 3] as follows: 1) at least 3-5 juvenile polyps in the colorectum, or 2) juvenile polyps throughout the gastrointestinal tract, or 3) any number of juvenile polyps in a person with a known family history of juvenile polyps. Each case was carefully reviewed by an experienced pathologist (GJAO) to confirm the histopathological diagnosis of JPS. The study was approved by the Johns Hopkins Institutional Review Board and carried out in accordance with the ethical guidelines of the research review committees of the institutions in Amsterdam and Utrecht.

DNA isolation

Genomic DNA was obtained from deparaffinized formalin-fixed paraffin-embedded non neoplastic colorectal tissue from patients with JPS using TK buffer (400 µg/mL of proteinase K and 0.5% Tween 20, 50 mmol/L Tris (pH=9), 1 mmol/L NaCl, 2 mmol/L EDTA). After overnight incubation in 50 µL TK buffer at 56°C, tubes were incubated at 95°C for 10 minutes to inactivate the proteinase K.[25]

Sequencing

Genomic DNA was amplified by PCR using Platinum® *Taq* DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA) and specific primers complementary to intronic sequences flanking all exons of *SMAD4*, *BMPR1A*, *ENG* and *PTEN.* Primer sequences and PCR conditions are available upon request. Amplification was performed using an initial denaturation step at 95°C for 10 min, followed by 35 cycles at 94°C for 15 sec, annealing temperature for 1 min and 72°C for 1 min.

The amplified fragments were first analyzed by agarose-gel electrophoresis. Subsequently, the PCR product was enzymatically purified using Shrimp Alkaline Phosphatase (USB Europe GmbH, Staufen, Germany) and Exonulcease I (New England Biolabs, Ipswich, MA, USA) according to the manufacturers' protocol. Samples were then subjected to direct sequencing of single strand PCR products using the BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism® 3130 genetic analyzer (Applied Biosystems). All products were sequenced in sense and anti-sense direction. Patient sequences were compared to wild type reference sequences using CodonCode Aligner software. The cDNA bases were numbered according to the reference sequence in Ensembl (*SMAD4*: NM_005359.3; *BMPR1A*: NM_004329.2; *ENG*: NM_000118.1; *PTEN*: NM_000314.3), where nucleotide 1 corresponds to the A of the ATG translation initiation codon. Each mutation/variation was confirmed by a second round of PCR amplification and sequencing. The possibility of missense mutations and intronic variations being polymorphic variants was excluded using a healthy control group. For human mutation nomenclature "Recommendations for Nomenclature System for Human Gene Mutations" were followed.[26, 27]

MLPA

Multiplex ligation dependent probe amplification was performed using the Juvenile Polyposis (Kit P158, containing probes for each of the *SMAD4* and *PTEN* exons and for all but one of the *BMPR1A* gene) and Hereditary Hemorrhagic Telangiectasia (Kit P093, containing probes for 13 different *ENG* exons, and for all *ACVRL1* and *BMPR2* exons) probe kits (MRC-Holland B.V., Amsterdam, the Netherlands). MLPA reactions were performed according to the manufacturer's instructions. In brief, 50 ng of genomic DNA in 5 µl TE was heat-denatured (5 min at 98°C) and incubated with the probe set for 16 hours at 60°C. Then the hybridized products were ligated (15 min at 54°C), PCR-amplified (35 cycles: 30 sec at 95°C; 30 sec at 60°C; 60 sec at 72°C; final elongation: 20 min at 72°C) and separated by electrophoresis on an automated sequencer. DNA from healthy individuals was used as normal control. Finally, MLPA data were evaluated using Coffalyser, a Microsoft Excel based program freely available at the MRC-Holland website. All samples were assayed in two independent MLPA reactions.

Table 1. Mutation detection rates in *SMAD4* and *BMPR1A* in 27 unrelated juvenile polyposis patients

	SMAD4	***BMPR1A***	**Total**
All patients			27
Point mutations	6 (22.2%)	3 (11.1%)	9 (33.3%)
Deletions	1 (3.7%)	3 (11.1%)	4 (14.8%)
All mutations	7 (25.9%)	6 (22.2%)	13 (48.1%)

RESULTS

Sequencing

By direct sequencing of *SMAD4*, *BMPR1A*, *ENG* and *PTEN* genes, nine germline mutations were found in 27 JPS patients (33.3%), including six (22.2%) *SMAD4* mutations and three (11.1%) *BMPR1A* mutations. (Table 1 and 2)

The *SMAD4* germline mutations included two missense mutations in exon 8 (c.970 T>C, p.C324R and c.989 A>G, p.E330G), and one non-sense mutation in exon 9 (c.1193 G>A, p.W398X). In addition, a 1bp deletion was found in exon 8 (c.971delG, p.C324FfsX12), a 25 bp deletion was found in exon 10 (c.1411_1435del25, p.G471FfsX25), and a single base pair duplication was found in exon 11 (c.1586_1587dupA, p.L529LfsX9).

In *BMPR1A* one missense mutation in exon 10 (c.1483 C>T, p.R480W) and one single base pair deletion in exon 8 (c.1061delG, p.G354EfsX10) were found. In addition, one intronic mutation was found in the splice acceptor site of intron 5 (c.531-2A>G). No mutations were found in *ENG* or *PTEN*. Several polymorphisms were found in *BMPR1A* and *ENG*. (Table 3)

Table 2. Germline defects in *SMAD4*, *BMPR1A* and *PTEN* found in juvenile polyposis patients

P	Gene	Exon	Nucleotide change	Predicted result	Controls	Method
7[1]	*SMAD4*	8	c.970 T>C	p.C324R	0/118	Seq.
14	*SMAD4*	8	c.989 A>G	p.E330G	0/117	Seq.
23	*SMAD4*	8	c.971delG	p.C324FfsX12		Seq.
19	*SMAD4*	9	c.1193 G>A	p.W398X		Seq.
4	*SMAD4*	10	c.1411-1435del25	p.G471FfsX25		Seq.
27	*SMAD4*	11	c.1586_1587dupA	p.L529LfsX9		Seq.
11[2]	*SMAD4*	1-11	Hemizygous deletion			MLPA
21	*BMPR1A*	Intron 5	c.531-2A>G	Splice site	0/116	Seq.
16	*BMPR1A*	8	c.1061delG	p.G354EfsX10		Seq.
20	*BMPR1A*	10	c.1483 C>T	p.R480W	0/134	Seq.
3	*BMPR1A*	10 and 11	Hemizygous deletion			MLPA
18	*BMPR1A/ PTEN*		Hemizygous deletion			MLPA
24	*BMPR1A/ PTEN*		Hemizygous deletion			MLPA

[1] mutation was confirmed in one affected family member
[2] deletion was confirmed in one affected family member
P: patient; Seq.: Sequencing

Table 3. Polymorphisms in *BMPR1A* and *ENG*

	Nucleotide	Amino acid change	Number of patients	Reference	refSNP ID
BMPR1A					
Exon 1	c.4 C>A	p.P2T	19/27	Howe[5]	rs17090779
Exon 5	c.435 G>A	p.P145P	2/27	Pyatt[7]	rs2230176
ENG					
Exon 1	c.14 C>T	p.T5M	1/27	Howe[13]	rs35400405
Exon 2	c.207 G>A	p.L69L	6/27	Howe[13]	rs16930129
Exon 5	c.572 G>A	p.G191D	1/27	Abdalla[28]	rs41322046
Exon 8	c.1029 C>T	p.T343T	1/27	Howe[13]	rs3739817
Exon 8	c.1060 C>T	p.L354L	1/27	Howe[13]	rs36092484
Exon 11	c.1374 A>G	p.P458P	1/27	Prigoda[29]	rs34828244
Exon 14	c.1794 T>C	p.G598G	3/27	Abdalla[28]	Rs41358947

MLPA

Using MLPA, a large genomic deletion was identified in 22.2% (4/18) of patients in whom no point mutation had been detected, or in 14.8% (4/27) of all patients examined. (Table 1 and 2) This included one hemizygous deletion of *SMAD4*, which was also found in an affected family member. In addition, one patient with a hemizygous deletion of exons 10 and 11 of *BMPR1A*, and two unrelated patients with a hemizygous deletion of both *BMPR1A* and *PTEN* were found. No deletions were found in *PTEN* alone, *ENG, ACVRL1* and *BMPR2*.
By sequencing and MLPA combined a germline defect was found in 48.1% (13/27) of JPS patients. MLPA identified 30.8% (4/13) of these germline defects.

DISCUSSION

SMAD4 and *BMPR1A* are the two best known juvenile polyposis genes. However, by direct sequencing, germline mutation of *SMAD4* or *BMPR1A* is found in only 30-40% of patients,[6-9] indicating that alternative ways of inactivation of these genes, or additional genes causing JPS may exist.
In the current study, a comprehensive genetic analysis of a group of 27 well documented JPS patients was performed using both direct sequencing and MLPA to investigate the role of large genomic deletions in the germline of JPS patients. By direct sequencing, germline mutations were found in 33.3% of JPS patients (22.2% in *SMAD4* and 11.1% in *BMPR1A*). This is consistent with previous studies reporting germline mutation of *SMAD4* in 18-24% and of *BMPR1A* in 11-24% of patients.[6-9]
Using MLPA, four unrelated patients with a large genomic germline deletion were identified, adding 14.8% to the total amount of germline defects in our cohort. Using both sequencing and MLPA a germline defect was identified in 48.1% of patients. Recently, a similar percentage (49%) was reported in a study that also combined sequencing and MLPA in JPS, but the role of *ENG* mutation in JPS was not addressed.[30]

Interestingly, two patients had deletion of both *BMPR1A* and *PTEN*, likely a contiguous gene deletion at 10q22-23. Deletion of this region has been reported in 11 patients.[12, 20-23, 31, 32] *PTEN* was deleted in all of these patients and *BMPR1A* in at least six and probably in another three of these patients. Clinically, most of these patients had juvenile polyposis of infancy as described by Sachatello et al.[33] and some also had symptoms of BRRS.[20, 21] Both patients in the current study had multiple juvenile polyps with the typical associated histology and also dysplasia. One of these patients was also diagnosed with thyroid carcinoma, raising the question of Cowden syndrome. Further studies would be needed to determine the exact size of the genomic deletion on 10q in these individuals.

In 51.9% of JPS patients in this cohort a germline defect was not found. Possibly, mutations in the promotor region or in intronic sequences that affect cryptic splice sites are responsible for some of these cases. However, it seems unlikely that the remaining 51.9% can be explained by undiscovered mutations in *SMAD4* of *BMPR1A* alone, suggesting that other genes predispose to JPS. Recently, germline *ENG* mutation was reported in two JPS patients and proposed as a potential novel JPS susceptibility gene.[12] However, others have not confirmed this finding,[13] and we did not detect any mutations or exon deletions of the *ENG* gene in the current study. Therefore, the role of *ENG* in JPS remains unclear. Several other TGF-β/BMP signaling molecules have also been studied but no mutations have been found.[6, 10, 11] These data suggest that there are other genes responsible for mutation negative JPS cases.

In summary, large genomic deletions in *SMAD4*, *BMPR1A* and *BMPR1A* and *PTEN* are not uncommon causes of JPS and these deletions are detectable using MLPA. In view of the substantial percentage of patients carrying a germline deletion (14.8%) as detected using MLPA, and given the reliability and user friendliness of this technique, we conclude that MLPA is a valuable adjunct in JPS diagnosis.

ACKNOWLEDGEMENTS

Supported by The Netherlands Digestive Disease Foundation (MLDS) grant WS 04-06, The John G Rangos, Sr. Charitable Foundation, The Clayton Fund, and NIH grants CA 53801, 63721, 51085 and P50 CA 93–16. We thank Linda Hylind and Jordy Coffa for technical assistance.

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9

NO *TGFBRII* GERMLINE MUTATIONS IN JUVENILE POLYPOSIS PATIENTS WITHOUT *SMAD4* OR *BMPR1A* MUTATION

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ABSTRACT

Background: Juvenile polyposis (JPS [MIM 174900]) is an autosomal dominant disorder characterized by multiple gastrointestinal juvenile polyps and an increased risk of colorectal cancer. JPS is caused by germline mutation of *SMAD4* or *BMPR1A*, both involved in TGF-β/BMP signaling. A germline defect in one of these genes is found in about half of JPS patients, suggesting that mutations in other genes may exist that predispose to JPS. TGFBRII is a member of the TGF-β signaling pathway and often somatically mutated in CRC. In this study, the role of TGFBRII in juvenile polyposis pathogenesis is investigated.

Methods: Genomic DNA from 19 patients with juvenile polyps, without germline *SMAD4* or *BMPR1A* mutation, was investigated for the presence of germline mutations and deletions in the *TGFBRII* gene.

Results: No pathogenic *TGFBRII* mutations or deletions were found in the germline of 19 patients with juvenile polyps.

Conclusion: No evidence was found for a role of TGFBRII in JPS pathogenesis, indicating that *TGFBRII* is unlikely to be a JPS susceptibility gene. Likely, other JPS causing genes exist in addition to *SMAD4* and *BMPR1A*.

INTRODUCTION

Juvenile polyposis is an autosomal dominant disorder characterized by the presence of multiple gastro-intestinal juvenile polyps and an increased risk of colorectal, and possibly gastric, small bowel and pancreatic cancer.[1, 2] In addition, JPS patients may have a range of extra-intestinal features.[3]

JPS is caused by germline mutation of *SMAD4* or *BMPR1*A, both involved in the Transforming Growth Factor–Beta/Bone Morphogenic Protein (TGF-ß/BMP) signaling pathway.[4, 5] A germline defect in one of these genes is found in approximately 50% of JPS patients, with 30-40% being a point mutation or small deletion and 10-15% a large genomic deletion.[6, 7] No germline defect is found in the remaining 50% of JPS patients. Although other ways of inactivation of *SMAD4* or *BMPR1A*, such as mutations in the promoter region or intronic sequences, may explain some of these cases, it is unlikely that the remaining 50% are due to undiscovered mutations in *SMAD4* or *BMPR1A* alone. Therefore other, yet undiscovered, genes may exist that predispose to JPS.

Several candidate genes have been investigated for a role in JPS pathogenesis (Table 1). Most are involved in the TGF-ß/BMP pathway, including *SMAD1*, *SMAD2*, *SMAD3*, *SMAD5*, *SMAD7*, *BMPR2*, *BMPR1B* and *ACVRL1*. Presently, no mutations have been found in these genes.[8-11] Recently, germline mutation of the TGB-β co-receptor Endoglin (*ENG*) was reported in two JPS patients.[12] However, no additional *ENG* mutations have been found in a total of 65 JPS patients, and the role of *ENG* as a JPS susceptibility gene is, therefore, unclear.[7, 11, 13] Also *PTEN*, the gene originally linked to Cowden syndrome (CS [MIM 158350]) and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]), has been suggested as a JPS gene,[14, 15] although this is not agreed upon by all.[16, 17] The current consensus is that *PTEN* mutations in patients with juvenile polyps likely represent CS or BRRS patients that have not (yet) developed extraintestinal clinical features specific to these conditions.[18] Moreover, patients with juvenile polyposis of infancy have been found to have both deletions of *BMPR1A* and *PTEN* genes, casting doubt on *PTEN* as the cause of JPS.[7, 19] The *CDX2* gene has also been investigated in juvenile polyposis, since mice with a

heterozygous mutation of *Cdx2* develop intestinal hamartomatous polyps.[20] But no pathogenic mutations were found in 37 *SMAD4* negative JPS families.[21]
The TGF-beta receptor type II (TGFBRII) is a component of the TGF-ß pathway and is mutated within a polyadenine tract in exon 3 in up to 90% of colorectal cancers with microsatellite instability and in 15% of microsatellite stable malignancies.[22-24] In addition, a germline mutation of *TGFBRII* has been reported in a patient with hereditary colorectal cancer.[25] Because TGFBRII plays a role in TGF-β signaling and it is important in colorectal carcinogenesis, we investigated whether germline mutation or deletion of this gene is involved in the pathogenesis of JPS. Therefore, all exons of the *TGFBRII* gene were investigated by sequencing and multiplex ligation-dependent probe amplification (MLPA) in 19 patients with juvenile polyps without germline *SMAD4*, *BMPR1A*, *PTEN* or *ENG* mutation.

Table 1. Candidate genes investigated in JPS pathogenesis

Gene	Patients studied/Mutations found	Author[ref]
BMPR1B (ALK6)	32/0	Howe[8]
BMPR2	59/0 *	Howe[8], van Hattem[7]
ACVR1 (ALK1)	66/0 **	Howe,[8] Gallione[11], van Hattem[7]
SMAD1	30/0	Bevan[9]
SMAD2	34/0	Bevan,[9] Roth[10]
SMAD3	34/0	Bevan,[9] Roth[10]
SMAD5	30/0	Bevan[9]
SMAD7	34/0	Bevan,[9] Roth[10]
CDX2	37/0	Woodford-Richens[21]
APC/MCC	1/0	Legget[26]

* 32 patients investigated by sequencing,[8] 27 by MLPA.[7]
** 39 patients investigated by sequencing,[8, 11] 27 by MLPA.[7]

METHODS

Patients

The *TGFBRII* gene was sequenced in 19 patients from 18 families with juvenile polyps without germline mutation or deletion of *SMAD4*, *BMPR1A*, *PTEN* and *ENG*.[7] Thirteen patients fulfilled the clinical criteria of juvenile polyposis defined as follows: 1) at least five juvenile polyps in the colorectum, or 2) juvenile polyps throughout the gastrointestinal tract, or 3) any number of juvenile polyps in a person with a known family history of juvenile polyposis.[1, 2] Each case was carefully reviewed by an experienced pathologist (GJAO) to confirm the histopathological diagnosis of JPS. In addition, six patients were included that did not fulfill JPS clinical criteria, but were suggestive for JPS. The study was approved by the Johns Hopkins Institutional Review Board and carried out in accordance with the ethical guidelines of the research review committee of the University Medical Center Utrecht.[27]

DNA isolation

Genomic DNA was obtained from deparaffinized formalin-fixed paraffin-embedded non neoplastic colorectal tissue from patients with JPS using TK buffer (400 µg/mL of proteinase K and 0.5% Tween 20, 50 mmol/L Tris (pH=9), 1 mmol/L NaCl, 2 mmol/L EDTA). After overnight incubation in 50 µL TK buffer at 56°C, tubes were incubated at 95°C for 10 minutes to inactivate the proteinase K.[28]

Sequencing and MLPA

Sequencing was performed as described previously.[7] Genomic DNA was amplified by PCR using Platinum® Taq DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA) and specific primers complementary to intronic sequences flanking all exons of *TGFBRII*. Primer sequences and PCR conditions are available upon request. Subsequently, the PCR product was enzymatically purified using Shrimp Alkaline Phosphatase (USB Europe GmbH, Staufen, Germany) and Exonulcease I (New England Biolabs, Ipswich, MA, USA). Samples were then subjected to direct sequencing of single strand PCR products using the BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism® 3130 genetic analyzer (Applied Biosystems). All products were sequenced in the sense and anti-sense direction. Patient sequences were compared to wild type reference sequences using CodonCode Aligner software.

The cDNA bases were numbered according to the reference sequence in Ensembl (*TGFBRII*: NM_001024847), where nucleotide 1 corresponds to the A of the ATG translation initiation codon. Each mutation/variation was confirmed by a second round of PCR amplification and sequencing. For human mutation nomenclature "Recommendations for Nomenclature System for Human Gene Mutations" were followed.[29, 30]

MLPA was performed using the P065-A1 MLPA kit (MRC-Holland BV, Amsterdam, the Netherlands), containing probes for all 7 *TGFBRII* exons. MLPA reactions were performed according to the manufacturer's instructions as described previously.[7]

RESULTS

All exons and intron-exon boundaries of the *TGFBRII* gene were PCR-amplified and analyzed by direct sequencing in 19 patients from 18 families without a *SMAD4*, *BMPR1A*, *PTEN* or *ENG* germline defect. No pathogenic germline mutations in *TGFBRII* were found in this cohort. Known polymorphic variations were found in intron 3 (rs1155705), intron 4 (rs11466512), exon 4 (rs2228048), and intron 7 (rs11466530) (Table 2). No germline deletions were found in the *TGFBRII* gene using MLPA.

Table 2. Polymorphisms found in *TGFBRII*

Location	Nucleotide	Amino acid change	No. of JPS patients	refSNP ID
Intron 3	c.338+7 A>G	intronic	9/18	rs1155705
Intron 4	c.530-4 T>A	intronic	7/18	rs11466512
Exon 4	c.1242 C>T	p.N414N	6/18	rs2228048
Intron 7	c.1600-8 C>T	intronic	1/18	rs11466530

DISCUSSION

In 50% of JPS patients, no germline mutation can be found in *SMAD4* or *BMPR1A*, suggesting that other JPS susceptibility genes exist.[6, 7] Multiple genes have been investigated for JPS pathogenesis, but no JPS causing mutations have been found (Table 1).[8-10, 21, 26]

TGFBRII is a key component of the TGF-β signaling pathway and has a well established role in colorectal carcinogenesis. It is frequently mutated in CRC with microsatellite instability and to a lesser extent in microsatellite stable tumors.[22-24] Furthermore, loss of TGFBRII in intestinal epithelium promotes invasion and malignant transformation of tumors in *Apc*$^{1638N/wt}$ mice, similarly as described in *Cis-Apc*$^{\Delta 716+/-}$*-Dpc4*$^{(+/-)}$ mice,[31] and mice with conditionally knocked out *TgfbrII* in fibroblasts develop intraepithelial neoplasia of the prostate and invasive squamous cell carcinoma of the forestomach.[32] In addition, a germline mutation in *TGFBRII* was found in a patient with atypical hereditary non-polyposis colorectal cancer (HNPCC).[25]

Because of its role in TGF-β signaling and CRC pathogenesis we hypothesized that *TGFBRII* may be a JPS susceptibility gene. Therefore, all exons and flanking intronic sequences of *TGFBRII* were sequenced in a group of JPS patients, but no germline mutations were found. In addition, no germline deletions were found using MLPA. These results indicate that mutation or deletion of *TGFBRII* is unlikely to cause JPS.

In 2004, *TGFBRII* germline mutations were reported to cause Marfan syndrome type 2.[33] Surprisingly, these patients do not have an increased risk of cancer.[34] Functional studies of the germline mutation found in the hereditary CRC case (944C>T, T315M, NM_003242) showed that the mutant protein is incapable of mediating TGF-β mediated growth inhibition, but did not loose the ability to induce extra-cellular matrix proteins.[35] In contrast, mutants found in MFS2 all suppressed extra-cellular matrix protein expression.[33] These diverging phenotypic effects of the different *TGFBRII* mutations, may explain the absence of malignancies in MFS2 patients carrying a *TGFBRII* mutation.[33]

Alternatively, the 944C>T variation could be a rare polymorphism without significance for CRC development. Although, Lu et al. did not find the same alteration in 119 subjects,[25] others found the 944C>T variation at a similar frequency in normal controls (7 of 492) and individuals with sporadic colorectal cancer (6 of 228).[33] Moreover, no additional germline mutations in *TGFBRII* have been found in HNPCC patients or in patients with familial or early onset CRC.[36 37]

In conclusion, no germline mutations or deletions in *TGFBRII* were found in this study, making it unlikely that *TGFBRII* is a JPS susceptibility gene and adding *TGFBRII* to the list of genes investigated for a potential role in JPS. Still, about half of JPS patients remain without a molecular diagnosis and the search for other JPS causing genes should continue apace. Candidate genes could include other, perhaps less obvious, components of the TGF-β/BMP pathway.

ACKNOWLEDGEMENTS

Supported by The Netherlands Digestive Disease Foundation (MLDS) grant WS 04-06, The John G. Rangos, Sr. Charitable Foundation, The Clayton Fund, and NIH grants CA 53801, 63721, 51085, and P50 CA 93-16.

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INCREASED CYCLOOXYGENASE-2 EXPRESSION IN JUVENILE POLYPOSIS SYNDROME

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Clinical Gastroenterology and Hepatology, in press

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ABSTRACT

Background: Gastrointestinal juvenile polyps may occur in juvenile polyposis syndrome (JPS) or sporadically. JPS is an autosomal dominant condition caused by a germline defect in *SMAD4* or *BMPR1A*, in 50-60% of cases, and characterized by multiple juvenile polyps, predominantly in the colorectum. JPS has an increased risk of gastrointestinal malignancy but sporadic juvenile polyps do not. Cyclooxygenase-2 (COX-2) expression is increased in gastrointestinal tumorigenesis and familial adenomatous polyposis (FAP). Inhibition of COX-2 leads to regression of colorectal adenomas in FAP patients and inhibits gastrointestinal tumorigenesis. To investigate the role of COX-2 in juvenile polyps, we compared expression of COX-2 in juvenile polyps from a well defined group of juvenile polyposis patients and sporadic juvenile polyps.

Methods: COX-2 expression was assessed in 65 polyps from 24 juvenile polyposis patients in whom the germline mutation status was been determined and 27 sporadic juvenile polyps using tissue microarray analysis. Two additional markers, HuR, a stabilizer of mRNA, and C/EBP-β, a transcription factor, both associated with increased COX-2 expression, were also investigated.

Results: Increased COX-2 expression in JPS patients was noted compared to patients with sporadic juvenile polyps ($p<0.001$). Also, JPS patients with a *BMPR1A* germline defect had higher COX-2 expression than did JPS patients in which no germline mutation was detected. High COX-2 levels correlated with increased cytoplasmic HuR expression in JPS polyps ($p=0.022$) but not in sporadic juvenile polyps.

Conclusion: Juvenile polyposis and sporadic juvenile polyps exhibit distinctive expression profiles of COX-2 that may have clinical implications.

INTRODUCTION

Juvenile polyps occur in about 1% of the pediatric population and are most often sporadic, solitary lesions of the colorectum.[1] These hamartomatous polyps are characterized by distorted and dilated crypts with reactive changes of the epithelium and an abundance of stroma. In contrast, juvenile polyposis syndrome is an autosomal dominant condition characterized by multiple juvenile polyps throughout the gastrointestinal tract.[2] In JPS, juvenile polyps often contain relatively less stroma, fewer dilated crypts and more epithelial proliferative activity than their sporadic counterparts.[3] Sporadic juvenile polyps are not associated with an increased risk of gastrointestinal malignancy.[1, 4] However, in juvenile polyposis, a recently performed person year analysis demonstrated a relative risk for colorectal cancer of 34 and a cumulative lifetime risk of 39%.[5]

Germline mutations in either *SMAD4* or *BMPR1A* are found in 50-60% of JPS cases.[6-9] The TGF-β co-receptor Endoglin (*ENG*) has been suggested as a predisposition gene for JPS, although this is still under debate.[9-11] *SMAD4*, *BMPR1A* and *ENG* are components of the Transforming Growth Factor-Beta (TGF-β)/Bone Morphogenetic Protein (BMP) signaling pathway which is involved in the regulation of cell proliferation and differentiation.[12] Patients with a germline *SMAD4* mutation may possess a more aggressive gastrointestinal JPS phenotype with higher incidence of neoplastic change compared to those with *BMPR1A* mutation.[13-15] But much remains unknown about the molecular-genetic phenotype of juvenile polyps. The increased risk of malignancy in JPS patients and the distinctive

histological appearance of JPS polyps suggest differences in molecular biology of JPS versus sporadic juvenile polyps.

Cyclooxygenase-2 (COX-2) is a key enzyme in the conversion of arachidonic acid to prostaglandins and affects several signal transduction pathways modulating inflammation and cell proliferation.[16, 17] COX-2 may play a crucial role in intestinal tumorigenesis through changes in cellular adhesion, local invasion, and inhibition of apoptosis, and is up-regulated in consecutive stages of the colorectal adenoma-carcinoma sequence in patients with sporadic colorectal cancer (CRC) and in familial adenomatous polyposis (FAP).[18-20]

HuR and C/EBP-β interact with COX-2 and may be involved in regulation of COX-2 expression in juvenile polyps. HuR is an mRNA-binding protein capable of inhibiting rapid mRNA degradation and is associated with COX-2 expression.[21] Nucleo-cytoplasmic translocation is necessary for HuR activation.[22] C/EBP-β is a transcription factor regulating proliferation and differentiation,[23] capable of inducing COX-2 expression.[24] Increased C/EBP-β correlates with invasiveness in human CRC.[25]

In this study we compare COX-2 protein expression in polyps of a well defined group of JPS patients with sporadic juvenile polyps using immunohistochemistry on tissue microarray (TMA). HuR and C/EBP-β expression were examined to investigate their relationship to COX-2 expression in JPS and sporadic juvenile polyps.

METHODS

Tissue selection

Eighty-two patients, diagnosed between 1985 and 2004 with one or more juvenile polyps, were identified in a retrospective search in the Department of Pathology databases of The Johns Hopkins Hospital in Baltimore, MD, and the Academic Medical Centre (AMC) Amsterdam, The Netherlands. The research was carried out in accordance with the ethical guidelines of the research review committee of these institutions.

Clinical and family history data were examined and polyps were histologically re-evaluated by an experienced pathologist (GJAO) to confirm the diagnosis of JPS or sporadic juvenile polyps. Also, all JPS patients underwent thorough genetic analysis through direct sequencing and MLPA analysis.[9] JPS was defined as patients with 3 or more juvenile polyps and/or a well established familial segregation and/or a germline mutation in one of the known JPS genes. Patients with sporadic juvenile polyps had a single sporadic polyp incidentally found and no family history of juvenile polyps. Sporadic juvenile polyps in patients with findings of colorectal mucosal inflammation were excluded.

A total of 50 patients (92 polyps) consisting of 24 JPS patients (median age at diagnosis 10 (range 2-32), 65 polyps) and 26 patients with sporadic juvenile polyps (median age at diagnosis 6 (range 1-61), 27 polyps) were selected for analysis. Of the 24 selected JPS patients, 7 (29%) had a *SMAD4* germline mutation and 6 (25%) carried a *BMPR1A* germline mutation, two of which had a contiguous *BMPR1A/PTEN* germline deletion.[9]

Tissue microarray

TMAs were constructed from formalin-fixed and paraffin-embedded specimens using a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). Three core biopsies (0.6 mm cylinders) were taken from the polyp tissue and, if present, also from

dysplastic foci within the polyp, in a standardized fashion, and arranged in a new recipient paraffin block. Normal mucosa was included separately when available.

Immunohistochemistry and scoring

Immunohistochemistry for COX-2 (160112, Cayman Chemical Co., Ann Arbor, MI, USA), HuR (19F12[26]) and C/EBP-β (sc-7962, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed as previously described.[27] Immunoreactivity of COX-2,[28] HuR,[29] and C/EBP-β[27] was quantified according to established systems as shown in table 1. The highest score found determined the overall polyp score. Similarly, patient scores were determined by the highest polyp score found in that particular patient.

Table 1. Scoring of immunohistochemistry

COX-2	Low	0 - no staining
		1 - very weak diffuse cytoplasmic staining
	High	2 - moderate to strong granular cytoplasmic staining in 10-50% of cells
		3 - strong intensity in >50% of cells
HuR		Nuclear and cytoplasmic staining was scored separately as positive (high) or negative (low) in epithelial cells.
C/EBP-β		Nuclear staining >25% of epithelial cells

Statistical analysis

Statistical analysis was performed using the SPSS 15.0 software package. A Chi-Squared (χ^2) Test, or, when appropriate, a Fisher's Exact Test was applied to determine whether the difference in expression between groups (JPS versus sporadic) or correlation between markers within a group were statistically significant ($P<0.05$). Overall patient scores were used when comparing JPS patients to patients with sporadic juvenile polyps for differences in expression of a certain marker. Correlations between markers were determined at individual polyp level using the overall polyp score.

RESULTS

Immunohistochemistry

A total of 50 patients (92 polyps), consisting of 24 JPS patients (65 polyps) and 26 patients with sporadic juvenile polyps (27 polyps), were analyzed. 81 Polyps were informative for all three markers. Immunohistochemical results for JPS and sporadic polyps are displayed in figure 1. Epithelial and stromal COX-2 was assessed separately. Stromal COX-2 staining was rare, with the exception of granulation tissue which formed a positive control. Therefore, only epithelial COX-2 data was included in our analysis. As nuclear HuR staining was positive in all polyps it was not included in statistical analysis.

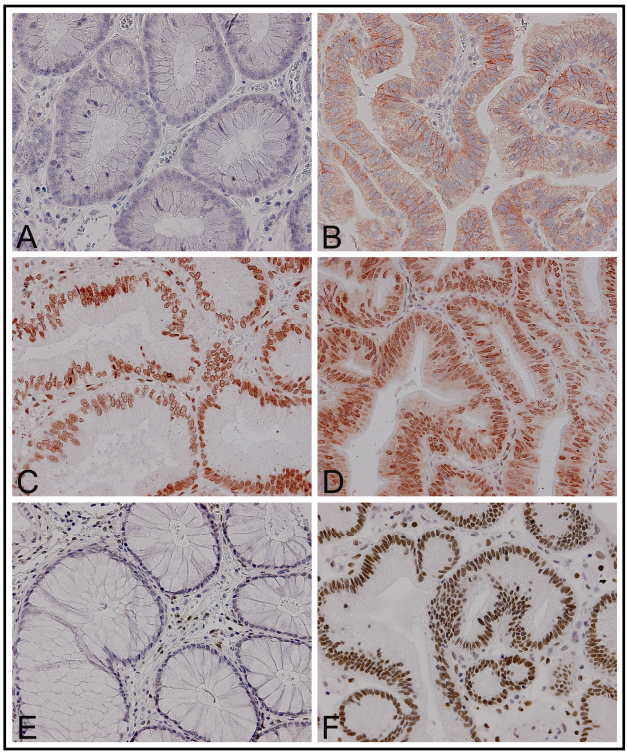

Figure 1. *Immunohistochemistry on tissue microarrays for COX-2. COX-2 (Low, A; COX-2 High, B), HuR (negative cytoplasmic staining, C; positive cytoplasmic staining, D) and C/EBP-β (negative, E; positive, F). Magnification 20X, counterstain hematoxylin.*

JPS versus sporadic juvenile polyps

COX-2 expression was significantly higher in JPS patients compared to patients with sporadic juvenile polyps (P<0.001) (Table 2). Of the 65 JPS polyps 14 (22%) contained dysplasia, but no dysplasia was found in sporadic juvenile polyps. To investigate a possible confounding effect of dysplasia, we determined whether dysplasia could be linked to high COX-2 expression. Although high COX-2 expression was relatively more common in dysplastic foci than in non-dysplastic polyp tissue, this difference was not significant (P=0.257). No statistically significant difference in JPS versus sporadic polyps was found in the expression of HuR (P=0.292) and C/EBP-β (P=0.234).

JPS patients carrying a *BMPR1A* germline mutation show a near significant increased COX-2 expression compared to JPS patients without germline mutation (P=0.086) whereas JPS patients with a SMAD4 germline defect did not (P=0.391) (Table 3).

Correlation of markers

Thirteen JPS polyps showed high expression of both COX-2 and cytoplasmic HuR. This correlation was statistically significant (P=0.022). No such correlation was seen in sporadic juvenile polyps (P=0.327). There was no correlation between COX-2 high phenotype and C/EBP-β positivity in either JPS polyps (P=0.984), or sporadic polyps (P=0.758).

Table 2. Immunohistochemical results: juvenile polyposis polyps versus sporadic juvenile polyps

	JPS		Sporadic juvenile		JPS versus sporadic
	n	IHC	n	IHC	
COX-2	24	54% high	24	4% high	P<0.001
HuR	23	57% high	24	50% high	P=0.292
C/EBP-β	22	96% pos	23	91% pos	P=0.234

n: Number of patients analyzed

Table 3. COX-2 expression in germline mutation carriers versus non-germline mutation carriers

Germline mutation			SMAD4		BMPR1A	
	COX-2		n	IHC	n	IHC
			7	57% high	6	83% high
No germline mutation	n	11	P=0.391		P=0.086	
	IHC	36% high				

n: Number of patients analyzed

DISCUSSION

COX-2 is up-regulated in consecutive stages of the adenoma-carcinoma sequence in sporadic CRC and FAP.[18-20] Chemoprevention using selective (e.g. celecoxib) and non-selective (e.g. sulindac) COX-2 inhibitors reduces the number and size of colorectal adenomas in these patients.[30, 31] Patients with juvenile polyposis syndrome have a markedly elevated relative and absolute risk for colorectal cancer.[5] In contrast, sporadic juvenile polyps are not considered to be precursors of colorectal malignancy.[1]

We examined and compared immunostaining of COX-2 and two additional molecular markers involved in the regulation of COX-2 expression, C/EBP-β and HuR, in 24 JPS patients and 26 patients with sporadic juvenile polyps. We found a significantly higher COX-2 expression in JPS patients compared to those with sporadic juvenile polyps. Interestingly, although not significant, *BMPR1A* germline mutation carriers showed an increase in COX-2 expression compared to JPS patients without a detected germline mutation. These findings are in line with Kurland et al. who recently described high COX-2 expression in one patient carrying a *BMPR1A* mutation.[32] JPS patients with a *SMAD4* germline mutation on the other hand did not have increased COX-2 expression, even though *SMAD4* germline mutation carriers have been described as possessing a more aggressive intestinal phenotype.[15] The number of patients in our study group in which a germline defect was found was limited, therefore these results need be interpreted with caution.

A subset of JPS patients had polyps with dysplastic foci but patients with sporadic juvenile polyps did not. Recently, Brasowski et al.[33] demonstrated progressively increasing COX-2 expression with increasing degree of dysplasia in JPS. Although a similar trend was seen in our JPS patients we did not find a statistical difference in COX-2 expression between

dysplastic foci and non-dysplastic polyp tissue. However, to rule out dysplasia as a potential confounding factor we calculated the difference in COX-2 expression in JPS versus sporadic juvenile polyps using polyp scores rather than the overall patient scores and stratified the results by dysplasia. In doing so, we excluded polyps containing dysplastic foci from the analysis, i.e. non-dysplastic JPS polyps versus sporadic juvenile polyps. We found that COX-2 remained significantly higher in JPS compared to sporadic juvenile polyps (data not shown).

With other studies showing intestinal polyp regression through COX-2 inhibition, our results may have clinical implications for JPS patients. Future in vivo testing should be performed to determine the effect of COX-2 inhibition on gastrointestinal polyp formation in JPS animal models.[34-37] Although COX-2 inhibition has proven effective in colorectal adenoma prevention, use of COX-2 inhibitors increases the risk of cardiovascular events and may thus not be suitable for routine prevention purposes.[38, 39] However, the patients in these studies were above middle age and the findings may therefore not be applicable to children and youths suffering from juvenile polyposis.

HuR is an mRNA-binding protein capable of inhibiting rapid mRNA degradation by selectively binding AU-rich-elements (AREs) in the 3' untranslated regions of mRNAs.[40] COX-2 mRNA contains HuR-binding AREs and cytoplasmic expression of HuR is associated with high COX-2 expression and poor prognosis in several human malignancies, including colorectal cancer.[29, 41, 42] Our data showed a correlation between high COX-2 expression and high cytoplasmic HuR expression in JPS but not in sporadic juvenile polyps. However, no difference was found in cytoplasmic HuR expression in JPS versus sporadic juvenile polyps. Therefore, the difference found in correlation between COX-2 and HuR expression in JPS and sporadic juvenile polyps may be explained mainly by the difference in COX-2 expression in both groups. Also, correlation between COX-2 and HuR was found in *SMAD4* but not in *BMPR1A* mutation carriers, whereas increased COX-2 expression was more common in *BMPR1A* mutation carriers. HuR expression was similar in patients with a *SMAD4* or *BMPR1A* germline mutation. Based on these results it remains unclear whether HuR is involved in up-regulation of COX-2 in JPS. It is feasible that regulation of COX-2 expression is governed by different mechanisms in *SMAD4* versus *BMPR1A* mutation carriers.

C/EBP-β is a transcription factor regulating proliferation and differentiation,[23] capable of inducing COX-2 expression and present in normal colorectal epithelial cells within the proliferative zone.[25] Generally, an increase in proliferative activity is seen in JPS compared to sporadic juvenile polyps. We found a C/EBP-β positive phenotype in more than 90% of both JPS and sporadic juvenile polyps. No correlation between C/EBP-β and COX-2 expression was observed.

In summary, evaluation of COX-2 status, and COX-2 regulating molecules HuR and C/EBP-β, showed a significantly higher COX-2 expression in JPS patients compared to patients with sporadic juvenile polyps. Also, our results suggest JPS patients carrying a *BMPR1A* germline defect may have higher COX-2 expression than those in which no germline defect was found. In this light, investigation of the effect of COX-2 inhibitors on polyp size and disease progression in JPS patients may be worthwhile. Additional research on the mechanisms of COX-2 regulation in juvenile polyps may be of interest.

ACKNOWLEDGMENTS

We are indebted to Mr. Folkert H. Morsink for technical support.

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SMAD4 IMMUNOHISTOCHEMISTRY REFLECTS GENETIC STATUS IN JUVENILE POLYPOSIS

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ABSTRACT

Background: Juvenile polyposis syndrome (JPS) can be caused by a germline defect in the *SMAD4* tumor suppressor gene. Biallelic inactivation of *SMAD4* occurs in advanced stages of pancreatic cancer and colorectal cancer, and is accurately reflected by SMAD4 immunohistochemistry (IHC) in tumor cells. In JPS the role of SMAD4 in both polyp formation and progression to dysplasia is poorly understood. We investigated SMAD4 protein expression by immunohistochemistry and *SMAD4* genetic status in juvenile polyps with a *SMAD4* germline defect.

Methods: Twenty juvenile polyps with a *SMAD4* germline defect were assessed for SMAD4 protein expression by IHC, and compared to 38 controls. Loss of heterozygosity (LOH) and sequence analysis was performed on laser microdissected polyp tissue. H&E slides were reviewed and graded for dysplasia and compared to SMAD4 protein expression.

Results: Nine of 20 polyps with a *SMAD4* germline defect had foci of aberrant epithelial SMAD4 protein expression. Of these 9 polyps, five showed loss of the remaining wild-type allele of *SMAD4* (LOH), two were non-informative, and two polyps with retention of heterozygosity revealed a somatic stop codon mutation upon sequencing, likely to result in a truncated protein. Low grade dysplasia was found in 9 of 20 polyps with a *SMAD4* germline defect, but no correlation was noted between aberrant SMAD4 expression and dysplasia.

Conclusion: Protein expression of SMAD4 assessed by immunohistochemistry accurately reflects *SMAD4* genetic status in JPS. Loss of SMAD4 protein expression occurs in the epithelium during advanced stages of polyp growth but does not correlate with dysplasia in juvenile polyps.

INTRODUCTION

Juvenile polyposis syndrome is an autosomal dominant disorder characterized by the presence of distinct juvenile polyps in the gastrointestinal tract and an increased colorectal cancer risk.[1-3] On histology, juvenile polyps are characterized by a prominent stromal compartment containing distorted and cystically dilated crypts often lined by reactive epithelium.[4] A germline mutation in the *SMAD4* or *BMPR1A* genes is found in 50% of patients.[5, 6] Both genes are involved in the Transforming Growth Factor–Beta/Bone Morphogenic Protein (TGF-ß/BMP) signaling pathway, regulating cell proliferation and differentiation.[7] SMAD4 is a cytoplasmic co-mediator which forms heteromeric complexes with various pathway restricted P-SMADs. These complexes are translocated to the nucleus where they regulate DNA transcription.[8, 9] Somatic inactivation of the *SMAD4* tumor suppressor gene occurs in up to 55% of pancreatic cancers, and in other types of cancer including colorectal cancer.[10-12] This occurs either through intragenic mutation with loss of the second allele (loss of heterozygosity, LOH) or deletion of both alleles (homozygous deletion).

In JPS the mechanism leading to polyp formation and the role of *SMAD4* or *BMPR1A* is not yet fully understood. One hypothesis is that juvenile polyps develop through a "landscaper" defect in which the defective cell population lies in the stromal compartment. Neoplasia of the epithelial cells may take place as a result of an abnormal microenvironment.[13, 14] Others suggest that inactivation of the second allele in the epithelial cell compartment is likely to initiate polyp formation.[15-17] Different mechanisms

of polyp formation may exist for polyps in individuals with either a *SMAD4* or *BMPR1A* germline mutation.[14]
In pancreatic cancer, immunohistochemistry mirrors the molecular status of *SMAD4*.[18] As such, it may also provide a valuable tool in the diagnosis of JPS. In addition, immunohistochemistry of SMAD4 could provide lead to the role of this gene in juvenile polyp development and disease progression. However, it is unclear whether immunohistochemistry accurately reflects *SMAD4* molecular status in JPS. Therefore, we investigated SMAD4 protein expression by immunohistochemistry and examined *SMAD4* genetic status in juvenile polyps.

METHODS

Patients and tissue

Archival material from patients with one or more juvenile polyps was collected from The Johns Hopkins Polyposis Registry and clinic (Baltimore, MD, USA) and two academic hospitals in the Netherlands (Academic Medical Center, Amsterdam, and University Medical Center, Utrecht). The study was carried out according to the guidelines of the ethical committee of these institutions and with their approval. Clinical and family history data were examined and polyps were carefully reviewed by an experienced GI pathologist (GJAO) to confirm the diagnosis of JPS or sporadic juvenile polyps. All JPS patients previously underwent genetic analysis through direct sequencing and MLPA analysis.[6]
Forty-one patients were included in this study, including 8 patients with a *SMAD4* germline defect, 6 patients with a *BMPR1A* germline defect and 27 patients with sporadic juvenile polyps. Polyp tissue was formalin-fixed and paraffinized to standard procedures

Immunohistochemistry

Immunohistochemistry was performed using a monoclonal antibody against SMAD4 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, cat. no. sc-7966, 1:400). Briefly, 4 µm sections were deparaffinized, blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed in Tris/EDTA buffer (10 mM/1 mM; pH=9.0) for 10 min at 120°C. Nonspecific binding sites were blocked in PBS with 10% normal goat serum for 10 min, followed by antibody incubation for 1h at room temperature. Antibody binding was visualized using the Powervision+poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA) and PowerDAB (Immunologic, Duiven, The Netherlands, cat. no. BS03-25) as chromogen. Sections were counterstained with hematoxylin.

Scoring of immunohistochemistry

On examination slides were scored as having either normal, reduced or loss of expression of SMAD4. Normal nuclear staining in the epithelial cells lining normal crypts, or inflammatory cells in the mesenchymal stroma on the same section served as an internal control, i.e. normal expression refers to the same expression as seen in these control cells. Loss of expression was defined as absence of nuclear staining. Reduced expression was graded when a weaker expression, but not a complete absence of nuclear staining was noted compared to the control cells (Figure 1). Also, all sections were reviewed for

dysplasia (GJAO and FJWK) using standard H&E stained reference slides. Dysplasia was graded according to the standard criteria.[19]

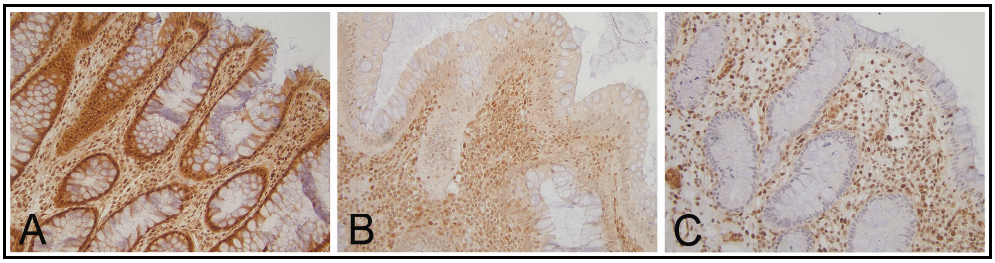

Figure 1. *SMAD4 Immunohistochemical Scoring. IHC slides were scored as normal (A), reduced (B) or loss of SMAD4 expression (C). Nuclear staining in the epithelial cells lining normal crypts or inflammatory cells in the mesenchymal stroma on the same section served as internal control. Magnification 20X.*

Laser microdissection and DNA isolation

Epithelium of interest was isolated by laser capture microdissection (LCM) using the PALM® Laser Microbeam Microdissection System (Microlaser Technologies, Bernried, Germany) on 8 µm sections counterstained with hematoxylin. DNA was obtained using TK buffer (400 µg/ml of proteinase K and 0.5% Tween 20, 50 mmol/l Tris (pH=9), 1 mmol/l NaCl, 2 mmol/L EDTA). After overnight incubation in 50 µl TK buffer at 56°C, tubes were incubated at 95°C for 10 minutes to inactivate the proteinase K.[20]

LOH analysis

Loss of heterozygosity was assessed using fluorescently labeled primers for the following microsatellites: D18S46, D18S474, D18S858 and D18S64.[17, 21, 22] Epithelium with aberrant SMAD4 expression was separated from normal SMAD4 stained epithelium using LCM. After PCR amplification the products were separated using the ABI Prism® 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). One µl of the PCR product was mixed with 23 µl formamide and 0.5 µl GeneScan™ ROX-500 (Invitrogen, Carlsbad, CA, USA) as a size marker.

Samples with two distinctly sized alleles of a particular marker were termed informative. For all informative markers, the allelic imbalance factor was calculated as described by Cawkwell et al.[23] LOH was assumed if the allelic imbalance factor was greater than 1.6 or less than 0.6. Observed losses were confirmed to exclude induced LOH. If retention of heterozygosity was found, microdissected material was sequenced to establish whether a somatic point mutation had taken place.

Mutation analysis

Sequencing of *SMAD4* was performed as described previously. In brief, genomic DNA was PCR amplified and sequenced using the ABI Prism® 3130 genetic analyzer. Primer sequences were described previously.[6]

RESULTS

Immunohistochemistry

A total of 58 polyps, including 20 polyps with a *SMAD4* germline defect, 11 with a *BMPR1A* germline defect and 27 sporadic juvenile polyps, were assessed for SMAD4 protein expression using immunohistochemistry (Figure 1). Results are summarized in Table 1. Of 20 polyps with a *SMAD4* germline defect, 9 showed focal reduction or loss of SMAD4 protein expression in the epithelium. In contrast, none of the 11 polyps carrying a *BMPR1A* germline mutation or any of the 27 sporadic juvenile polyps had aberrant SMAD4 expression (data not shown).

Table 1. Results SMAD4 immunohistochemistry

Patient	Polyp	Exon	Mutation	Effect	SMAD4 IHC normal	reduced	loss
1	1.1	10	c.1411-1435del25	p.G471FfsX25		■	
	1.2	10	c.1411-1435del25	p.G471FfsX25	■		
2*	2.1	8	c.970 T>C	p.C324R	■		
	2.2	8	c.970 T>C	p.C324R	■		
	2.3	8	c.970 T>C	p.C324R		■	
3*	3.1	8	c.970 T>C	p.C324R		■	
	3.2	8	c.970 T>C	p.C324R	■		
4*	4.1	1-11	hemizygous deletion				■
5*	5.1	1-11	hemizygous deletion				■
	5.2	1-11	hemizygous deletion		■		
6	6.1	8	c.989 A>G	p.E330G	■		
	6.2	8	c.989 A>G	p.E330G	■		
	6.3	8	c.989 A>G	p.E330G	■		
7	7.1	9	c.1193 G>A	p.W398X	■		
	7.2	9	c.1193 G>A	p.W398X	■		
	7.3	9	c.1193 G>A	p.W398X			■
8	8.1	8	971delG	p.C324FfsX12			■
	8.2	8	971delG	p.C324FfsX12			■
	8.3	8	971delG	p.C324FfsX12	■		
	8.4	8	971delG	p.C324FfsX12			■

*Patient 2 and 3 and patient 4 and 5 were from the same family

LOH and mutation analysis

To assess the implication of aberrant epithelial SMAD4 protein expression, we investigated whether reduction or loss of SMAD4 expression correlates with the occurrence of a somatic event in *SMAD4*, i.e. LOH or a somatic point mutation, in polyps with a *SMAD4* germline mutation. LOH analysis of the *SMAD4* locus was performed using 4 microsatellite markers. Nine polyps were assessed, all carrying a germline mutation in *SMAD4*, and all of which had aberrant SMAD4 expression. Results are seen in Table 2.

Polyp 2.3, 3.1, 8.1, 8.2a and 8.4a with reduction or loss of nuclear SMAD4 expression showed LOH in two or more markers surrounding *SMAD4*, including at least one of two markers closest to the *SMAD4* locus. Retention of heterozygosity was found in polyp 1.1 and 7.3 even though SMAD4 expression was reduced or lost. Subsequent sequence analysis revealed a somatic stop codon mutation in exon 1 (1.1) and exon 2 (7.3) of *SMAD4*, likely to result in truncation of the protein. In polyp 4.1 and 5.1 with a hemizygous germline deletion of *SMAD4* and immunohistochemical loss of the SMAD4 protein, LOH markers closest to *SMAD4* were non-informative, although more distant markers did show LOH.

Table 2. Results LOH and mutation analysis

Patient	Polyp	SMAD4 IHC	D18s46	SMAD4	D18s474	D18s858	D18s64	Somatic mutation
1	1.1	reduced	ROH		NI	ROH	ROH	c.170 T>A p.L57X
2*	2.3	reduced	NI		LOH	LOH	NI	
3*	3.1	reduced	LOH		LOH	LOH	NI	
4*	4.1	loss	NI		NI	LOH	NI	
5*	5.1	loss	NI		NI	LOH	LOH	
6	6.2	normal	NI		NI	ROH	NI	
7	7.1	normal	ROH		ROH	NI	ROH	
	7.3	loss	ROH		ROH	NI	ROH	c.403 C>T p.R135X
8	8.1	loss	LOH		NI	LOH	LOH	
	8.2a	loss	LOH		NI	LOH	ROH	
	8.2b	normal	ROH		NI	ROH	ROH	
	8.4a	loss	LOH		NI	LOH	LOH	
	8.4b	normal	ROH		NI	ROH	ROH	

ROH: retention of heterozygosity (white); LOH: Loss of heterozygosity (black); NI: not-informative
*Patient 2 and 3 and patient 4 and 5 are from the same family

Dysplasia and genetic status of *SMAD4*

With aberrant epithelial SMAD4 protein expression reflecting the occurrence of a somatic event in the *SMAD4* tumor suppressor gene, we investigated whether this could be linked to neoplastic change in juvenile polyps by reviewing all corresponding H&E slides for dysplasia. In 9 of 20 polyps with a *SMAD4* germline defect, foci of low grade dysplasia were found, two of which contained focal high grade dysplasia. Four polyps were called indefinite and 7 were found negative for dysplasia.

Intriguingly, the presence of dysplasia did not consistently correlate with reduction or loss of nuclear SMAD4 protein expression in juvenile polyps (Table 3). Polyp 6.2, 6.3 and 7.1 were found dysplastic even though nuclear SMAD4 expression of the epithelium was normal (Figure 2a), whereas, polyp 4.1 and 5.1 showed loss of epithelial SMAD4 expression but appeared to be non-dysplastic (Figure 2b). Polyp 1.1 and 3.1 had foci of low grade dysplasia within a larger area of reduced epithelial nuclear SMAD4 expression

(Figure 2c) but in polyp 7.3 areas of low grade dysplasia extended beyond the area showing loss of expression of SMAD4 (Figure 2d).
Remarkably, polyp 8.2 and 8.4 both showed loss of SMAD4 expression in non-dysplastic epithelium (8.2a and 8.4a) but on the same sections contained low grade dysplasia with normal SMAD4 expression (8.2b and 8.4b) (Figure 2e).
To confirm that SMAD4 immunohistochemistry accurately mirrors the molecular status of *SMAD4*, we aimed to exclude somatic inactivation of *SMAD4* in dysplastic juvenile polyp tissue with a normal SMAD4 staining pattern. Therefore, dysplastic epithelium with normal nuclear SMAD4 expression was microdissected and analyzed for LOH using non-dysplastic epithelium with normal nuclear SMAD4 expression as a reference. As is shown in Table 2, polyp 6.2, 7.1, 8.2a and 8.4a all had retention of heterozygosity of the SMAD4 locus. Also no somatic mutations were found.

Table 3. Dysplasia in juvenile polyps

Patient	polyp	Dysplasia	SMAD4 IHC
1	1.1	low grade	reduced
	1.2	negative	normal
2*	2.1	indefinite	normal
	2.2	negative	normal
	2.3	indefinite	reduced
3*	3.1	low grade	reduced
	3.2	indefinite	normal
4*	4.1	negative	loss
5*	5.1	negative	loss
	5.2	negative	normal
6	6.1	negative	normal
	6.2	high grade	normal
	6.3	high grade	normal
7	7.1	low grade	normal
	7.2	low grade	normal
	7.3	low grade	loss
8	8.1	indefinite	loss
	8.2a	negative	loss
	8.2b	low grade	normal
	8.3	negative	normal
	8.4a	negative	loss
	8.4b	low grade	normal

*Patient 2 and 3 and patient 4 and 5 were from the same family

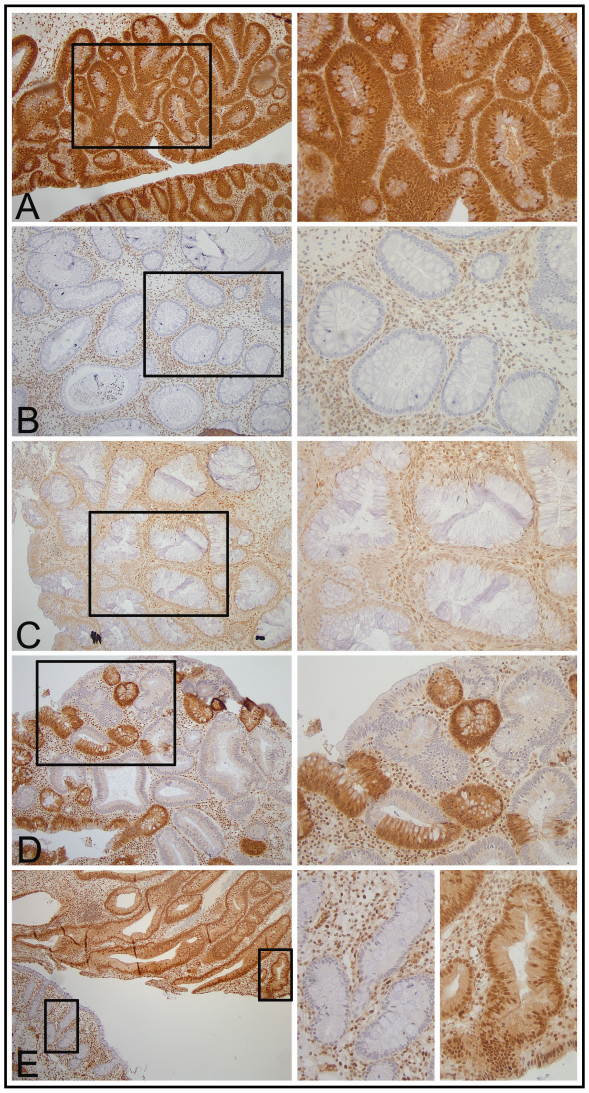

Figure 2. *SMAD4 IHC and Dysplasia. Dysplasia with normal epithelial SMAD4 expression (A); non-dysplastic epithelium with loss of SMAD4 expression (B); dysplasia within area of reduced SMAD4 expression (C); dysplasia extending beyond area of SMAD4 loss; dysplasia with normal SMAD4 expression and non-dysplastic epithelium with loss of SMAD4 expression adjacent on one section (E). Magnification left panel 10X (figure 2E 5X) with 20X zoom (right panel).*

DISCUSSION

SMAD4 is one of two known genes which give rise to juvenile polyposis syndrome when a mutation occurs in the germline. *SMAD4* is a tumor suppressor gene and is frequently inactivated in advanced stages of pancreatic cancer and in other cancers including colorectal malignancies. In pancreatic cancer, loss of immunohistochemical labeling in tumor cells reflects with high accuracy the molecular status of *SMAD4*.[18]

In JPS, the mechanism leading to polyp formation and the role of SMAD4 is not well understood. Some investigators postulate that juvenile polyps may arise through a "landscaper" mechanism in which the defective cells lie in the stromal compartment.[13] However, previous studies show inactivation of the second allele of *SMAD4* to occur

through LOH in the epithelial compartment of juvenile polyps.[15, 17] In fact, it is deemed likely that a second hit of the wild type allele initiates growth of JPS polyps, fitting the classic tumor suppressor model.[24]

In this study we investigated SMAD4 protein expression by immunohistochemistry and examined *SMAD4* genetic status in juvenile polyps. In about half of the polyps with a *SMAD4* germline defect focal reduction or loss of nuclear SMAD4 expression in the epithelium was seen. In contrast, no aberrant SMAD4 expression was seen in polyps from patients with a *BMPR1A* mutation or in any of the sporadic juvenile polyps.

We then assessed whether aberrant epithelial nuclear SMAD4 immunostaining of juvenile polyps with a *SMAD4* germline defect could be linked to somatic inactivation of the *SMAD4* gene. Out of 9 polyps with aberrant epithelial nuclear SMAD4 protein expression five polyps showed LOH in several markers including at least one of two markers flanking *SMAD4* and two polyps had a somatic stop codon mutation, likely resulting in truncation of the SMAD4 protein. Two remaining polyps with loss of epithelial SMAD4 expression had a hemizygous germline deletion of all 11 exons of *SMAD4* as was previously established using MLPA analysis.[6] It has, however, proven difficult to assess LOH status using microsatellite technique because the full extent of the germline deletion was not known, leading to unreliable or non-informative results. Markers located further away from the *SMAD4* gene locus did show LOH in both polyps.

Our results show that the observation of reduction or loss of epithelial SMAD4 protein expression in the polyps of individuals with JPS accurately predicts the presence of a *SMAD4* germline defect, ranging from missense mutations to hemizygous deletions. Moreover, we linked aberrant nuclear SMAD4 protein expression to somatic inactivation through LOH or somatic mutation.

Other studies have reported LOH of *SMAD4* in stromal fibroblasts, and targeted inactivation of *SMAD4* in stromal T-cells leads to polyp formation in mice.[17, 25] Although loss of SMAD4 expression in the stromal cells in juvenile polyps was not observed in this study, we cannot rule out that haploinsufficiency of *SMAD4* in the stroma contributes to juvenile polyp initiation as per the "landscaper" theory. However, we demonstrated that somatic inactivation of *SMAD4* appears to occur not in the stroma but in the epithelium.

Nevertheless, only a subset of polyps with a *SMAD4* germline mutation showed focal loss of epithelial SMAD4 protein expression, meaning that a normal SMAD4 immunostaining does not exclude the possibility of a *SMAD4* germline defect. Since SMAD4 expression reflects somatic inactivation of the *SMAD4* gene, this implies that inactivation of the wild type allele of *SMAD4* in the epithelium is not required for polyp initiation but rather occurs as a late event in polyp growth. This concurs with the findings of Xu et al., describing LOH of *SMAD4* only in larger antral tumors in *Smad4*$^{+/-}$ mice as opposed to smaller tumors, indicating a late event in tumor progression but not an obligate step in tumor initiation.[26]

Remarkably, aberrant epithelial SMAD4 protein expression could not be linked to neoplastic change in juvenile polyps with a *SMAD4* germline defect. Not only did dysplastic foci occur with normal epithelial SMAD4 expression (Figure 2a), we also found cases with abnormal epithelial SMAD4 expression but no dysplastic features (Figure 2b). In contrast to the classical molecular tumor progression models, these findings suggest that neoplastic change in the epithelium of juvenile polyps is not necessarily initiated by inactivation of the second allele of the *SMAD4* tumor suppressor gene. Rather somatic inactivation of *SMAD4* in the epithelium occurs as a late event during neoplastic progression, and as

such, SMAD4 does not appear to act as a "gatekeeper" for neoplasia in JPS. Similarly, biallelic inactivation of the *BRCA2* tumor suppressor gene in patients with a germline mutation in *BRCA2* has been described to occur as a relatively late event in pancreatic tumorigenesis.[27]

On the other hand, in some cases, neoplasia was preceded by somatic inactivation of *SMAD4*, as shown by reduced or lost protein expression on IHC. Consequently, *SMAD4* inactivation seems to occur independently of neoplastic change in juvenile polyps, or perhaps, true neoplasia is microscopically not being recognized as such.

In summary, we found that SMAD4 immunohistochemistry accurately reflects *SMAD4* genetic status in polyps of the juvenile polyposis syndrome and may provide a reliable screening tool in the molecular diagnosis of juvenile polyposis syndrome. We demonstrate that somatic inactivation of *SMAD4* appears to occur in the epithelium and is not likely to initiate polyp formation but is a late event during polyp growth. Also, biallelic epithelial inactivation of *SMAD4* may take place independent of neoplastic change in juvenile polyps, although the exact role and timing of tumorigenesis remains unclear.

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SUMMARY AND DISCUSSION

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SUMMARY AND DISCUSSION

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SUMMARY AND DISCUSSION

Studies on inherited disorders with an increased risk of cancer have been very important for our understanding of cancer pathogenesis in general.[1] FAP and HNPCC are the two classical syndromes that represent cancer pathogenesis of the colorectum. These two syndromes were the inspiration for the "gatekeeper" and "caretaker" theories that are still valuable models to describe colorectal carcinogenesis.[2] A hypothesis for a third pathway, the "landscaper" defect, arose after studies in juvenile polyposis and suggested that disrupted mesenchymal-epithelial communication, as a result of defective BMP signaling, causes tumorigenesis.[3, 4] This thesis describes clinical and molecular studies on familial adenomatous polyposis and juvenile polyposis.

Part I

The focus of the first part of this thesis is on clinical aspects of FAP, in particular management and chemoprevention of duodenal polyposis. FAP is the classical polyposis syndrome. It is caused by germline mutation of the *APC* gene and serves as the model for pathogenesis of most sporadic colorectal cancers: the adenoma-carcinoma sequence.[2] FAP patients are mainly troubled by the development of colorectal adenomatous polyps and the primary cause of death of these patients is colorectal cancer. However, with improved management of colorectal disease and increased life-expectancy of FAP patients, duodenal polyps and adenocarcinoma have emerged as major health problems in these patients. Duodenal adenocarcinoma is now the second leading cause of death in FAP.[5] **Chapter 4** discusses the clinicopathological features, management and prevention of duodenal neoplasia in FAP. Frequent endoscopic surveillance and targeted treatment of duodenal polyposis are the main treatment options for duodenal polyposis, but these modalities can not guarantee a polyp-free duodenum. Surgical intervention, such as duodenotomy or duodenectomy, may be necessary in severe duodenal disease. The Spigelman classification of severity of polyposis should be used to guide continued surveillance after base-line examination and treatment of duodenal polyps. In addition, the value of chemoprevention of duodenal polyposis using non-steroidal anti-inflammatory drugs (NSAIDs) was investigated. Colorectal adenomas in FAP regress after treatment with these agents,[6] but their value for duodenal polyposis was unclear. Literature review showed that results of chemoprevention studies of duodenal adenomas are disappointing.

The striking difference in effect of NSAID chemoprevention on colorectal versus duodenal polyps led us to compare expression of the main target of these drugs, cyclooxygenase-2 (COX-2), between colorectal and duodenal tissues. Levels of COX-2 appeared to be significantly higher in the duodenum than in the colorectum of FAP patients. This result may explain the poorer response of duodenal adenomas to COX-2 inhibition and raise the possibility that higher dosages of COX-2 inhibitors may be needed for treatment of duodenal adenomas (**Chapter 5**). In **chapter 6**, expression of the mRNA stability protein HuR is investigated in the colorectum of patients with FAP. Levels of HuR increased in consecutive stages of the adenoma-carcinoma sequence and a correlation was found with COX-2 expression. This suggests that HuR is associated with progression to malignancy by stabilizing COX-2 mRNA and, thereby, increasing COX-2 levels in the colon.

Part II

The second part of this thesis describes clinical and molecular studies in juvenile polyposis syndrome. JPS is a very rare disorder and, compared to FAP, less well characterized with regard to clinical manifestations and molecular pathogenesis. Despite its rarity, JPS is an interesting model to study because of the "landscaper" mechanism that may underlie tumorigenesis in this syndrome.[3, 4] JPS is characterized by the presence of multiple histological distinctive hamartomatous polyps and it is associated with an increased risk of cancer, notably colorectal, gastric, small bowel and pancreatic cancer. However, evidence for this comes from a limited number of case series and collections of literature case reports and no formal risk assessment of cancer in JPS patients existed.[7-13] Therefore we performed person-year analysis to define the magnitude of risk for gastrointestinal cancer in these patients (**Chapter 7**). A relative risk of colorectal cancer of 34 and a cumulative life-time risk of 38.7% were found in JPS patients. No extra-colonic malignancies were found in our cohort and further studies have to be performed to define risk of these cancers in JPS. In addition, genotype-phenotype studies are needed to clarify differences between JPS patients with *SMAD4* and *BMPR1A* mutations, in particular with regard colorectal and gastric cancer risk. However, for appropriate risk assessment and genotype-phenotype studies, numbers of JPS patients should be increased, necessarily by a combined effort from multiple institutions.

JPS is caused by germline mutation of the *SMAD4* or *BMPR1A* gene.[14, 15] In addition, *ENG* has been proposed as a JPS susceptibility gene, but this has not been confirmed.[16, 17] Germline mutation of *SMAD4* or *BMPR1A* is found in only 30-40% of JPS patients, suggesting that alternative ways of inactivation or additional JPS susceptibility genes may exist.[18-21] The 30-40% mutation detection rate is based on techniques that only detect point mutations or small deletions in *SMAD4* or *BMPR1A*, but not larger deletions, such as deletion of whole a exon or an entire gene. Therefore, the role of large germline deletions of one or more exons of *SMAD4*, *BMPR1A, ENG* or *PTEN* as the cause of JPS was investigated by multiplex ligation-dependent probe amplification (MLPA). In a comprehensive genetic analysis of 27 unrelated JPS patients we identified a germline defect in *SMAD4* or *BMPR1A* in almost 50% of the patients by combining both direct sequencing and MLPA. Nine mutations (33.3%) were detected by direct sequencing, whereas MLPA identified four additional patients (14.8%) with a germline hemizygous large genomic deletion. The role for *ENG* in JPS remains unclear, as we did not find any defects in this gene (**Chapter 8**). Because 50% of JPS patients remain without a known germline defect, other genes predisposing to JPS may exist. In **chapter 9**, the potential role of the *TGFBRII* gene in JPS was investigated by sequence analysis and MLPA in JPS patients without germline mutation of *SMAD4*, *BMPR1A*, *ENG* or *PTEN. TGFBRII* is involved in TGF-β signaling and germline mutation of this gene has been described in a patient with hereditary colorectal cancer.[22] We did not find any pathogenic germline mutations or deletions in the *TGFBRII* gene in our cohort and therefore conclude that *TGFBRII* is unlikely to be a JPS susceptibility gene. Still, about 50% of JPS patients remain without a molecular diagnosis. This number may be increased up to about 60% using stringent diagnostic criteria,[23] but it is likely that (an) other gene(s) exists that predispose to JPS, making further studies to identify these genes necessary.

Prevention of polyp and neoplasia development in juvenile polyposis using chemoprevention, similar as described in FAP (first part of this thesis), may be a useful

asset in the treatment of these patients. To investigate the potential value of chemoprevention by (selective) COX-2 inhibition we measured expression of COX-2 in juvenile polyps (**Chapter 10**). Increased levels of COX-2 were found in juvenile polyps from JPS patients compared to sporadic juvenile polyps. This suggests that chemoprevention using (selective) COX-2 inhibition could be beneficial in JPS, but its value requires further investigation. Mouse models of JPS would be appropriate candidates for an initial exploration and may be followed by randomized clinical trials in patients with juvenile polyposis. Anecdotally, sulindac was effective in suppressing polyp development after surgery in two JPS patients.[24]

In **chapter 11**, molecular aspects of juvenile polyps from patients with a germline defect of *SMAD4* were studied. Using immunohistochemistry, we showed that focal reduction or loss of SMAD4 expression was present in the epithelium of about half of the juvenile polyps from *SMAD4* mutation carriers. Stromal SMAD4 expression was normal. Complete loss of immunostaining was seen in cases with a germline stop codon mutation, whereas reduced SMAD4 expression was seen in cases with a missense germline mutation. No loss of SMAD4 expression was seen in sporadic juvenile polyps and juvenile polyps from patients with a germline *BMPR1A* mutation. In cases with aberrant SMAD4 protein expression we found either LOH or somatic mutation of the wild type *SMAD4* allele. Surprisingly, loss of SMAD4 immunostaining did not correlate with dysplasia in polyps, i.e. non-dysplastic areas with loss of SMAD4 expression, as well as dysplastic areas with normal SMAD4 expression were observed.

The finding of loss of SMAD4 in the epithelium is in line with previous studies and does not support a "landscaper" mechanism for SMAD4. However, our observation that aberrant expression was often focally and not observed in all polyps from *SMAD4* germline mutation carriers, contrasts with previous studies.[25-27] The late occurrence of LOH of *SMAD4* suggests that haploinsufficiency of *SMAD4* in the epithelium is sufficient for polyp initiation, compatible with previous findings in *Smad4* deficient mice.[28, 29] In addition, we did not find a consistent correlation between loss of epithelial SMAD4 and the presence of dysplasia, suggesting that loss of the wild type allele is not obligatory for dysplastic change in juvenile polyps. This observation does not fit with a typical tumor suppressor/"gatekeeper" role for *SMAD4*, as was previously suggested.[25] Accordingly, biallelic inactivation of *BRCA2* has been described as a relatively late event in pancreatic carcinogenesis in carriers of a germline *BRCA2* mutation.[30]

Although we and others only found epithelial loss of SMAD4,[25-28] it might be possible that stromal haploinsufficiency or loss of *SMAD4* does have a role in polyp initiation and that epithelial loss only occurs at a later stage in polyp progression. A recent study in favor of this "landscaper" mechanism for SMAD4 in juvenile polyposis, showed that selective loss of Smad4-dependent signaling in T-cells leads to spontaneous epithelial gastrointestinal cancers in mice, whereas epithelial specific deletion of *Smad4* did not.[31] In addition, in a report advocating the "gatekeeper" mechanism, LOH of *SMAD4* was not only found in the epithelium but also in stromal fibroblasts and pericryptal myofibroblasts, although the authors suggested that the epithelial and stromal cells could have a common origin.[25] These observations suggest that stromal alterations in SMAD4 signaling might have a role in JPS pathogenesis and possibly select for LOH within the epithelium.[31] However, the fact that we and others did not observe loss of protein expression in the stroma of juvenile polyps is conflicting.[26,27]

Similar issues about "gatekeeper" or "landscaper" defects hold for mechanisms underlying polyp development caused by *BMPR1A* germline mutation. Originally, the "landscaper" model was proposed in response to the finding of LOH at chromosome 10q22 (*BMPR1A* locus) in stromal lymphocytes and macrophages of juvenile polyps. However, these results have not been confirmed and have been questioned.[15, 25] Moreover, a mouse model that phenocopies juvenile polyposis, showed that BMP-4 localizes exclusively to the mesenchymal compartment of the intestine in mice and disruption of BMP signaling resulted in development of polyps resembling juvenile polyps, suggesting that disrupted mesenchymal-epithelial communication, by defective BMP signaling, causes the "landscaper" defect.[4] Additional mouse models of JPS have been established by tissue wide conditional inactivation of *BMPR1A*[32] and conditional inactivation of *BMPRII* in intestinal stroma.[33]

In conclusion, mechanisms of polyp formation and carcinogenesis in juvenile polyps remain unresolved and may be different in a *BMPR1A* or *SMAD4* background. These different pathogenic mechanism may explain differences between *SMAD4* and *BMPR1A* mutation carriers with regard to upper GI tract polyps and cancer and the more aggressive phenotype in patients with *SMAD4* germline mutation.[20, 23] In this regard, we observe distinctive histopathological characteristics of polyps from patients with a germline *SMAD4* mutation compared to *BMPR1A* mutation carriers. Polyps from *BMPR1A* mutation carriers have a more classical juvenile polyp appearance with a marked stromal component with inflammation, crypt dilatation and surface erosion, whereas *SMAD4* mutation carriers show a more proliferative epithelium, with less stromal expansion and little surface erosion (preliminary results).

Further studies of polyps from patients with *BMPR1A* and *SMAD4* germline defects are needed to shed light on the pathogenic mechanisms underlying tumorigenesis in JPS. For instance, LOH of *BMPR1A* should be further investigated. Reliable immunohistochemical staining for BMPR1A would be helpful to confirm LOH data and to localize expression or loss of BMPR1A into the stromal or epithelial compartment (or both). In addition, status of BMP signaling should be studied in *SMAD4* and *BMPR1A* mutation carriers by detection and quantification of downstream targets, such as Smad1-5-8. Also, further genotype-phenotype studies are needed to confirm differences in histopathological and clinical expressions of juvenile polyposis in patients with *SMAD4* or *BMPR1A* germline mutation.

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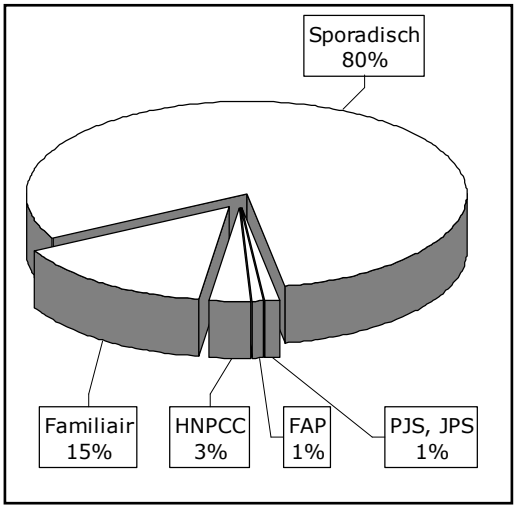
SUMMARY IN DUTCH

NEDERLANDSE SAMENVATTING

Dikkedarmkanker wordt in Nederland jaarlijks bij ongeveer 10.000 mensen gediagnosticeerd en is hiermee de derde meest voorkomende vorm van kanker in Nederland. Ongeveer de helft van de patiënten met darmkanker overlijdt hieraan.

Darmkanker kan worden ingedeeld in sporadische (niet-erfelijke) of familiaire/erfelijke darmkanker. Bij het overgrote deel van de patiënten (~80%) met darmkanker zijn er geen aanwijzingen voor een onderliggende erfelijke aanleg, dit wordt ook wel sporadische darmkanker genoemd. Bij ongeveer 20% van de patiënten zijn er aanwijzingen dat erfelijkheid een rol speelt bij het krijgen van darmkanker. In de meerderheid van deze gevallen (~15%) is er sprake van een zogenaamde familiaire aanleg, maar is er geen onderliggende genetische afwijking bekend. Slechts bij circa 5% van de patiënten met darmkanker kan een erfelijke afwijking als oorzaak van de verhoogde kans op kanker worden aangetoond (Figuur 1). Patiënten hebben in dit geval een erfelijke verandering (mutatie) in een specifiek gen dat een specifiek syndroom veroorzaakt dat gepaard gaat met een verhoogde kans op darmkanker. Er kan dan sprake zijn van hereditair non-polyposis colorectaal carcinoom (HNPCC), familiare adenomateuze polyposis (FAP), of een van de zeldzamere syndromen zoals juveniele polyposis (JPS) of Peutz-Jeghers (PJS) (Tabel 1).



Figuur 1. *Oorzaken van dikkedarmkanker. Sporadisch: niet-erfelijk; HNPCC: hereditair non-polyposis colorectaal carcinoom; FAP: familiaire adenomateuze polyposis; PJS: Peutz-Jeghers syndroom; JPS: juveniele polyposis syndroom.*

Tabel 1. Syndromen met een verhoogde kans op darmkanker en de betrokken genen

Syndroom	Gen
Hereditair non-polyposis colorectaal carcinoom (HNPCC)	Mismatch repair (MMR) genen
Familiaire adenomateuze polyposis (FAP)	*APC*
Juveniele polyposis syndroom (JPS)	*SMAD4*, *BMPR1A*
Peutz-Jeghers syndroom (PJS)	*LKB1*

In **hoofdstuk 3** worden de klinische en genetische aspecten van familiaire adenomateuze polyposis, juveniele polyposis en Peutz-Jeghers syndroom uitvoerig besproken. Elk van deze syndromen wordt gekenmerkt door de aanwezigheid van tientallen tot honderden goedaardige gezwellen (poliepen) in de darm (polyposis) en elk syndroom gaat gepaard met een specifiek type poliep (Figuur 1, pagina 21).
Het bestuderen van syndromen die gepaard gaan met een verhoogde kans op kanker heeft veel inzicht opgeleverd over de ontstaanswijze van kanker in het algemeen. De syndromen HNPCC en FAP staan model voor het ontstaan van de overgrote meerderheid van alle darmtumoren.
FAP wordt veroorzaakt door een erfelijke mutatie in het *APC* gen. Niet-erfelijke mutatie (ofwel somatische mutatie) van dit gen treedt op in circa 80-85% van de niet-erfelijke gevallen van darmkanker. Mutatie van het *APC* gen is meestal de eerste stap die tot het ontstaan van darmkanker leidt. Vanwege deze cruciale functie wordt dit gen ook wel getypeerd als een "gatekeeper" of "poortwachter" gen. Als gevolg van de erfelijke mutatie van het *APC* gen ontwikkelen patiënten met FAP een zeer groot aantal poliepen in de dikke darm. Deze zogenaamde adenomateuze poliepen zijn stuk voor stuk voorlopers van darmkanker en het is vrijwel onvermijdelijk dat een van deze voorlopers zich daadwerkelijk tot darmkanker zal ontwikkelen. Door de aanwezigheid van deze vele adenomateuze poliepen hebben patiënten met FAP praktisch 100% kans om darmkanker te krijgen, vaak al op zeer jonge leeftijd. Verwijderen van de gehele dikke darm is de enige behandeling die darmkanker bij deze patiënten kan voorkomen. Naast poliepen in de dikke darm, krijgen veel FAP patiënten echter ook last van poliepen in de twaalfvingerige darm (het duodenum). Behandeling van de poliepen in het duodenum wordt uitvoerig besproken in **hoofdstuk 4**.
Bij HNPCC en ongeveer 15% van de niet-erfelijke vormen van darmkanker ligt disfunctioneren van een van de mismatch repair (MMR) eiwitten ten grondslag aan het ontstaan van darmkanker. MMR eiwitten spelen een belangrijke rol bij het herstellen van fouten die kunnen optreden tijdens de vermeerdering van het DNA. Omdat de MMR eiwitten met het herstellen van DNA schade een verzorgende functie hebben voor het DNA, worden deze genen ook wel "caretaker" genen genoemd. Een niet goed functionerend MMR eiwit leidt tot een sterk verhoogd aantal mutaties in genen die betrokken zijn bij het ontstaan van kanker, met als gevolg een sterk verhoogde kans op kanker. Als gevolg van de erfelijke mutatie in een van de MMR genen is bij patiënten met HNPCC niet het ontstaan van poliepen, maar de ontwikkeling van poliepen tot kanker versneld. HNPCC patiënten hebben daarom, in tegenstelling tot FAP patiënten, geen groot aantal poliepen in de darm, maar wel een sterk verhoogde kans op darmkanker.
Een derde route die tot het ontstaan van darmkanker zou kunnen leiden is gebaseerd op bevindingen in juveniele polyposis syndroom. Net zoals patiënten met FAP, hebben patiënten met JPS vele poliepen in de dikke darm. De poliepen van JPS patiënten worden juveniele poliepen genoemd en zien er onder de microscoop anders uit dan de poliepen van FAP patiënten. JPS wordt veroorzaakt door een erfelijke mutatie in het *SMAD4* of het *BMPR1A* gen. De hypothese over het ontstaan van darmkanker in dit syndroom kwam tot stand nadat genetische veranderingen in juveniele poliepen werden gevonden in het onderliggende steunweefsel (het stroma) en niet in de cellen die de darm aan de binnenzijde bekleden (het epitheel), zoals in de meeste gevallen van darmkanker. Er werd geopperd dat darmkanker in juveniele polyposis syndroom zou kunnen ontstaan als gevolg

van verstoorde communicatie tussen de cellen in het stroma en in het epitheel. Dit effect van het stroma op het epitheel wordt het "landscaper" mechanisme genoemd. De "landscaper" theorie staat echter ter discussie aangezien andere studies hebben aangetoond dat de genetische veranderingen vooral in het epitheel optreden en niet in het stroma, wat meer bij een "gatekeeper" mechanisme past. Daarnaast is de manier waarop poliepen en darmkanker ontstaan mogelijk anders bij juveniele polyposis patiënten met een erfelijke mutatie van het *SMAD4* gen dan bij patiënten met een mutatie in het *BMPR1A* gen.

Deel I

Het eerste deel van dit proefschrift gaat over FAP. Als gevolg van de verbeterde behandeling van dikke darm poliepen zijn poliepen in het duodenum (de twaalfvingerige darm) en duodenum kanker een belangrijk gezondheidsprobleem geworden voor patiënten met FAP. In **hoofdstuk 4** worden klinische en genetische aspecten en behandeling en preventie van duodenum poliepen en kanker besproken. Regelmatige endoscopische controle en doelgerichte behandeling van duodenum poliepen zijn de belangrijkste behandelopties. Deze behandelwijze kan echter geen poliep-vrij duodenum garanderen. Chirurgische behandeling kan noodzakelijk zijn in ernstige gevallen van duodenum polyposis. Als leidraad bij de behandeling van duodenum polyposis kan de Spigelman classificatie voor ernst van polyposis gebruikt worden (Tabel 2, pagina 52 en Tabel 3, pagina 53). Daarnaast wordt in dit hoofdstuk het nut van behandeling van duodenum poliepen met behulp van aspirine-achtige middelen (chemopreventie) besproken. Aspirine-achtige middelen (ofwel niet-steroidale anti-inflammatoire drugs; NSAIDs) remmen de werking van een enzym dat betrokken is bij de ontwikkeling van kanker (het COX-2 enzym). Dikke darm poliepen worden kleiner en/of verdwijnen (regressie) na behandeling met deze middelen, maar het nut van deze middelen voor de behandeling van duodenum poliepen was onduidelijk. Literatuuronderzoek toonde aan dat het effect van chemopreventie met NSAIDs in het duodenum teleurstellend is.
Het verschil in effect van NSAIDs op regressie van poliepen in de dikke darm vergeleken met de dunne darm was voor ons aanleiding om te onderzoeken of er een verschil bestond in de hoeveelheid van het belangrijkste doelwit (COX-2) van deze middelen tussen de dikke en de dunne darm. Het bleek dat COX-2 significant meer voorkomt in de dunne darm vergeleken met de dikke darm van patiënten met FAP. Dit resultaat kan mogelijk het mindere effect van NSAIDs in het duodenum verklaren en suggereert dat wellicht een hogere dosering NSAIDs noodzakelijk is voor chemopreventie van duodenum poliepen (**hoofdstuk 5**). In **hoofdstuk 6** hebben we de expressie van een bepaald eiwit (HuR) onderzocht dat betrokken is bij stabiliseren van mRNA en hiermee de hoeveelheid van andere eiwitten kan reguleren. We hebben aanwijzingen gevonden dat HuR een rol speelt bij het reguleren van het COX-2 eiwit in de dikke darm.

Deel II

Het tweede deel van dit proefschrift gaat over juveniele polyposis. JPS is een zeldzame aandoening waarbij patiënten vele kenmerkende poliepen (juveniele poliepen) in de darm en een verhoogde kans op kanker hebben. Ondanks de zeldzaamheid van JPS, is het een zeer interessant onderzoeksmodel aangezien de poliepen en kanker volgens het zogenaamde "landscaper" mechanisme lijken te ontstaan.

Het kankerrisico bij JPS is nooit formeel vastgesteld. Wij hebben daarom een risicoanalyse (person-year analysis) uitgevoerd (**hoofdstuk 7**). Patiënten met JPS bleken een 34 keer groter risico op darmkanker te hebben dan gemiddeld. De kans dat een JPS patiënt darmkanker krijgt gedurende het leven is 38.7%. In onze studie vonden we geen gevallen van maag-, dunne darm- of alvleesklierkanker, terwijl deze tumoren in de literatuur wel zijn beschreven bij JPS patiënten. Toekomstige studies zullen moeten uitwijzen of JPS patiënten daadwerkelijk een verhoogde kans hebben op deze tumoren.
JPS wordt veroorzaakt door een erfelijke mutatie in het *SMAD4* of het *BMPR1A* gen. Door gebruik te maken van een bepaalde techniek (sequencing) kan bij ongeveer 30-40% van de JPS patiënten een mutatie in deze genen worden aangetoond. Deze techniek kan echter alleen kleine veranderingen in deze genen aantonen. In **hoofdstuk 8** hebben we, door middel van een relatief nieuwe techniek (MLPA), onderzocht of grotere veranderingen (deleties) in *SMAD4* of *BMPR1A* ook ten grondslag kunnen liggen aan JPS. Door sequencing en MLPA te combineren konden we bij bijna 50% van de JPS patiënten een erfelijke afwijking in het *SMAD4* of *BMPR1A* gen aantonen; 33% werd gevonden met behulp van sequencing en 15% met behulp van MLPA. De veranderingen die met behulp van MLPA werden gevonden konden niet worden aangetoond met sequencing. Omdat nog steeds bij 50% van de JPS patiënten geen erfelijke afwijking kan worden gevonden, is het waarschijnlijk dat er nog andere genen zijn die JPS kunnen veroorzaken. In **hoofdstuk 9** hebben we onderzocht of het gen *TGFBRII* wellicht een rol speelt bij het ontstaan van JPS. Hiervoor hebben we bij 19 JPS patiënten, zonder mutatie in *SMAD4* of *BMPR1A*, het *TGFBRII* gen onderzocht door middel van sequencing en MLPA. We vonden geen afwijkingen in dit gen in deze groep JPS patiënten en het is daarom niet waarschijnlijk dat *TGFBRII* en rol speelt bij het ontstaan van JPS. Verdere studies naar andere genen die JPS kunnen veroorzaken zijn noodzakelijk.
Chemopreventie van poliepen en kanker met behulp van aspirine-achtige middelen (NSAIDs) zou bij JPS, net zoals bij FAP, een waardevolle aanvulling kunnen zijn op bestaande therapieën voor JPS. Aspirine-achtige middelen remmen de werking van een enzym dat betrokken is bij de ontwikkeling van kanker (COX-2 enzym). Om het potentiële effect van chemopreventie door middel van COX-2 remming in JPS te bepalen, hebben wij in **hoofdstuk 10** onderzocht of het COX-2 enzym in juveniele poliepen aanwezig is. We vonden een toegenomen hoeveelheid van het COX-2 enzym in poliepen van JPS patiënten vergeleken met niet-erfelijke juveniele poliepen. Dit resultaat laat zien dat JPS patiënten mogelijk baat zouden kunnen hebben bij chemopreventie door middel van COX-2 remming door het gebruik van aspirine-achtige middelen. Toekomstige studies met muismodellen van JPS en met JPS patiënten zullen moeten laten zien of er ook daadwerkelijk een klinisch effect is van chemopreventie door middel van COX-2 inhibitie in JPS.
In **hoofdstuk 11** hebben we moleculaire aspecten van juveniele poliepen van JPS patiënten met een erfelijke mutatie in het *SMAD4* gen onderzocht. Met behulp van een bepaalde techniek (immuunhistochemie, eiwit kleuring) hebben we aangetoond dat bij ongeveer de helft van de poliepen van JPS patiënten met een mutatie in het *SMAD4* gen er minder of geen SMAD4 eiwitten aanwezig zijn in de cellen die de binnenzijde van de dikke darm bekleden (het epitheel). De hoeveelheid SMAD4 was normaal in het onderliggende steunweefsel. Totale afwezigheid van het SMAD4 eiwit werd gezien in poliepen van patiënten met een bepaalde mutatie in het DNA die veroorzaakt dat de productie van het SMAD4 eiwit wordt gestopt (stop-mutatie). Indien een patiënt een mutatie had die

veroorzaakt dat er een niet werkzaam eiwit ontstaat (missense-mutatie) werd er een verminderde hoeveelheid SMAD4 eiwitten gevonden. Poliepen van patiënten met een mutatie in het andere gen dat JPS veroorzaakt (*BMPR1A* gen) en niet-erfelijke juveniele poliepen lieten normale hoeveelheid van SMAD4 zien. De eiwitkleuring kan dus een indicatie geven of het *SMAD4* gen gemuteerd is en wat voor soort mutatie dit is.
Vervolgens hebben we onderzocht wat het onderliggende moleculaire mechanisme voor veranderde aanwezigheid van SMAD4 was. Van ieder gen zijn twee exemplaren (allelen) aanwezig in het DNA, een van de moeder en een van de vader. Patiënten met JPS hebben een erfelijke mutatie in een van de twee allelen. Verlies of verminderde aanwezigheid van het SMAD4 eiwit wijst er op dat ook het andere (normale ofwel wild-type) exemplaar van het *SMAD4* gen gemuteerd is raakt. In alle gevallen met verlies of verminderde aanwezigheid van het SMAD4 eiwit konden we ofwel verlies (deletie, loss of heterozygosity), ofwel niet-erfelijke (somatische) mutatie van het wild-type exemplaar van het *SMAD4* gen aantonen. Theoretisch zou men verwachten dat verlies/mutatie van het wild-type exemplaar van het *SMAD4* gen een van de eerste stappen in de ontwikkeling van kanker in deze poliepen zou zijn. Tot onze verrassing bleek er echter geen relatie te bestaan tussen verlies/mutatie van het wild-type allel van het *SMAD4* gen en het ontstaan van veranderingen in de poliep die uiteindelijk tot kanker kunnen leiden (dysplasie).
De bevinding dat verlies of verminderde aanwezigheid van het SMAD4 eiwit optreedt in het epitheel, komt overeen met eerdere bevindingen en is een argument tegen de "landscaper" theorie, waarbij het idee juist is dat het defect gelegen is in het onderliggende stroma en dat de stromale afwijking leidt tot veranderingen in het epitheel die uiteindelijk kunnen leiden tot kanker van het epitheel. Onze bevinding dat veranderde aanwezigheid van het SMAD4 eiwit vaak slechts plaatselijk in het epitheel van de poliepen aanwezig is, is in strijd met eerdere studies. Het relatief laat optreden van somatisch verlies van het tweede allel (wild-type allel) suggereert dat verlies van één allel in het epitheel voldoende is om juveniele poliepen te doen ontstaan (haploinsufficiëntie), wat in overeenstemming is met bevindingen in *Smad4* mutante muismodellen. Het feit dat we geen relatie vinden tussen vermindering of afwezigheid van SMAD4 eiwit in het epitheel en dysplasie doet tevens vermoeden dat verlies van het wild-type allel niet noodzakelijk is voor het ontstaan van dysplasie in het epitheel. Deze bevinding past echter niet bij een typische tumor-suppressor/"gatekeeper" rol voor SMAD4. Een vergelijkbare bevinding is ook beschreven bij een ander vermeend tumor-suppressor gen (*BRCA2*) waarbij, bij patiënten met een erfelijke *BRCA2* mutatie, inactivatie van het wild-type allel pas laat in het ontstaan van alvleesklierkanker optreedt.
Hoewel wij (en overigens ook anderen) verminderde aanwezigheid van SMAD4 in het epitheel hebben gevonden, zou het toch mogelijk kunnen zijn dat haploinsufficiëntie of verlies van *SMAD4* in het stroma ook een rol speelt bij het ontstaan van juveniele polyposis en dat epitheliaal verlies van het wild-type allel van SMAD4 pas in een later stadium optreedt. Een recente studie ten faveure van een dergelijk "landscaper" mechanisme voor SMAD4, liet zien dat selectief verlies van Smad4 in T-cellen in het stroma van muizen tot spontane epitheliale tumorvorming leidde, terwijl verlies van Smad4 specifiek in het epitheel niet leidde tot tumorvorming. In een andere studie werd, naast verlies van SMAD4 in het epitheel, ook verlies in stromale cellen gevonden. Deze bevindingen laten zien dat veranderingen in de aanwezigheid van SMAD4 in het stroma wel degelijk een rol zouden kunnen spelen bij het ontstaan van JPS. De normale

aanwezigheid van SMAD4 in het stroma die wij en anderen hebben gevonden ondersteunt deze "landscaper" theorie voor SMAD4 echter niet.
Vergelijkbare vragen wat betreft het "landscaper" of "gatekeeper" mechanisme spelen ook rondom het ontstaan van poliepen bij patiënten met een mutatie van het *BMPR1A* gen. Het "landscaper" model is oorspronkelijk ontstaan als gevolg van de bevinding van verlies van het *BMPR1A* gen in cellen in het stroma van juveniele poliepen van JPS patiënten. Deze bevindingen zijn echter nooit bevestigd en er zijn vraagtekens geplaatst bij de betrouwbaarheid van de data. Daarnaast is doormiddel van een muismodel van JPS aangetoond dat verstoorde communicatie tussen epitheel en stroma, via *BMPR1A,* het "landscaper" effect veroorzaakt. Hoewel verdere studies noodzakelijk zijn, lijkt het er dus op dat er bij patiënten met een mutatie in het *BMPR1A* gen sterkere argumenten bestaan die een "landscaper" mechanisme voor het ontstaan van poliepen en kanker ondersteunen dan bij patiënten met een mutatie in het *SMAD4* gen.
De exacte mechanismen waardoor poliepen en kanker in JPS ontstaan blijven onduidelijk. Bovendien zijn de mechanismen mogelijk verschillend bij een mutatie van het *SMAD4* gen vergeleken met en mutatie van het *BMPR1A* gen. Deze verschillende mechanismen zouden mogelijk ook sommige verschillen kunnen verklaren die worden gezien tussen patiënten met een mutatie in het *SMAD4* en het *BMPR1A* gen, zoals de schijnbaar hogere kans op (maag)kanker bij patiënten met een *SMAD4* mutatie. Hierbij is het interessant om vermelden dat wij ook een verschil zien tussen de structuur van de poliepen van patiënten met een *SMAD4* en een *BMPR1A* mutatie.
Verdere studies met poliepen van *SMAD4* en *BMPR1A* mutatiedragers zijn nodig om het inzicht te vergroten in de ontstaansmechanismen van poliepen en kanker in JPS. Onderzoek naar de aan- of afwezigheid van verlies van het wild-type allel van *BMPR1A* en de lokalisatie hier van (in het epitheel of in het stroma) zijn belangrijke volgende stappen in het juveniele polyposis onderzoek.

ABOUT THE AUTHOR AND LIST OF PUBLICATIONS

ABOUT THE AUTHOR

Lodewijk Brosens is born on the 2nd of June 1979 in Amsterdam, the Netherlands. After high school at the St. Ignatius Gymnasium in Amsterdam, Lodewijk started Medical Biology at the Free University in Amsterdam in 1998. One year later he also started Medical School at the University of Amsterdam (AMC).

In 2003, Professor Offerhaus (then AMC) gave Lodewijk the opportunity to visit the Johns Hopkins Hospital in Baltimore, Unites States. Supported by a grant from the Dutch Cancer Society, Lodewijk worked in 2003/2004 for 10 months as a student research fellow at the departments of Pathology and Gastroenterology of the Johns Hopkins Hospital (Supervisors Prof. dr. F.M. Giardiello and Dr. M. Goggins). At Hopkins, the studies on familial adenomatous polyposis and juvenile polyposis described in this thesis were initiated.

Upon his return from the United States, Lodewijk obtained his Masters of Science degree in Medical Biology in August 2004. He finished medical school in December 2006 and continued as per the 1st of January 2007 his studies on polyposis syndromes in the laboratory of Professor Offerhaus at the department of Pathology in the UMC Utrecht. In May 2008, Lodewijk started his residency in Pathology at the department of Pathology of the University Medical Center in Utrecht (Opleider: Prof. dr. J.G. van den Tweel).

Lodewijk received the Johns Hopkins Young Investigator Award 2004 during his stay at Hopkins, the Hippocrates Award 2006 for the research conducted at Johns Hopkins, and the KNAW/Van Walree conference-awards 2005 and 2008 to visit to the Digestive Disease Week to present results of his research.

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"If I lived twenty more years and was able to work, how I should have to modify the Origin, and how much the views on all points will have to be modified! Well it is a beginning, and that is something …."

Charles Darwin to J. D. Hooker, 1869

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