Juvenile polyposis: aspects of molecular genetics and histology on the pathogenesis of a precancerous syndrome
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Juvenile polyposis
Aspects of molecular genetics and histology on the pathogenesis
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Arnout van Hattem

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Juvenile polyposis

Aspects of molecular genetics and histology on the pathogenesis of a precancerous syndrome
Cover: Juvenile polyposis is a disease of childhood. And although juvenile polyps at first glance may appear innocent, a closer look will reveal their underlying malignant potential.

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

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Juvenile polyposis

Aspects of molecular genetics and histology on the pathogenesis of a precancerous syndrome

Academisch proefschrift

Ter verkrijging van de graad doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus prof.dr. D.C. van den Boom ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Agnietenkapel der Universiteit op vrijdag 17 april 2009 te 14.00 uur

door

Willem Arnout van Hattem

Geboren te Amersfoort
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General introduction
and outline of the thesis

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**General introduction and outline of the thesis**

W. Arnout van Hattem

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**Colorectal cancer** Colorectal cancer (CRC) is the second leading cause of cancer-related death in Western society, with incidence rates increasing annually in both the United States and the European Union. The estimated 5-year survival averages around 53% depending on the extent of the disease.\(^1\), \(^2\)

The lifetime risk of developing colorectal cancer in the absence of personal or family history for the disease is 5-6%. These sporadic cases constitute the vast majority (80%) of all individuals diagnosed with colorectal cancer. A positive family history, i.e. an affected first degree and/or second degree relative, may increase the lifetime risk up to 20% and accounts for around 15% of all colorectal cancer diagnoses. Despite evident familial predisposition, these patients have no verifiable inherited genetic defect.

Approximately 5% of all newly diagnosed colorectal cancer cases are attributable to hereditary colorectal cancer susceptibility syndromes, comprising familial adenomatous polyposis (FAP) (1%), hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome (3%) and a variety of other conditions (1%) including the hamartomatous polyposis syndromes such as Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS). These hereditary syndromes may have a lifetime risk of developing colorectal cancer reaching up to 80-100% depending on the underlying genetic defect.\(^3\)

**Adenoma-carcinoma sequence** Colorectal cancer develops along a series of histopathological steps. Normal intestinal epithelium may undergo dysplastic change, forming small adenomas, and proceed to larger adenomas containing high grade dysplasia. Eventually high grade dysplasia may progress into invasive carcinoma.\(^4\)

Investigation of the inherited cancer susceptibility syndromes has proven invaluable to our understanding of colorectal tumorigenesis. It has provided evidence of accumulating genetic changes underlying the stepwise colorectal cancer development. This has ultimately led to what is currently known as the “adenoma-carcinoma sequence”. The notion of a sequential colorectal adenoma-carcinoma progression model is fundamental to the contemporary approach of colorectal cancer research.\(^5\)

**Familial adenomatous polyposis** Different mechanisms driving the adenoma-carcinoma sequence have been proposed. Initiation may occur in a direct manner through the inactivation of tumor suppressor genes. \(APC\) is such a gene and is a key component of the Wnt signalling pathway. Inactivation of \(APC\) leads to active Wnt signalling and Wnt target gene transcription. Wnt target genes directly modulate cell growth by repressing apoptosis and inducing proliferation. FAP patients carry a germline mutation in the \(APC\) gene. Subsequent somatic inactivation of the wild-type allele initiates neoplastic growth. As a result FAP patients develop countless adenomas. These tumors
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progress to invasive cancer at a normal pace as dictated by the adenoma-carcinoma sequence. It is due to the sheer number of adenomas that FAP patients are at an increased risk of colorectal cancer. Biallelic inactivation of APC is also the rate limiting step in about 85% of sporadic colorectal cancers. APC thus seems to act as “gatekeeper” for neoplastic growth.(6)

Hereditary non-polyposis colorectal cancer Patients with HNPCC on the other hand, initiate adenomas at a rate similar to that of the general population, and yet have a markedly increased risk of these tumors progressing to invasive cancer. HNPCC is caused by an inherited defect of one of the cancer susceptibility genes involved in DNA repair known as the mismatch repair (MMR) genes, including MLH1 and MSH2. Inactivation of these genes leads to a greatly increased mutation rate resulting in microsatellite instability (MSI). The massive accumulation of mutations in the growth regulatory genes causes an accelerated progression to malignancy. Their role in DNA repair has prompted the MMR genes to become known as “caretaker” genes.(6)

Juvenile polyposis A third CRC pathway is also one of indirectly acting cancer susceptibility genes and is largely based on studies of juvenile polyposis syndrome.

Historically, the first documentation of a juvenile polyp by Diamond in 1939, described two polyps of the rectum in a 30-month-old girl that he considered to be of “congenital” origin and referred to as adenoma.(7) Subsequent accounts continued to designate these lesions as adenomas, as did Kerr in 1948. He reported on polyps of the colon in children resembling adenomatous polyps in adults, albeit colonic polyps in children had numerous more goblet cells and had smaller nuclei. He also noted inflammatory reaction of the stroma may be present but none the less considered polyps of the colon in children to be true neoplasms and not the result of local or diffuse inflammation.(8)

Although the term adenoma was also used by Harris in 1953, he believed the immaturity and hyperactivity of the glands causing the adenomatous appearance, were, in fact, attributable to “youth” of the tissue rather than to neoplasia.(9)

In 1957 Horrileno et al. agreed that development of colorectal polyps in children is not a neoplastic event. Rather, they argued, mechanical irritation by the feces of traumatized redundant mucosal folds may lead to hyperplasia of the mucous glands, ulceration of the surface epithelium and superimposed chronic inflammation. Increase in polyp size was thought to originate in proliferation of granulation tissue, distension of mucus-retention cysts and influx of inflammatory cells.(10)

Hamartoma Morson in 1962 first described juvenile polyps as malformation or hamartoma of the tissues of the intestinal mucosa, in particular of the mucosal connective tissue or lamina propria.(11)

This view regarding the hamartomatous nature of juvenile polyps became widely accepted. In 1981, Lipper et al. described two types of hamartomatous juvenile polyps namely type 1 with surface ulceration, oedematous and chronically inflamed stroma and glands of greater length, tortuosity and complexity, and type 2 which were multilobulated with compact tubular glands lined by taller epithelium and void of surface erosion.(12)

However, both phenotypes had foci suggestive
of adenomatous transformation which was substantiated by a third polyp type that was also encountered, containing a mix of the type 1 and type 2 phenotypes in a single juvenile polyp. Indeed, an increasing number of studies reported focal adenomatous transformation in juvenile polyps or even cancer together with juvenile polyps elsewhere in the gastrointestinal tract. Several case series and literature case reports have since described an increased cancer risk of the colorectum, small bowel, stomach and pancreas to be associated with JPS.

Meanwhile, the term juvenile polyposis had been introduced in 1964 by McColl and colleagues who in 1966 proposed a genetic predisposition to the disease. In 1998, Howe et al. performed linkage analysis on a large JPS kindred and found germline mutation of deleted in pancreatic cancer 4 (DPC4) or SMAD4 in all affected members. Three years later the same group identified bone morphogenic protein receptor 1a (BMPR1A) as second gene causing JPS.

**Landscaper theory** In an attempt to reconcile the hamartomatous, i.e. non-neoplastic, origin of juvenile polyps with the observation of an expanded stromal compartment comprising a mixture of mesenchymal and inflammatory elements in juvenile polyps and their limited potential of becoming malignant, Kinzler and Vogelstein in 1998 launched the “landscaper” theory. They proposed that the increased cancer susceptibility due to inherited mutations in JPS is the product of an abnormal stromal environment.

In support of the landscaper theory, it has recently been shown that transgenic inhibition of stromal BMP signalling in the intestine of mice leads to a phenocopy of JPS. Also, one study reported somatic loss of the BMPR1A region exclusively in the lamina propria and not in the epithelium suggesting inactivation of BMPR1A might indeed be a stromal event.

**Smad4** heterozygous mice also developed hamartomatous polyps but with less prominent stromal features. Loss of heterozygosity (LOH) was found specifically in the epithelium of larger polyps. Likewise, LOH of the SMAD4 locus was demonstrated in the polyp epithelium of JPS patients with a SMAD4 germline mutation suggesting SMAD4 may act as gatekeeper rather than as landscaper in JPS pathogenesis.

**OUTLINE OF THE THESIS**

Our increasing knowledge of the pathogenesis of inherited gastrointestinal polyposis syndromes has proven elucidative in early detection and treatment of sporadic colorectal cancer. Chapter 2 provides an overview of the three best known gastrointestinal polyposis syndromes: FAP, JPS and Peutz-Jeghers syndrome. The clinical manifestations, molecular genetics, pathogenesis and management of these syndromes are reviewed. This thesis deals with the clinical and molecular aspects of juvenile polyposis syndrome.

JPS is associated with an increased cancer risk of colorectum, stomach, small bowel and pancreas, but no formal risk analysis exists. In chapter 3 we perform a person-year analysis to define the magnitude of risk for gastrointestinal cancer in JPS patients.

In 30-40% of individuals diagnosed with JPS a germline mutation in SMAD4 or BMPR1A of the TGF-ß/BMP signalling pathway is found using conventional sequencing techniques. Multiplex ligation-dependant probe amplification (MLPA)
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is a novel technique that can be used to detect copy number changes in genomic DNA sequences. Chapter 4 provides a comprehensive genetic analysis in a group of well documented JPS patients to determine the role of large genomic deletions in JPS pathogenesis, as revealed by MLPA. In chapter 5 we propose TGFBRII as JPS candidate gene and search for germline mutations in a group of JPS patients without germline mutations in the established JPS causing genes.

Although in appearance juvenile polyps share common features, phenotypes may vary considerably. Increasing knowledge of the genetics underlying the disease raise the possibility of investigating a potential genotype-phenotype correlation, the results of which are described in chapter 6.

In chapter 7 we address the role of SMAD4 in polyp formation and subsequent progression to dysplasia by validating SMAD4 protein expression as marker of SMAD4 status in juvenile polyps with a SMAD4 germline defect.

Lastly, in chapter 8 we explore the potential value of chemopreventive therapy in targeting juvenile polyp formation and neoplastic progression through investigation of COX-2 expression in JPS polyps compared to sporadic juvenile polyps.

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

Lodewijk A. A. Brosens, W. Arnout van Hattem, Marnix Jansen, Wendy W. J. de Leng, Francis M. Giardiello, G. Johan A. Offerhaus

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Gastrointestinal polyposis syndromes

Lodewijk A. A. Brosens, W. Arnout van Hattem, Marnix Jansen, Wendy W. J. de Leng, Francis M. Giardiello, G. Johan A. Offerhaus

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

FAMILIAL ADENOMATOUS POLYPOSIS

Introduction

Familial adenomatous polyposis (FAP) 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. Histologically, adenomas in FAP resemble sporadic adenomas.

**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%.

*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. Duodenal adenomas can be found in 30-70% of FAP patients and the lifetime risk of duodenal adenoma development is nearly 100%.

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, tubulovillous, 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.

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.

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.

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. The risk of developing duodenal malignancy increases with higher Spigelman stages. Stage II and III disease...
Gastrointestinal polyposis syndromes

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

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<td>Polyp number</td>
<td>1-4</td>
<td>5-20</td>
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Stage 0, 0 points; stage I, 1-4 points; stage II, 5-6 points; stage III, 7-8 points; stage IV, 9-12 points.

Table 1. Spigelman classification for duodenal polyposis in FAP.
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 (HNPPC), but tumors in these patients are usually astrocytomas or glioblastomas and present later in life. The association of glioblastoma with HNPPC 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 \textit{APC} gene was identified.(3-5) The \textit{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 \textit{APC} gene mutations have been reported in FAP. Germline mutations are mainly found in the 5’ half of the \textit{APC} gene, particularly in codons 1061 and 1309. Most mutations are frameshifts due to insertions or deletions, or nonsense mutations, leading to truncated \textit{APC} proteins.(1, 34) A high frequency of somatic \textit{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 \textit{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 \textit{MYH} were identified in patients with both classic and attenuated FAP in which no germline \textit{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. \textit{MYH} has a repair function critical for \textit{APC}, and the \textit{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 \textit{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 \textit{APC} gene tend to present as attenuated FAP, characterized by less than 100 colorectal polyps and malignant transformation occurring 10-20 years after diagnosis.

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**Figure 2.** Schematic representation of the \textit{APC} gene, consisting of 15 exons and 2843 codons. Most germline mutations are found in the 5’ half the \textit{APC} gene, particularly in codons 1061 and 1309. A high frequency of somatic \textit{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 \textit{APC} gene cause attenuated FAP.
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, 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

**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.

**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
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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)

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)

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-through (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)

<table>
<thead>
<tr>
<th>Table 2. Indications for APC gene testing.</th>
</tr>
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<tbody>
<tr>
<td>≥100 colorectal adenomas</td>
</tr>
<tr>
<td>First-degree relatives (&gt;10 years old) of FAP patients</td>
</tr>
<tr>
<td>≥20 cumulative colorectal adenomas (suspected for AFAP)</td>
</tr>
<tr>
<td>First-degree relatives (&gt;10 years old) of AFAP patients</td>
</tr>
</tbody>
</table>

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 and stage I every four years, stage II every 2 to 3 years, stage III every 6 to 12 months and for stage IV surgery should be considered (Table 3).(10, 51)

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

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)

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

<table>
<thead>
<tr>
<th>Spigelman Stage</th>
<th>Endoscopic frequency</th>
<th>Chemoprevention</th>
<th>Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>4 years</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Stage I</td>
<td>2-3 years</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Stage II</td>
<td>2-3 years</td>
<td>+/-</td>
<td>No</td>
</tr>
<tr>
<td>Stage III</td>
<td>6-12 months</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Stage IV</td>
<td>6-12 months</td>
<td>+/-</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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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 characterised by multiple juvenile polyps primarily in the colorectum but also elsewhere in the gastrointestinal tract. It is the most common hamartomatous polyposis syndrome, occurring in 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. JP 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-Ruvalcaba-Riley, 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 oedematous 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) JP 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 JP 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 harbour 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 JP, (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 JP, and these calculations vary widely. Jass et al. found colorectal cancer in 18 of 87 (20.7%) JP 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.(66)

A review of medical records of the Iowa JP 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 JP 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 JP patients carry an increased risk of colorectal and possibly gastric cancer.

**Extra-intestinal manifestations.** A variety of extra-intestinal manifestations in JP have been described, although most are based on case reports. Also, extra-intestinal features in JP are difficult to interpret due to an unclear distinction between JP, Cowden, and Bannayan-Ruvalcaba-Riley syndrome.

Extra-intestinal manifestations have been reported in about 10 to 78% of JP patients.(66,
Anomalies described, include hypertelorism, macrocephaly, digital clubbing, polydactyly, mental retardation, hydrocephalus, congenital cardiac anomalies, pulmonary arteriovenous malformations, pulmonary stenosis, telangiectasias, Meckel’s diverticulum, intestinal malrotation, cryptorchidism, and bifid uterus and vagina. (59, 65, 71, 77, 78)

An association between JP and hereditary hemorrhagic telangiectasias (HHT) exists. Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome) is an autosomal dominant disorder of vascular dysplasia affecting many organs. Characteristic features are telangiectasias 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). (79)

Case reports of juvenile polyposis patients with symptoms of HHT, including arteriovenous malformations in the lung, (80-83) liver, (82, 83) and skin (83) and gastrointestinal telangiectasias, (81, 82) 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, (84) and germline mutations in ENG were recently described in two JP patients. (85)

In JP 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. (82, 84) In addition, digital clubbing and pulmonary osteoarthropathy are frequently described in combination with arteriovenous malformations. (78, 80)

**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. (86) 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. (87)

Intestinal polyposis is not pathognomonic 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. (86) Clinical features of CS include mucocutaneous lesions (facial trichilemmoma, acral keratoses, papillomatous papules and mucosal lesions are pathognomonic), 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. (87)

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. (88, 89) 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. (89)
Genetic defect

Currently alterations in two genes, SMAD4 and BMPR1A, are identified causes of JP. 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 JP. (90) Germline mutations in SMAD4 are found in 16-24% of JP patients, most in the 3’ half of the gene, encoding the highly conserved MH2 domain, which is involved in SMAD oligomerization and transcriptional activation. (90-93) In 2001, bone morphogenetic protein receptor 1A (BMPR1A), located on chromosome 10q22.3, was identified as a second JP gene. (94) Germline BMPR1A mutations are noted in 17-24% of JP patients. (91-93) Since germline mutations in SMAD4 or BMPR1A are identified in a minority of patients with clinically defined juvenile polyposis, (91) other components of the TGF-β signaling pathway have been studied. Other SMAD genes, including SMAD1, SMAD2, SMAD3, SMAD5 and SMAD7 were analysed, but no germline mutations in these genes were found in JP patients. (95, 96) In addition, germline BMPR2, BMPR1B and ACVRL1 mutations were excluded as a cause for JP. (91) Recently, germline mutations in the HHT gene ENG, encoding the TGF-β co-receptor endoglin, were reported in two patients with JP. (85)

The role for germline PTEN (chromosome 10q23.3) mutations in juvenile polyposis is unclear. Some investigators suggested that PTEN could be involved in JP (97) while others disagree. (98) Discriminating between JP and CS can be difficult since the penetrance of CS is less than 10% below age 15. (63) Currently, PTEN mutations in patients with JP likely represent CS or BRRS patients that have not yet expressed the clinical features of these conditions. (99)

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 JP, >10 polyps, and higher frequency of family history of gastrointestinal cancer) compared to those without an identified germline mutation. (92) 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. (100)

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. (92, 93, 100) Finally, the combined syndrome of JP and hereditary hemorrhagic telangiectasia has been associated with germline mutations in SMAD4. (84)

Cancer pathogenesis

Malignant transformation in JP likely occurs in adenomatous foci within juvenile polyps, and/or in adenomas that arise as separate lesions. Reports describing adenomatous foci in juvenile polyps (61, 67, 72-74) suggest that these polyps can undergo neoplastic change. In addition, carcinoma in situ and adenocarcinoma have been described within juvenile polyps. (68, 101, 102)
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. 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).

Although alterations in genes with a function in the TGF-β/BMP signaling pathway cause JP, 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).
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, they hypothesized that stromal changes provide an abnormal environment influencing behaviour of adjacent epithelium and ultimately leading to an increased risk of neoplastic transformation. 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. Consequently, disrupted mesenchymal-epithelial communication, by defective BMP signaling, is hypothesized to cause the “landscaper” defect. 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. Lastly, disturbed stromal TGF-β signaling ultimately leads to epithelial neoplasia of the prostate in mice.

Whether germline SMAD4 mutations in JP also act via a landscaper mechanism is unclear. Historically, SMAD4 is known as a tumor-suppressor gene in several cancer types. One study found homozygous SMAD4 deletions primarily in the epithelium, but also in stromal fibroblasts and pericryptal myofibroblasts, of juvenile polyps of JP 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.

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

Management

Screening. Patients at risk or with a high suspicion of JP should have endoscopic screening of the colon and upper gastrointestinal tract at age 15 or at the time of first symptoms. At the time of diagnosis of JP, the entire gastrointestinal tract should be examined for the presence of polyps. 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 JP and can be enrolled in screening programs for the general population.

Surveillance and treatment. Endoscopic examination of the colon and upper
gastrointestinal tract is recommended every two to three years in patients with JP. In patients with polyps, endoscopic screening should be performed yearly, until the patient is polyp free. (6, 113-115) Patients with mild polyposis can be managed by frequent endoscopic examinations and polypectomy. (115)

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. (114-116) Surgical options include subtotal colectomy with ileorectal anastomosis (IRA) or total proctocolectomy with pouch. (115, 116) It is unclear which type of surgery is preferable but, in analogy with FAP, may depend on the extent of rectal polyposis. (117) However, no relationship was found between rectal polyp burden and the type of surgery. (116) Moreover, recurrence of rectal polyps in patients with subtotal colectomy is frequent (115) and recent data show that about half of these individuals require subsequent proctectomy. (116) Therefore, total proctocolectomy has been advocated as the initial surgery for patients with massive juvenile polyposis, unable to be managed endoscopically. (115) Although the surgery of choice in JP remains debatable, patients need frequent post-operative endoscopic surveillance because of high recurrence rates of polyps in the remnant rectum and the pouch. (116)

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 JP polyps than in sporadic juvenile polyps and correlates with polyp size and dysplasia. (118) This observation suggests that chemoprevention using selective or non-selective COX-2 inhibitors could be beneficial in JP. Currently, NSAID chemoprevention in JP has not been studied systematically. Anecdotally, Oncel et al. described the use of sulindac 300 mg per day in two JP 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. (116) The value of NSAID chemoprevention in JP requires further investigation.

PEUTZ-JEGHERS SYNDROME

Introduction

Peutz-Jeghers syndrome (PJS) 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, 119, 120) 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. (119, 121)

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. (119, 122)

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. (123) 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. (122, 124)

**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. (120, 125) Symptoms usually occur during the first decade of life, and 50-60% of patients will have complaints before the age of 20. (126) In addition, PJS patients can present with gastrointestinal malignancy. (120)

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 perianal 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. (120)

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). (120, 125) 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. (123)

**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. (127-130) 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. (127)

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
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cancer was 48% at age 57.(129)

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.(130)

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).(128)

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.(131)

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 (127, 128, 130) and the cumulative risk between 80-90% at age 70.(128, 131)

Genital tract tumors. Several gonadal malignancies occur in PJS patients.(132, 133) 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.(132)

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.(134, 135)

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.(132, 133)

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.(136, 137) LKB1 is a tumor-suppressor involved in intracellular signal transduction and cellular polarity.(138) Mutations in LKB1 result in inactivation of the kinase activity,(139) although germline mutations that allow retention of kinase activity have recently been reported.(140)

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. 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.

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. A possible second locus was proposed at chromosome 19q13.4. However, no germline mutations in this region have been identified. In addition, several LKB1/STK11-interacting proteins, including STRAD on chromosome 17q23.3, BRG1, MO25, and LIP1 (LKB1-interacting protein) on chromosome 2q36, 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.

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. However, a recent large multi center study did not find such correlation.

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. 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. A recent large collaborative study, however, did not find a correlation between type or site of LKB1 mutation and cancer risk.

Cancer pathogenesis

The pathogenic mechanisms involved in gastrointestinal polyph 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. 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.
Consequently, LKB1 was thought to act as a typical tumor-suppressor gene. 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.

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. 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. 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. Loss of polarity function may also affect asymmetric stem cell division in PJS and lead to expansion of the stem cell pool. 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. 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.

Surveillance and treatment. Most authors recommend endoscopic surveillance of the upper gastrointestinal tract at a two year interval starting at age 10. However, others recommend upper gastrointestinal endoscopic surveillance every three years, starting at age 25. 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. 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.

Multiple bowel resections due to gastrointestinal complications of polyps may eventually result in short-bowel syndrome. Therefore, prevention of surgery is key in the
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. 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. In addition, pelvic ultrasound, and cervical smears should be performed yearly. 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 feminising symptoms.

Chemoprevention: Several investigators showed increased levels of COX-2 in hamartomas and carcinomas 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 \( LKB^{+/−} \) 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.

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. 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

FAP, JP 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 JP 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 polygenosis and carcinogenesis, and improved management of patients afflicted by these disorders.

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Chapter two


Risk of colorectal cancer in juvenile polyposis

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**ABSTRACT** Juvenile polyposis is an autosomal dominant syndrome characterized by development of hamartomatous gastrointestinal polyps and 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 juvenile polyposis patients were compared with the general population through person-year analysis with adjustment for demographics. **Results.** In juvenile polyposis patients, the relative risk (RR) of colorectal cancer was 34.0 (95% confidence limits [CL], 14.4, 65.7). Similar risks were noted in both males (30.0, CL, 9.6, 68.6) and females (43.7, CL, 8.8-125). The cumulative life time risk for colorectal cancer was 38.7%. The mean age of colorectal cancer was 43.9±10.4 (SD). Other gastrointestinal malignancies were not noted in this cohort. **Conclusions.** Patients with juvenile polyposis have a markedly elevated relative 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. Gut 2007 Jul;56(7):965-7

**INTRODUCTION**

Juvenile polyposis (JPS) is an autosomal dominant syndrome characterized by development of histopathological juvenile polyps in the gastrointestinal tract. Polyps usually occur by the third decade of life and primarily affect the colorectum. Recently, investigators have discovered that germline mutations in the BMPR1A and SMAD4 gene cause this disorder in a minority (39%) of patients. Also, mutation of the ENG gene may or may not be a cause of young-onset juvenile polyposis.

Juvenile polyposis 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 colorectal cancer risk. Also, other malignancies including gastric, small bowel and pancreatic cancer have been noted in some studies. However, a formal risk assessment of gastrointestinal cancer risk in JPS patients has not been reported.

The purpose of this study was to define the magnitude of risk for colorectal cancer in juvenile polyposis. The occurrence of colorectal cancer in patients with juvenile polyposis from The Johns Hopkins Polyposis Registry and clinic were compared with the general population of the
United States through person year analysis.

**METHODS**

Patient data were collected from The Johns Hopkins Polyposis Registry and clinic. Patients were defined as having juvenile polyposis according to the accepted clinical criteria (1,6) as follows: 1) at least 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 polyposis. This study was approved by the Johns Hopkins Joint Committee on Clinical Investigation (institutional review board).

A risk assessment for colorectal adenocarcinoma risk was performed. Computation of person-years at risk for colorectal cancer started January 1, 1970 until July 1, 2005. Patients were considered 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.

Person-years at risk were calculated for ages 0 to 84 years according to sex, race and age-specific categories during subsequent 5- years calendar time periods 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 (SEER) data for the US population were employed.(14) The ratio of observed carcinomas over the expected number was computed with a test of significance and 95% confidence limits assuming a Poisson distribution. This ratio forms the relative risk (RR) and compares cancer risk in the study population with that in the general population. Using the absolute rates for each 5 year age group, the cumulative risk was calculated according to: cumulative risk = 1-exp (-cumulative rate).(13)

**RESULTS**

The study population for the person-year analysis consisted of 84 patients with juvenile polyposis 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>
<thead>
<tr>
<th>Age at diagnosis of CRC (y)</th>
<th>Sex</th>
<th>Race</th>
<th>Prior partial colectomy (age, y)</th>
<th>Death from CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>F</td>
<td>W</td>
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<td>No</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>W</td>
<td>Yes (28)</td>
<td>No</td>
</tr>
<tr>
<td>37</td>
<td>M</td>
<td>W</td>
<td>Yes (18)</td>
<td>Yes</td>
</tr>
<tr>
<td>41</td>
<td>M</td>
<td>W</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>48</td>
<td>F</td>
<td>W</td>
<td>No</td>
<td>No</td>
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<td>M</td>
<td>W</td>
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<td>Yes</td>
</tr>
<tr>
<td>53</td>
<td>M</td>
<td>W</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>58</td>
<td>M</td>
<td>W</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1. lists the juvenile polyposis patients with colorectal adenocarcinoma. The mean age of diagnosis of colorectal cancer was 43.9±10.4 (SD). Two patients with prior prophylactic colectomy with ileoanal anastomosis and one with colectomy and Hartman’s pouch developed subsequent cancer in the retained rectum. Five of eight (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 relative risk for colorectal cancer in juvenile polyposis patients was 34.0 (CL, 14.4, 65.7). This statistically significantly elevated risk was similar in males (RR 30.0; CL, 9.6, 68.6) and females (RR 43.7; CL, 8.8, 125). Based on an 80 year life span, the absolute risk for colorectal cancer was 38.7/100 persons.

DISCUSSION

In this study performed by person-year analysis, both male and female patients with juvenile polyposis had a statistically significantly elevated relative risk (34.0) of colorectal cancer compared with the general population. The lifetime risk of colorectal cancer was calculated at 39%. These findings are consistent with several case series (5,6,8-12) and a publication which compiled literature reports (7) estimating a 13-38% frequency of colorectal cancer in JPS patients. Jass et al. reported a 68% cumulative risk of colorectal cancer in patients from the St. Mark’s Registry but, details were not provided.(15)

In juvenile polyposis, as in other inherited syndromes of colorectal neoplasia, elevated risk of colon malignancy appears 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 diagnosed at 30 years old. In two previous reports, the mean age of colorectal cancer was 34 years with one individual diagnosed as early as 15 years of age.

Extracolonic gastrointestinal cancers (less than 30 confirmed literature cases) have been reported in other studies of juvenile polyposis patients. 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 reveals gastric and small bowel tumors together occurring at about one-fifth the frequency of colorectal cancer in this patient group.

A caution raised by comparison of a registry based population to the general United States population is detection bias- that is, surveillance of the population in the registry may lead to higher diagnosis of certain disorders compared to the general population. Though this concern cannot be discounted, none of the patients with malignancy came to registry attention secondary to the diagnosis of colorectal cancer. Also, the
risks generated for colorectal cancer in juvenile polyposis patients in this analysis are consistent with the prevailing literature. Moreover, our colorectal cancer risk estimates are likely conservative, since patients with partial colectomy were not censored from the analysis at the time of surgery. In this regard, 3 of the patients with colorectal cancer developed malignancy in the retained rectum several years after 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.

In summary, this study, taken together with other literature data, argues for a marked increased risk of colorectal cancer in patients with juvenile polyposis. These finding are in concert with expert opinion which recommends the commencement of screening for juvenile polyposis in at risk individuals at age 15 (or earlier if the patient is symptomatic).

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. Also, others advise periodic upper endoscopy and evaluation of the small intestine. 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.

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Large genomic deletions of SMAD4, BMPR1A and PTEN in juvenile polyposis

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ABSTRACT Juvenile polyposis syndrome (JPS) 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 (48.2%) 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. Conclusions. 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 48.2% 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. Gut 2008 May;57(5):623-7

INTRODUCTION

Juvenile polyposis (JPS) is an autosomal dominant disorder characterized by multiple gastrointestinal juvenile polyps and an increased risk of colorectal cancer. 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. 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 TGF-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 polyplps. 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
Large genomic deletions in juvenile polyposis

(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. 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 electrophoreses. Subsequently, the PCR product was enzymatically purified using Shrimp Alkaline Phosphatase (USB Europe GmbH, Staufen, Germany) and Exonuclease 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; BMPRIA: 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

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

<table>
<thead>
<tr>
<th></th>
<th>SMAD4</th>
<th>BMPR1A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Point mutations</td>
<td>6 (22.2%)</td>
<td>3 (11.1%)</td>
<td>9 (33.3%)</td>
</tr>
<tr>
<td>Deletions</td>
<td>1 (3.7%)</td>
<td>3 (11.1%)</td>
<td>4 (14.8%)</td>
</tr>
<tr>
<td>All mutations</td>
<td>7 (25.9%)</td>
<td>6 (22.2%)</td>
<td>13 (48.2%)</td>
</tr>
</tbody>
</table>
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.

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 nonsense 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.L529FfsX9).

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)

**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
Large genomic deletions in juvenile polyposis

**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 48.2% (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 48.2% 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.(28)

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, 29, 30) 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.(31) 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

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Nucleotide</th>
<th>Amino acid change</th>
<th>Number of patients</th>
<th>Reference</th>
<th>refSNP ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPR1A</td>
<td>1</td>
<td>c.4 C&gt;A</td>
<td>p.P2T</td>
<td>19/27</td>
<td>Howe, 2001</td>
<td>rs17090779</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>c.14 C&gt;T</td>
<td>p.T5M</td>
<td>01-01-27</td>
<td>Howe, 2007</td>
<td>rs35400405</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>c.207 G&gt;A</td>
<td>p.L69L</td>
<td>01-06-27</td>
<td>Howe, 2007</td>
<td>rs16930129</td>
</tr>
<tr>
<td>ENG</td>
<td>8</td>
<td>c.1029 C&gt;T</td>
<td>p.T343T</td>
<td>01-01-27</td>
<td>Howe, 2007</td>
<td>rs3739817</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>c.1005 C&gt;T</td>
<td>p.L354L</td>
<td>01-01-27</td>
<td>Howe, 2007</td>
<td>rs36092484</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>c.1794 T&gt;C</td>
<td>p.G598G</td>
<td>01-03-27</td>
<td>Abdalla, 2005</td>
<td></td>
</tr>
</tbody>
</table>
Chapter four

would be needed to determine the exact size of the genomic deletion on 10q in these individuals.

In 51.8% 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 51.8% can be explained by undiscovered mutations in SMAD4 or 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 signalling 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.

REFERENCES
Large genomic deletions in juvenile polyposis


No TGFBRII germline mutations in juvenile polyposis patients without SMAD4 or BMPR1A mutation

Gut 2009 Jan;58(1):154-6

Lodewijk A. A. Brosens, W. Arnout van Hattem, Marijke C. E. Kools, Chantal Ezendam, Folkert H. Morsink, Wendy W.J. de Leng, Francis M. Giardiello, G. Johan A. Offerhaus

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No TGFBRII germline mutations in juvenile polyposis patients without SMAD4 or BMPR1A mutation

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ABSTRACT

Juvenile polyposis (JPS) is an autosomal dominant disorder characterized by the presence of multiple gastro-intestinal 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 in the TGFBRII gene. Results. No pathogenic TGFBRII variations were found in the germline of 19 patients with juvenile polyps. Conclusions. 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. Gut 2009 Jan;58(1):154-6

Juvenile polyposis (JPS) is an autosomal dominant disorder characterized by the presence of multiple gastro-intestinal juvenile polyps and an increased risk of colorectal (CRC).(1) JPS is caused by germline mutation of SMAD4 or BMPR1A, both involved in the Transforming Growth Factor–β/Bone Morphogenic Protein (TGF-β/BMP) signaling pathway. A recent study in this journal (Gut 2008;57:623-7) showed that 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. Since no germline defect is found in ~50% of JPS patients, it is likely that other JPS causing genes exist.(2) Several candidate genes, mostly involved in TGF-β/BMP signaling, have been investigated for a role in JPS pathogenesis. No mutations have been found in these genes.(3-6) (Table 1) Recently, the TGF-β co-receptor Endoglin was proposed as a JPS susceptibility gene, but other studies could not confirm this.(2) Also PTEN, the gene originally linked to Cowden syndrome (CS) and Bannayan–Riley–Ruvalcaba syndrome (BRRS), has been suggested as a JPS gene. The current consensus, however, is that PTEN
In TGF-β signaling and in (colorectal) carcinogenesis, we investigated whether germline mutation or deletion of the TGFBRII gene is involved in JPS pathogenesis.

Nineteen JPS patients from 18 families, in whom germline mutation or deletion of SMAD4, BMPR1A, PTEN or ENG was previously excluded, were investigated for germline defects in the TGFBRII gene. JPS was defined according to accepted clinical criteria.(1) All exons and intron-exon boundaries of the TGFBRII gene were analyzed by direct sequencing and the possibility of germline deletion of (parts of) the TGFBRII gene was investigated by MLPA (P065 MLPA kit, MRC-Holland BV, Amsterdam, The Netherlands). No pathogenenic germline mutations or deletions in TGFBRII were found in this cohort. Known polymorphic variations were found in intron 3, intron 4, exon 4, and intron 7 (Table 2).

TGFBRII germline mutation is linked to Marfan syndrome type 2.(13) Surprisingly, these patients do not have an increased risk of cancer. (14) Possibly, diverging phenotypic effects of the different TGFBRII mutations are responsible for

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**Table 1. Candidate genes investigated in the pathogenesis of juvenile polyposis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Patients studied/ Mutations found</th>
<th>Reference (first author and year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPR1B (ALK6)</td>
<td>32/0</td>
<td>Howe 2004</td>
</tr>
<tr>
<td>BMPR2</td>
<td>59/0*</td>
<td>Howe 2004, van Hattem 2008</td>
</tr>
<tr>
<td>ACVR1 (ALK1)</td>
<td>66/0**</td>
<td>Howe 2004, Gallione 2004, van Hattem 2008</td>
</tr>
<tr>
<td>SMAD1</td>
<td>30/0</td>
<td>Bevan 1999</td>
</tr>
<tr>
<td>SMAD2</td>
<td>34/0</td>
<td>Bevan 1999, Roth 1999</td>
</tr>
<tr>
<td>SMAD3</td>
<td>34/0</td>
<td>Bevan 1999, Roth 1999</td>
</tr>
<tr>
<td>SMAD5</td>
<td>30/0</td>
<td>Bevan 1999</td>
</tr>
<tr>
<td>SMAD7</td>
<td>34/0</td>
<td>Bevan 1999, Roth 1999</td>
</tr>
<tr>
<td>CDX2</td>
<td>37/0</td>
<td>Woodford-Richens 2001</td>
</tr>
</tbody>
</table>

*32 patients investigated by sequencing, 27 by MLPA.
** 39 patients investigated by sequencing, 27 by MLPA.
the absence of malignancies in Marfan patients carrying a TGFBRII mutation. (13) Alternatively, the germline variation (944C>T) found in the patient with hereditary CRC could be a rare polymorphism without significance for CRC development. Although, this alteration was not found in 119 control subjects, (10) others found it at a similar frequency in normal controls (7 of 492) and individuals with sporadic CRC (6 of 228). (13) Moreover, no additional germline mutations in TGFBRII have been found in HNPCC patients or in patients with familial or early onset CRC. (15, 16)

Because of its role in TGF-β signaling and CRC pathogenesis we hypothesized that TGFBRII may be a JPS susceptibility gene. Linkage analysis could not be performed due to the lack of large JPS kindreds in our cohort. It is nevertheless felt that TGFBRII is unlikely to be involved in JPS pathogenesis since no germline mutations or deletions in TGFBRII were found in the current study. Still, about half of JPS patients remain without 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.

### References

15. Shin KH, Park YJ, Park JG. Mutational analysis of the

Histological variations in juvenile polyp phenotype: A link to patient genotype

Manuscript submitted

W. Arnout van Hattem, Daniëlle Langeveld, Wendy W. J. de Leng, Folkert H. M. Morsink, Fiebo F. W. ten Kate, Paul J. van Diest, Christine A. Iacobuzio-Donahue, Francis M. Giardiello, G. Johan A. Offerhaus, Lodewijk A. A. Brosens

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Department of Pathology and Medicine, The Johns Hopkins School of Medicine, Baltimore, Maryland, USA.
Histological variations in juvenile polyp phenotype: A link to patient genotype

W. Arnout van Hattem, Daniëlle Langeveld, Wendy W. J. de Leng, Folkert H. M. Morsink, Fiebo F. W. ten Kate, Paul J. van Diest, Christine A. Iacobuzio-Donahue, Francis M. Giardiello, G. Johan A. Offerhaus, Lodewijk A. A. Brosens

ABSTRACT Juvenile polyps are distinct hamartomatous malformations of the gastrointestinal tract and occur sporadically or as part of the heritable juvenile polyposis syndrome (JPS). Juvenile polyps are histologically characterized by a marked increase of the stromal cell compartment, but an epithelial phenotype has also been reported. In the majority of JPS patients a germline mutation of either SMAD4 or BMPR1A of the TGF-β/BMP pathway is found. We compared the histological phenotype of juvenile polyps in patients with a SMAD4 or BMPR1A germline mutation and those with sporadic juvenile polyps. **Methods.** H&E slides of 65 JPS polyps and 25 sporadic juvenile polyps were reviewed for histological features including dysplasia. The crypt-stroma ratio was determined using an unbiased stereologic approach. All polyps were subsequently categorised as type A (crypt-stroma ratio <1.00) or type B (crypt-stroma ratio >1.00), the latter referring to the epithelial phenotype. Cell cycle activity was assessed using immunohistochemistry for the proliferation marker Ki67. **Results.** Patients with a SMAD4 germline mutation had predominantly type B juvenile polyps, whereas individuals with a BMPR1A germline mutation had primarily type A polyps. No distinction could be ascribed to differences in cell cycle activity. Dysplasia was equally common in JPS polyps with either a SMAD4 or BMPR1A germline mutation. **Conclusions.** Juvenile polyps in the setting of JPS may exhibit distinct phenotypes correlating with the underlying patient genotype. Manuscript submitted.

INTRODUCTION

Juvenile polyposis syndrome (JPS) is a rare autosomal dominant disorder characterized by the presence of multiple juvenile polyps in the gastrointestinal tract. Patients present usually by the second decade of life and have a markedly increased risk of colorectal cancer in middle age. (1, 2) Clinical diagnosis is made when any one of the following criteria is met: (1) more than 3-5 juvenile polyps in the colorectum; (2) juvenile polyps throughout the intestinal tract; or (3) any number of juvenile polyps in combination with a positive family history of JPS.(3-5) Sporadic juvenile polyps are a more common finding, occurring in up to 2% of the pediatric population, and are considered benign solitary lesions of the
colorectum.(3, 6)

Juvenile polyps most often have a spherical appearance with extensive surface erosion and a marked increase of the stromal cell compartment, inflammatory and reactive changes of the epithelium, and distorted and dilated crypts. Several reports have noticed a more lobulated and epithelial phenotype in a subset of juvenile polyps.(3)

JPS is caused in 50-60% of patients by a germline defect in SMAD4 or BMPR1A of the TGF-β/BMP pathway.(7-9) Inactivation of these genes in mice leads to a JPS-like phenotype. Smad4 mutant mice develop gastrointestinal polyps characterized by elongated and dilated tubular structures lined by hyperplastic or serrated epithelium and, at best, a moderate expansion of the stromal cell compartment. Although mostly hyperplastic, the epithelium may show some atypia with occasional foci of dysplasia.(10, 11) In Bmpr1a mutant mice, polyps have cystically dilated and distorted glands filled with mucin and inflammatory cells surrounded by fibrous stroma.(12) Moreover, inhibition of BMP signalling in mice by transgenic expression of noggin, a BMP inhibitor, under control of a villin promoter, leads to branching and budding of the intestinal epithelium, crypt dilation and reactive inflammatory changes. At later stages these mice develop foci of dysplastic epithelium and adenomatous change.(13)

These mice display features reminiscent of JPS, but it seems differences may exist in the phenotype depending on the genetic background.

In this study we compared the histological phenotype of juvenile polyps with a SMAD4 or BMPR1A germline mutation and sporadic 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 conducted with approval of the institutional review boards of these institutions. 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 were analysed for germline defects of SMAD4, BMPR1A, PTEN, ENG and TGFBRII through direct sequencing and MLPA.(9, 14) Thirty-nine patients (90 polyps) were included in this study, of which 8 patients (21 polyps) had a SMAD4 germline defect, 6 patients (44 polyps) had a BMPR1A germline defect, and 25 patients had a sporadic juvenile polyp.

| Table 1. Features of the classic juvenile polyp and the epithelial juvenile polyp variant |
|----------------------------------|----------------------------------|
| Classic juvenile polyp           | Epithelial juvenile polyp         |
| Spherical                        | Lobulated                        |
| Eroded and granular surface      | Villous-like surface              |
| Stromal compartment expanded     | Stromal compartment not expanded  |
| Low crypt density                | High crypt density                |
| Flattened reactive epithelium    | Columnar hypermucinous epithelium |

JPS is caused in 50-60% of patients by a germline defect in SMAD4 or BMPR1A of the TGF-β/BMP pathway.(7-9) Inactivation of these genes in mice leads to a JPS-like phenotype. Smad4 mutant mice develop gastrointestinal polyps characterized by elongated and dilated tubular structures lined by hyperplastic or serrated epithelium and, at best, a moderate expansion of the stromal cell compartment. Although mostly hyperplastic, the epithelium may show some atypia with occasional foci of dysplasia.(10, 11) In Bmpr1a mutant mice, polyps have cystically dilated and distorted glands filled with mucin and inflammatory cells surrounded by fibrous stroma.(12) Moreover, inhibition of BMP signalling in mice by transgenic expression of noggin, a BMP inhibitor, under control of a villin promoter, leads to branching and budding of the intestinal epithelium, crypt dilation and reactive inflammatory changes. At later stages these mice develop foci of dysplastic epithelium and adenomatous change.(13) These mice display features reminiscent of JPS, but it seems differences may exist in the phenotype depending on the genetic background. In this study we compared the histological phenotype of juvenile polyps with a SMAD4 or BMPR1A germline mutation and sporadic juvenile polyps.
**Histological characterization**

Histological characterization of the juvenile polyps was performed on standard H&E stained slides. Based on initial screening of the slides, histological features were grouped together creating two categories describing the classic juvenile polyp phenotype and a more epithelial juvenile polyp variant as shown in Table 1 and Figure 1. All polyps were classified according to these two categories and were graded for dysplasia according to the standard criteria (GJAO and FJWtK).(15)

**Stereology**

To determine the crypt-stroma ratio in juvenile polyps, points of a Weibel grid overlying crypts or stroma were counted in systematically at random selected fields of vision at a 10x magnification using Q-PRODIT (Leica, Cambridge, UK) as previously described.(16) A ratio <1.00 indicating a low crypt density was designated type A and a ratio >1.00 type B. All polyps were categorized according to these criteria and results were compared to the histological classification.

**Immunohistochemistry and scoring**

Tissue was formalin-fixed and paraffinized by standard procedures. Immunohistochemistry was performed using a monoclonal antibody for Ki67 (DAKO MIB-1, Cat.no. M7240, 1:200). Briefly, 4 µm sections were deparaffinized, blocked for endogenous peroxidase activity by immersion in 0.3% H₂O₂ 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) with 3,3-diamino-benzidine (DAB, Sigma D5637) as chromogen. Slides were counterstained with hematoxylin.
Ki67 is a nuclear proliferation marker expressed during all phases of actively growing but not quiescent cells. Normal colon mucosa shows a distinctive nuclear Ki67 staining pattern with positive cells limited to the bottom third of the crypt, i.e. the proliferative compartment. Slides were scored in a dichotomous manner describing retention or loss of compartmentalisation, the latter indicating an expansion of cell cycle activity (Figure 2).

**Statistical analysis** Statistical analysis was performed with the SPSS 15.0 software package. The chi-square test was used to determine whether correlation between phenotypes and Ki67 expression were statistically significant.

## RESULTS

**Histological characterization** H&E stained slides of all juvenile polyps were reviewed. Several variations in histological appearance were encountered, most notably regarding crypt density and surface erosion (Figure 1). Therefore, two categories were created (Table 1). The classic juvenile polyp phenotype was found in 39 of 65 JPS polyps (60%) and the epithelial juvenile polyp variant in 26 polyps (40%). The epithelial juvenile polyp variant was more common in polyps with a SMAD4 germline mutation compared to polyps with a BMPR1A germline mutation (p<0.001). (Table 2) All of the 25 sporadic juvenile polyps were of the classic juvenile polyp phenotype.

**Crypt-stroma ratio** The crypt-stroma ratio was significantly higher in juvenile polyps with a SMAD4 germline mutation compared to juvenile polyps with a BMPR1A germline mutation (p=0.001) (Figure 3). Juvenile polyps with a BMPR1A germline mutation had a higher ratio than sporadic juvenile polyps (p<0.001). Out of 65 JPS polyps 38 (58%) were of type A (crypt/stroma ratio < 1) and 27 (42%) were of type B (crypt/stroma ratio > 1). Classification according to crypt-stroma ratio confirmed observations made based on histological screening (p<0.001). (Table 2) Of the sporadic juvenile polyps evaluated all but one were classified as type

<table>
<thead>
<tr>
<th>Germline mutation</th>
<th>Histological classification</th>
<th>Crypt-stroma classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classic</td>
<td>Epithelial</td>
</tr>
<tr>
<td>SMAD4</td>
<td>6 (29%)</td>
<td>15 (71%)</td>
</tr>
<tr>
<td>BMPR1A</td>
<td>33 (75%)</td>
<td>11 (25%)</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>26</td>
</tr>
</tbody>
</table>
Dysplasia All polyps were graded for dysplasia. The frequency in which different categories of dysplasia were seen was similar for polyps with either a SMAD4 or BMPR1A germline mutation (Table 3). However, evaluation of dysplasia by polyp type revealed a distinct pattern in polyps with a SMAD4 or BMPR1A background. Focal dysplasia in a SMAD4 setting was found only in type B polyps, but in a BMPR1A setting focal dysplasia was seen in both type B and type A polyps, with a somewhat reduced frequency in the latter. (Table 4) All sporadic polyps were negative for dysplasia.

Immunohistochemistry To investigate whether variation in crypt density in juvenile polyps could be attributed to differences in proliferative activity, immunostaining of the Ki67 proliferation marker was performed. Focal loss of compartmentalisation of Ki67, indicating expanded cell cycle activity, was observed in 12 of 21 juvenile polyps with a SMAD4 germline mutation (57%) and in 25 of 44 juvenile polyps with a BMPR1A germline mutation (57%). Evaluation of the immunostaining per polyp type and germline defect showed a correlation between

<table>
<thead>
<tr>
<th>Table 3. Dysplasia in juvenile polyps</th>
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<tbody>
<tr>
<td>Dysplasia</td>
</tr>
<tr>
<td>SMAD4</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Indefinite</td>
</tr>
<tr>
<td>Low grade</td>
</tr>
<tr>
<td>High grade</td>
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<td>Total</td>
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<tr>
<th>Table 4. Dysplasia in juvenile polyps organised by phenotype and germline defect</th>
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<tr>
<td>Dysplasia</td>
</tr>
<tr>
<td>SMAD4</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Indefinite</td>
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<tr>
<td>Low grade</td>
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<td>High grade</td>
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<td>Total</td>
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<th>Table 5. Results Ki67 immunohistochemistry on juvenile polyps organised by phenotype and germline mutation</th>
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</thead>
<tbody>
<tr>
<td>Compartmentalisation of Ki67</td>
</tr>
<tr>
<td>SMAD4</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Loss</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
a B phenotype and focal loss of Ki67 compartmentalisation in juvenile polyps with a SMAD4 germline mutation (p=0.006), but not in those with a BMPR1A mutation (p=0.131) (Table 5). However, when stratified by presence or absence of dysplasia no correlation between de-compartmentalisation of Ki67 and the B phenotype was seen in juvenile polyps with either a SMAD4 (p=1.000) or BMPR1A germline mutation (0.668). Focal loss of compartmentalisation was found in 2 sporadic juvenile polyps.

DISCUSSION

JPS is caused by a germline defect in SMAD4 or BMPR1A.(7, 8) Transgenic mice develop distinct JPS-like phenotypes depending on which of the JPS causing genes is altered. Smad4 mutant mice show hyperplastic or serrated epithelium and minor stromal overgrowth, whereas, Bmpr1a mutant mice (or inhibition of BMP signalling through transgenic expression of noggin) leads to polyps with reactive changes of the epithelium, crypt dilation and a prominent stromal compartment.(10-13) We investigated and compared the histological phenotype of juvenile polyps in patients with either a SMAD4 or BMPR1A germline mutation.

An overview of our findings organized per patient is provided in Table 6. In line with earlier reports, histological evaluation revealed a subset of JPS polyps featuring an epithelial phenotype (40%), different from classic juvenile polyps characterized by a prominent stromal compartment (60%).(3, 6) (Table 1) The epithelial phenotype was more prevalent in patients with a SMAD4 germline mutation whereas juvenile polyps from those with a BMPR1A germline mutation predominantly had classic juvenile polyps (p<0.001).

Evaluation of the crypt-stroma ratio revealed that juvenile polyps from patients with a SMAD4 germline mutation had a significantly higher crypt-stroma ratio compared to polyps from those with a BMPR1A germline mutation (p=0.001) indicating a higher crypt density in the former. (Figure 2) Subsequent classification into type A (crypt-stroma ratio<1.00) or type B (crypt-stroma ratio ≥1.00) confirmed our initial findings based on histology (p<0.001). (Table 2)

Similar frequencies of indefinite, low grade and high grade dysplasia in juvenile polyps from patients with either a SMAD4 or BMPR1A germline defect were found (Table 3), contradicting earlier reports of a more dysplasia prone intestinal phenotype in polyps with a SMAD4 germline mutation.(17, 18) Interestingly, 50% of all foci of low grade or high grade
Histological variations in juvenile polyposis

dysplasia in juvenile polyps with a *BMPR1A* germline defect were found in type A polyps whereas none of the type A polyps with a *SMAD4* germline defect contained dysplasia. (Table 4)

Evaluation of the Ki67 proliferation marker demonstrated that focal loss of compartmentalization of Ki67 could not be linked to an A or B phenotype when stratified by dysplasia. This is consistent with the notion that loss of compartmentalization of the proliferative zone is a general feature of dysplasia regardless of the underlying genetic defect.

*SMAD4* and *BMPR1A* are both key components of the TGF-ß/BMP signalling pathway maintaining homeostasis of the intestinal lining through processes of cellular proliferation (TGF-ß) and differentiation and apoptosis (BMP). Signal transduction takes place through phosphorylation of the type 1 transmembrane receptor kinase (i.e. BMPR1A) by the type 2 receptor. The activated type 1 receptor phosphorylates the pathway restricted SMAD2 and 3 (TGF-ß) or SMAD1,5 and 8 (BMP) which in complex with the common mediator SMAD4 are translocated to the nucleus where target gene transcription is regulated.(19)

Individuals with germline defects in *SMAD4* or *BMPR1A* and consequent disrupted TGF-ß/BMP signalling develop multiple hamartomatous malformations in the gastrointestinal tract. These hamartomas are often characterized by an abnormal stromal component suggesting a prominent role for the stroma in polyp formation. The polyp epithelium initially shows normal maturation, although inflammation is common and may cause reactive changes. Subsequent dysplastic progression of the epithelium has been proposed to be the result of the altered microenvironment.(20)

Recent murine studies provide evidence that conditional inactivation of Bmpr2 in the intestinal mesenchyme leads to development of hamartoma-like polyps, whereas conditional deletion of Bmpr1a in the epithelium showed elongation of the villi, but no de-novo crypt or polyp formation was observed.(21, 22) Consistent loss of heterozygosity (LOH) of the *BMPR1A* locus has thus far not been detected in the epithelium or stroma of JPS polyps carrying a *BMPR1A* germline mutation(8), although one study reports somatic loss of the 10q22 region exclusively in the lamina propria and not in the epithelium suggesting inactivation of *BMPR1A* might be a stromal event.(23)

Selective loss of Smad4-dependant signalling in T cells leads to a JPS-like phenotype reminiscent of what we described as an A type polyp with cystic spaces lined by columnar epithelium surrounded by abundant stroma.(24) Smad4 heterozygous mice on the other hand develop polyps with an epithelial phenotype (type B) and show LOH specifically in the epithelium of larger polyps.(10, 25) Likewise, LOH of the *SMAD4* locus occurs in the epithelium of juvenile polyps with a *SMAD4* germline mutation. The exact role of SMAD4 and timing of *SMAD4* inactivation in polyp initiation and progression remains poorly understood but it seems likely that these polyps develop mainly through an epithelial defect.(26)

Although the number of polyps in this assay is limited and findings may, therefore, be difficult to interpret, we propose that juvenile polyps in patients with a *SMAD4* germline defect may have a higher crypt density regardless of the dysplastic status. Alternatively, juvenile polyps in patients...
with a BMPR1A defect have a more as a classic juvenile polyp with a prominent stromal compartment. Crypt density in these polyps is initially low but may increase due to neoplastic change of the epithelium.

Investigation of the proliferative activity by Ki67 immunohistochemistry indicated that the difference in crypt density we encountered in juvenile polyps with a SMAD4 or BMPR1A germline mutation is not a result of altered proliferative activity.

We conclude that juvenile polyps in the setting of juvenile polyposis syndrome may exhibit distinct phenotypes. Patients with a SMAD4 germline mutation are more likely to juvenile polyps with an epithelial phenotype typified by a relatively high crypt density, whereas, individuals with a BMPR1A mutation are more often of the classic juvenile polyp phenotype with a prominent stromal compartment. Importantly, we find similar rates for all grades of dysplasia in juvenile polyps with either a SMAD4 or BMPR1A background.

REFERENCES

SMAD4 protein expression in polyps of juvenile polyposis mirrors genetic status but does not reflect neoplastic progression

Manuscript in preparation

W. Arnout van Hattem, Lodewijk A. A. Brosens, Wendy W. J. de Leng, Folkert H. Morsink, Fiebo J. W. ten Kate, Christine A. Iacobuzio-Donahue, Francis M. Giardiello, G. Johan A. Offerhaus

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SMAD4 Protein expression in polyps of juvenile polyposis mirrors genetic status but does not reflect neoplastic progression

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ABSTRACT Juvenile polyposis syndrome (JPS) can be caused by a germline defect in the SMAD4 tumor suppressor gene. Somatic inactivation of SMAD4 occurs in advanced stages of pancreatic cancer and colorectal cancer, and is accurately reflected by loss of SMAD4 immunohistochemical staining in tumor cells. We addressed the role of SMAD4 in polyp formation and subsequent progression to dysplasia by validating SMAD4 protein expression as marker of SMAD4 status in juvenile polyps with a SMAD4 germline defect. Methods. Twenty juvenile polyps with a SMAD4 germline defect and 38 controls were assessed for SMAD4 protein expression by immunohistochemistry (IHC). Areas of aberrant SMAD4 expression were laser microdissected and analysed for loss or somatic mutation of the SMAD4 wild-type allele using well established LOH and sequencing techniques. All polyps were graded for dysplasia and in similar fashion SMAD4 status was determined in microdissected dysplastic epithelium. Results. Nine polyps with a SMAD4 germline defect (45%) had focal loss of epithelial SMAD4 protein expression, five of which showed LOH in the remaining wild-type allele of the SMAD4 locus and two polyps with retention of heterozygosity revealed a somatic stop codon mutation upon sequencing. Remarkably, somatic inactivation of epithelial SMAD4 did not coincide with dysplastic change. Conclusions. SMAD4 IHC mirrors genetic status and may provide a highly specific screening tool in the molecular diagnosis of JPS. However, epithelial SMAD4 inactivation is not required for polyp formation and, moreover, occurs seemingly independent of dysplastic progression, conflicting with the proposed “gatekeeper” function of SMAD4 in JPS pathogenesis. Manuscript in preparation.

INTRODUCTION

Juvenile polyposis syndrome (JPS) 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.
A germline mutation in the SMAD4 or BMPR1A genes is found in 50% of patients. SMAD4 is a cytoplasmic co-mediator which forms heteromeric complexes with various receptor dependent SMADs. These complexes are translocated to the nucleus where they regulate DNA transcription. 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. This occurs either through intragenic mutation with loss of the wild-type allele (loss of heterozygosity, LOH) or deletion of both alleles (homozygous deletion).

The role of SMAD4 or BMPR1A in JPS polyp formation is poorly understood. According to the landscaper theory juvenile polyp growth is generated by a stromal defect. Homeostasis of the epithelium is disrupted by the abnormal microenvironment leading to de novo crypt formation and crypt distortion. On the other hand, SMAD4 seems to act in a true tumor suppressor fashion with evidence suggesting somatic inactivation of the SMAD4 wild-type allele in the epithelium drives polyp formation.

Different mechanisms of polyp formation may exist for polyps in individuals with either a SMAD4 or BMPR1A germline mutation.

In pancreatic cancer, somatic inactivation of SMAD4 is accurately mirrored by loss of immunohistochemical staining. As such, SMAD4 immunohistochemistry (IHC) may prove a valuable tool in the molecular diagnosis of JPS and, at the same time, could provide lead to the role of this gene in juvenile polyp formation and disease progression i.e., dysplasia. This is hampered by lack of studies demonstrating in a systematic manner a correlation between SMAD4 protein expression and SMAD4 status in JPS. For this reason we investigated SMAD4 protein expression by IHC and determined the corresponding SMAD4 status in juvenile polyps carrying a SMAD4 germline defect. We addressed the implications of our findings with regard to the role of SMAD4 in polyp formation and disease progression.

**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.

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% H2O2 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 haematoxylin.

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)

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 haematoxylin. 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 micosatellites: 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 GeneScanTM 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 IHC (Figure 1). Results are summarized in Table 1. Of 20 polyps with a SMAD4 germline defect, 9 (45%) showed focal reduction or loss of nuclear SMAD4 protein expression in the epithelium. In contrast,

<table>
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<tr>
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*Patient 2 and 3 and **patient 4 and 5 were from the same family*
none of the 11 polyps carrying a \textit{BMPR1A} germline mutation or any of the 27 sporadic juvenile polyps had aberrant SMAD4 expression (data not shown).

\textbf{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 \textit{SMAD4}, i.e. LOH or a somatic point mutation, in polyps with a \textit{SMAD4} germline mutation. LOH analysis of the \textit{SMAD4} locus was performed using 4 microsatellite markers. Nine polyps were assessed, all carrying a germline mutation in \textit{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 \textit{SMAD4}, including at least one of two markers closest to the \textit{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 \textit{SMAD4}, likely to result in truncation of the protein. In polyp 4.1 and 5.1 with a hemizygous germline deletion of \textit{SMAD4} and immunohistochemical loss of the SMAD4 protein, LOH markers closest to \textit{SMAD4} were non-informative, although more distant markers did show LOH.

\textbf{Dysplasia and genetic status of \textit{SMAD4}.} With aberrant epithelial SMAD4 protein expression reflecting the occurrence of a somatic event in the \textit{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

<table>
<thead>
<tr>
<th>Patient</th>
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ROH, retention of heterozygosity (white); LOH, loss of heterozygosity (black); Ni, non-informative (gray)

*Patient 2 and 3 and **patient 4 and 5 were from the same family
20 polyps with a SMAD4 germline defect low grade dysplasia was 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 and 6.3 had focal high grade dysplasia 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 IHC accurately mirrors SMAD4 status, we aimed to exclude somatic inactivation of SMAD4 in dysplastic juvenile polyp tissue with a normal SMAD4 staining pattern. 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.

**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 colorectal cancer. In pancreatic cancer, loss of immunohistochemical labeling in tumor cells reflects with high accuracy the genetic status of SMAD4.(18)

The role of SMAD4 in JPS polyp formation is poorly understood. Investigators supporting the

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**Table 3. Dysplasia in juvenile polyps**

<table>
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<td>8.4b</td>
<td>low grade</td>
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*Patient 2 and 3 and **patient 4 and 5 were from the same family*
landscaper theory postulate that juvenile polyps arise primarily due to a stromal defect. The abnormal stroma causes disruption of normal development and regeneration of the overlying epithelium.\(^{(14,15)}\) In contrast, other studies provide evidence that LOH of \textit{SMAD4} in the epithelium initiates polyp growth suggesting \textit{SMAD4} acts in a classic tumor suppressor fashion in JPS polyps.\(^{(16,17)}\)

In this study we illuminate the role of \textit{SMAD4} in juvenile polyp formation by investigating \textit{SMAD4} protein expression and \textit{SMAD4} status in juvenile polyps. In almost half of all polyps with a \textit{SMAD4} germline defect focal reduction or loss of nuclear \textit{SMAD4} expression in the epithelium was seen. In contrast, no aberrant \textit{SMAD4} expression was seen in polyps from patients with a \textit{BMPR1A} mutation, or in any of the sporadic juvenile polyps.

Aberrant \textit{SMAD4} immunostaining in JPS showed clear correlation with somatic inactivation of the \textit{SMAD4} gene. Out of 9 polyps with aberrant \textit{SMAD4} expression five polyps had LOH and two polyps had a somatic stop codon mutation resulting in truncation of the \textit{SMAD4} protein. Two remaining

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{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).}
\end{figure}
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. 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.

These results demonstrate that aberrant nuclear SMAD4 protein expression in a juvenile polyp is indicative of somatic inactivation through LOH or somatic mutation as has previously been shown in pancreatic cancer. Furthermore we showed that the observation of reduction or loss of epithelial SMAD4 expression in the polyps of individuals with JPS is highly specific for the presence of a SMAD4 germline defect, ranging from missense mutations to hemizygous deletions. Nevertheless, since focal loss of epithelial SMAD4 expression was found only in a subset of juvenile polyps with a SMAD4 germline mutation it is implied 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 during polyp growth. This concurs with the findings of Xu et al, describing LOH of SMAD4 only in larger antral tumors in Smad4 heterozygous mice as opposed to smaller tumors, indicating a late event in tumor progression but not an obligate step in tumor initiation.

One study by Kim et al. reported that targeted inactivation of Smad4 in stromal T-cells leads to a JPS-like phenotype and epithelial cancers of the gastrointestinal tract in mice, whereas inactivation of Smad4 in the epithelium does not. Although our results argue that inactivation of SMAD4 occurs in the epithelium and not in the stroma of juvenile polyps, we cannot rule out that haploinsufficiency of SMAD4 in cells of the stromal compartment contributes to juvenile polyp initiation as per the landscaper theory. In fact, our finding that epithelial inactivation of SMAD4 is not required for polyp initiation renders it possible that this is indeed the case.

With regard to the role of SMAD4 in disease progression, we showed that in juvenile polyps with a SMAD4 germline defect neoplastic change of the epithelium is not necessarily initiated by inactivation of the second allele of the SMAD4 tumor suppressor gene (Figure 2a), disagreeing with the proposed gatekeeper function of SMAD4 in JPS. Rather these results are suggestive of a pathway leading to dysplasia in JPS with somatic inactivation of SMAD4 as a late event during neoplastic progression. 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.

On the other hand, somatic inactivation of SMAD4 also occurred in epithelium without morphological features of dysplasia (Figure 2b). In some cases this was observed on the very same section containing areas of low grade dysplasia with normal SMAD4 expression (Figure 2e).

Consequently, the role of SMAD4 in neoplastic progression of juvenile polyps remains unclear. Although SMAD4 inactivation is seen in a clonal pattern it occurs seemingly independent of recognizable dysplastic change. Perhaps the most likely scenario is one in which two pathways leading to dysplasia occur parallel. In juvenile polyps carrying a SMAD4 germline defect there
exists an increased selective pressure that could lead to early stage inactivation of this gene. This molecular neoplasia can be visualized by loss of SMAD4 immunohistochemical staining but may on microscopy of the H&E section not yet be recognizable as such. Alternatively, selective pressure may also be increased on other pathway genes capable of initiating neoplastic change. This could be a direct result of the SMAD4 germline defect or be due to the abnormal microenvironment present in juvenile polyps. Somatic inactivation of SMAD4 may then occur at later stage, possibly leading to acceleration of the neoplastic progression.

In summary, we found that SMAD4 immunohistochemistry accurately reflects SMAD4 status in polyps of the juvenile polyposis syndrome and may provide a reliable screening tool in the molecular diagnosis of juvenile polyposis syndrome. Somatic inactivation of SMAD4 occurs in the epithelium but is not likely to initiate polyp formation. Dysplasia of the epithelium may take place independent of epithelial SMAD4 inactivation. The exact role and timing of SMAD4 inactivation in tumorigenesis requires further investigation.

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Increased cyclooxygenase-2 expression in juvenile polyposis
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Ari Ristimäki, Francis M. Giardiello, G. Johan. A. Offerhaus

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Increased cyclooxygenase-2 expression in juvenile polyposis

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ABSTRACT 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 24 genetically well defined JPS patients and 26 patients with 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.

Conclusions. 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. 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 (JPS) is an autosomal dominant condition characterized by multiple juvenile polyps throughout the gastrointestinal tract. In JPS, juvenile polyps often contain relatively less stroma, fewer dilated crypts and more epithelial proliferative activity than their sporadic counterparts.
Chapter eight

Juvenile polyps are not associated with an increased risk of gastrointestinal malignancy. 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%. Germline mutations in either SMAD4 or BMPR1A are found in 50-60% of JPS cases. The TGF-β co-receptor Endoglin (ENG) has been suggested as a predisposition gene for JPS, although this is still under debate. 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. 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. 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. 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).

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. Nucleo-cytoplasmic translocation is necessary for HuR activation. C/EBP-β is a transcription factor regulating proliferation and differentiation, capable of inducing COX-2 expression. Increased C/EBP-β correlates with invasiveness in human CRC.

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 Center (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. JPS was defined as patients with 3 or more
juvenile polyposis and/or a well established familial segregation and/or a germline mutation in one of the known JPS genes. Patients with sporadic juvenile polyposis had a single sporadic polyp incidentally found and no family history of juvenile polyposis. Sporadic juvenile polyposis 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 polyposis (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 germ-line 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 (0.6mm 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.

**Table 1. Scoring of immunohistochemistry**

<table>
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<th>Antibody</th>
<th>Scoring</th>
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<tr>
<td>COX-2</td>
<td>low 0 - no staining</td>
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<td></td>
<td>1 - very weak diffuse cytoplasmic staining</td>
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<td></td>
<td>2 - moderate to strong granular cytoplasmic staining in 10-50% of cells</td>
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<td>high 3 - strong intensity in &gt;50% of cells</td>
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<td>HuR</td>
<td>Nuclear and cytoplasmic staining was scored separately as positive (high) or negative (low) in epithelial cells.</td>
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<tr>
<td>C/EBP-β</td>
<td>Nuclear staining &gt;25% of epithelial cells</td>
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**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 deter-mined by the highest polyp score found in that particular patient.

**Statistical analysis.** Statistical analysis was performed using the SPSS 15.0 software package. A Chi-Squared (χ2) 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 polyposis 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 analysed. 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.

**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 significantly higher in JPS patients compared to 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

<table>
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<th>JPS</th>
<th>Sporadic juvenile polyps</th>
<th>JPS versus sporadic</th>
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<tr>
<td>COX-2</td>
<td>n = 24</td>
<td>IHC = 54% high</td>
<td>n = 24</td>
</tr>
<tr>
<td>HuR</td>
<td>n = 23</td>
<td>IHC = 57% high</td>
<td>n = 24</td>
</tr>
<tr>
<td>C/EBP-β</td>
<td>n = 22</td>
<td>IHC = 96% pos</td>
<td>n = 23</td>
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n, number of patients; IHC, immunohistochemistry

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

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).
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 increase in 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 markers.** 13 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).

**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.

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.

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<th>Table 3. COX-2 expression in germline mutation carriers versus non-germline mutation carriers</th>
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<td><strong>Germline mutation</strong></td>
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<td>SMAD4</td>
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<td>BMPR1A</td>
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n, number of patients analyzed; IHC, immunohistochemistry
A subset of JPS patients had polyps with dysplastic foci but patients with sporadic juvenile polyps did not. Recently, Brasowski et al. 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. 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. 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. 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. 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 noted only 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 capable of inducing COX-2 expression and present in normal colorectal epithelial cells within the proliferative zone. 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.

REFERENCES
Summary and concluding remarks

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Department of Pathology, University Medical Center, Utrecht, The Netherlands.
Summary and concluding remarks

W. Arnout van Hattem

Studies on the hereditary colorectal cancer susceptibility syndromes have contributed considerably to our current understanding of colorectal tumorigenesis. In particular FAP and HNPCC (Lynch syndrome) have provided insight into two major pathways leading to colorectal cancer. The genes and corresponding mechanisms underlying these hereditary conditions have come to be known as “gatekeeper” and “caretaker” respectively. A third pathway has been proposed and is based largely on observations of juvenile polyposis syndrome.

JPS is a rare condition characterized by the presence of multiple histologically distinct juvenile polyps in the colorectum. Juvenile polyps are hamartomatous malformations of the intestinal mucosa featuring a large stromal compartment which in cellular structure is reminiscent of the lamina propria but without muscularis mucosae. The stroma is often inflamed and crypts may be dilated (retention cysts) and distorted. The epithelial lining of the crypts which is generally rich in mucus secreting goblet cells shows normal maturation and is in essence non-neoplastic, contrary to the adenomatous polyps as seen in FAP patients. The epithelium may however progress to neoplasia. In fact, variable estimates of gastrointestinal cancer risk in JPS have been provided based on evidence from a limited number of case series and collections of literature case reports. However, a formal risk assessment of gastrointestinal cancer in JPS patients has not been reported.

In chapter 3 we performed a person-year-analysis to define the magnitude of gastrointestinal cancer risk in a cohort of JPS patients. A significantly elevated relative risk (34.0) of colorectal cancer was found in male and female patients. Perhaps more illustrative was the cumulative life time risk for colorectal cancer of 39%. With a mean age of 43.9 the elevated colorectal cancer risk appeared to be associated with a younger age of onset. Although extracolonic gastrointestinal cancers, including gastric, small bowel and pancreatic cancer have been reported in JPS, no such cases were present in our cohort. Consequently, a risk assessment for these malignancies could not be performed. Further investigation is necessary to define the risk of gastrointestinal cancer in relation to the underlying genetic defect.

JPS is an autosomal dominant condition caused by germline mutation of SMAD4 or BMPR1A of the TGF-β/BMP signalling pathway. One study reported mutation of the TGF-β co-receptor ENG in two patients with JPS but this has not been confirmed. Also PTEN, originally linked to Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS), has been associated with JPS, but others have disputed this. Most likely, PTEN mutations in patients with a juvenile polyposis phenotype represent CS or BRRS patients that have not yet expressed the extraintestinal clinical features of these conditions.

Based on conventional sequencing techniques, germline mutations are found in an estimated 30-40% of JPS cases. These techniques however,
detect only point mutations and small deletions, ignoring large scale genomic deletions of SMAD4 or BMPR1A as possible cause of JPS. Multiplex ligation-dependant probe amplification (MLPA) is a novel technique capable of detecting copy number changes in genomic DNA sequences at exon level. Chapter 4 describes a comprehensive genetic analysis of a well documented group of unrelated JPS patients using both conventional techniques as well as MLPA to address the role of large scale deletions of SMAD4, BMPR1A, ENG and PTEN in JPS pathogenesis.

In nearly 50% of patients a germline defect was found in SMAD4, BMPR1A or BMPR1A and PTEN. Two thirds of these defects were detected by direct sequencing and one third by MLPA, indicating that large genomic deletions are not uncommon in JPS. Taking into consideration the user friendliness and reliability of this technique, MLPA provides a valuable adjunct in JPS diagnosis. Germline mutations in ENG were not found in this assay and its potential role in JPS pathogenesis thus remains doubtful.

Although the detected mutation rate in JPS was increased to 50% by use of MLPA analysis, there still remains a large group of patients diagnosed with JPS based on clinical and histological findings but in whom no germline defect could be found. This number can in part be reduced by applying more stringent diagnostic criteria but the notion of other genes predisposing to JPS seems inevitable. Recent studies have ruled out several candidate genes most of which are active in the TGF-β/BMP pathway, including SMAD1, SMAD2, SMAD3, SMAD5, SMAD7, BMPR2, BMPR1B and ACVRL1.

TGFBRII is a key component of the TGF-β signalling pathway and has a well established role in colorectal carcinogenesis. In chapter 5 we hypothesized TGFBRII may be involved in JPS pathogenesis. Therefore, direct sequencing and MLPA analysis of TGFBRII was performed on JPS patients without documented germline mutations in SMAD4, BMPR1A or BMPR1A and PTEN.

We detected no pathogenic mutations of the TGFBRII gene. Although sequencing of the promoter region or investigation of protein expression via immunohistochemistry may prove interesting, we are inclined to conclude that, based on these results, it is unlikely TGFBRII mutations in the germline are causally involved in JPS pathogenesis.

Despite advances at the molecular level, detection of a germline mutation in SMAD4 or BMPR1A may still only confirm clinical suspicion. Histological findings remain of crucial importance in the identification of juvenile polyps and setting the initial diagnosis. Although juvenile polyps generally share common histological features such as stromal expansion, surface erosion, and cystically dilated glands, phenotypical variations have been described. Most notably reports have been made of a more adenovillous appearance.

Interestingly, targeted inactivation of the JPS causing genes in mice revealed that distinctions in phenotype may be related to the underlying genetic defect. In JPS this relation has thus far remained unclear, as well as the implications thereof with regard to the mechanisms of polyp formation and subsequent dysplastic change. Chapter 6 provides a phenotype analysis of JPS polyps with a documented SMAD4 or BMPR1A germline defect.

In line with existing literature, we found a majority of polyps presented as “classic” juvenile
polyps with an expanded stromal compartment and low crypt density, whereas a subset of polyps contained relatively less stroma and higher crypt density. The latter “epithelial” phenotype was found predominantly in juvenile polyps with a SMAD4 germline mutation whereas the classic phenotype was more common among juvenile polyps with a BMPR1A germline mutation. The distinction in crypt density could not be ascribed to expanded cell cycle activity as revealed by immunostaining of the proliferation marker Ki67.

Frequencies of low grade or high grade dysplasia were similar in juvenile polyps with a SMAD4 or BMPR1A background contradicting earlier reports of a more aggressive intestinal phenotype in polyps with a SMAD4 germline defect. Importantly, in polyps carrying a BMPR1A germline defect, focal low grade and high grade dysplasia was equally present in the classic juvenile polyps as well as in epithelial type juvenile polyps, stressing in particular the neoplastic potential of the, in histological appearance, more “innocent” classic juvenile polyp.

Inhibition of stromal BMP signalling in the intestines of mice leads to the formation of polyps with an expanded stromal compartment, ectopic crypt formation and crypt dilation much like the classic juvenile polyps found in JPS. In line with the proposed landscaper model, these murine polyps eventually display focal dysplasia of the overlying epithelium. Although consistent loss of heterozygosity (LOH) of the BMPR1A locus has thus far not been detected in the epithelium or stroma of JPS polyps carrying a BMPR1A germline mutation, one study provides evidence that somatic inactivation of BMPR1A occurs in the lamina propria. However, further investigation is needed to illuminate BMPR1A status during consecutive stages of JPS pathogenesis.

SMAD4 is generally considered a classic tumor suppressor gene and is inactivated in advanced stages of pancreatic and colorectal cancer. Given the initial non-neoplastic nature of juvenile polyps it seems unlikely that somatic inactivation of SMAD4 is a prerequisite for polyp formation. However, it is conceivable neoplastic change in juvenile polyps with a SMAD4 germline mutation is initiated by somatic inactivation of SMAD4 thus acting as a gatekeeper for neoplasia rather than as a landscaper. In chapter 7 we addressed the role of SMAD4 in polyp formation and subsequent progression to dysplasia by validating SMAD4 protein expression as marker of SMAD4 status in juvenile polyps with a SMAD4 germline defect.

Loss of SMAD4 expression was observed in the epithelium of a subset of juvenile polyps with a SMAD4 germline mutation, but not in the stroma. Molecular analysis revealed that aberrant SMAD4 immunohistochemistry accurately reflected SMAD4 status, i.e. LOH or somatic mutation of the wild-type allele, indicating that somatic inactivation of SMAD4 is indeed not necessary for the initiation of juvenile polyp growth.

Remarkably, somatic inactivation of epithelial SMAD4 did not consistently coincide with visible dysplastic change. Loss of SMAD4 expression was observed in several polyps that did not appear dysplastic and, conversely, focal high grade dysplasia in some instances occurred without accompanying loss of SMAD4 expression.

Thus the role of SMAD4 in dysplastic progression of juvenile polyps with a germline mutation in SMAD4 remains unclear. It seems
more than one pathway may be involved. Increased selective pressure on \textit{SMAD4} could lead to early epithelial inactivation of this gene. This molecular neoplasia can be visualized by loss of \textit{SMAD4} immunohistochemical staining but may on microscopy of the H&E section not yet be recognizable as such.

Alternatively, in some cases somatic inactivation of \textit{SMAD4} may occur in later stages of dysplasia, perhaps leading to acceleration of the neoplastic progression. Dysplastic change may in this scenario be initiated by other genes experiencing increased selective pressure as a result of the \textit{SMAD4} germline defect. Which genes are implied here and whether they act via the gatekeeper or landscaper mechanism remains to be determined. Certainly, focus should lie on genes of the conventional adenoma–carcinoma sequence, i.e. \textit{APC} and downstream targets thereof.

In chapter 8 we explored COX-2 expression in juvenile polyps. COX-2 is a well known target for chemoprevention. Up-regulation of COX-2 occurs in consecutive stages of the adenoma–carcinoma pathway and is a direct effect of Wnt activation, which, in the normal colonic mucosa is suppressed by BMP signalling. Using selective COX-2 inhibitors, FAP patients experience a reduction in number and size of colorectal adenomas.

We found that COX-2 expression was significantly higher in JPS polyps compared to sporadic juvenile polyps, in particular in polyps carrying a \textit{BMPR1A} germline mutation. In this light, investigation of the effect of COX-2 inhibitors on polyp size and disease progression in existing mouse models mimicking JPS, and subsequently in JPS patients may prove exciting.

In conclusion, although subject of debate in past decades, polyps of juvenile polyposis syndrome are undoubtedly preneoplastic lesions and should in the clinical setting be approached as such. Additional investigation, however, is needed to define the risk of extra-colonic gastrointestinal cancer in JPS and also to determine the cancer risk in relation to the underlying germline defect.

\textit{SMAD4} and \textit{BMPR1A} are attributable for a substantial percentage of JPS cases. Many other genes have been excluded as cause of JPS, including \textit{TGFBRII}, but this does not rule out the possibility of additional genes predisposing to JPS. Genes associated with conditions that show considerable overlap with JPS in a clinical and histological sense, such as IBD, should, in this search, be considered.

Histological characterization reveals distinct juvenile polyp phenotypes associated with the underlying genetic defect, suggesting different mechanisms of disease progression may be involved depending on the affected gene. As part of the TGF-\(\beta\)/BMP signalling pathway \textit{BMPR1A} and \textit{SMAD4} are involved in maintaining the homeostasis of the intestinal lining through processes of cellular proliferation, differentiation and apoptosis. Haploinsufficiency of either of these genes is likely to promote polyp formation resulting in numerous juvenile polyps in the gastrointestinal tract. Subsequent dysplastic change of the polyp epithelium may develop indirectly as result of an altered microenvironment or directly through somatic inactivation of the remaining wild-type allele of the affected gene in the polyp epithelium.

Evidence indicates both mechanisms may be applicable to juvenile polyps with a \textit{SMAD4}
germline mutation. Somatic inactivation of SMAD4 occurs in some cases as early event, even before morphologic features of dysplasia become microscopically evident. In other instances, however, high grade dysplasia occurs without early somatic inactivation of SMAD4. The genes and mechanisms driving dysplastic change in these polyps remains to be determined. The conventional adenoma-carcinoma sequence is in this regard a likely candidate and future investigation should focus on the APC gene and its downstream targets.

Investigation of the genetic status of BMPR1A in juvenile polyps with a BMPR1A germline mutation has proven more difficult and is hampered by the lack of a reliable immunohistochemical staining of the protein product of this gene. Immunohistochemistry of the pSMADs1, 5 and 8, however, provides a functional read out of active BMP signalling and may be of aid in this matter.

Finally, the effect of COX-2 inhibiting chemoprevention should be examined as this may have considerable clinical implications with regard to disease burden during infancy and childhood, and the risk of gastrointestinal cancer later in life.
Nederlandse samenvatting
Dikkedarmkanker is de derde meest voorkomende vorm van kanker in Nederland. Jaarlijks wordt darmkanker bij ongeveer 10.000 mensen gediagnostiseerd. De helft hiervan komt te overlijden aan de gevolgen van deze ziekte.

Zoals bij andere vormen van kanker komt ook darmkanker voor in niet-erfelijke vorm (sporadisch) en erfelijke vorm (familiair). In tegenstelling tot wat vaak wordt verondersteld leidt een ruime meerderheid van de patiënten met darmkanker aan sporadische, dus niet-erfelijke darmkanker. Slechts bij 20% van de patiënten is er sprake van familiaire darmkanker. En ondanks familiaire aanleg wordt bij driekwart van deze patiënten geen erfelijke afwijking gevonden. Bij het overige kwart, dus slechts in 5% van de gevallen, kan de aandoening worden herleid tot een aantoonbare erfelijke afwijking.

Zo’n erfelijke afwijking betekent meestal een verandering (mutatie) in een specifiek gen. Afhankelijk van welk gen is aangedaan ontwikkelen patiënten een specifiek ziektebeeld (syndroom). Tot nu toe is een klein aantal syndromen bekend dat gepaard gaat met een verhoogde kans op darmkanker. Familiaire adenomateuze polyposis (FAP) en hereditaire non-polyposis colorectaal carcinoom (HNPCC) zijn daarvan de meest voorkomende. Zeldzamer syndromen zijn Peutz-Jeghers syndroom, vernoemd naar de Haagse internist Jan Peutz (1886-1957), en juveniele polyposis syndroom (JPS). Hoofdstuk 2 geeft een overzicht van de klinische en genetische aspecten van FAP, JPS en Peutz-Jeghers syndroom.

De darm wordt aan de binnenzijde (naar de darmholte toe) bekleed door een cellaag met een laagdikte van een cel (het epitheel). Hieronder bevindt zich een dikke laag steunweefsel (het stroma). Deze twee celcompartimenten worden van elkaar gescheiden door het basaal membraan. Een tumor (gezwel) in de darm groeit vrijwel altijd vanuit het epitheel compartiment. In eerste instantie groeit een tumor op goedaardige wijze, wat wil zeggen dat de tumor in zijn groei beperkt blijft tot het compartiment waarbinnen deze is ontstaan. Dit betekent dat een tumor die uitgaat van het epitheel dus niet het onderliggende stromale compartiment in groeit, met als gevolg dat de tumor, naarmate deze in omvang toeneemt, gaat uitstulpen in de darmholte. Zo’n uitstulping heet ook wel een poliep.

Een poliep kan uitgroeien tot een kwaadaardig (maligne) tumor, ofwel kanker. Dit gebeurt wanneer de tumor door het basaal membraan heen groeit en het stromale compartiment binnendringt (invasieve groei). De tumorcellen kunnen in deze fase tevens bloed- en lymfevaten binnendringen en zich zodoende uitzaaien naar andere organen.

Kanker ontstaat dus niet plotseling maar ontwikkelt zich gedurende een stapsgewijs proces. Ten grondslag aan dit jarenlange proces ligt een aantal (vijf tot zeven) zich in de loop der tijd opstapelende genmutaties. Welke genen zijn aangedaan bepaalt hoe de tumor zich ontwikkelt.
Van elk gen erf een kind twee kopieën (allelen); een allel van de vader en een allel van de moeder. Er bestaat een groep genen, tumor suppressor genen, welke een remmende werking hebben op de celdeling. De twee allelen van een tumor suppressor gen kunnen worden vergeleken met de twee remmen van een fiets. Als een van de twee allelen inactief wordt als gevolg van een mutatie is er nog altijd een allel over dat als rem functioneert. Echter, de druk op het resterende allel neemt als gevolg hiervan wel toe. Als dan het tweede allel wegvalt is er in feite geen rem meer op de celdeling en kan de cel waarin dit plaatsvindt een groeivoordeel ondervinden ten opzichte van de omliggende cellen. In de klinische pathologie wordt de term “dysplasie” gebruikt om deze snel delende cellen aan te duiden. Door het groeivoordeel ontstaat er een tumor. In de tumorcellen kunnen nieuwe mutaties ontstaan. Elke additionele mutatie zorgt voor extra groeivoordeel (en een hogere graad van dysplasie) ten opzichte van omliggende cellen. Wanneer een tumor voldoende mutaties heeft ondergaan kan deze een invasief groeipatroon gaan vertonen. Een niet-invasieve tumor in de darm heet ook wel een adenoom. Wordt deze invasief, dan heet dat carcinoom (kanker).

Het idee van een dergelijk tumor-progressie-model is grotendeels gebaseerd op bevindingen bij het bestuderen van de erfelijke darmkanker-syndromen FAP en HNPCC en staat bekend als de “adenoma-carcinoma sequentie”.

Patiënten die lijden aan een erfelijk syndroom dat gepaard gaat met een verhoogde kans op kanker erven van een van hun ouders meestal een gemuteerd allel van een specifiek tumor suppressor gen. Zij zijn dus bij geboorte als het ware al een “rem” kwijt. Er bestaan meerdere tumor suppressor genen en elk van deze genen leidt via een verschillend mechanisme tot tumorgroei. Het bestuderen van erfelijke darmkankersyndromen biedt inzicht in het werkwingsmechanisme van deze genen, welke vaak ook een rol spelen bij het ontstaan van sporadische darmkanker.

FAP patiënten hebben een aangeboren mutatie in een allel van het APC gen. Als het tweede allel van dit gen wegvalt leidt dit direct tot een toename in celdeling en dus tumorgroei. FAP patiënten ontwikkelen daardoor vaak ontelbare adenomen in de dikke- en dunne darm. Het verhoogde risico op darmkanker schuilt hem in het grote aantal tumoren in de darm van deze patiënten. De kans dat een van deze tumoren kwaadaardig wordt is groter dan ware het slechts enkele tumoren, hetgeen regelmatig voorkomt in de normale bevolking. Dat het wegvallen van beide allelen van APC direct leidt tot tumorgroei maakt het een klassiek tumor suppressor gen. Het wordt ook wel de “atekeeper” (poortwachter) genoemd van de adenoma-carcinoma sequentie.

Een ander type tumor suppressor gen is gemuteerd in patiënten met HNPCC. Deze patiënten erven een gemuteerd allel van een van de zogenaamde “mismatch repair” genen (MMR). Deze genen zijn, zoals de naam reeds suggereert, betrokken in het DNA-schade-herstel systeem, dat
fouten (mutaties) in het DNA moet voorkomen tijdens de celdeling. Door hun rol in het
onderhoud van DNA worden de MMR genen ook wel “caretaker” genen genoemd. Inactivatie
van een van de MMR genen resulteert in een verslechtering van het DNA-schade-herstel
mechanisme hetgeen tot gevolg heeft dat er een grote toename is in het aantal mutaties dat
optreedt in het DNA tijdens de celdeling. Deze mutaties kunnen plaatsvinden in genen die een
belangrijke rol spelen bij de stapsgewijze tumorprogressie. Patiënten met HNPCC ontwikkelen,
in tegenstelling tot FAP patiënten, tumoren die in aantal vergelijkbaar zijn met de normale
bevolking. Echter, door het grote aantal mutaties in hun DNA groeien deze tumoren veel sneller
uit tot kanker.

Een mogelijk derde mechanisme dat leidt tot darmkanker wordt beschreven aan de hand van
onderzoek naar het juveniele polyposis syndroom (JPS). Patiënten met JPS ontwikkelen vaak op
zeer jonge leeftijd (in de eerste tien tot twintig levensjaren) vele poliepen in de dikke darm.
Deze poliepen zijn echter van andere aard dan de adenomateuze poliepen van FAP patiënten.
FAP patiënten erven een gemuteerd allel van het APC gen. Pas als het tweede allel wegvalt is er
geen rem meer op celdeling en ontstaat er een tumor (adenoom). JPS wordt veroorzaakt door
een aangeboren mutatie in een allel van SMAD4 of BMPR1A. Het hebben van een gemuteerd
allel van een van deze twee tumor suppressor genen lijkt voldoende om een tumor te doen
ontstaan. Het tweede allel is dus nog intact. Deze tumor ontstaat niet door ongeremde
celdeling (immers, een rem functioneert nog naar behoren), maar is eerder een architecturale
afwijking van het, in principe uit normaal delende en uitrijpende cellen bestaande,
darmweefsel. Dergelijke tumoren worden hamartomen genoemd. Het onderliggende
mechanisme waardoor hamartomen ontstaan, en hoe het kan dat patiënten met JPS een
verhoogde kans op kanker hebben is grotendeels onbekend.

Een gangbare hypothese is het “landscaper” model. In tegenstelling tot adenomen (die uitgaan
van het epitheel), bevatten hamartomen relatief veel stroma. Verondersteld wordt dat door een
defect in het stroma (bijvoorbeeld het verlies van het tweede allel van SMAD4 of BMPR1A) de
communicatie tussen stromacellen en epitheelcellen verstoord wordt. Hierdoor kunnen
epitheelcellen zich abnormaal gaan gedragen en dysplastisch worden. Dit in eerste instantie
laaggradig dysplastisch epitheel kan dan via het eerder beschreven tumor progressie model
vorderen tot hooggradige dysplasie en vervolgens invasieve kanker. Of het daadwerkelijk op
deze wijze verloopt is echter nog niet duidelijk, net als nog niet bekend is welke genen
betrokken zijn bij de stapsgewijze progressie. In dit proefschrift proberen we een aantal van
deze vragen te beantwoorden.

Dat JPS patiënten een verhoogd risico hebben om darmkanker te ontwikkelen was reeds
bekend. Echter, de grootte van dit kankerrisico was nog niet eerder vastgesteld. Om die reden
hebben wij een risicoanalyse uitgevoerd (een person-year analysis), beschreven in hoofdstuk 3.
Hieruit bleek dat patiënten met JPS een verhoogd risico op darmkanker hebben van bijna 39% ten opzichte van de normale bevolking. Net als bij andere erfelijke darmkankersyndromen ontwikkelen JPS patiënten darmkanker op relatief jonge leeftijd (gemiddeld 43,9 jaar). Deze resultaten hebben directe klinische implicaties met betrekking tot het screenen en behandelen van JPS patiënten.

Juveniele polyposis syndroom wordt veroorzaakt door erfelijke mutaties in SMAD4 of BMPR1A. Maar op basis van eerdere studies kan slechts 30-40% van de gevallen worden toegeschreven aan een mutatie in een van deze genen tezamen. In de overige 60% van klinische JPS patiënten wordt vooralsnog geen genetisch defect gevonden. In deze studies is gebruik gemaakt van conventionele technieken om de DNA sequentie van de betreffende genen te ontrafelen, om zodoende vast te stellen of er een mutatie in een van de allelen heeft plaatsgevonden. Deze techniek (sequencen) is uitstekend geschikt om kleine mutaties mee op te sporen, maar laat grote veranderingen onderbelicht. In hoofdstuk 4 beschrijven wij de rol van grote genomische veranderingen in het ontstaan van juveniele polyposis syndroom aan de hand van het conventionele sequencen en een nieuwe techniek, MLPA. Op deze manier hebben wij in de helft van 27 JPS patiënten een mutatie in SMAD4 of BMPR1A kunnen aantonen, waarvan een derde werd gevonden door middel van MLPA.

Ondanks deze verbetering met betrekking tot het in beeld brengen van de genetische defecten die ten grondslag kunnen liggen aan juveniele polyposis syndroom, wordt niet verholpen dat bij 50% van JPS patiënten geen onderliggend defect gevonden wordt. Mogelijk zijn er andere genen, naast SMAD4 en BMPR1A, die juveniele polyposis syndroom kunnen veroorzaken. In hoofdstuk 5 onderzochten we een mogelijke rol van het TGFBRII gen bij het ontstaan van juveniele polyposis syndroom. TGFBRII is een gen dat deel uitmaakt van de TGF-β/BMP signaleringsroute, waarin ook SMAD4 en BMPR1A actief zijn. Door middel van sequencen en MLPA hebben wij 19 patiënten onderzocht die op basis van klinische gegevens verdacht zijn voor JPS, maar waarin geen mutatie in SMAD4 of BMPR1A is gevonden. Wij hebben geen mutatie gevonden in TGFBRII en het is daarmee onwaarschijnlijk dat TGFBRII een rol van betekenis speelt bij het ontstaan van juveniele polyposis syndroom.

Weefselleer (histologie) vult een sleutelpositie in de moderne diagnostiek. In toenemende mate vormt de moleculaire genetica een aanvulling hierop. Deze ontwikkeling maakt het mogelijk nieuwe inzichten te verwerven met betrekking tot de histologie, in het licht van de moleculair genetische achtergrond. Juveniele poliepen zijn histologisch goed herkenbaar door een aantal overeenkomstige eigenschappen. Zo is er in een juveniele poliep meestal sprake van een duidelijke toename in omvang van het stroma ten opzichte van het epitheel, vormen zich cysteuze holtes die gevuld zijn met slijm, en is er ontsteking van het stroma en epitheel. Bovendien is het oppervlak van juveniele poliepen vaak geërodeerd (vergelijkbaar met een
oppervlakkige schaafwond met van de huid). Ondanks overeenkomsten blijkt uit eerdere studies dat juveniele poliepen histologisch toch sterk kunnen verschillen. Als variant op het klassieke beeld zoals hierboven beschreven wordt ook melding gemaakt van een juveniele poliep type met een hogere epitheel dichtheid en zonder erosie van het oppervlak. Bevindingen op basis van experimenten met muizen waarin ofwel Smad4 of Bmpr1a kunstmatig gedeactiveerd is, lijken te suggereren dat deze histologische verschillen gerelateerd zouden kunnen zijn aan het onderliggend genetisch defect. Hiertoe bestuderen we in hoofdstuk 6 het histologisch beeld en de epitheel-stroma verhouding van juveniele poliepen afkomstig van een groep JPS patiënten waarvan de gen mutatie bekend is. Hieruit blijkt dat JPS patiënten met een BMPR1A mutatie met name poliepen lijken te ontwikkelen die voldoen aan het klassieke beeld, terwijl JPS patiënten met een SMAD4 mutatie voornamelijk poliepen hebben die doen denken aan de variant met een hogere epitheel dichtheid. De kenmerken van deze laatste groep poliepen doen vermoeden dat deze poliepen veel eens een agressiever groeipatroon zouden kunnen vertonen. Echter, beide juveniele poliep typen blijken in gelijke mate verschillende stadia van dysplasie te ontwikkelen, hetgeen suggereert dat beide typen vergelijkbare potentie hebben om tot kanker uit te groeien.

In hoofdstuk 7 onderzoeken we de moleculaire status van SMAD4 in poliepen van JPS patiënten met een aangeboren mutatie in een van de allelen van het SMAD4 gen. Zoals beschreven, is in juveniele polyposis syndroom, anders dan bij FAP, het hebben van een gemuteerd allel voldoende om poliepen te doen ontstaan. Deze poliepen zijn (nog) niet dysplastisch, maar kunnen dat wel worden. Onduidelijk is hoe in deze poliepen de overgang van niet-dysplastisch- naar dysplastisch epitheel tot stand komt. Voor de hand liggend zou zijn, gezien de tumor suppressor functie van SMAD4, dat het wegvallen van het intacte tweede allel hieraan ten grondslag ligt. Weinig is bekend over dit inderdaad het geval is, en zo ja, of dit in het stroma dan wel het epitheel plaatsheeft (denk aan de landscaper theorie).

In normaal darmweefsel bevindt het SMAD4 eiwit, het product waarvoor het SMAD4 gen codeert, zich in de cellen van zowel het epitheel als het stroma. Door middel van een eiwitspecifieke kleuring (immuunhistochemie, IHC) kan het SMAD4 eiwit in de cellen zichtbaar worden gemaakt en bekeken worden onder de microscoop. Op deze manier hebben wij aan kunnen tonen dat in ongeveer de helft van de poliepen van JPS patiënten met een erfelijke mutatie in het SMAD4 gen, minder of geen SMAD4 eiwitten meer aanwezig zijn in gedeeltes van het epitheel. In het stroma werd geen verandering in SMAD4 eiwit expressie gezien. Het ontbreken van SMAD4 eiwit in de cellen van het epitheel duidt erop dat in deze cellen geen SMAD4 gen meer aanwezig is, dus dat in deze cellen het tweede allel van SMAD4 is weggevallen. Theoretisch is te verwachten dat in dit geval het wegvallen van het tweede allel van SMAD4 een eerste stap is de adenoma-carcinoma tumor progressie. Immers, ook van
andere tumor suppressor genen (bijvoorbeeld het APC gen bij FAP patiënten) is bekend dat bij het wegvallen van beide allelen de adenoma-carcinoma progressie geïnitieerd wordt (de gatekeeper functie). Dit zou betekenen dat cellen die geen SMAD4 eiwit expressie hebben herkenbaar zouden moeten zijn als zijnde dysplastisch. Dit bleek verrassend genoeg echter niet het geval. Een aantal van de door ons onderzochte poliepen bevatten delen waarin het epitheel dysplastisch is, maar deze gebieden kwamen niet altijd overeen met de gebieden waarin epitheliaal verlies van SMAD4 werd gezien. Zo was er in sommige poliepen sprake van dysplasie maar werd geen epitheliaal verlies van SMAD4 waargenomen, terwijl in andere poliepen SMAD4 verlies in het epitheel werd gezien zonder dat hier op het oog sprake was van dysplasie. Het verlies van SMAD4 eiwit expressie in het epitheel is een argument tegen de landscaper theorie. Immers, deze bepleit een genetisch defect primair in het onderliggende stroma welke invloed uitoefent op het bekledende epitheel, waardoor kanker zou kunnen ontstaan. Het ontbreken van een correlatie tussen verlies van epitheliaal SMAD4 eiwit expressie en dysplasie doet vermoeden dat het verlies van het tweede allel van SMAD4 niet noodzakelijk is bij het ontstaan van dysplasie in juvéniele poliepen met een erfelijke mutatie in SMAD4. Bovendien dringt de opmerkelijke conclusie zich op dat moleculaire dysplasie (wanneer twee allelen van een gen zijn gedeactiveerd en er zodoende groeivoordeel ontstaat, is er op moleculair niveau per definitie sprake van dysplasie) niet altijd met het oog herkenbaar is als zodanig. Een gatekeeper functie van SMAD4 lijkt hiermee omstreden in op zijn minst een gedeelte van juvéniele poliepen, en de zoektocht zal zich moeten toespitsen op andere genen die betrokken zouden kunnen zijn bij het ontstaan van dysplasie.

**Hoofdstuk 8** behandelt de aanwezigheid van het enzym cyclooxygenase-2 (COX-2) in juvéniele poliepen en de mogelijke implicaties daarvan met betrekking tot de behandeling van JPS. In poliepen van FAP patiënten is COX-2 in verhoogde mate aanwezig ten opzichte van normaal (gezond) weefsel en vormt een belangrijke stimulans voor tumor progressie. Aspirine-achtige middelen (ofwel niet-steroidale anti-inflammatoire drugs; NSAIDs) remmen de werking van COX-2. Bij FAP patiënten waarvan COX-2 wordt geremd door middel van behandeling met NSAIDs wordt een afname gezien in de hoeveelheid en in de grootte van darmpoliepen. Wij vonden een verhoogde aanwezigheid van COX-2 in juvéniele poliepen, met name in de poliepen van JPS patiënten met een erfelijke mutatie in BMPR1A. Dit betekent dat deze patiënten mogelijk baat kunnen hebben bij chemopreventie waarbij COX-2 wordt geremd door middel van NSAIDs. Of dit daadwerkelijk het geval is zal eerst moeten blijken uit studies naar de gevolgen van behandeling met NSAIDs van muizen met een kunstmatig gedeactiveerd Smad4 of Bmpr1a en een klinisch beeld gelijkend juvéniele polyposis syndroom.
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Nu ik achteraf mijn periode in Baltimore op waarde kan schatten moet ik bekennen dat deze periode niet in de laatste plaats op wetenschappelijk, maar vooral ook op persoonlijk gebied, van ongelooflijk belang voor mij is geweest. Hiervoor ben ik je intens dankbaar. Ik deel je voorliefde voor dit waanzinnige land, Baltimore incluis, en heb genoten van de paar dagen die we er deelden na afloop van de DDW.

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During my stay in Baltimore I was fortunate enough to work in the pancreatic cancer lab under supervision of Micheal Goggins. Your trust and inspiring enthusiasm made me feel right at home. An important part of my deciding to pursue a PhD in the first place is the joy I experienced in working in your lab. Therefore, I owe you and all the people of the pancreatic cancer lab who helped me on my way many thanks.

Frank, although we met in person only last summer in San Diego, I feel we’ve worked closely together over the past couple of years. Your thoughts and ideas on the subject are invaluable. You are a warm and generous person and it is an honor to have worked with you. I hope I will be able to continue to work with you in the future. Many, many thanks for all you’ve done.

Maar natuurlijk was er van dit alles niets terecht gekomen zonder hulp van de GE-groep. Lodewijk, jij bent een fantastisch onderzoeker en ik prijs me gelukkig dat ik een beetje bij je heb mogen afkijken. Ik heb het idee dat we elkaar goed aan vullen en ik heb altijd heel fijn met je samengewerkt. Ik hoop dat ook in de toekomst te blijven doen. Of dat als patholoog zal zijn laat ik nog even in het midden. Wendy, je geeft geweldige invulling aan jouw centrale rol in de
About the author

Arnout van Hattem was born in Amersfoort, The Netherlands, on the second of September, 1981. Having lived in Arnhem and Heemstede the family moved to Oakville, Ontario in Canada in the early 1990’s. Upon return to The Netherlands two years thereafter Arnout started high school at Atheneum College Hageveld in Heemstede. He graduated in 1999 and moved to Delft to study Architecture. All too quickly though, he realized this was not meant to be and set out to study Medicine in Amsterdam.

In September 2001 he enrolled Medical School at the University of Amsterdam (AMC). In 2005 he was provided the opportunity by Prof Johan Offerhaus of the department of Pathology of the AMC to embark on a 10 month research elective at the Johns Hopkins Medical Institutions in Baltimore, MD, USA. Supported by a grant from the Dutch Cancer Society, Arnout worked in the Pancreatic Cancer Lab under supervision of Dr Michael Goggins.

In August of 2006 Arnout received his doctoral degree in Medicine and, postponing his clinical rotations, commenced his PhD in the Gastrointestinal Research Group of Prof Offerhaus on the subject of juvenile polyposis syndrome. After his PhD defense in April 2009, Arnout will focus on continuing and hopefully finishing his medical training. His rotations are due to start in May 2009.
Publications
Publications


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