MOLECULAR GENETIC BACKGROUND OF JUVENILE POLYPOSIS

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Academic Dissertation

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ABBREVIATIONS

AAPC	attenuated adenomatous polyposis coli
ActR-IB	activin A receptor, type I B
ALK1	activin receptor-like kinase 1 gene
APC	adenomatous polyposis coli gene
bp	base pair
BRCA1	breast and ovarian cancer 1 gene
BRCA2	breast and ovarian cancer 2 gene
BMP	bone morphogenetic protein
BRR	Bannayan-Riley-Ruvalcaba syndrome
BZS	Bannayan-Zonana syndrome
CS	Cowden syndrome
CDKN2	cyclin dependent kinase inhibitor gene 2
cDNA	complementary deoxyribonucleic acid
CEPH	Centre dÉtudes du Polymorphisme Humain
CGH	comparative genomic hybridization
CHRPE	congenital hypertrophy of the retinal pigment epithelium
cM	centiMorgan
c-*MYC*	cellular proto-oncogene homologous to myelocytomatosis virus oncogene
CRC	colorectal carcinoma
dATP	deoxyadenosine triphosphate
DCC	deleted in colorectal cancer gene
dCTP	deoxycytosine triphosphate
DGGE	denaturate gradient gel electrophoresis
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DPC4	deleted in pancreatic carcinoma 4 gene
dpp	decapentaplegic gene (*Drosophila* BMP homolog)
dTTP	deoxythymidine triphosphate
EDTA	ethylene dinitrilotetra-acetic acid
FAP	familial adenomatous polyposis
GI	gastrointestinal
GS	Gardner syndrome
HHT1	hereditary haemorrhagic telangiectasia type 1
HHT2	hereditary haemorrhagic telangiectasia type 2
HMPS	hereditary mixed polyposis syndrome
HNPCC	hereditary non-polyposis colorectal cancer
JP	juvenile polyposis
JP1	putative juvenile polyposis locus 1
kb	kilobase
KRAS2	Kirsten rat sarcoma 2 viral oncogene homolog
LKB1	serine/threonine protein kinase gene (also known as *STK11*)
lod	logarithm of odds
LOH	loss of heterozygosity
Mad	*Drosophila mother against dpp* gene
MCC	mutated in colorectal cancer gene
MH1	Mad homology domain 1

MH2	Mad homology domain 2
MLH1	mutator L homolog 1 gene
MMAC1	mutated in multiple advanced cancers 1 gene
mRNA	messenger ribonucleic acid
M-MLV	Moloney murine leukemia virus
MSH2	mutator S homolog 2 gene
MSH6	mutator S homolog 6 gene
MSI	microsatellite instabile
MSS	microsatellite stable
N-CAM1	neural cell adhesion molecule 1
p	short arm of the chromosome
p16	gene encoding protein 16 also know as *CDKN2*
PAGE	polyacrylamide gel electrophoresis
PJS	Peutz-Jeghers syndrome
PCR	polymerase chain reaction
PHTS	*PTEN*-hamartoma-tumor syndrome
PI-3	phoshatidylinositol 3
PKB	protein kinase B
PMS1	postmeiotic segregation 1 gene
PMS2	postmeiotic segregation 2 gene
Ptd-Ins (3,4,5)P_3	phosphotidylinositol-3,4,5-triphosphate
PTEN	phosphatase and tensin homolog deleted on chromosome ten gene
q	long arm of the chromosome
RACE	rapid amplification of cDNA ends
RB1	retinoblastoma 1 gene
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
Sma	*C. elegans* homolog of *Drosophila Mad* gene
SMAD	human homolog of *Drosophila Mad* gene
Smad3	mouse homolog of *SMAD3* gene
SRP19	ribosomal signal recognition particle gene
SSCP	single strand conformation polymorphisms
STK11	serine-threonine kinase 11 gene
TB2	gene locating in 5q21, also known as *DP1* (deleted in polyposis) gene
Tcf-4	T-cell factor-4
TGFβ	transforming growth factor beta
TGFβRII	transforming growth factor beta type II receptor gene
TP53	tumor protein p53 gene
UTR	untranslated region
VHL	von Hippel-Lindau syndrome gene

INTRODUCTION

Colorectal cancer is the second leading cause of cancer deaths in Finland. In 1995 there were 2022 new cases of colorectal cancers and 970 deaths from this disease (Cancer Incidence in Finland 1995, Finnish Cancer Registry; Causes of Death 1996, Statistics Finland). In general the incidence of colorectal cancer is high in Western populations and low in Africa, Asia and South America.

Apart from its social and economical relevance, colorectal cancer represents an ideal biological model for studying the molecular events responsible for tumor initiation and progression. This feature has improved the identification of a number of genetic and epigenetic changes such as hypo- and hypermethylation, activation of the oncogenes and inactivation of the tumor suppressor genes, which are characteristic for the different progression stages of tumorigenesis (Fearon and Vogelstein, 1990).

There are also a number of well-known inherited syndromes that are associated with a high risk for the development of colorectal cancer. Among these are the hereditary polyposis syndromes, which account for approximately 1% of all colorectal cancer cases. Even rare, these syndromes represent an excellent model for studying the tumorigenesis. First, they have premalignant lesion, the polyp, which may progress through hamartoma/adenoma-carcinoma sequence. It is therefore possible to study molecular genetic events at different stages of carcinogenesis. It is increasingly recognized that the molecular genetic events responsible for these syndromes may also lead to the better understanding of the mechanisms underlying the development of sporadic colorectal cancer. Second, because these syndromes are inherited in an autosomal dominant manner, linkage analysis can be efficiently used for the localization of these genes.

From a more clinical point of view, the isolation of these genes allows presymptomatic diagnosis of the disease in affected families, and the establishment of genotype-phenotype correlation between the type and site of the mutation and its corresponding clinical consequence.

The aim of this thesis is to clarify the molecular genetic background of juvenile polyposis. First, the clinical features and genetic background of other known hereditary polyposis syndromes will be reviewed.

REVIEW OF THE LITERATURE

1. CLINICAL FEATURES AND CANCER RISK OF HEREDITARY POLYPOSIS SYNDROMES

The hereditary polyposis syndromes are a heterogeneous group of diseases characterized by multiple intestinal polyps. Most of these syndromes predispose to colorectal cancer, and they are divided into two groups on the basis of their pathology and clinical features. The first group has a high risk of colorectal cancer and is associated with the presence of adenomas of the bowel (adenomatous polyposis) and the second group is associated with hamartomas, although adenomas may also occur (hamartomatous polyposis).

1.1 Adenomatous polyposes

Hereditary adenomatous polyposes have been traditionally divided into three syndromes; familial adenomatous polyposis (FAP), Gardner syndrome (GS) and Turcot's syndrome. Although FAP, GS and Turcot's syndrome were originally considered as three separate conditions, they are now generally thought to be different manifestations of the same disorder, particularly as retinal lesions have been reported in each of these syndromes (Stein and Brady, 1988; Berk et al., 1988; Munden et al., 1991).

1.1.1 Familial adenomatous polyposis, Gardner syndrome and Turcot's syndrome

FAP was first reported by Chargelaigue in 1859, when he described this condition in two patients (Chargelaigue, 1859). FAP is characterized by an early onset of multiple adenomatous polyps of the colon and rectum. The high risk of colorectal cancer related to FAP was described as early as 1887 by Smith, and a few years later Handford recognized that sporadic adenomas could give rise to cancers (Smith, 1887; Handford, 1890). FAP patients develop colorectal cancer usually by the fourth decade of life and they are also at increased risk for several extracolonic malignancies, like cancers of the thyroid, small intestine, stomach, and brain (Jagelman et al., 1988; Giardiello, 1995). Varieties of benign extracolonic features have also been reported, the most common being polyps in the upper gastrointestinal tract (Ranzi et al., 1981; Järvinen et al., 1983). Other important diagnostic features reported in FAP patients are

ocular, cutaneous, and skeletal manifestations (Bussey 1975; Krush et al. 1988). Cutaneous lesions include epidermoid cysts, fibromas, lipomas and sebaceous cysts. Desmoid tumors are fibromatous lesions occuring in the extremities, abdominal wall and the mesentery of approximately 10% of FAP patients (Clark and Phillips, 1996; Gardner and Richards, 1953). The most common skeletal manifestations are osteomas which develop in the skull, long bones and characteristically in the mandible at the angle of the jaw (Brett et al., 1994).

Although, approximately 1 in 10,000 individuals are affected with FAP, less than one percent of all colon cancer cases occur in FAP patients, in part because of prophylactic colectomies (Boland et al., 1995; Kinzler and Vogelstein, 1998).

The milder variant of FAP is the so-called attenuated adenomatous polyposis coli (AAPC) which is characterized by a reduced number of intestinal polyps (less than 100 polyps) and delayed age of onset (approximately 10 to 15 years later than those observed in patients with classical FAP), although the lifetime risk for colorectal cancer remains unchanged (Leppert et al., 1990; Spirio et al., 1992).

The most common variant of FAP is the Gardner syndrome (GS). This syndrome was first reported by Gardner and his colleagues when they described a group of patients with multiple colonic adenomas and familial colon cancer like those in FAP, but who also had osteomas of the skull and multiple epidermal cysts and other skin lesions (Gardner and Richards, 1953; Gardner, 1962). Soft–tissue tumors, dental abnormalities, and congenital hypertrophy of the retinal pigment epithelium (CHRPE) were later added to the manifestations of the syndrome (Gardner and Richards, 1953; Gardner, 1962; Lewis et al., 1984; Stein and Brady, 1988).

In 1959, Turcot and colleagues reported two siblings with adenomatous polyposis coli, one of them also developed a medulloblastoma of the spinal cord and the other developed glioblastoma of the frontal lobe (Turcot, 1959). Turcot's syndrome is characterized by adenomas of the colon, tumors of the central nervous system and multiple skin lesions (Turcot et al., 1959; Everson and Fraumeni, 1976). The majority of Turcot's syndrome are thought to be variants of FAP, but in most cases patients have less than 100 colonic adenomas which is not typical for FAP (Hamilton et al., 1995).

1.2 Hamartomatous polyposes

Hamartomas are developmentally disorganized, benign tumors, which may occur in many organ systems. Hamatomatous polypose syndromes are traditionally classified into four subgroups; Peutz-Jeghers syndrome (PJS), juvenile polyposis (JP), Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRR), although most recent studies have suggested that CS and BRR might be phenotypic variants of the same disorder.

1.2.1 Peutz-Jeghers syndrome

In 1896 Hutchinson described twins with mucocutaneous pigmentation; one of them died of an intussusception and the other developed breast cancer (Hutchinson, 1896). The disease was later more carefully described by Peutz and Jeghers (Peutz, 1921; Jeghers et al., 1949). The main feature of a Peutz-Jeghers syndrome (PJS) is the hamartomatous polyps of the gastrointestinal tract. The polyps occur primaly in the small intestine, but they can also be found in the colon and stomach. The characteristic feature of Peutz-Jeghers polyp is a core of smooth muscle arising from the muscular mucosa that extends into the polyp like the trunk and branches of a tree. Another characteristic feature of PJS is mucocutaneous pigmentation. Usually these melanin spots are flat and dark brown, and they are located on the lips, buccal mucosa and hands (Spiegelman et al., 1995).

In the early literature cancer was not associated with PJS. Later, reports indicating an increased risk of gastrointestinal cancer in PJS appeared (Utsunomiya et al., 1975; Folley et al., 1988). It is now well documented that PJS patients are at an increased risk for both gastrointestinal and extra-gastrointestinal cancer. It has been estimated that PJS patients have an 18-fold increased risk of adenocarcinoma as compared with the general population (Giardiello et al., 1987). The most common gastrointestinal cancers in PJS are cancers of colon, stomach and small intestine, whereas cancers of the pancreas, breast, ovary and testis are frequently diagnosed extraintestinal cancers (Dozois et al., 1970; Wilson et al., 1986; Giardiello et al., 1987; Spigelman et al., 1989, Hizawa et al., 1993; Boardman et al., 1998).

1.2.2 Cowden syndrome

Cowden syndrome (CS), also known as multiple hamartoma syndrome or multiple hamartoma and neoplasia syndrome, was first described by Lloyd and Dennis (Lloyd and Dennis 1963). CS is characterized by multiple hamartomatous lesions, especially of the skin, mucous membranes, breast, thyroid and gastrointestinal tract, and by a high incidence of malignant tumors of the breast and thyroid gland (Starink et al., 1986; Eng, 1997). The life time risk of CS patients to develop breast cancer is estimated to be 25-50% and the risk for thyroid cancer is 3-10% (Brownstein et al., 1978; Starink et al., 1986; Hanssen and Fryns, 1995; Longy and Lacombe, 1996). CS may also include central nervous system manifestations, like macrocephaly, Lhermitte-Duclos disease or dysplastic gangliocytoma of the cerebellum, and sometimes also mental retardation (Albrecht et al., 1992; Eng et al., 1994).

1.2.3 Bannayan-Riley-Ruvalcaba syndrome

This disorder was first described in a single patient by Bannayan and colleagues in 1971. Few years later Zonana and colleagues described the same disorder in a father and 2 sons (Zonana et al., 1976). Bannayan-Riley-Ruvalcaba syndrome (BRR) which is also known as Bannayan-Zonana syndrome (BZS), Ruvalcaba-Myhre-Smith syndrome and Riley-Smith syndrome, is a congenital syndrome with characteristic features of macrocephaly, cognitive and motor dysfunction, subcutaneous and visceral lipomas and hemangiomas, pigmentary spotting of the penis, and juvenile type of polyps in the colon (Bannayan, 1971; Zonana et al., 1976; Gorlin et al., 1992). Unlike in CS, an increased risk of malignancy has not been documented of patients with BRR (Marsh et al., 1998a).

Although CS and BRR have distinct phenotypic features, the presence of macrocephaly, intestinal polyposis, and lipomas in both diseases suggests a partial clinical overlap. Moreover, the presence of features common in CS and BRR within the same family has been reported in a few kindred (Fargnoli et al., 1996).

1.2.4 Juvenile polyposis

Juvenile polyposis syndrome was first described by McColl and colleagues in 1964. Two years later Smilow and colleagues reported the first familial cases of this syndrome (McColl et al., 1964; Smilow et al., 1966). Juvenile polyposis (JP) is a rare

condition characterized by the occurrence of multiple juvenile polyps in the gastrointestinal tract. It is estimated that JP affects approximately 1 in 100,000 people (Burt et al., 1993). The most widely accepted definition for JP requires any one of the following: (a) more than five juvenile polyps of the colorectum; (b) juvenile polyps throughout the gastrointestinal tract; (c) any number of juvenile polyps with a family history of juvenile polyposis (Jass et al., 1988). The number of polyps in JP patients can vary between few to few hundred, which is clearly less than in FAP (Veale et al., 1966, Stemper et al., 1975; Järvinen et al., 1993). Macroscopically, the polyps are 5-50 mm in size and have a spherical head and a narrow stalk. Microscopically, a polyp contains dilated or cystic epithelial tubules and an excess of lamina propria. The muscularis mucosa does not reach to the stalk, in contrast to Peutz-Jeghers polyps (Järvinen, 1993; Desai et al., 1995).

Juvenile polyposis differs from PJS by the absence of consistent extraintestinal manifestations, which could serve as diagnostic markers in symptom-free family members. However, a variety of associated malformations have been detected in many patients with juvenile polyposis. These include arteriovenous malformations, porphyria, psoriasis, mental retardation, congenital heart disease, cleft lip/palate, epilepsy, hereditary haemorrhagic telangiectasia (HHT), digital clubbing, hypertrophic pulmonary osteoarthropathy and malrotation of the gut (Restrepo et al., 1978; Cox at al., 1980; Järvinen et al., 1993; Desai et al., 1998, Inoue et al., 1999).

Juvenile polyps were originally defined as non-neoplastic hamartomatous epithelial tumors with no potential for malignant or premalignant transformation, and this is still largely true for solitary juvenile polyps (Rozen and Baratz, 1982; Järvinen and Franssila, 1984; Jass et al., 1988; Nugent et al., 1993). The adenomatous and carcinomatous changes in juvenile polyps were originally considered to be occasional findings (Stemper et al., 1975; Liu et al., 1978; Billingham et al., 1980; Friedman et al., 1982). The malignant potential of juvenile polyposis was first recognized in the 1970s and, at present, it is agreed that juvenile polyposis is a precancerous condition. The risk of JP patients to develop gastrointestinal malignancy has been estimated to be from 9 percent to as high as 50 percent (Järvinen and Franssila, 1984; Jass et al., 1988; Howe et al., 1998a; Agnifili et al., 1999). Recently, 24 studies reporting gastrointestinal cancers among JP patients were reviewed and it was concluded that among 133 familial JP patients, there were 42 cases of colorectal cancer (31.5%), 15 cases of stomach cancer (11.3%), and one case each of pancreatic and duodenal

cancer (0.75%). Overall, 58 of these 133 patients (44.4%) developed gastrointestinal cancer (Howe et al., 1998a).

1.3 Hereditary mixed polyposis sydrome

Hereditary mixed polyposis syndrome (HMPS) is a rare condition in which patients develop characteristic polyps of the large bowel. These polyps closely resemble juvenile polyps, but show significant histological differences. Also adenomatous and hyperplastic polyps may occur in affected individuals. Typically less than 15 polyps are found at colonoscopy and there is no extracolonic disease associated with the development of the polyps (Whitelaw et al., 1997). HMPS is also known to predispose to colorectal cancer. It is still unclear whether HMPS is a variant of juvenile polyposis or a distinct disease (Murday and Slack, 1989).

2. MOLECULAR GENETIC BACKGROUND OF HEREDITARY POLYPOSIS SYNDROMES

2.1 Familial adenomatous polyposis, Gardner syndrome and Turcot's syndrome

The inherited nature of FAP was first noted by Bickersteth in 1890, when he described a mother and son with the condition (Bickersteth, 1890). It took almost one hundred years before Herrera and colleagues described a constitutional interstitial deletion of chromosome arm 5q in a patient with Gardner syndrome (Herrera et al., 1986). Like Herrera et al., also Kobayashi et al. (1991) described an interstitial deletion of 5q in a boy with Gardner syndrome, mental retardation and multiple minor anomalies. This deletion involved chromosome band 5q22.1-q31.1 (Kobayashi et al., 1991). Similarly, Hockey and colleagues (1989) described an interstitial deletion of 5q15-q22 in two brothers with FAP (Hockey et al., 1989). These observations suggested that a tumor suppressor gene on chromosome 5q could be responsible for the condition of these patients. This proposal was later confirmed by linkage analysis, which established the linkage of FAP to chromosome 5q21 markers in all the kindreds analyzed (Leppert et al., 1987; Bodmer et al., 1987). At the same time, as the linkage to chromosome 5q was found, Nakamura and colleagues (1988) made a linkage study with three FAP and three Gardner syndrome families, and succeeded in refining the genetic localization of the polyposis locus to a position at 5q21-q22 (Nakamura et al.,

1988). Four genes were later mapped to this region; *MCC*, *TB2*, *SRP19* and *APC* (Kinzler et al., 1991; Joslyn et al., 1991).

One of these genes, *APC*, was found to be mutated in the germline of FAP patients (Nishisho et al., 1991; Groden et al., 1991) and later also in sporadic colorectal tumors (Nishisho et al., 1991, Miyoshi et al., 1992; Powell et al., 1992). The *APC* gene has been examined in over 500 FAP kindreds and coding region mutations are detected in majority of them (Nagase and Nakamura, 1993; Powell et al., 1993). *APC* is a large gene and the majority of mutations seen in FAP patients occur in the first half of the last exon (the last exon contains a 6579 bp uninterrupted open reading frame) (Miyoshi et al., 1992). The manifestations of FAP can vary considerably and in some cases this is due to a specific mutation. CHRPE for example is associated with truncating mutations between codons 463 and 1387 (Olschwang et al., 1993; Wallis et al., 1994; Caspari et al., 1995). Another example is AAPC. Spirio and colleagues (1993) have shown that terminating mutations located close to the 5' end of *APC* gene result in a milder phenotype of FAP (Spirio et al., 1993). However, patients with identical mutations can develop dissimilar clinical features. For example some patients with identical truncating mutations develop features of GS while others do not (Giardiello et al., 1994).

Mutations in *APC* gene have also been found in patients with Turcot's syndrome, although the correlation between *APC* mutations and Turcot's syndrome is not straightforward (Van Meir, 1998). Hamilton and colleagues (1995) studied 14 Turcot syndrome families and they detected germline *APC* mutation in ten of them. In addition, germline mutations in the mismatch-repair gene *MLH1* or *PMS2* were found in two families. Their findings indicate that Turcot's syndrome can result from two distinct types of germline defects: mutation of the *APC* gene or mutation of a mismatch-repair gene (Hamilton et al., 1995).

The *APC* gene encodes a cytoplasmic protein that can bind to and promote the degradation of β-catenin. β-catenin binds to members of the Tcf family of transcription factors and activates gene transcription. Recently, the c-*MYC* oncogene was identified as a target gene in this pathway (He et al., 1998). It was further proposed that in normal colorectal epithelial cells, wild type APC prevents β-catenin from forming a complex with Tcf-4 and activating c-*MYC*. In colorectal tumors with *APC* mutations or activating *β-catenin* mutations an increased β-catenin/Tcf-4 activity

leads to overexpression of c-*MYC*, which then promotes neoplastic growth (He et al., 1998).

2.2 Peutz-Jeghers syndrome

There has been few attempts to localize the gene for PJS. In 1996, Markie and colleagues reported a pericentric inversion of chromosome 6 in a patient with PJS (Markie et al., 1996), but a linkage was not detected in PJS families (Tomlinson et al., 1996). Another locus was suggested on chromosome band 1p32-34, a result that was obtained by the linkage analysis of two families. However, the linkage study with extended pedigrees conflicted the previous results (Tomlinson and Houlston, 1997) and the analysis of new set of families excluded linkage to 1p (Tomlinson et al., 1996).

The PJS gene was finally localized in 1997 by Hemminki and colleagues. A novel strategy was used for the localization. Multiple PJS polyps from a single PJS patient were used in comparative genomic hybridization (CGH). It revealed a subtle loss at 19p in 6 of 16 polyps examined. LOH analysis using microsatellite markers from 19p confirmed the CGH results. 12 PJS families of different ethnic origin were used for linkage analysis and all families showed the linkage to 19p13.3. Other studies confirmed 19p13.3 as the PJS predisposing locus (Amos et al., 1997; Mehenni et al., 1997; Olschwang et al., 1998).

The gene predisposing to PJS was identified in 1998 (Hemminki et al., 1998). First, the PJS region was narrowed down to 800 kilobases (kb) by meiotic recombination mapping in PJS families (around markers *D19S886* and *D19S883)*. Transcripts in this interval were identified by database searches and direct cDNA selection. The 27 transcripts identified in this region were screened for mutations, and truncating germline mutations were identified in *LKB1(STK11)* gene locating in 19p13.3. At the same time, Jenne and colleagues (1998), demonstrated mutations in *LKB1 (STK11)* in five PJS patients (Jenne et al., 1998). These finding were confirmed also by other groups. Resta and colleagues reported four germline mutations in nine PJS cases and Wang and colleagues reported germline mutations in seven out of 12 PJS patients, respectively (Resta et al., 1998; Wang et al., 1998a). Gruber and colleagues studied six families with PJS from the John's Hopkins Polyposis Registry, and they confirmed linkage to 19p13.3. They also identified germline mutations in *LKB1* in all six families studied (Gruber et al., 1998).

LKB1 was originally identified as a serine/threonine protein kinase expressed in human fetal liver (Nezu, GenBank accession number U63333). LKB1 shows strong homology with the cytoplasmic serine/threonine kinase XEEK1 of *Xenopus laevis* (Su et al., 1996) and shows weaker similarity to many other protein kinases. Most of the *LKB1* mutations identified in PJS are truncating and are located in the kinase core domain of the protein (Hemminki et al., 1998; Jenne et al., 1998; Mehenni et al., 1998; Ylikorkala et al., 1999). It is likely that *LKB1* is a tumor-suppressor gene, since loss of the normal allele was observed in the polyps from a PJS patient with germline mutation on the other allele (Hemminki et al., 1998). However, the frequency of *LKB1* somatic mutations in a wide range of sporadic tumors seem to be low (Avizienyte et al., 1998; Avizienyte et al., 1999). Only a small number of somatic changes have been reported in sporadic colorectal, breast and gastric cancers (Resta et al., 1998; Bignell et al., 1998; Park et al., 1998). An exception was the study by Dong and colleagues (1998), who reported frequent mutations in *LKB1* in left-sided colon cancers (Dong et al., 1998). *LKB1* promoter hypermethylation has been demonstrated in a few cases of colorectal and testicular tumors, but in general, it seems to be a rare event in sporadic cancers (Esteller et al., 1999, submitted). *LKB1* appears to be restricted to the genetic pathway of tumorigenesis in gastrointestinal hamartomas and adenocarcinomas of PJS patients and other tumor types developing in PJS patients.

2.3 Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome

CS and BRR are both autosomal dominantly inherited syndromes. The gene responsible for CS was localized by a genome wide search using dinucleotide repeat markers at 10-20 cM intervals. Nelen and colleagues (1996) examined 12 CS families and a maximum lod score of 8.92 at theta = 0.02 was obtained with the marker *D10S573*, locating on chromosome band 10q22-q23 (Nelen et al., 1996). It was suggested that the susceptibility gene for CS is likely a tumor suppressor gene as evidenced indirectly by loss of heterozygosity in the CS critical interval on 10q22-23 in various CS related tumors (Marsh et al., 1997; Marsh et al., 1998b).

At the time as the CS gene was localized to 10q22-23, a new putative tumor suppressor gene was identified at this same region. *PTEN/MMAC1* (phosphatase and tensin homolog deleted on chromosome ten) was originally isolated from cancer cell lines harboring homozygous deletions on the chromosome region 10q23. *PTEN* encodes a dual-specificity protein phophatase, which shows homology to the focal

adhesion molecules tensin and auxillin (Li et al., 1997; Steck et al., 1997; Myers., 1998). One of PTEN's major endogenous substrates is phosphotidylinositol-3,4,5-triphosphate [Ptd-Ins(3,4,5)P_3], a phospholipid in the phoshatidylinositol 3-kinase (PI-3 kinase) pathway, which previously has been shown to be important in cell growth signaling (Stambolic et al., 1998; Myers et al., 1998; Dahia et al., 1999; Maehama and Dixon, 1998). In this pathway, PTEN may act as a 3-phosphatase to dephosphorylate Ptd-Ins(3,4,5)P_3 to Ptd-Ins(3,4)P_2. Mutant or decreased PTEN leads to the accumulation of Ptd-Ins(3,4,5)P_3, which is required for activation of protein kinase B (PKB)/Akt, a known cell survival factor (Stambolic et al., 1998; Myers et al., 1998; Dahia et al., 1999; Li et al., 1998).

Several *PTEN* mutations have been identified in sporadic tumors and cancer cell lines from various tissues including brain, endometrium, prostate, breast, thyroid, and melanoma (Risinger et al., 1997; Cairns et al., 1997; Rhei et al., 1997; Dahia et al., 1997). Germline mutations of *PTEN* were first found to be associated with CS (Tsou et al., 1997; Liaw et al., 1997; Nelen et al., 1997; Lynch et al., 1997) and later also with BRR (Marsh et al., 1997). Up to now, germline mutations in the *PTEN* have been found in 13-81% of CS patients (Tsou et al., 1997; Liaw et al., 1997; Nelen et al., 1997; Lynch et al., 1997; Marsh et al., 1998a) and in 57-60% of BRR cases (Marsh et al., 1998a; Longy et al., 1998). The *PTEN* mutation frequency in CS patients varies widely in different studies. One possible explanation is that some patients screened in these studies are not true CS patients. Marsh and colleagues (1998c) analyzed mutations in the *PTEN* gene in 64 unrelated Cowden syndrome-like families and identified only one mutation, in a male with follicular thyroid carcinoma. It was concluded that germline *PTEN* mutations play a relatively minor role in Cowden syndrome-like families (Marsh et al., 1998c).

Celebi and colleagues (1999) were the first to describe a family with two female members phenotypically fulfilling the criteria for CS and two male members with the phenotypic findings of BRR that all associated with a single germline mutation in the *PTEN* coding sequence (Celebi et al., 1999). Marsh and colleagues (1999) analyzed *PTEN* mutations among 43 unrelated BRR cases, including a subset of 11 families with both BRR and CS, and they detected mutations in 26 of them (60%) (Marsh et al., 1999). Interestingly, ten of 11 BRR/CS (91%) overlap families were shown to have germline *PTEN* mutations. So, the overlap of a number of clinical features and the sharing of identical *PTEN* mutations in CS and BRR patients suggests that CS and

BRR are different presentations of a single syndrome, which could be named "PTEN hamartoma-tumor syndrome" (PHTS) (Marsh et al., 1999).

2.4 Juvenile polyposis

Linkage studies in JP families have been limited. There is one report excluding *APC* and *MCC* as the genes for JP (Leggett et al., 1993). Other genetic studies, originally stimulated by the finding of an interstitial deletion in the chromosomal region 10q22-24 in an infant with multiple colonic juvenile polyps and several congenital abnormalities, have focused on the region of the *PTEN* gene (Jacoby et al., 1997a). Evaluation for LOH in 10q22 within juvenile polyps revealed somatic deletions within the lamina propria in 83% of polyps derived from 16 unrelated patients (13 familial and 3 patients with sporadic JP). Maps indicating the contiguous extent of deletion for each individual polyp were constructed from LOH data, showing that the shortest overlapping region was the 3-cM interval between microsatellite markers *D10S219* and *D10S1696.* This interval was considered as a putative JP locus and was named JP1 (Jacoby et al., 1997b).

To further clarify the genetic background of JP, Howe and colleagues (1998b) performed a focused genome screen to identify a gene locus predisposing to JP. The genotyping was performed on 43 individuals, of whom 13 were affected (the Iowa kindred). The linkage strategy involved the typing of markers at loci known to play an important role in colorectal polyposis or cancer (including regions where *MSH2, MLH1, MCC*, *APC, HMPS, PTEN, KRAS2, TP53, DCC* and *LKB1* genes are located). Linkage to JP was established with several markers from chromosome 18q21.1. The maximum lod score was 5.00, with marker *D18S1099*. There was no evidence of linkage to other markers. Analysis of critical recombinants placed the JP gene in an 11.9 cM interval between markers *D18S1118* and *D18S487* (Figure 1), a region that also contains the tumor-suppressor genes *DCC* and *SMAD4 (DPC4),* both of these being good candidates for the JP gene (Howe et al., 1998b).

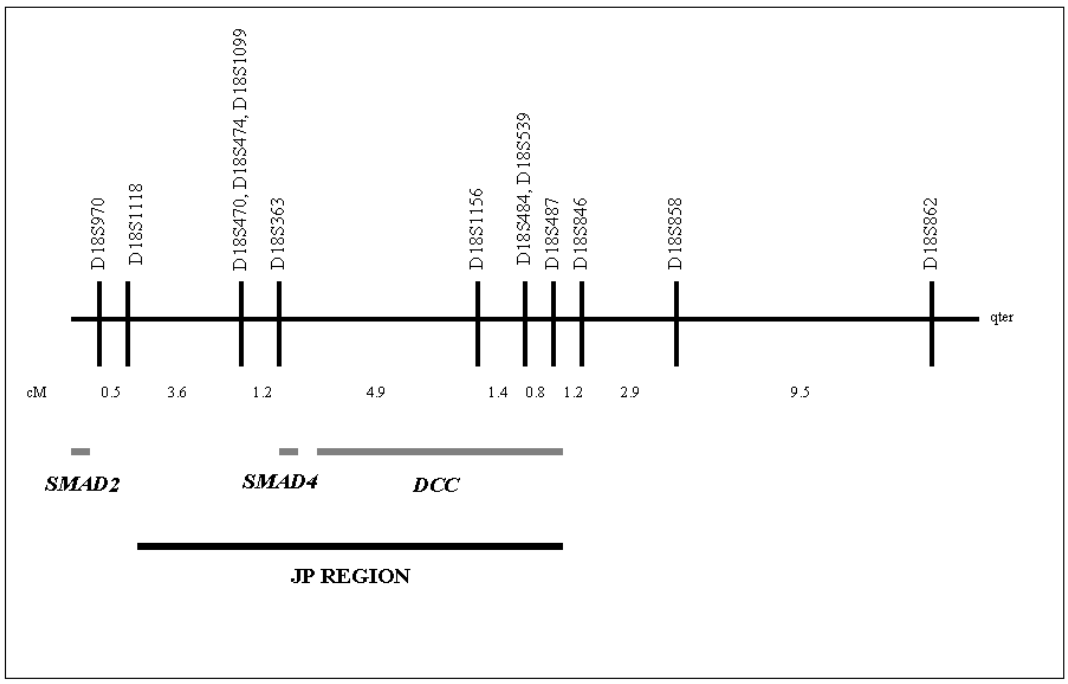


Figure 1. Schematic representation of markers and genes in 18q21 (modified from Howe et al., 1998b). JP gene is located between markers D18S1118 and D18S487. This region includes two genes: DCC and SMAD4. SMAD2 is located outside of the JP region.

2.5 Hereditary mixed polyposis syndrome

Like JP, hereditary mixed polyposis syndrome (HMPS) is inherited in an autosomal dominant manner. It is still unclear whether HMPS is a variant of JP or a distinct disease (Murday and Slack, 1989). To clarify the molecular genetic background of HMPS, Thomas and colleagues (1996) genotyped one large HMPS family. As a result, the linkage of HMPS to the *APC*, *MSH2*, *TP53* and *DCC* loci were excluded and evidence of linkage was found in chromosome 6q. Multipoint linkage analysis gave a maximum lod score of 3.93 between markers *D6S468* and *D6S283*. (Thomas et al., 1996). Also Whitelaw and colleagues (1997) have reported that HMPS is unlinked to candidate loci with importance in colorectal tumorigenesis, such as *APC*, *MSH2* and *MLH1* (Whitelaw et al., 1997). The gene for HMPS has not yet been identified and the genetic events behind HMPS are still unclear.

AIMS OF THE STUDY

1. To localize the gene/genes predisposing to JP
2. To identify the gene/genes predisposing to JP
3. To analyze the role of JP gene/genes in hereditary and sporadic colorectal tumorigenesis

MATERIALS AND METHODS

1. Patients and tumor samples

1.1 JP families and patients (studies I, II and III)

Family 1 (Pedigree 1, Figure 2). Two JP patients were reported in this Finnish family. Multiple colonic juvenile polyps were observed in both of these patients, one of them was also diagnosed with colorectal and pancreatic carcinomas at ages 42 and 50, respectively (JP 4/1 in study II, family 1 in study III).

Family 2. This Finnish family (Pedigree 2, Figure 2) includes four members with JP, two of whom were diagnosed with colorectal carcinoma (at ages 40 and 50). Among all JP cases, the juvenile polyps were observed in the large intestine and in one patient also in the stomach. One additional member of this family had been diagnosed with a colonic adenoma without other evidence of JP. This individual had been operated on for aortic stenosis of unknown etiology at the age of 25. One JP patient and two at risk individuals (ages 11 and 17) displayed a ventricular septal defect (family 2 in study III).

Family 3. Family 3 (Pedigree 3, Figure 2) originates from Texas, the United States, and includes five members with JP. One of these JP patients was diagnosed with a colorectal carcinoma at the age of 30. Multiple colonic polyps including adenomatous changes were reported in all five JP cases (family 3 in study III).

Family 4 (Pedigree 4, Figure 2). This family originates from Finland and includes six members with JP, two of whom are also diagnosed with colorectal carcinoma (at ages 34 and 53). Among all JP cases, juvenile polyps were observed in colon and in two patients also in the stomach (JP2/13 in study II, family 4 in study III).

Family 5 (Pedigree 5, Figure 2). Family 5 originates from Finland and includes eight members with JP. One of them is also diagnosed with colorectal carcinoma. There are two other cancer cases in this family, one with acute myeloid leukemia and other with gastric cancer (JP5/1 in study II).

Sporadic case 1 (female). At birth, a hamartoma of the renal pelvis was diagnosed and removed. At the age of seven the patient was diagnosed with Wilms' tumor, and the right kidney was removed. At the age of 12, Ebstein's anomaly (displacement of the tricuspid valve) was diagnosed and surgically corrected. At the age 22, the patient

was operated on for bowel obstruction caused by a poorly differentiated adenocarcinoma in the colon ascendens. In addition to the carcinoma, 20 to 30 polyps were observed in the proximal colon up to hepatic flexure. Several polyps were examined by an experienced pathologist, and all were hamartomatous polyps, which could be designated as juvenile polyps. In one polyp, some features of Peutz-Jeghers polyp were also seen. This patient died at the age of 25 (sporadic case 1 in study III).

Sporadic case 2 (female). This patient has congenital panhypopituitarism. JP was diagnosed at the age of 38. Juvenile polyps were observed in the large and small intestine and in stomach. Biological parents and relatives are unknown and the patient has no children.

Sporadic case 3 (male). JP was diagnosed at the age of 13. Juvenile polyps were observed in the large and small intestine. This patient has also been diagnosed with empty cella-syndrome, Osler's disease, and epilepsy. There is no family history of JP (JP 1/1 in study II, sporadic case 3 in study III).

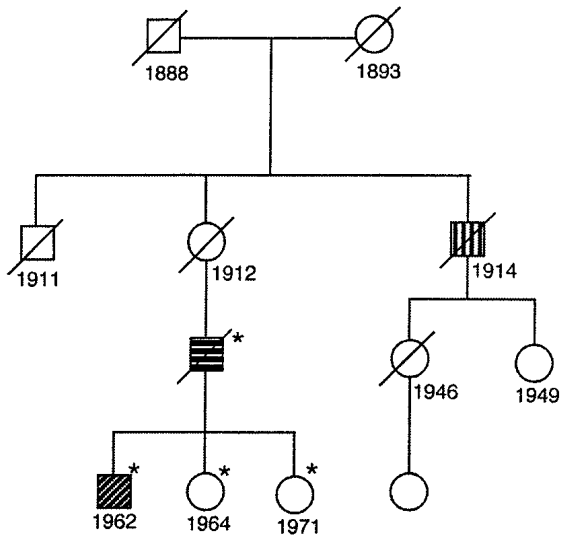
Sporadic case 4 (male). This patient was diagnosed with 30 to 40 colonic juvenile polyps at age of 6, but there is no family history of JP (JP 10/1 in study II).

Sporadic case 5 (female). At the age of 18 ulcerative colitis (UC) was diagnosed, later the diagnosis was confirmed as JP (age of 29). In addition to JP, this patient is also mentally retardated. There is no clear evidence of family history of JP, however the patient's father has a history of gastrointestinal symptoms, but has not been clinically evaluated.

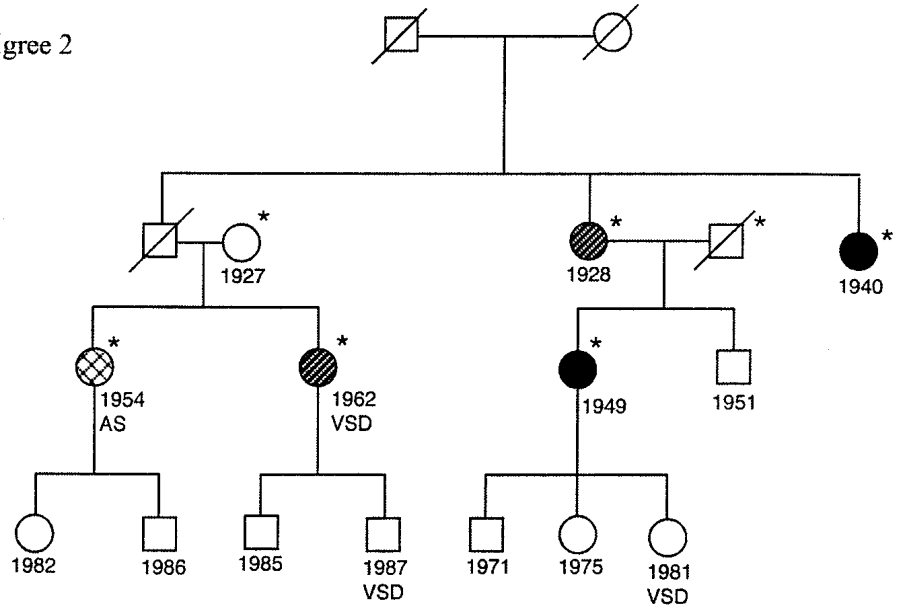
In addition to patients listed above, studies I and II included JP families and sporadic cases from United Kingdom (study I) and United States (studies I and II). Clinical features and pedigree of the Iowa kindred (family I-13 in study II) have been previously reported; first by Stemper and colleagues (1975) and later by Howe and colleagues (1998a).

This study was approved by the Ethical Committee of the Department of Medical Genetics, University of Helsinki.

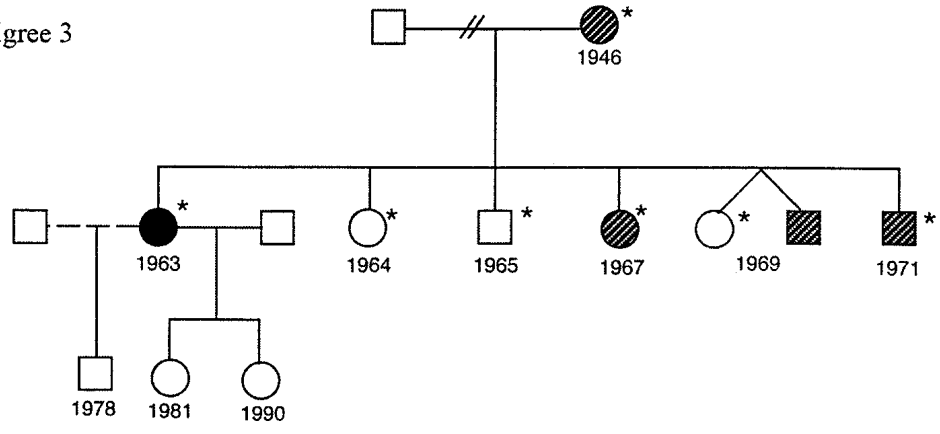
Pedigree 1



Pedigree 2



Pedigree 3



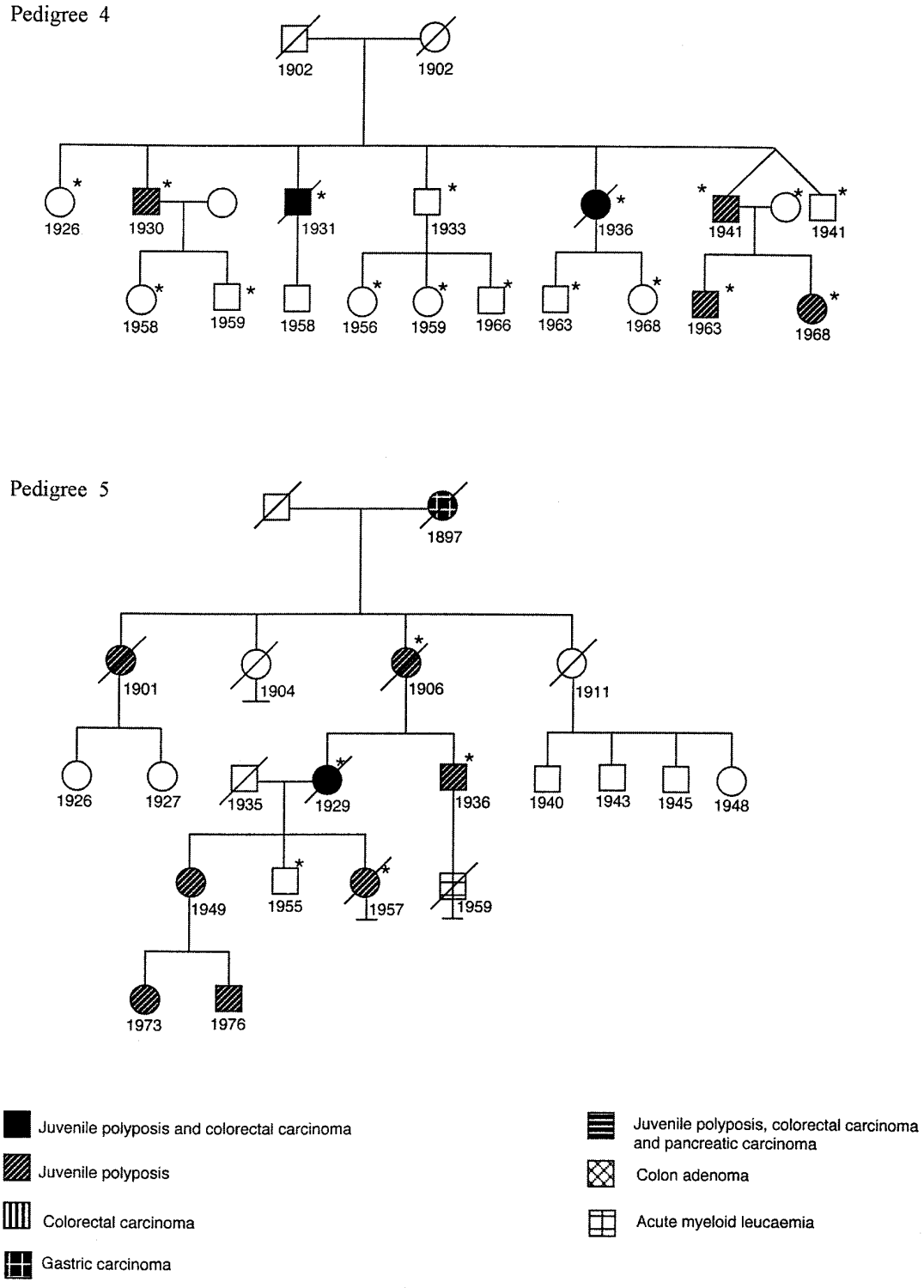


Figure 2. Pedigrees of JP families. Those samples where DNA was available are marked with an asterisk.

1.2 Hereditary non-polyposis colorectal cancer families (study IV)

Fourteen Finnish hereditary non-polyposis colorectal cancer (HNPCC) kindreds from whom lymphoblastoid cell lines were available were selected for this study. One affected individual per family was included in the study. Six kindreds fulfilled the Amsterdam criteria for HNPCC (Vasen et al., 1991). Other patients represent familial HNPCC-like colorectal cancer (CRC). The number of patients with CRC or endometrial cancer ranged from 2 to 6 per family. All kindreds selected for this study have been previously shown to be *MLH1* and *MSH2* mutation negative (Nyström-Lahti et al., 1996; Holmberg et al., 1998). All except three kindreds displayed microsatellite stable tumors (MSS). In these three kindreds DNA from tumor tissue has not been available.

1.3 Tumor samples (study V)

Between May 1994 and June 1998 over one thousand fresh-frozen specimens of colorectal adenocarcinoma have been collected at the Department of Medical Genetics, Haartman Institute, University of Helsinki (Aaltonen et al., 1998; Salovaara et al., in press). Among those, 26 microsatellite instable (MSI) and 16 MSS tumors were selected for *SMAD4* methylation analysis and 15 MSI and 7 MSS tumors for *SMAD4* mutation screening, respectively.

2. DNA and RNA extraction

In studies I and II, DNA was extracted from JP patient cell lines (cell pellets) or blood samples with standard procedure. DNA extraction from paraffin-embedded tumor or normal tissue was performed using the phenol and chloroform procedure described in Kannio et al. (1996).

In studies III and IV, total cellular RNA was extracted from lymphoblasts by RNA extraction kit (QIAGEN).

In study V, the tumor DNA was extracted from fresh frozen tumor specimens by the standard procedure described by Lahiri and Nürnberger (1991). The corresponding normal DNA was extracted from normal mucosa or blood.

3. cDNA synthesis (studies III and IV)

20 µl of cDNA was created from 0.8 µg of RNA using standard random priming methods with 200 units of M-MLV reverse transcriptase (Promega), 1 × reaction buffer (Promega), 10 µM random hexamer and 60 units of RNAse inhibitor (Promega). The reaction was carried out at 42°C for 1 h and then 95°C for 10 min.

4. Polymerase chain reaction (PCR)

4.1 *PTEN* gene (study I)

Primers for genomic *PTEN* amplification have been previously described (Liaw et al., 1997; Steck et al., 1997) except for primers for amplification and sequencing of exons 2 and 4 which are shown in study I. The detailed PCR conditions are described in study I.

4.2 SMAD4 gene (studies II, III, IV, V)

Primers used for genomic amplification of the *SMAD4* gene have been previously described (Moskaluk et al., 1997) except for new primers for exons 4, 7 and 8, which are described in study III. Those primers were designed by using the Primer3 program. PCR conditions for genomic *SMAD4* amplification are shown in study III.

The cDNA sequence for *SMAD4* was derived from the GenBank (accession number U44378). The gene was amplified in five fragments and PCR primers for cDNA amplification were designed using the Primer3 server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and they are listed in study IV. The PCR reactions were carried out as described in studies II, III, IV and V.

4.3 *SMAD2*, *SMAD3* and *SMAD7* genes (studies III and IV)

The cDNA sequences for *SMAD2*, *SMAD3* and *SMAD7* were derived from GenBank database (accession numbers U65019, U76622 and AF010193, respectively). The genes were amplified in five fragments and PCR primers for cDNA amplification were designed using the Primer3 server. Primer sequences and PCR conditions are shown in studies III and IV.

4.4 *ALK1* and *endoglin* genes (unpublished data)

The genomic sequences for *ALK1* and *endoglin* were derived from the GenBank database (accession numbers U77707-U77713 for *ALK1* and U37439-47, U17156-7, and AF036969-71 for *endoglin*, respectively). The primers and PCR conditions for amplification of all exons of *ALK1* and *endoglin* genes have been previously published. Those primers and conditions were also used here (Berg et al., 1997; Gallione et al., 1998).

5. Denaturating gradient gel electrophoresis (DGGE) (study I)

DGGE was performed for all exons of *PTEN*, with the exception of exons 2 and 4, in probands from 10 JPS families and 8 sporadic cases. The rest of the samples (4 JP families and 3 sporadic cases) and exons 2 and 4 in all cases, were directly sequenced. For DGGE conditions, see study I.

6. Single strand conformation polymorphism (SSCP) (study II)

In study II, the germline *DCC* and *SMAD4* mutations were initially screened by SSCP assay. The SSCP procedure is described in study II.

7. Automated sequencing (studies I, II, III, IV and V)

The PCR products were purified using the QIAquick PCR purification Kit (QIAGEN). Direct sequencing of the purified PCR products was performed using the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing kits (PE/ABI). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC Bioproducts) and analyzed on an Applied Biosystems model 373A or 377 DNA sequencer (PE/ABI).

8. Restriction enzyme analysis (studies III, IV and V)

In study III, restriction enzyme digestion was used to screen for the presence of two base substitutions in *SMAD4* in control individuals. *Eco*RI (New England BioLabs) digestion was used to detect a C to G change at codon 177 (exon 4). For the analysis of an A to C change at codon 353 *Alw*I (New England BioLabs) digestion was performed. The detailed digestion procedure is described in study III.

In study IV, the presence of an A to G change at codon 170 (*SMAD3* exon 3) in control individuals was analyzed with *Hga*I (New England BioLabs) digestion. For the detailed procedure, see study IV.

In study V, *NsiI* (New England BioLabs) digestion was used to detect a G to A change at codon 118 (*SMAD4* exon 2). The PCR was performed as described is study V. The detailed digestion procedure is described in study V.

9. Polyacrylamide gel electrophoresis (PAGE) (study III)

In study III, PAGE analysis was performed to analyze the presence of 4-base-pair deletion in control individuals. A new set of primers was designed for amplification of *SMAD4* exon 9. Primers were forward: GGTTGCACATAGGCAAAGGT and reverse: TTGGGTAGATCTTATGAACAGCA (5' to 3'). With these primers, a 156-bp fragment, containing the site of the 4-base-pair deletion, was amplified from exon 9. For PCR conditions, see study III. 10 µl of denaturing loading buffer (95% formamide, 20 mM EDTA, 0.05 % bromphenol blue, 0.05% xylene cyanole FF) was added to 10 µl of PCR sample, and the sample was denaturated for 5 min at 80^{o}C. A 5 µl aliquot of the mixture was loaded in 6% polyacrylamide gels containing 8.3 M urea and run at 2.5 kV for 50 min. Finally, the gels were dried and autoradiographed.

10. Linkage analysis (studies I and III)

Eight JP families originating from the United States and the United Kingdom were included in the linkage analysis of study I. The microsatellite markers, PCR conditions and statistical methods used for this analysis are described in detail in the original study (study I).

In study III, those two JP families, in which the germline mutations of *SMAD4* gene were not detected, were tested for linkage to 18q21. Microsatellite markers *D18S970*, *D18S474*, *D18S1099*, *D18S851*, *D18S484*, *D18S858* and *D18S977* (*SMAD4* is located close to markers *D18S474* and *D18S1099*) were used for the analysis. PCR reactions were carried out in a volume of 10 µl containing 50 ng DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of dATP, dGTP, dTTP and 0.7 µl of [α-32P] dCTP (3000Ci/mmol, Amersham), 0.5 µM of each primer and 0.25 units of AmpliTaqGOLD polymerase (PE/ABI). After PCR amplification in standard conditions, PAGE analysis was performed (see above).

Multipoint linkage analyzes were performed using the GENEHUNTER program (Kruglyak et al. 1996). The JP locus was defined as an affection status locus with dominant inheritance. Three liability classes and age dependent penetrances were set as follows: liability class 1 assigned to age <21 years, with heterozygote penetrance of 50%; liability class 2, age 21-50 years, heterozygote penetrance 70%; liability class 3, >50 years heterozygote penetrance 80%. The JP locus frequency was assumed to be 1/50,000 and the marker loci frequencies and their genetic distances were obtained from the CEPH database V8.1 (http://www.cephb.fr and http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map).

11. Methylation analysis (study V)

In study V, the methylation status of the *SMAD4* promoter was studied in tumors from patients with CRC. The fragment selected for this analysis was CG-rich region, including the non-coding exon 1. To determine whether this particular promoter region was hypermethylated, a PCR-based *HpaII* and *MspI* restriction enzyme assay was used. Both tumor and normal DNA were digested and the reactions contained either no enzyme, 25 units of *HpaII*, or 20 units of *MspI.* Samples were incubated for 16 h at 37°C. To analyze cleavage of the *SMAD4* promoter region, 12.5 ng of DNA from each digest was analyzed by PCR in 25 µl reaction volume. Primers were designed (Primer3) to amplify a 408 bp fragment of *SMAD4* promoter containing six *HpaII/MspI* restriction sites and the primer sequences were: forward: 5'-CAAGTTGGCAGCAACAACAC; and reverse: 5'- ACATGGCGCGGTTACCT.

RESULTS

Linkage analysis to 10q22-24 (study I)

Four microsatellite markers flanking the *PTEN* locus (markers *D10S219*, *D10S551*, *D10S579*, and *D10S541*) were used to generate haplotypes for 47 individuals of eight informative JP families originating from the United States and the United Kingdom. For the microsatellite markers *D10S219* and *D10S541*, the maximum two-point lod score was 0 at a recombination fraction of $\theta = 0.5$ for both models (model 1: allele frequency 0.002 and penetrance 0.5; model 2: allele frequency 0.0002 and penetrance 0.85). For markers *D10S551* and *D10S579*, the maximum two-point lod scores were 0.50 and 0.72, respectively, at $\theta = 0$ for the first model and 0.63 and 0.20 at $\theta = 0$ and $\theta = 0.4$, respectively for the second model. Multipoint analysis revealed lod scores < -2.0 over the entire region, so the linkage of JP to *PTEN* locus was excluded in the eight families studied.

PTEN mutation analysis (study I)

PTEN mutations were analyzed among probands from 14 JP families and 11 sporadic cases by either direct sequencing or DGGE analysis and mutations were not detected. A frequent polymorphism in intron 8 was observed in 36 % (4 of 11) of sporadic JPs cases. The presence of this sequence variant in its heterozygous state in four cases excludes whole gene deletion as a cause of JP in these patients.

Mutation analysis of *SMAD4* gene in JP (studies II, III)

To localize the gene predisposing to JP, a whole genome wide search was done in three Finnish JP families (families 2, 4 and 5, data not shown). Simultaneously and independently, Howe and colleagues (1998b) succeeded in localizing the gene predisposing to JP in chromosome band 18q21.1 (Howe et al., 1998b). This chromosomal region contains two putative tumor suppressor genes: *DCC* and *SMAD4*. Since both of these genes were good candidates for JP, the mutation screening was performed for both of them. After sequencing 14 *DCC* exons and 11 *SMAD4* exons, a 4 base pair (bp) deletion was detected in *SMAD4* exon 9 (between nucleotides 1372 and 1375, GenBank accession number U44378). This mutation was

first detected in one affected individual from the Iowa kindred. Next, the *SMAD4* exon 9 was screened from all 46 members of the Iowa JP kindred, the same 4 bp deletion being present in all 13 affected and 4 of 26 at risk individuals, but not in any of 7 spouses (study II).

Then, eight additional unrelated JP patients were analyzed for mutations of all exons of *SMAD4* by SSCP or genomic sequencing, and the mutation was found in four of them. Two JP kindreds (originating from United States and Finland) were segregating the same 4 bp deletion in exon 9 that was detected in the Iowa kindred. This deletion causes a frameshift that creates a new stop codon at codon 434. In total, 242 controls were analyzed for the presence of this alteration and the altered allele was not observed in any of them (study II). Mutations were also detected in two sporadic JP cases. The first one was a 2 bp deletion in exon 8 at nucleotides 1170 to 1171 (codon 348). This deletion causes a frameshift that creates a stop codon at codon 350. The change was not detected in any of 101 controls analyzed. Another patient was found to have a 1 bp insertion between nucleotides 815 and 820 of exon 5, this change added a guanine to a stretch of six sequential guanines in the wild-type sequence and created a frameshift and a new stop codon at codon 235. For this change, 107 controls were analyzed and again, this change was not detected in any of them (study II).

In study III, seven unrelated JP families or sporadic patients were analyzed for mutations of all exons of *SMAD4* by genomic sequencing. Four out of these seven cases were previously analyzed for *SMAD4* mutations in study II (by SSCP), and were then reported mutation negative (JP1/1, JP2/13, JP4/1, and JP6/1, study II). In this study, three different germline defects were detected. In family 3, we detected the same 4-base-pair deletion in exon 9, which has been previously described in three JP kindreds. In the sporadic case number 2 (JP6/1 in study II) a C to a G transversion at nucleotide 661 was detected. This mutation changes serine to a stop codon at codon 177. Forty-nine controls were analyzed (*Alw*I digestion) and the change was not detected in any of them. The change detected in JP family 1 (JP 4/1 in study II) was an A to C transition at nucleotide 1186, which is predicted to convert tyrosine to serine at amino acid 353. This variant was present in both cases with JP (see Material and Methods, Patients) but also in one unaffected at risk individual. For this variant, 55 Finnish controls were analyzed by restriction enzyme digestion (*Eco*RI), and none of them displayed the change. These two base pair changes were missed in study II,

because they did not show in SSCP analysis. No *SMAD4* mutations were detected in families 2 and 4, or in sporadic cases number 1 and 3.

In total, a set of 12 independent JP cases were analyzed for *SMAD4* mutations (studies II and III). Among these, mutations were detected in 5 families and 3 sporadic cases.

Linkage analysis to 18q21 (study III)

Linkage analysis using markers *D18S970*, *D18S474*, *D18S1099*, *D18S851*, *D18S484*, *D18S858* and *D18S977* (*SMAD4* is located close to markers *D18S474* and *D18S1099*) resulted in a clear exclusion ($Z \leq -2$) of the *SMAD4* region in family 2 (see Materials and Methods, Patients). However, formal exclusion could be obtained only at, and in the near vicinity of, marker *D18S858* in family 4 while the rest of the marker map produced inconclusive lod scores.

Mutation analysis of *SMAD2*, *SMAD3* and *SMAD7* in JP patients (study III)

SMAD2, *SMAD3* and *SMAD7* mutations were analyzed among those JP families or sporadic cases where *SMAD4* or *PTEN* mutations had not been detected (families 2 and 4 and patients 1 and 3). Mutation analysis was performed by automated sequencing covering the translated region of these genes. No *SMAD2*, *SMAD3* or *SMAD7* mutations were detected in any of these patients. The only variant identified was an A to G change at the third position of codon 103 in the *SMAD3* gene. The change was homozygous in all of our four samples. This polymorphism has been reported earlier and the variant does not cause any amino acid change (Arai et al., 1998).

Mutation analysis of *SMAD2, SMAD3 and SMAD4* in HNPCC patients (study IV)

SMAD2, *SMAD3* and *SMAD4* mutations were analyzed among 14 familial colon cancer kindreds, eleven of these displaying at least one MSS tumor. Previous studies had evaluated *MLH1* and *MSH2* mutations in these families, all of them being mutation negative (Nyström-Lahti et al., 1996; Holmberg et al., 1997). *SMAD* gene mutation analysis was performed by automated sequencing covering the translated region of the genes. Genetic alterations were not detected in *SMAD2* or *SMAD4* genes in any of these patients.

In the *SMAD3* gene, three discrepancies were detected between GenBank sequence (U76622) and sequences from our patients. The first was an A to G change at the third position of codon 103 (exon 2), this silent change has been reported earlier (study II). A second, silent, change detected was C to T transition at nucleotide 907 (exon 6). The frequency of these two variants in the normal population was not analyzed, as the changes were silent.

The third change was an adenine to guanine transition at nucleotide 545, which is predicted to convert isoleucine to valine at amino acid 170. This change was detected in two patients. For this variant, 110 Finnish controls were analyzed by restriction enzyme digestion (*Hga*I). Seven out of 110 control individuals displayed the change (6.4 %). To further compare the frequency of this polymorphism in colon cancer patients versus control individuals, 132 patients were included in analysis. Taken together the 14 HNPCC patients and 132 colon cancer patients the frequency of this polymorphism was 8.9% (13/146). From those 13 cancer patients who had valine instead of isoleucine at codon 170, four turned out to be familial. Segregation of the polymorphism was analyzed in two of these families where DNA from multiple family members was available, and the polymorphism was not segregating with cancer in these families.

SMAD4 promoter methylation (study V)

In this study, the possible hypermethylation of the *SMAD4* promoter was analyzed by using a *HpaII* and *MspI* digestion. Using this assay, we examined the methylation status for *SMAD4* promoter region in a group of 26 MSI and 16 MSS colorectal tumors. The fragment selected for this analysis was a CG-rich region published by Hagiwara and colleagues (submitted), and the amplified sequence contained altogether 55 CpG dinucleotides. It was possible to determine the methylation status for six CCGG sites by restriction. No PCR product was detected from any of *HpaII* digested DNA, suggesting that the *SMAD4* promoter is unmethylated in all the cases studied.

Mutation analysis of *SMAD4* in sporadic colorectal carcinomas (study V)

Twenty-two primary colon cancers were analyzed for mutations of all exons of *SMAD4* by genomic sequencing. The only change detected was a G to A transition at

the third position of codon 118 (exon 2). This silent change was present in one tumor sample and also in corresponding normal DNA.

Mutation analysis of *SMAD4* promoter in JP patients (unpublished data)

Mutations in *SMAD4* 5'-untranslated region (331 bp fragment downstream from the transcription start site) were analyzed among those JP families/cases where *SMAD4* or *PTEN* mutations had not been detected (families 2 and 4 and sporadic cases 1 and 3). Mutation analysis was performed by automated sequencing, and no mutations were detected in any of patients analysed.

Mutation analysis of *ALK1* and *endoglin* genes (unpublished data)

Genomic *ALK1 and endoglin* mutation screening was performed among those four JP patient, where *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7* or *PTEN* mutations were not detected. Mutations were not detected in either one of these genes in any of those four patients.

DISCUSSION

Exclusion of *PTEN* gene as the gene predisposing to JP (study 1)

Previous studies have suggested that the gene predisposing to JP is located in chromosomal band 10q22-24, in the region where the *PTEN*, gene associated with CS and BRR is located. Jacoby and colleagues (1997) first reported a *de novo* interstitial deletion of 10q22.3-24.1 in a single JP patient with multiple congenital abnormalities (Jacoby et al., 1997a). To further analyze the relevance of this finding, they studied allelic loss at 10q22 in 47 juvenile polyps from 16 unrelated JP patients. As a result, 83% of these polyps were found to have somatic deletion at 10q22 (Jacoby et al., 1997b). These findings were interpreted as evidence for a tumor suppressor gene on 10q.

In our study, *PTEN* mutations were analyzed among probands from 14 JP families and 11 sporadic cases and no mutations were detected. The lack of germline *PTEN* mutations in a total of 25 unrelated JP patients argues that *PTEN* is not the JP susceptibility gene. Also the possibility of whole gene deletion was excluded among those four cases in which heterozygous sequence variant in intron 8 of *PTEN* was present.

The possible linkage of JP to chromosomal region 10q22-24, which includes *PTEN* gene and the putative JP locus (JP1, Jacoby et al., 1997b) was also analyzed in this study. Four microsatellite markers covering the *PTEN* locus and flanking 20 cM were used to generate haplotypes for members of eight JP families. The linkage analysis excluded linkage to *PTEN* locus and, hence excluded the possibility of both a *PTEN* promoter and intron mutations in at least these eight families. Thus, our data suggested that *PTEN* is not the gene responsible of JP.

Later, another negative study was published by Riggins and colleagues (1997). In their study, 11 JP cases were analyzed for *PTEN* mutations and no mutations were found (Riggins et al., 1997).

There are however two reports where *PTEN* mutations have been described in JP patients. Lynch and colleagues (1997) reported one family, which was thought to have both JP and CS, as having nonsense mutation in *PTEN* (Lynch, et al., 1997).

In the study by Olschwang and colleagues (1998) 14 JP patients were screened for *PTEN* mutations and three variants were observed in three JP patients. The first

variant was a 1 bp deletion at codon 232. This frameshift mutation leads to a stop codon at position 255. The patient having this variant was a 74 year old man diagnosed with anemia, hypoalbuminaemia, polyps in the GI tract and laryngeal cancer. The second variation was a T to G transversion at codon 35, predicted to substitute arginine for methionine. This patient was ten years old and had a history of rectal bleeding. Juvenile polyps were found throughout the GI tract. The third variant was a T to G transversion at the second position of the consensus splicing sequence of the donor site of exon 6 (intronic variant). This change was predicted to lead to the skipping of at least exon 6 in the mRNA, resulting in a shift of the translational reading frame. The patient with this variant was 14 years old when he underwent colonoscopy that revealed juvenile polyps (Olschwang et al., 1998).

These reports showing that four individuals with juvenile polyposis had *PTEN* germline mutations would appear to confirm that *PTEN* is the predisposing gene on 10q in some families with JP (Lynch et al., 1997; Olschwang et al., 1998). However, one of these patients was described as having both CS and JP (Lynch et al., 1997), whereas the other three had no family history of JP (Olschwang et al., 1998). This raises the question whether these patients were truly affected with JP or CS (Eng and Ji, 1998).

SMAD4 is a gene predisposing to JP (studies II and III)

To localize the gene predisposing to JP, a whole genome wide search was performed in three Finnish JP families (families 2, 4 and 5, data not shown). Simultaneously and independently, Howe and colleagues succeeded in localizing the gene predisposing to JP on chromosome band 18q21.1, between markers *D18S1118* and *D18S487* (Howe et al., 1998b). This interval contains two putative tumor suppressor genes, *DCC* and *SMAD4* (Figure 1).

The *DCC* gene was cloned in 1990, and it encodes a cell-surface protein containing homology with N-CAM (Fearon et al. 1990). There have been many reports on the loss of heterozygosity at the *DCC* gene locus in human colon cancers (Kikuchi-Yanoshita et al., 1992; Thiagalingam et al., 1996), suggesting that *DCC* might be a tumor suppressor gene. However, mutations in the coding region of *DCC* seem to be rare (Cho et al. 1994) and the position of *DCC* as a candidate tumor suppressor is not clear.

SMAD4 (DPC4) was first identified through deletion mapping in sporadic pancreatic cancers (Hahn et al., 1996). It is inactivated in nearly half of sporadic pancreatic carcinomas and in a subset of breast, ovarian, and colorectal tumors (Schutte et al., 1996; Thiagalingam et al., 1996). The *SMAD4* gene was first called *DPC4* (deleted in pancreatic carcinoma, locus 4), since it was the fourth marker to be investigated for homozygous deletions in pancreatic cancer. Later the name *SMAD4* has been used in addition to *DPC4,* as this gene is a member of the *SMAD* family of genes related to the *Drosophila Mad* and *C. elecans Sma* genes (Sekelsky et al., 1995; Hahn et al., 1996; Savage et al., 1996).

The mutation screening was performed for both of these genes simultaneously. After sequencing 14 *DCC* exons and all 11 *SMAD4* exons, a 4 base pair deletion in exon 9 of *SMAD4* was detected. After this finding the mutation screening was limited only to the *SMAD4* gene. In total, a set of 12 independent JP cases were analyzed for *SMAD4* mutations (studies II and III). Among these, mutations were detected in 5 families and 3 sporadic cases (62%).

Interestingly, the mutation was the same in four out of five familial cases (4 bp deletion in exon 9). This deletion causes a frameshift that creates a new stop codon at codon 434. In the recent study *SMAD4* mutations were analyzed from 11 unrelated JP patients and mutations were found in three of them (Friedl et al., 1999). In two of these patients the mutation was the same 4 bp deletion in exon 9, described earlier in four unrelated patients (studies II and III). According to the haplotype analysis, these two patients did not share common alleles for the markers *D18S363* and *D18S1110* (markers located close to *SMAD4*) (Friedl et al., 1999). Since this 4 bp deletion has been detected in one kindred originating from Finland, in three kindreds originating from the United States, and in two kindreds originating from Germany, the defect seems to represent a mutational hotspot rather than an ancestral founder mutation.

Other germline *SMAD4* mutations detected in JP are a 1 bp insertion in exon 5 (study I), a 2 bp deletion in exon 8 (study I), an A to C transition in exon 8 (study III), a C to G transversion in exon 4 (study III), a 2 bp deletion in exon 6 (Friedl et al., 1999) and a C to T transition in exon 8 (Houlston et al., 1998). Mutations detected in studies II and III, or reported in the literature are summarized in Table 1.

Patient	Type	Codon (exon)	Nucleotide change	Predicted effect	Study
I-13	F	414-416 (9)	4 bp deletion	stop at codon 434	II
M-1	F	414-416 (9)	4 bp deletion	stop at codon 434	II
JP 5/1	F	414-416 (9)	4 bp deletion	stop at codon 434	II
JP 11/1	S	348 (8)	2 bp deletion	stop at codon 350	II
JP 10/1	S	229-231 (5)	1 bp insertion	stop at codon 235	II
F3	F	414-416 (9)	4 bp deletion	stop at codon 434	III
F1	F	353 (8)	A→C transversion	Tyr →Ser	III
C2	S	177 (4)	C→G transversion	Ser →Stop at codon 177	III
FJP15	F	277-278 (6)	2 bp deletion	Pro →Stop at codon 278	Friedl et al., 1999
FJP4	F	414-416 (9)	4 bp deletion	stop at codon 434	Friedl et al., 1999
FJP12	F	414-416 (9)	4 bp deletion	stop at codon 434	Friedl et al., 1999
AF	F	361 (8)	C →T transition	Arg → Cys	Houlston et al., 1998

F= familial case, S= sporadic case

Table 1. SMAD4 germline mutations in JP families/patients reported in the literature.

On the basis of structural and functional criteria, the SMAD family can be divided into three subgroups. The first group includes so called receptor regulated SMADs that act as direct substrates for receptors (in human those are SMADs 1, 2, 3, 5 and 8) (Liu et al., 1996; Eppert et al., 1996; Chen et al., 1997; Nishimura et al., 1998). The second group includes SMADs that are not direct receptor substrates, but whose function is essential for signalling by the receptor regulated SMADs. SMAD4 belongs to the second group. The third group includes the inhibitory SMADs (SMADs 6 and 7) (Hata et al., 1998; Hayashi et al., 1997; Nakao et al., 1997). All these SMAD proteins have a domain structure composed of highly conserved N-terminal and C-terminal domains known as MH1 and MH2 (Mad homology domains 1 and 2) which are joined by a linker region (Figure 3). The receptor regulated SMADs are directly phosphorylated at their carboxyl terminus by type I TGFβ superfamily receptors. SMAD4 lacks this carboxy-terminal phosphorylation sequence and thus can not behave as direct substrate for the type I receptors (Macias-Silva et al., 1996; Zhang et al., 1996; Krezschmar et al., 1997). In the inactive state, SMAD4 is located in the cytoplasm as a homo-oligomer and remains in an inactive conformation through an interaction between the MH1 and MH2 domains. After ligand stimulation and phosphorylation of the receptor regulated SMADs, SMAD4 forms hetero-oligomers with receptor regulated SMADs and translocates into the nucleus (Lagna et al., 1996; Liu et al., 1997; Whitman, 1998). In the nucleus this SMAD hetero-oligomer complex may specifically associate with a nuclear transcription factor, which then targets the SMAD transcriptional activation function to a specific promoter (Liu et al., 1997). In addition to targeting promoters via trancription factors, SMADs have a site-specific DNA-binding activity. It has been demonstrated that the MH1 domain of SMAD protein may directly bind to the promoter or enhancer region of the genes, thus regulating the transcription (Whitman, 1998). The third mechanism for SMAD protein signalling in cells is transcriptional activation through MH2 domain (Liu et al., 1997) A summary of the domain structure of SMADs and their interaction is presented in Figure 3.

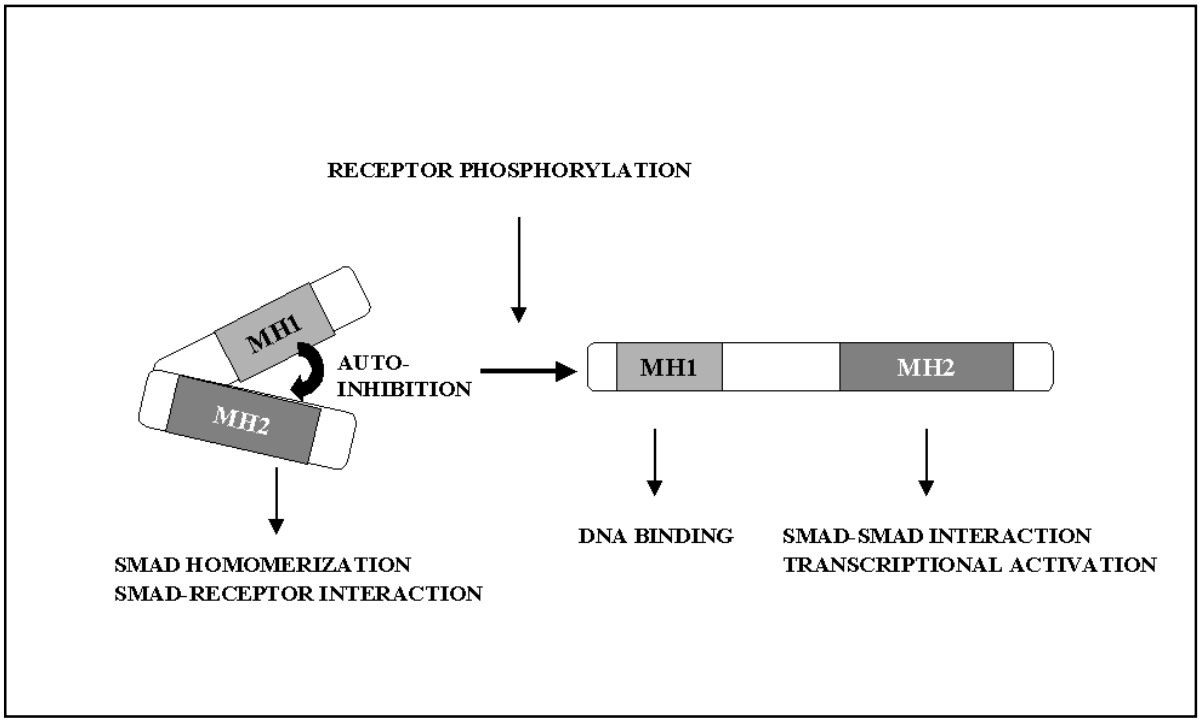


Figure 3. The domain structure of SMADs and their interactions (modified from Hata et al., 1998). In basal state, SMADs form homo-oligomers and remain in an inactive conformation through an interaction between the MH1 and MH2 domains.

The majority of somatic, as well as germline, mutations described in *SMAD4* map to the carboxyl-terminus between codons 330-526 (MH2 domain), and half of mutations are located in exons 8 and 9 (Hahn et al., 1996, Hahn et al., 1998; Kim et al., 1996; Schutte et al., 1996; Takagi et al., 1996) (Figure 3). The tumorigenic mutations in SMADs fall in three major categories according to their effect on protein function. The missense mutations in the MH1 domain interrupt DNA binding function, truncating mutations of the MH2 domain remove its ability to activate gene expression, and the missense mutations of the MH2 domain appear to interfere with its nuclear localization (Dai et al., 1998). The location of *SMAD4* germline mutations and the hotspots of sporadic *SMAD4* mutations are shown in Figure 4.

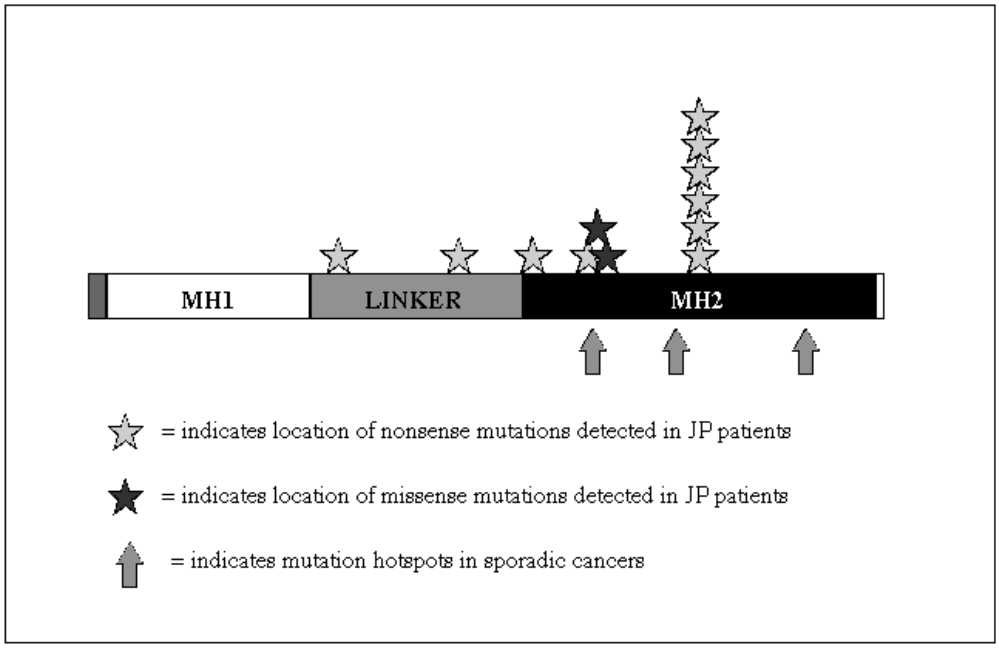


Figure 4. Summary of the locations of germline SMAD4 mutations in JP patients. The hotspots of somatic mutations in sporadic cancer are also shown.

Other candidate genes for JP (study III and unpublished data)

So far, 12 *SMAD4* mutations have been identified in 46 familial and sporadic JP cases (26%) (studies II and III; Friedl et al., 1999; Houlston et al., 1998). In our studies (studies II and III) *SMAD4* mutations were detected in eight of 12 JP patients analysed (66%). However, in two recent studies a considerably lower proportion of *SMAD4* involvement in JP were reported. Friedl and colleagues analysed 11 unrelated patients with JP and found *SMAD4* mutation in three of them (27%). Similarly, Houlston and colleagues analysed 21 JP patients and found a mutation only in one of them (5%). Taken together these results suggest that mutations in *SMAD4* predispose to JP, but account only for approximately 25% of cases. Further studies will hopefully clarify the true frequency, though issues such as diagnosis of JP and different mutation detection method utilized (genomic sequencing in studies II, III, and in the study by Friedl and colleagues and conformation-specific gel elctrophoresis in the study by Houlston) may contribute to the discrepancy.

Other explanation for the relatively low *SMAD4* mutation frequency among JP patients may be the existence of other gene/genes predisposing to JP. Houlston and colleagues (1998) analyzed eight JP families for linkage to *SMAD4* and found no evidence for linkage. It could be excluded in two of the eight kindreds and was unlikely in two others. Of those four families which provided good evidence against linkage to 18q21.1, two were also unlinked to the *PTEN* locus (Houlston et al., 1998), one was compatible with *PTEN* linkage but no *PTEN* mutation was detected (study I) and one has not been tested for *PTEN* mutations. None of these families had notable features of CS or BRR.

In our studies (studies II and III) *SMAD4* mutations were not detected in two families (families 2 and 4; family 2 also excluding linkage to 18q21) and two sporadic cases (sporadic cases 1 and 3), these being negative also in the previous *PTEN* mutation analysis (study I). Two of these (one family and one sporadic case) display a JP phenotype with heart anomalies; the sporadic case also has a history of Wilms' tumor. The other sporadic case was diagnosed with hereditary hemorrhagic telangiectesia (HHT) and epilepsy.

There is good evidence for the importance of the TGF-β signaling pathway in colorectal carcinogenesis. A large proportion of sporadic colon cancers with MSI have *TGF-β type II receptor* mutations (Myeroff et al., 1995) and germline variants in the *TGF-β type II receptor* may predispose to colon cancer (Lu et al., 1998). Also the

downstream targets of TGF-β have been implicated in colorectal carcinogenesis. Somatic mutations of *SMAD2* and *SMAD4* have been found in colorectal cancer in humans and germline *Smad3* mutations cause colon cancer in mice (Zhu et al., 1998). It has been hypothesized that the germline mutations in genes (other than *SMAD4*) encoding different components of the TGF-β signaling pathway may be present in JP.

To study this hypothesis further, we focused on *SMAD* genes, which are involved in TGF-β signaling via TGF-β type II and I receptors (TGFβR-II and TGFβR-I). SMAD4 is known to be the co-SMAD and its function is essential for signaling by the receptor-regulated SMADs. This group includes SMADs 2 and 3, which are substrates of the TGF-β and activin type I receptors (TGFβR-I and ActR-IB) and mediators of TGF-β and activin signals (Whitman, 1998; Hata et al., 1998). The same pathway also includes SMAD7, which is an inhibitory SMAD. Expression of SMAD7 inhibits the receptor-regulated phosphorylation of SMAD2 or SMAD3 (Hayashi et al., 1997; Nakao et al., 1997). In this study a cDNA based mutation screening was performed for *SMAD2*, *SMAD3* and *SMAD7* genes and no mutations were detected in these genes in any of our patients (study III). Recently, similar results were reported by Bevan and colleagues (1999). In their study, 30 unrelated JP patients, who were negative in previous *SMAD4* mutation analyses (Houlston et al., 1998) were screened for germline mutations at *SMAD1*, *SMAD2*, *SMAD3* and *SMAD5.* No mutations were found in any of the patients screened (Bevan et al., 1999).

Previous results together with the occurrence of other genetic syndromes with juvenile polyposis, suggest genetic heterogeneity in the etiology of JP. BRR, Gorlin syndrome and HHT are genetic syndromes, which commonly are associated with JP. The genes responsible for these conditions may also be considered as candidate genes for JP. The gene predisposing to BRR (and CS) is *PTEN*, and as discussed in previous chapters it is unlikely that it would also be the gene predisposing to JP. Gorlin syndrome is characterized by multiple basal cell carcinomas of the skin. The gene for Gorlin syndrome has been localized to chromosome 9 and shown to be the human homolog of the Drosophila developmental gene *patched* (Johnson et al., 1996). The third syndrome, which is occasionally described among JP patients, is HHT (also known as Osler's disease or Osler-Rendu-Weber disease). HHT is an autosomal dominant disorder characterized by multisystemic vascular dysplasia and recurrent

haemorrhage (Guttmacher et al., 1995). Two predisposing genes have been identified for HHT. *Endoglin*, which maps to chromosome 9q33-34 has been identified as the gene for HHT1 (HHT type 1), and *ALK1* (*activin receptor–like kinase 1* gene), which maps to chromosomal locus 12q11-14, is the gene for HHT2 (HHT type 2) (McDonald et al., 1994; Shovlin et al., 1994; Johnson et al., 1995; Vincent et al., 1995).

One of our JP patients was diagnosed with HHT (study III, sporadic case 3). Similar cases have previously been reported in some other studies. In 1980 two patients (mother and daughter) with HHT and polyposis were reported (Cox et al., 1980). Two years later an autosomal dominant syndrome with juvenile gastrointestinal polyposis, cutaneous telangiectesia, and pulmonary arteriovenous malformations in a father and two children were described. The father died from colon cancer at the age of 36 (Conte et al., 1982). Recently, Desai and colleagues (1998) described one JP patient with HHT and two patients with some features of HHT (Desai et al., 1998).

The association of HHT with juvenile polyposis in a subset of patients raises the possibility that JP could occur as part of a 9q or 12q microdeletion syndrome in some cases, or JP with HHT might be a unique syndrome due to mutation in a single gene. To further study this hypothesis, *endoglin* and *ALK1* mutation screening was performed among those JP patient, where *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7* or *PTEN* mutations were not detected. Both endoglin and ALK1 are members of the TGF-β receptor superfamily and are therefore cosidered as good candidates for JP gene. No mutations were detected in either of these genes (unpublished data). Our data suggest that there are still unidentified genes predisposing to JP, perhaps associated with developmental defects such as cardiovascular anomalies.

Mutation analysis of *SMAD* genes in HNPCC patients (study IV)

HNPCC is an autosomal dominant cancer susceptibility syndrome. The genes responsible for the disease are *MSH2*, *MLH1*, *PMS1*, *PMS2* and *MSH6* (Leach et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994; Nicolaides et al., 1994, Miyaki et al., 1997). Inactivation of both alleles of one of these genes results in microsatellite instability (MSI) that is a hallmark of HNPCC tumors. The genes responsible for

microsatellite stable (MSS) HNPCC are still largely unknown (Peltomäki et al., 1997; Aaltonen et al., 1993, Nyström-Lahti et al., 1996).

Loss of growth inhibition by transforming growth factor β(TGF-β) is an important step in colorectal tumorigenesis. In HNPCC tumors with MSI, this is mainly due to frameshift mutations within a polyadenine sequence repeat in the *TGFβRII* gene. It has been proposed that germline mutations in *TGFβRII* underlie a subset of MSS HNPCC cases (Lu et al., 1998). Other genes involved in the TGF-β pathway could also be candidates for MSS HNPCC (Grady et al., 1999).

SMAD proteins play a key role in intracellular TGF-β signaling and mutations in some of the *SMAD* genes disrupt this pathway. To investigate whether germline mutations in *SMAD2, SMAD3* or *SMAD4* could predispose to MSS HNPCC, we analyzed mutations in these genes in 14 HNPCC kindreds in which germline mutations in *MSH2* and *MLH1* had been ruled out (Nyström-Lahti et al., 1996; Holmberg et al., 1997). The only changes detected were three polymorphisms in *SMAD3*. One of these was an A to G transition at nucleotide 545, which changes isoleucine to valine at amino acid 170, in the so called linker-region of SMAD3 protein. This variant has not been reported earlier. We analyzed 110 Finnish controls and 132 additional colon cancer patients by restriction enzyme digestion to clarify the frequency of this polymorphism. In total, seven control individuals (6.4%) and 13 colon cancer patients (8.9 %, including two HNPCC cases) displayed the variant, suggesting that this polymorphism is rather neutral than related to colorectal cancer. Even *SMAD2* and *SMAD4* genes are mutated in a subset of human colorectal tumors, our results suggest that these genes are not involved in the MSS HNPCC. To date, *SMAD3* mutations have not been reported in human cancers. In a recent study *SMAD3* mutations were analysed among 35 sporadic colorectal and 15 HNPCC cancers and no mutations were found (Arai et al., 1998). Further work is necessary to unravel the molecular background of MSS HNPCC.

SMAD4 is infrequently hypermethylated and mutated in sporadic colorectal cancers (study V)

SMAD4 seems to be mutated in only less than 10% of cancers analysed. The only exception is pancreatic cancer, where the mutation frequency is approximately

20% (Takagi et al., 1996; Thiagalingham et al., 1996; Schutte et al., 1996; Hahn et al., 1996; Nagatake et al., 1996; Rozenblum et al., 1997).

Knudson's hypothesis that two hits are required for the full inactivation of a tumor-suppressor gene has been shown to be correct in most human cancers. Traditionally, it has been thought that tumor suppressor genes are inactivated by intragenic mutations and loss of chromosomal material. However, the fact that methylation of CpG islands located in the promoters of genes can cause transcriptional silencing, has led to the suggestion that hypermethylation of tumor-suppressor gene promoters may be one of the mechanisms leading to malignant transformation (Jones and Laird, 1999). Several tumor suppressor genes contain CpG islands in their promoters and some of them, like *RB1* and *VHL* also show evidence of methylation silencing (Herman et al. 1994; Sakai et al., 1991). On the other hand, promoters of some tumor suppressor genes are very rarely hypermethylated. For example, *TP53* is mostly inactivated by point mutations, whereas *p16* is generally inactivated by homozygous deletion (Hussain and Harris, 1998; Kamb et al. 1994).

There has been two studies on the possible *SMAD4* promoter region. The first candidate for the *SMAD4* promoter was reported by Minami and colleagues (1998), when they cloned a 1.4 kb fragment of the *SMAD4* 5'-flanking region from a phage library by using the first coding exon's sequence as a primer. This *SMAD4* promoter lacks typical TATA boxes and CpG islands, but contains some TATA-like structures (TAAAAT) as well as some binding sites for transcription factors (Minami et al., 1998). Later, another candidate for the *SMAD4* promoter was published by Hagiwara and colleagues (submitted). First, they identified a new non-coding exon (exon 1) by using the 5'-rapid amplification of the cDNA ends (5'-RACE) (Hagiwara et al., submitted). Then *SMAD4* promoter was cloned using the exon 1 sequence as a probe. The *SMAD4* promoter published by Hagiwara and colleagues is a typical CpG island having a high G+C content (Hagiwara et al., submitted).

In study V, we examined whether hypermethylation of the promoter could be an alternative mechanism for *SMAD4* inactivation. The CpG island near non-coding exon 1 was selected for this analysis, since it is well documented that methylation has important regulatory effects especially when involving these CpG rich areas (Bird, 1986). In total, 26 MSI and 16 MSS tumors and corresponding normal DNAs were selected for the analysis and no evidence of hypermethylation was found.

In the recent study *SMAD4* mutations were analysed from 176 colorectal tumors and *SMAD4* mutation frequencies were found to be 0% in adenoma, 10% in intramucosal carcinoma, 7% in primary invasive carcinoma without metastasis, 35% in primary invasive carcinoma with metastasis and 31% in distant metastasis. So, the frequency of *SMAD4* mutations was strongly correlating with the stage of colorectal cancer (Miyaki et al., 1999). This correlation was further confirmed by Koyma and colleagues (1999). They analysed *SMAD4* mutations among 64 advanced colorectal cancers and found them in seven tumors. They also demonstrated that in all these seven tumors carrying intragenic mutations in one allele, LOH analysis showed the loss of the other allele. Their results suggested that inactivation of both alleles of the *SMAD4* gene occurs in a substantial portion of advanced colorectal cancers (Koyama et al., 1999).

In our study (study V), 22 primary CRCs were taken into *SMAD4* mutation screening and the only change detected was a polymorphism in exon 2. The tumors included in our mutation screening study were mainly classified as Dukes' stages A and B (15/22,) and one explanation for the lack of *SMAD4* mutations could be that the tumors were mainly limited to mucosa and submucosa and were not metastasizing.

There are many studies reporting *SMAD4* mutation analyses for different tumors. In most of them, the mutation screening is limited to the coding exons and small fragments of the introns around these exons. In one recent study, Zhou and colleagues (1999) analysed six endometrial tumors that do not express normal *SMAD4*, and found mutation in the 5'-untranslated region in two of them (Zhou et al., 1999). The region included in mutation screening was a 331 bp long fragment, which spanned nucleotides –262 to +69 from the transcription start site of *SMAD4*. This fragment contains most of the important transcription factor binding sites (Minami et al., 1998). To further study whether this 5'-untranslated region is also mutated in colorectal carcinomas, we sequenced the same fragment from 15 MSS and 7 MSI tumor samples. We however found no changes.

It has generally been considered that the genes involved in hereditary cancer syndromes are also mutated in the same type of sporadic tumors (Fearon, 1998). For example *APC*, the gene responsible for FAP, is involved in up to 80% of all sporadic colon cancers (Beroud and Soussi, 1996). There is good evidence that the hamartomas in PJS, JP and CS can progress to colorectal carcinoma. However, mutations in *PTEN*, *LKB1* or *SMAD4* genes in the sporadic colon cancers occur at very low frequencies

(Kong et al., 1997; Avizienyte, et al., 1998; Wang et al., 1998b; MacGrogan et al., 1997; Takagi et al., 1996).

There are also examples of cancer syndromes, where a gene, which is mutated in germline is not frequently mutated in the corresponding somatic cancers. The familial breast/ovarian cancer genes, *BRCA1* and *BRCA2*, cause cancer when mutated in the germline, but are only seldom mutated in sporadic cancers (Futreal et al., 1994; Miki et al., 1996; Teng et al., 1996). There is, however, evidence that *BRCA1* is inactivated by promoter methylation in some sporadic breast cancers (Dobrovic and Simpfendorfer, 1997). *TP53* gene is an another example of discrepancy in somatic and germline mutations. The *TP53* mutations were first described in sporadic colorectal carcinomas (Baker et al., 1989), in which they are frequent, however families with germline *TP53* mutations (Li-Fraumeni syndrome) are not predisposed to colorectal carcinomas (Knudson, 1993).

Our results together with previously published data strongly suggests that the *SMAD4* is not frequently mutated in primary colorectal carcinoma and that the hypermethylation of *SMAD4* promoter region is not a crucial mechanism in colorectal tumorigenesis.

SUMMARY

The aims of this study were to localize and identify the gene predisposing to JP, and to evaluate its role in hereditary and sporadic colorectal tumorigenesis.

In study I, we analysed whether *PTEN*, gene frequently mutated in CS and BRR, could also be the gene predisposing to JP. In total, 25 unrelated JP patients were screened for the presence of *PTEN* mutations and no mutations were found. Furthermore, linkage analysis in eight informative families excluded linkage to *PTEN*, and hence, excluded the possibility of *PTEN* promoter or 3'-UTR mutations and whole gene deletions at least these eight JP families. Thus, although germline mutation has been detected both in CS and BRR (also characterized by hamartomatous gastrointestinal polyps), our results suggest that JP is not allelic to these syndromes.

To localize the gene predisposing to JP, a whole genome wide search was done in three Finnish JP families (families 2, 4 and 5, data not shown). However, simultaneously and independently, Howe and colleagues (1998b) succeeded in localizing the gene predisposing to JP in chromosome band 18q21.1 (Howe et al., 1998b). This chromosomal region contains two putative tumor suppressor genes: *DCC* and *SMAD4,* and in collaboration with Howe and colleagues (**study II**) we managed to identify germline mutations in *SMAD4* gene in JP patients. Conclusively, *SMAD4* mutations were found in 8 of 12 JP cases studied (5 familial and 3 sporadic). *SMAD4* mutations were not detected in two families and in two sporadic cases, that were negative also in the previous *PTEN* mutation analysis. JP is obviously a genetically heterogeneous condition, as evidenced by the fact that not all families are linked to 18q markers and not all patients studied had germline *SMAD4* mutations. It has been hypothesized that the germline mutations in genes (other than *SMAD4*) encoding different components of the TGF-β signalling pathway may be present in JP. To study this hypothesis further, we focused our screening on *SMAD* genes, which are involved in TGF-β signaling via TGF-β type II and I receptors. **In study III** cDNA based mutation screening was performed for *SMAD2*, *SMAD3* or *SMAD7* genes and no mutations were detected in these genes in any of our patients.

One syndrome, which is occasionally detected among JP patients, is HHT. Two genes have been identified for HHT. *Endoglin*, which maps to chromosome 9q3 is identified as the gene for HHT1 and *ALK1*, which maps to chromosomal locus 12q11-14 is the gene for HHT2, respectively. Both *endoglin* and *ALK1* are members of *TGF-β receptor* superfamily and are therefore considered as good candidates for JP gene. We screened *endoglin* and *ALK1* mutations among those JP patients, where *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7* or *PTEN* mutations have not been detected. No mutations were detected in either one of these genes in any of patients analyzed (**unpublished data**).

Loss of growth inhibition by transforming growth factor β (TGF-β) is an important step in colorectal tumorigenesis. As SMAD proteins play a key role in intracellular TGF-β signaling, mutations in some of the *SMAD* genes may disrupt this pathway. To investigate whether germline mutations in *SMAD2, SMAD3* or *SMAD4* could predispose to HNPCC, we analyzed mutations in these genes from 14 HNPCC kindreds in which germline mutations of *MSH2* and *MLH1* had been ruled out. *SMAD* gene mutations were not found in any of patient analyzed, suggesting that these genes do not have a major role in HNPCC (**study IV**).

It is generally considered that the genes involved in hereditary cancer syndromes are also mutated in the same type of sporadic tumors. However, *SMAD4* mutations seem to be relatively rare in the sporadic colon cancers. **In study V**, we examined whether hypermethylation of the promoter could be an alternative mechanism for *SMAD4* inactivation in colorectal cancers. In total, 26 MSI and 16 MSS tumors and corresponding normal DNAs were analyzed and no evidence of hypermethylation was found. *SMAD4* mutation screening was also performed among 22 primary CRCs and the only change detected was a polymorphism in exon 2. Our results together with previously published data suggests that *SMAD4* is not frequently mutated in primary colorectal carcinoma and that the hypermethylation of *SMAD4* promoter region is not a crucial mechanism in colorectal tumorigenesis.

CONCLUSIONS AND FUTURE PROSPECTS

Although it has been reported that some patients with JP have germline *PTEN* mutations, this connection remains controversial and until now the only proven cause of JP, is germline mutation in the *SMAD4* gene. However, *SMAD4* mutations seem to account for only less than half of the JP cases suggesting that juvenile polyposis is a genetically heterogeneous disease. The identification of further JP loci by linkage analysis might be problematic, since most of the existing JP families are relatively small, and it is possible that there is still more than one gene to be identified. There have been a few attempts to identifying other JP genes by "candidate gene"- strategy, but so far these efforts have failed.

Identification of further gene/genes predisposing to JP will, however, be important, since it may help in the diagnosis, and especially asymptomatic at-risk individuals may benefit from gene testing. In particular, when a mutation in a predisposing gene (like *SMAD4*) is known to be segregating in the family, at-risk persons who are negative for the mutation, may be relieved of years of colonoscopic surveillance. Similarly, persons who are test positive for the mutation, may approach the screening regimen with a greater willingness to participate.

Further work related to the function of *SMAD4* gene in juvenile polyposis is also needed. Especially the role of the 4 bp deletion detected in six JP families is highly interesting, since it seems to represent a mutational hotspot rather than an ancestral founder mutation. On the other hand, the fact that a single mutation in *SMAD4* is responsible for 50% of the clinically classified JP cases might simplify the genetic testing.

The role of the *SMAD4* gene in colorectal tumorigenesis is still largely unknown. There is some evidence suggesting that the *SMAD4* gene is mutated in advanced stages of human colorectal cancers, but otherwise the *SMAD4* mutations seem to be rare in these cancers. Our results also suggest that the hypermethylation of the *SMAD4* promoter is not a crucial mechanism in colorectal carcinogenesis. However, final conclusions about the significance of *SMAD4* in colorectal tumorigenesis can not be drawn before the expression of *SMAD4* has been carefully studied in these tumors.

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