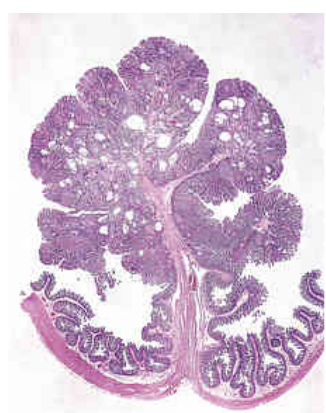
UNIVERSITA' DI NAPOLI FEDERICO II

**DOTTORATO DI RICERCA IN BIOCHIMICA
E BIOLOGIA MOLECOLARE E CELLULARE
XXIII CICLO**

HAMARTOMATOUS POLYPOSIS SYNDROMES: MOLECULAR MECHANISMS AND GENETIC TESTING.



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«Non sono i frutti della ricerca scientifica che elevano un uomo ed arricchiscono la sua natura, ma la necessità di capire e il lavoro intellettuale.»

Albert Einstein

SUMMARY

Hamartomatous polyposis syndromes are a rare group of hereditary autosomal dominant disorders that comprise less than 1% of all hereditary colorectal cancers. However, these hamartomatous polyposis syndromes have a malignant potential for the development of colorectal cancer as well as extracolonic cancers. The hamartomatous polyposis syndromes include juvenile polyposis syndrome (JPS); PTEN hamartoma tumor syndrome, which includes Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS), and Peutz-Jeghers syndrome (PJS). Due to the rarity of these conditions, a thorough understanding of their clinical presentation, including extraintestinal manifestations, and genetics is important. For pediatric gastroenterologists, understanding how to recognize and establish the appropriate diagnosis and cancer risk and following appropriate screening and surveillance guidelines is crucial for early detection to minimize the risk of carcinoma as children reach adulthood.

Peutz-Jeghers syndrome (PJS) is an autosomal dominantly inherited syndrome characterized by mucocutanoeus pigmentation, multiple hamartomatous polyps in the gastrointestinal tract and an increased risk of cancer at a young age. Inactivating germ-line mutations in the tumor suppressor gene STK11/LKB1 have been detected in approximately 80% of patients.

The aim of this work is to clarify the molecular basis of the disease in Italian PJS patients.

We investigate the *STK11/LKB1* gene mutations in a well-characterized series of 9 unrelated Italian PJS patients, by using a combination of PCR, RT-PCR, DNA sequencing, Southern blot analysis and real-time polymerase chain reaction techniques.

We have characterized the specific STK11 mutation in 6 probands, and identified 2 truncating mutations (1 novel and 1 known mutation), one missense known mutation in the exon four, and two novel small in-frame deletions in the exon six. Finally, we have found an intra-exonic in-frame deletion encompassing exons 2 and 4: the possible mechanism leading to this genomic rearrangement is most likely an Alu-Alu homologous recombination.

In our study point mutations, small scale deletions/insertions and exonic STK11 deletions account for about 67% of PJS; mutations in other STK11 related genes examined have not be found. Other gene

inactivating methods, such as chromosomal rearrangements mediated by Alu-Alu homologous recombination, which cannot be detected by routinely molecular biology screening methods, might be responsible for PJS in mutations negative population subset. However, the existence of genetic heterogeneity cannot be excluded.

The “PTEN hamartomatous tumor sindrome” (PHTS), include a group af syndromes that are caused by germline mutations of the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome ten). They belong to hamartomatous polyposis syndromes family, a rare and heterogeneous group of hereditary autosomal dominant disorders characterized by multiple polyps in the gastrointestinal tract and greatly increased risk of developing malignant tumours in multiple tissues.

The PTEN tumor suppressor gene affects multiple cellular processes including cell growth, proliferation, and cell migration by antagonizing phosphatidylinositol 3-kinase (PI3K)/Akt phatway.

we have first screened the PTEN coding region in three italian patients with clinical diagnosis of PHTS by using a combination of RT-PCR, direct sequencing of the amplified fragments and real-time polymerase chain reaction techniques. Afterwards, in periferal blood cells of these patients, we have defined the expression profile of other genes directly related to PI3K/Akt phatway, as cMYC, COX2, CCND1 and TNFa, or involved in colorectal cancer onset, as APC, DKC1 and hTERT.

We have characterized the specific PTEN mutation in 1 subject, the c406C->T (C136R) mutation, a missence mutation of the catalytic domain just described in literature before. The others two patients showed a low level of PTEN mRNA expression, respectively of 0,3 and 0,4 fold change, related to a healthy controls. All three patients were characterized by an high level of COX2, TNFa and CCND1 genes expression and decrease expression of APC gene.

Our data represent the first evidence of a PI3K/Akt phatway dysregulation in periferal blood cells of PHTS patients that probably determine a pro inflammation attivation. Knowledge of specific molecular phatways costitutively dysregulated in this syndrome could be helpful in optimizing molecular targeted therapy and preventative care.

RIASSUNTO

Le sindromi amartomatose sono un gruppo di disordini autosomici dominanti molto rari, rappresentate dalla poliposi giovanile (JPS), dalla sindrome di Peutz-Jeghers (PJS), e dalle sindromi amartomatose associate al gene PTEN (PHTS); queste includono a loro volta la sindrome di Cowden (CS) e la sindrome di Bannayan-Riley-Ruvalcaba (BRRS).
Pur essendo molto rare, esse sono tuttavia associate ad un aumento significativo del rischio di sviluppare un tumore del colon, così come di altri tipi di tumori extracolici, le cui sedi variano tra le varie sindromi. Appare, quindi, evidente l'importanza di poter preventivamente diagnosticare,ciascuna sindrome al fine di indirizzare i pazienti ad una specifica ed adeguata sorveglianza. L'analisi molecolare dei geni candidati svolge un ruolo cruciale sia nella diagnostica, che nella ricerca dei processi molecolari alla base delle sindromi amartomatose. Inoltre lo studio delle alterazioni dei pathways molecolari coinvolti nell'insorgenza di tali sindromi rappresenta un importante mezzo, per chiarirne i meccanismi molecolari e individuare nuovi e più mirati bersagli terapeutici.
A tal fine sono stati analizzati 9 pazienti affetti da sindrome di Peutz-Jeghers e 3 soggetti affetti da sindrome di Cowden provenienti da altrettante famiglie non imparentate tra loro.
Mutazioni germinali nel gene oncosoppressore STK11/LKB1, che codificano per una proteina della famiglia delle serine-treonine-chinasi, la serina-treonina-chinasi 11 (STK11), sono responsabili dell'insorgenza della PJS. La chinasi LKB1 fosforila ed attiva 14 chinasi a valle, attraverso le quali regola numerosi processi cellulari tra cui quello apoptotico, della regolazione della proliferazione cellulare, della polarità cellulare e della regolazione del metabolismo.
Le mutazioni a carico del gene STK11/LKB1 rendono conto di una percentuale di casi di PJS che varia tra il 30 e 70% ed è quindi di grande importanza chiarire le basi molecolari dell'insorgenza della malattia nei casi che non presentano mutazioni puntiformi a carico del gene STK11/LKB1.
L'analisi molecolare della regione codificante del gene LKB1/STK11, eseguita in 9 individui con diagnosi clinica di PJS ha

permesso l'identificazione e la caratterizzazione di tre mutazioni non descritte in letteratura e di due già precedentemente descritte. Nella nostra casistica la mutazione è stata identificata nel 60% dei casi, coerentemente a quanto indicato in letteratura. Nei soggetti affetti da PJS, negativi per la presenza di mutazioni puntiformi, nel gene STK11 è stata effettuata un'analisi sia qualitativa che quantitativa del messaggero utilizzando le tecniche di RT-PCR e di real-time-RT-PCR. Per valutare la presenza di eventuali delezioni intrageniche sono stati effettuati esperimenti di long-range PCR sul DNA genomico e di Souther Blotting. I frammenti di peso molecolare inferiore a quello atteso, evidenziati in seguito ad elettroforesi dei prodotti di PCR, sono stati caratterizzati mediante sequenziamento diretto dei frammenti amplificati. In fine è stata poi eseguita l'analisi *in silico* della sequenza del gene STK11/LKB1 utilizzando il programma RepeatMasker al fine di effettuare una precisa localizzazione delle sequenze ripetute presenti all'interno della sequenza genomica.

In uno dei soggetti analizzati è stata identificata, a livello del messaggero, la perdita degli esoni 2 e 3 Attraverso l'analisi di sequenza è stato possibile identificare la presenza di due sequenze Alu, appartenenti alla stessa sotto-famiglia (AluY), in corrispondenza dei "break-point" della delezione che si trovano proprio all'interno della ripetizione di 26 coppie di basi che costituisce il "core" di queste sequenze.

Nei tumori umani l'elevata densità di sequenze Alu è stata spesso associata ad un'elevata frequenza di riarrangiamenti genomici, quali delezioni o inversioni, che non sempre vengono rilevati mediante le comuni tecniche di biologia molecolare, e che potrebbero giustificare la percentuale di casi di PJS, nei quali non viene identificata la specifica mutazione a carico del gene STK11/LKB1.

La sindrome di Cowden è dovuta a mutazioni nel gene oncosopressore PTEN, che codifica per una proteina con attività tirosina-fosfatasica che determina l'inibizione della via PI3K/Akt.

I pazienti affetti da noi analizzati, presentavano caratteristiche fenotipiche tipiche di tale sindrome. L'analisi molecolare è stata condotta mettendo a punto in primo luogo una reazione di RT-PCR e sequenza diretta del prodotto di PCR, a cui

successivamente è seguita la valutazione dei livelli di espressione del messaggero del gene PTEN. In uno dei tre pazienti è stata dunque identificata una mutazione puntiforme a livello dell'esone 5 del gene, successivamente confermata mediante amplificazione e sequenziamento diretto sul DNA genomico del soggetto. A questa prima analisi è seguita la quantizzazione relativa dei livelli d'espressione del gene, effettuata mediante real-time PCR, che ha evidenziato una marcata diminuzione di espressione del gene PTEN nei due pazienti negativi per la presenza di mutazioni nella regione codificante del gene.
Infine, per meglio comprendere i meccanismi molecolare coinvolti nell'insorgenza delle sindromi PHTS, è stato effettuato uno studio sui livelli di espressione di alcuni geni correlati al pathway molecolare PI3K/Akt o comunque coinvolti nell'insorgenza dei CRC, quali APC, cMYC, CCND1, COX-2 e TNFα, in linfociti estratti da sangue periferico. Tale analisi ha evidenziato un significati aumento nell'espressione nei pazienti affetti da CS dell'espressione della COX-2, CCD1 e TNFα, mentre risulta diminuita l'espressione del gene APC. Queste indicazioni, che certamente necessitano di ulteriori approfondimenti, rappresentano però la prima evidenza di una possibile attivazione del pathway PI3K/Akt in linfociti di sangue periferico provenienti da pazienti con mutazioni del gene PTEN. Mutazioni di tale gene dunque determinare un'attivazione molto precoce di segnali pro-infiammatori che a lungo andare possono alterare la crescita cellulare, spingendo così la cellula verso la trasformazione neoplastica.

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INTRODUCTION

A more individualized approach to cancer treatment and prevention will depend upon the ability to identify and understand the molecular changes that drive the tumorigenic process in each individual tumor. The advancement of DNA sequencing technologies spurred by the human genome sequencing project is allowing researchers to determine how many somatic mutations exist in a given tumor *(Heinen C. D., 2009)*. The number of mutations may be as high as 80 or more *(Sjoblom T. et al., 2006; Wood L.D. et al., 2007)* or it may be as small as eight to ten *(Ley T.J. et al., 2008);* these differences likely are due to the tumor type, the methods involved for detecting alterations and the threshold for predicting whether an alteration is significant to phenotype. Determining which variations drive tumor phenotype will depend upon an ability to ascertain the cellular function affected by each somatic change. The relationship between genotype to phenotype remains the great challenge of basic cancer research and the key to developing effective targeted therapies. Nothing, perhaps, demonstrates this challenge better than contemplating how much, yet how little, we understand about the relationship between genotype and phenotype in hereditary colorectal cancer (CRC) *(Heinen C. D., 2009).* Because of the high frequency of cases in the population, of the ability to identify and isolate benign precursor lesions in the colon, and of hereditary diseases with increased predisposition to CRC development, colon cancer has been one the most studied cancers and it serves as a model for understanding basic principles of tumorigenesis that may apply to all tumor types *(Fearon E.R., Vogelstein B., 1990).*

More than 1 million new cases of colorectal cancer (CRC) are diagnosed worldwide each year. The CRC is the 3rd most common malignancy and the 4th most common cause of cancer mortality worldwide. *(Tenesa A. et al. 2009).*

Approximately 5% of CRC cases are associated with highly penetrant inherited mutations and clinical presentations that have been well-characterized. Kindred and twin studies estimated that approximately 30% of all CRC cases are an inherited form of the disease, are not completely understood. They are likely to be caused by alterations in single genes that are less penetrant but more common than those associated with the well-characterized syndromes. Inherited CRCs are also likely to be caused by alterations in multiple susceptibility loci that have additive effects. A precise understanding of the genetics of inherited CRCs is important for identifying at-risk individuals, improving cancer

surveillance and prevention strategies, and developing better diagnostic and therapeutic approaches. (*Lichtenstein P, et al.* 2000; *Grady WM. 2003).*

Between 2% to 5% of all colon cancers arise in the setting of well-defined inherited syndromes. Each is associated with a high risk of colon cancer. Clarification of predisposing genes allows for accurate risk assessment and more precise screening approaches. (TAB 1). The syndromes of CRC are defined on the basis of clinical, pathological, and, more recently, genetic findings. Conditions that express adenomatous polyps include Lynch syndrome (also called hereditary nonpolyposis colorectal cancer) familial adenomatous polyposis (FAP), and MUTYH-associated polyposis (MAP) *(Jasperson KW et al., 2010).*

Lynch syndrome, accounting for 2%–4% of all CRCs *(Stoffel E, et al. 2009)*, is the result of a germline mutation in a class of genes involved in DNA Mismatch Repair (MMR), including hMSH2, hMLH1, hMSH6, and hPMS2, and it is characterized by a high level of microsatellite instability (MSI-H). The lifetime risk is 40%–60%, and it is many greater than the estimated risk for CRC in general population. Lynch syndrome is also responsible for approximately 2% of all endometrial cancers *(Hampel H. et al, 2006).*

FAP is the second-most common inherited CRC syndrome, with a prevalence of 1 in 10,000 individuals. Characteristic features of FAP include development of hundreds to thousands of colonic adenomas beginning in early adolescence, and inevitable CRC in untreated individuals. The average age of CRC diagnosis if untreated is about 39 years; nearly 7% develop CRC by age 21 and about 95% by age 50. FAP and attenuated FAP are caused by germline mutations in APC gene , which encodes a tumour suppressor that is part of the WNT signalling pathway. The characterization of *APC* mutation in a proband confirms the diagnosis, allowing precise identification of at risk-relatives who have inherited the disease *(Jasperson KW et al., 2010).* Hamartomatous polyps are the primary lesions in hamartomatous syndromes; they include juvenile polyposis syndrome (JPS), PTEN hamartoma tumor syndrome, which includes Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS); and Peutz-Jeghers syndrome (PJS). Finally, hyperplastic polyposis (HPP) is an unusual condition that has a substantial cancer risk and must be distinguished from the other conditions. All of these conditions are inherited, autosomal-dominant disorders, except MYH associated polyposis (MAP), which is autosomal-recessive, and HPP, which is rarely inherited. Attenuated FAP is

associated with small number of adenomas so his phenotype can be confused with Lynch syndrome, or sporadic polyps. Although clinical similarities do exist, each syndrome has distinct cancer risks, characteristic clinical features, and separate genetic etiologies. Diagnosis and management recommendations are based on these divergent features *(Jasperson KW et al., 2010).*

Disease	Gene	Incidence	Mutation identified (%)	Inheritance	No. of polyps	Distribution of polyps	Polyp histology	Other symptoms	Remarks
Classical familial adenomatous polyposis (FAP)	*APC*	1 : 10 000	80–90	AD	100 to >5000	Large bowel, duodenum, (stomach)	Adenoma	Desmoids, osteomas, CHPRE, epidermoid cysts, hepatoblastoma, medulloblastoma	Mutation detection rate rises with severity of disease
Attenuated FAP (AFAP)	*APC*	<1:10 000	20–30	AD	10–100	Large bowel, duodenum, (stomach)	Adenoma	Rare	On a continuous spectrum with classical FAP
MUTYH-associated polyposis (MAP)	*MUTYH*	<1:10 000	15–20	AR	20 to hundreds	Large bowel, duodenum, (stomach)	Adenoma	Increased incidence of extraintestinal malignancies, rarely sebaceous gland tumors	Low risk of recurrence in offspring (AR)
Hereditary nonpolyposis colorectal cancer (HNPCC)	*MLH1, MLH2, MSH6, PMS2*	1 : 500 ?	60–80	AD	0 to >30	Large bowel	Adenoma	Endometrial carcinoma, gastric carcinoma, sebaceous gland tumors, etc.	If few adenomas, HNPCC is an important DD for AFAP/MAP
Birt–Hogg–Dubé syndrome (BHD)[*1]	*BHD (FLCN)*	Rare	80–90	AD	Several, multiple	Large bowel	Adenoma	Specific skin tumors, renal tumors, pulmonary cysts (pneumothorax)	Not usually associated with gastrointestinal polyps
Peutz–Jeghers syndrome (PJS)	*STK11 (LKB1)*	1:150000	90	AD	<20	Small bowel, large bowel, stomach	PJ polyps	Mucocutaneous/perioral hyperpigmentation, ovarian tumors (SCTAT), breast cancer	Pigmentations often fade over the course of life; colorectal adenomas are also frequent
Juvenile polyposis syndrome (JPS)	*SMAD4, BMPR1A*	1:16 000–1:100 000	60	AD	~ 5 to hundreds	Small bowel, large bowel, stomach	Juvenile polyps	SMAD4 mutation carriers: hereditary hemorrhagic telangiectasia (HHT) in ~ 20%, gastric polyps and gastric cancer	Polyps often histologically misinterpreted, in case of doubt refer to expert pathologist
Juvenile polyposis of infancy	*BMPR1A + PTEN*	Very rare	100	AD	Numerous	Small bowel, large bowel, stomach	Juvenile polyps	Symptomatic in first years of life, symptoms of JPS.	Often fulminant course with high mortality
Cowden syndrome (CS)	*PTEN*	1:200 000	80	AD	Multiple	Small bowel, large bowel, stomach	JP, HP, lipomas, ganglioneuromas, etc.	Mucocutaneous tumors, breast cancer, endometrial carcinoma, thyroid cancer, other hamartomatous tumors	Colorectal hamartomas manifest late and are not the main focus for the diagnosis
Bannayan–Ruvalkaba–Riley sindrome (BRRS)	*PTEN*	1:200 000	60	AD	Multiple	Small bowel, large bowel, stomach	JP, HP, lipomas, ganglioneuromas, etc.	Macrocephaly, developmental delay, intestinal hamartomas, lipomatosis, hemangiomatosis, speckled penis in males	Colorectal hamartomas manifest late and are not the main focus for the diagnosis

Table 1: Hereditary colon cancer syndromes

JP, juvenile polyps; HP, hyperplastic polyps; PJ, Peutz–Jeghers; BRRS, Bannayan–Ruvalkaba–Riley syndrome;CHRPE, congenital hypertrophy of the retinal pigment epithelium; CRC, colorectal carcinoma; SCTAT, sex cord tumors with annular tubules; AD, autosomal dominant; AR, autosomal recessive; ?, unknown at present; DD, differential diagnosis; [*1] HNPCC and BHD are not themselves polyposis syndromes, but are included in the DD of polyposis syndromes. According to recent studies, BHD is not associated with intestinal polyps;

From: *Arztebl Int. 2010 March; 107(10): 163–173.*

1.1 Hereditary Hamartomatous Polyposis Syndromes.

Hamartomatous polyposis syndromes are a rare group of hereditary autosomal dominant disorders that comprise less than 1% of all hereditary colorectal cancers. *(Wirtzfeld DA et al., 2001)* Hamartomatous polyps are benign entities comprised of cells that are indigenous to the area in which they are found (ie, all cell layers with a mesenchymal predominance). However, these hamartomatous polyposis syndromes have a malignant potential for the development of colorectal cancer as well as extracolonic cancers. The progression of hamartomatous polyps to carcinoma is still being elucidated. Unlike adenomatous polyps, in which malignant transformation progresses through the adenoma-carcinoma sequence via a gatekeeper or caretaker defect, in hamartomatous polyps, a proposed hamartoma-carcinoma sequence hypothesis involves a landscaper defect in which stromal elements create a local environment that promotes epithelial dysplasia and ultimately leads to carcinoma *(Kinzler KW et al.,1998).* The hamartomatous polyposis syndromes include juvenile polyposis syndrome (JPS); PTEN hamartoma tumor syndrome, which includes Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS), and Peutz-Jeghers syndrome (PJS). Due to the rarity of these conditions, a thorough understanding of their clinical presentation, including extraintestinal manifestations, and genetics is important. For pediatric gastroenterologists, understanding how to recognize and establish the appropriate diagnosis and cancer risk and following appropriate screening and surveillance guidelines is crucial for early detection to minimize the risk of carcinoma as children reach adulthood *(Manfredi M, 2010).*

Juvenile polyps are the most common type of pediatric gastrointestinal polyps. Solitary juvenile polyps can develop at any age, though they appear most frequently in preschool children and have an incidence of 2% in children under 10 years of age. In JPS, affected individuals develop multiple gastrointestinal juvenile polyps, predominantly in the colon, though the condition may also affect the rest of the gastrointestinal tract *(Desai DC. Et al., 1995)*. Multiple extraintestinal manifestations have been reported in approximately 11–20% of cases with JPS, and they can include heart defects, polydactyl, clubbing, intestinal malrotation, Meckel diverticulum, hydrocephalus, macrocephaly, hypertelorism, cleft lip, cleft palate, double renal pelvis and ureter, bifid uterus and vagina, undescended testes, and supernumery teeth. *(Chow E. et al. 2005)* Individuals with JPS are at risk for the development of colorectal, gastric,

small intestinal, and pancreatic cancers. The risk of developing colorectal cancer from solitary juvenile polyps is thought to be negligible or nonexistent. However, individuals with JPS are at risk for developing adenomatous change and carcinoma. The incidence of colorectal cancer has been reported to be to 20%, with a mean age of 34 years (age range, 15–59 years) and an estimated cumulative colorectal cancer risk of 68% by 60 years of age *(Schreibman IR. et al., 2005).* The surveillance protocol for this disease recommends the continue screening until 70 years of age if a genetic mutation is found, and no polyps are detected at the time of the initial endoscopy; however, if no genetic mutation is found and no polyps are detected at the initial endoscopy, repeat endoscopy should be performed every 1–2 years until 35 years of age. *(Manfredi, 2010).*

The PTEN hamartoma tumor syndromes (PHTS) are a collection of rare clinical syndromes characterized by germline mutations of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10. They includes Cowden's syndrome (OMIM 158350), Bannayan–Riley–Ruvalcaba syndrome (OMIM 153480), and all syndromes that are caused by germline mutations of the tumor suppressor gene PTEN (*Wirtzfield et al. 2001).* The PHTS are a spectrum of syndromes with variable clinical manifestations characterized by aberrant growth. Hamartomas are a histologically distinct subtype of benign tumors in which cells maintain normal differentiation but are disorganized with respect to architecture. Cowden syndrome (CS) is the prototypic syndrome, characterized by mucocutaneous lesions, benign hamartomas, macrocephaly, and increased predisposition to breast, thyroid, and endometrial carcinoma. Lhermitte–Duclos (LD), a variant of CS, is characterized by dysplastic gangliocytomas of the cerebellum, which can lead to hydrocephalus, ataxia, and seizures *(Hobert JA, Eng C., 2009).* CS is an autosomal dominant syndrome, with a reported incidence of 1 in 200,000 individuals. This most likely remains an underestimation, as CS is associated with a high degree of phenotypic variability and its hallmark features are under-recognized within the medical community (*GM Blumenthal and PA Dennis 2008).* Hamartomatous polyps throughout the gastrointestinal tract are associated with this syndrome but are not as common as the extraintestinal findings associated with the syndrome, Gastrointestinal polyps in CS are typically asymptomatic, and can occur anywhere in the GI tract, with colonic polyps present in 60–90% of patients. Their incidence varies in the literature, ranging anywhere from 30% to 85% *(Marra C. et al. 1994; Starink TM, et al. 1986).* It is

generally thought that the incidence of gastrointestinal polyps in CS is less than that of BRRS, though this belief is debated in the literature. Benign thyroid lesions occur in up to 75% of patients with CS, including adenomas, hamartomas, multinodular goiter, and Hashimoto's thyroiditis. Up to half of women with CS are afflicted with benign breast disease, which can be extensive and bilateral. Cowden syndrome patients are at increased risk to develop breast, thyroid, and endometrial cancer. The lifetime risk of breast cancer in women with CS is estimated to be as high as 50%, as compared to 11% within the general population. In addition to breast, thyroid, and endometrial cancers, other malignancies have anecdotally been reported to be increased with CS, including melanoma, renal cell carcinoma, and gliomas *(Eng C, 2003*).

Bannayan–Riley–Ruvalcaba sindrome is characterized by macrocephaly, benign hamartomas, pigmented macules of the glans penis, lipomas, hemangiomas, and the developmental delay, or mental retardation. Other phenotypic features of BRRS disorder include thyroid abnormalities such as Hashimoto's thyroiditis, high-arched palate overgrowth of prenatal or postnatal onset, macrosomia, hypotonia, joint hyperextensibility, downward slanting palpebral fissures, frontal bossing, hypoglycemia, seizures, and cafe´ au lait spots. These phenotypic features are highly variable, although they appear to cluster within a given family. There is significant phenotypic overlap between CS and BRRS with common features including hamartomas, macrocephaly, and thyroid abnormalities. Interestingly, identical PTEN mutation shave been found in patients who present with phenotypic manifestations characteristic of either BRRS or CS. Even individuals within a single family that have the same germline PTEN mutation can have phenotypic features more consistent with either BRRS or CS. Same authors have suggested that BRRS and CS represent a single disorder with variable phenotypic expression and age-related penetrance, and have questioned whether the distinction between BRRS and CS is clinically relevant. (*Pilarski R, Eng C., 2004)*

Peutz–Jeghers syndrome (OMIM 175200) is an autosomal dominant hamartomatous polyposis syndrome characterized by melanotic mucocutaneous hyperpigmentation and GI hamartomas, which occur anywhere from the stomach to the anus with a prevalence of approximately one in 200,000. The pigmentation, by melanin, has the appearance of freckle-like spots on the face, lips, mouth, and anal region, presenting in more than two thirds of patients with PJS *(Winship IM, 2008).* The numbers of polyps present in each case are usually fewer than 20, and these polyps vary in size from several millimeters to more than 5

cm in diameter. In contrast to JPS, in which the polyps occur in the colon, Peutz–Jeghers hamartomatous polyps are most prevalent in the small intestine (64%), but they may also be present in the colon (53%), the stomach (49%), and rectum (32%). The disease, associated with inactivating mutations in lkb1/Stk11 gene, has variable penetrance, even within families; some patients only manifest with hyperpigmentation, while others may manifest with both pigmentation and intestinal polyps *(McGarrity TJ, Amos C 2006).* The diagnosis of PJS is based upon clinical findings and the histologic appearance of the polyps. Individuals with PJS are at risk for the development of colorectal, gastric, small intestinal, esophageal, and pancreatic cancers. PJS patients are also at risk for extraintestinal cancer such as lung, breast, ovarian, testicular, and endometrial cancers. A meta-analysis showed that the risk of developing any type of cancer by 64 years of age was 93% (relative risk of 15) and found that the relative risk of developing any type of cancer was 47% by 65 years of age in PJS patients with known genetic mutations in *LKB1/STK11***.** More recently, a study looking at 419 PJS patients, 297 of whom had documented mutations, showed the risk of cancer to be 60% by 60 years of age and 85% by 70 years of age. This same study reported the risks of developing gastrointestinal cancer (31%), breast cancer (31%), gynecologic cancer (18%), pancreatic cancer (7%), and lung cancer (13%) by 60 years of age. Individuals with PJS are also at risk for developing rare sex cord tumors. Women are at risk for sex cord tumors with annular tubules that are benign, and men are at risk for developing Sertoli cell tumors, which result in feminization *(Jasperson KW, et al. 2010).*

1.2 Genetic of the Hereditary Hamartomatous Polyposis Syndromes.

Juvenile polyposis syndrome (JPS) has been commonly associated with three genes: *SMAD4*, *BMPR1A*, and *ENG*, all of which are part of the transforming growth factor-β (TGFβ) superfamily of proteins. The *SMAD4* gene, located on chromosome 18q21.1, was first identified; germline mutations in the *SMAD4* gene have a prevalence of 20% in JPS patients. *(Jasperson KW, et al. 2010).* Patients with the *SMAD4* mutation are more likely to have upper gastrointestinal polyps. Multiple types of mutations have been reported in the *SMAD4* gene, including missense, nonsense, deletions, and insertions; however, the most common mutation is the 4-base pair deletion in exon 9.

The bone morphogenetic protein receptor type IA (*BMPR1A*) gene is located on chromosome 10q22-23 and was reported by Howe and co-worker in 2001. (*Howe JR et al. 2001).* Germline mutations in the *BMPR1A* gene have a prevalence of 20% in JPS patients. Mutations of the endoglin gene (*ENG)* have recently be identified in 2 patients with JPS; however, its role as a predisposition gene still requires additional confirmation. The *ENG* gene is located on chromosome 9q34.1.47 *ENG* encodes the protein endoglin, which is an accessory protein of the TGF-β signalling pathway *(Jasperson KW, et al. 2010).*

The involvement of the *PTEN* gene mutation in patients with juvenile polyposis is a controversial topic, they are present in five percent of familial JPS which initially led to speculation that PTEN mutations might lead to some cases of JPS. However, upon further study, it was found that these patients had unrecognized Cowden's syndrome (*Howe JR et al. 2001; Eng C. 2001).* This finding may not exclude PTEN involvement in some cases of JPS, and it might indicate a biological synergy of the BMPR1A and PTEN genes. In addition, several infants with very aggressive phenotype of JPS, characterized by generalized polyposis and often diagnosed before 2 years of age, were found to have germline deletions that encompass both PTEN and BMPR1A. These children's clinical manifestations often overlap with those seen in Cowden's syndrome or Bannayan– Riley–Ruvalcaba syndrome (BRRS). Given the high occurrence of breast and thyroid neoplasms in patients with Cowden's syndrome, the screening for these cancers should be undertaken if a PTEN mutation is identified in JPS patients **(***Hui-Min Chen & Jing-Yuan Fang 2009).*

Approximately 85% of patients with Cowden syndrome and more than 60% of patients with BRRS were noted to carry germline mutations in the PTEN tumor suppressor gene, located on chromosome 10q23 *(Eng C. 2000).* The PTEN protein inhibits cell growth and proliferation by acting as a negative regulator of the AKT pathway *(Zhou XP, et al. 2003)* and somatic PTEN mutations are prevalent in various malignancies *(Eng C. 2003).* Mutations have been detected in the promoter region of PTEN in patients with Cowden's syndrome, whereas deletions of all or part of PTEN (not normally detectable by conventional polymerase chain reaction) have been documented in patients with BRRS. Several BRRS patients do not have a PTEN mutation; however, it has recently been demonstrated that a significant proportion of these patients have germline PTEN deletions**.** Therefore, point mutations or deletions in the different regions of the PTEN gene might be corresponding to the different levels

of possibility of developing BRRS or Cowden's syndrome. Until recently, no gene mutation for Proteus syndrome has been identified. Although mouse models of PTEN deficiency are available, their function in helping to dissect the pathogenesis of human PHTS syndrome is limited. Heterozygous PTEN +/− mice often develop thymic and peripheral lymphomas and prostate cancer at a young age. They also develop hamartomatous polyps of the gastrointestinal tract, but the spectrum of neoplasia and malignancies observed bears little resemblance to that seen in cases of human PHTS syndrome *(Chen H and Fang J, 2009).*
Germline mutations in the serine/threonine kinase 11 gene (STK11/LKB1) are documented in approximately up to 70-80% of the PJS patients; of these about 15% have germline deletions in all or part of the gene. *(Volikos E, et al. 2006; Aretz S, et al 2005; Hearle NC, 2006)* In the remaining 20-30 % of PJS patients, defects in other genes or not yet identified ways of LKB1 inactivation might be responsible for PJS. Several putative candidate genes have been studied, including genes encoding LKB1 interacting proteins; so far a second PJS gene has not been identified. (*Alhopuro P et al. 2005).* The *STK11* gene is localized on chromosome 19 at position p13.3, spans 23 Kb in the genome and includes 10 exons, of which, nine are coding exons and 1 is an untranslated exon localized at 3'-UTR. (*Hemminki A, Tomlinson I, et al. 1997).* It is an ubiquitously expressed gene encoding a serine/threonine kinase involved in the transduction of intracellular growth signals. LKB1/STK11 has an essential role in G1 cell cycle arrest, cell polarity, p53-dependent apoptosis, and cellular energy levels. *(Manfredi M., 2010).* Genotype–phenotype correlation suggests that patients with PJS, who have a truncating mutation in STK11/LKB1, have a significantly earlier age of onset than those who have a missense mutation or when no mutation is detected in STK11/LKB1 *(Amos CI, et al. 2004).* A follow-up study on patients with PJS who had a germline mutation in this tumor suppressor gene confirmed that these patients have a very high risk of developing cancer *(Chen H and Fang J, 2009).*

1.3 Hamartomatous Syndromes and their molecular pathways.

Transforming growth factor beta (TGF-β) patway in hamartomatous syndromes:

The TGF-β signalling pathway is involved in the control of several biological processes, including cell proliferation, differentiation,

migration and apoptosis *(Massague J. et al., 2000)* (Fig.1). It is one of the most commonly altered cellular signalling pathways in human cancers *(Elliott RL, Blobe GC., 2005)*. TGF-β signalling is activated by the binding of TGF-β ligands to type II TGF-β receptor (TGFBR2). Three TGF- β isoforms (TGFB1, TGFB2 and TGFB3) are expressed in mammalian epithelium, and each is encoded by a unique gene and expressed in both a tissue-specific and developmentally regulated manner. TGFB1 is the most abundant and ubiquitously expressed isoform. Once TGFBR2 bound to TGF-β, recruits and phosphorylates the type I TGF-β receptor (TGFBR1), which stimulates TGFBR1 protein kinase activity. Activated TGFBR1 phosphorylates two downstream transcription factors, SMAD2 and SMAD3, allowing them to bind to SMAD4. BMPR1A is a type 1 serine/threonine kinase receptor protein that is bound to a type II serine/threonine kinase receptor protein; it acts upstream of SMAD4, phosphorylates SMAD proteins that then bind to SMAD4. The resulting binds SMAD complexes translocate into the nucleus and interact with other transcription factors in a cell-specific manner to regulate the transcription of a multitude of TGF-β-responsive genes *(Elliott RL, Blobe GC., 2005)*. It is increasingly apparent that TGF-β-related proteins initiate the activation not only of SMADs but also of other signalling pathways. These pathways regulate SMAD-mediated responses and also induce SMAD-independent responses. Some of the downstream targets of TGF-β signalling are important cell-cycle checkpoint genes, including CDKN1A (p21), CDKN1B (p27) and CDKN2B (p15), and their activation leads to growth arrest *(Derynck R, Zhang YE, 2003)*. Therefore, TGF-β serves as a tumor suppressor in the normal intestinal epithelium by inhibiting cell proliferation and inducing apoptosis. Many colorectal cancers escape the tumor-suppressor effects of TGF-β and are resistant to TGF-β-induced growth inhibition *(Derynck R, Zhang YE, 2003)*. However, during the late stages of colorectal carcinogenesis, TGF-β acts as a tumor promoter and is usually highly expressed. High levels of TGFB1 in the primary colorectal tumor are associated with advanced stages and a greater likelihood of recurrence and decreased survival *(Xu Y, Pasche B., 2007)*. Experimentally, prolonged exposure to high levels of TGF-β promotes neoplastic transformation of intestinal epithelial cells and TGF-β stimulates the proliferation and invasion of poorly differentiated and metastatic colon cancer cells *(Sheng H, et al. 1999)*. Although the mechanism by which TGF-β switches its growth inhibitory effect into growth stimulatory effect is not well understood, TGF-β has been shown to increase the production of several mitogenic growth factors including

TGF-α, FGF and EGF. In addition, TGF-β can activate SMAD-independent pathways, such as Ras/MAPK pathway, JNK pathway and PI3 kinase/Akt pathway *(Elliott RL, Blobe GC., 2005)*. Thus, TGF-β may drive the proliferation of colorectal cancer cells in conjunction with these oncogenic pathways. TGF-β is also a potent regulator of cell adhesion, motility and the extracellular matrix composition, which are involved in tumor invasion and metastasis. In addition, TGF-β signalling promotes angiogenesis and immuno-suppression *(Elliott RL, Blobe GC., 2005)*. Therefore, it is likely that cancer cells achieve resistance to the tumor-suppressor effects of TGF-β but remain responsive to the tumor-promoter effects of TGF-β via selective alterations of this signalling pathway *(Xu Y, Pasche B., 2007)*.

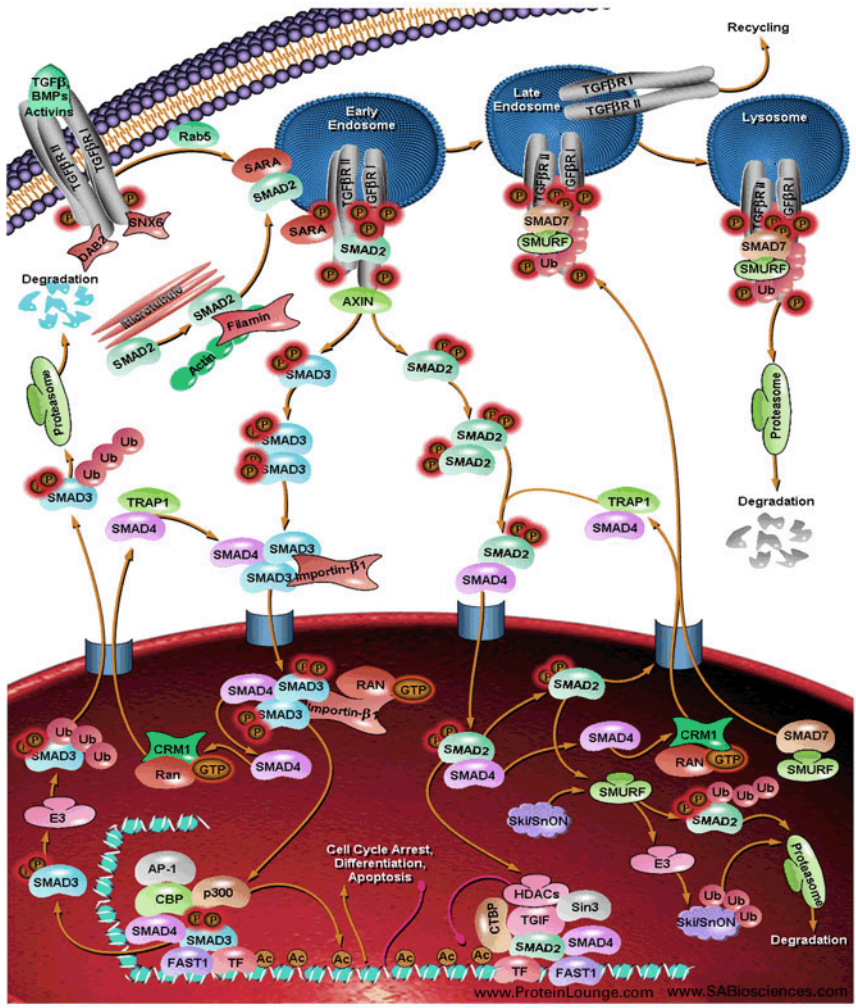


Figure 1: TGFβ molecular pathways.

PI3K/PTEN/AKT Pathway:

Phosphoinositide 3-kinase (PI3K) plays a crucial role in effecting alterations in a broad range of cellular functions in response to extracellular signals. A key downstream effector of PI3K is the serine-threonine kinase Akt which, in response to PI3K activation, phosphorylates and regulates the activity of a number of targets, including kinases, transcription factors and other regulatory molecules (Fig. 2) . A major role for PI3K pathway activation in human tumors has been more recently established following both the positional cloning of the *PTEN* tumor suppressor gene, and the discovery that the PTEN protein product was a lipid phosphatase that antagonizes PI3K function and consequently inhibits downstream signalling through Akt. Subsequently a number of the components of the pathway have been found mutated or deregulated in a wide variety of human cancers highlighting the key role of this pathway in cellular transformation.

PI3K belongs to a large family of PI3K-related kinases or PIKK. Other members of the family include mTOR (mammalian target of rapamycin), ATM (ataxiatelangiectasia mutated), ATR (ATM and RAD3 related), DNA-PK (DNA-dependent protein kinase). All possess the characteristic PI3K-homologous kinase domain and a highly conserved carboxyl-terminal tail *(Kuruvilla & Schreiber, 1999)*. However, only PI3K is known to have an endogenous lipid substrate. Importantly, all members of the PIKK family have been implicated in human cancer both as oncogenes, this is the case of type I PI3K or as tumor suppressor genes in the case of ATM and ATR. The PI3K family comprises eight members divided into three classes according to their sequence homology and substrate preference *(Fruman et al., 1998; Vanhaesebroeck & Waterfield, 1999)*. All mammalian cells express representatives of the three groups.

PTEN (phosphatase and tensin homolog deleted on chromosome 10)/MMAC1 (mutated in multiple advanced cancers)/TEP-1(TGFb-regulated and epithelial cell enriched phosphatase) antagonizes signalling through the PI3K pathway. Indeed, cells lacking PTEN function exhibit a two fold increase in PtdIns-3,4,5-P3 levels *(Stambolic et al., 1998)*. PTEN can also dephosphorylate tyrosine-, serine-, and threonine-phosphorylated peptides *(Myers & Tonks, 1997)*. This activity may be related to regulation of cell adhesion and spreading. Literature findings suggest that this activity is not sufficient to block tumor development. Indeed, the preponderance of the published data suggests that PTEN's role as a tumor suppressor is mediated largely through its lipid phosphatase activity.

The serine-threonine protein kinase Akt (also known as protein kinase B, PKB) mediates many of the downstream effects of PI3K and consequently plays a central role in both normal and pathological signalling by the PI3K pathway. There are three closely related enzymatic isoforms Akt1 (PKBa), Akt2 (PKBb)and Akt3 (PKBg), they are similar both in structure and size and are thought to be activated by a common mechanism. To date, no differences in substrate preference have been established are currently assumed to have identical or similar substrate specificity. The three isoforms are widely expressed though Akt3 tissue distribution seems to be more restricted than 1 and 2, being primarily expressed in brain and testis *(Okano, et al. 2000)*.

Activation of Akt is a multi-step process involving both membrane binding and phosphorylation. Upon PI3K activation and production of PtdIns-3,4,5-P3 and PtdIns-3,4-P2, Akt is recruited to the plasma membrane where it binds to these phosphoinositides through its PH domain. Activation is then thought to involve a conformational change and phosphorylation on two residues. Growth factor stimulation of PI3K activity leads to Akt activation. Conversely, PI3K inhibition (i.e. using chemical inhibitors such as wortmannin or LY294002) and PTEN mediated dephosphorylation of PtdIns-3,4,5-P3 and PtdIns-3,4-P2 results in inhibition of Akt. After activation, Akt can phosphorylate a number of substrates both in the cytoplasm and in the nucleus.

Akt phosphorylates a variety of substrates involved in the regulation of key cellular functions, including cell growth and survival, glucose metabolism and protein translation. These targets include GSK3, IRS-1 (insulin receptor susbtrate-1), PDE- 3B (phosphodiesterase-3B), BAD, human caspase 9, Forkhead and NF-kB transcription factors, mTOR, eNOS, Raf protein kinase, BRCA1, and p21Cip1 /WAF1 *(Altiok et al., 1999; Montagnani, et al., 2001; Zhou et al., 2001; Zimmermann & Moelling, 1999)*. One common mechanism through which Akt-mediated phosphorylation results in substrate inhibition is through the regulation of subcellular localization by interaction with 14-3-3 proteins (i.e. BAD, forkhead transcription factors). 14-3-3 proteins are cytoplasmic proteins that bind specifically to phosphoproteins and retain them in the cytoplasm away from their targets. In particular the Akt consensus phosphorylation site is also a consensus 14-3-3 binding site (*Yaffe et al., 2001)*. FKHR, FKHRL1 and AFX transcription factors (henceforth referred to as Forkhead) belong to the winged helix/forkhead transcription factors family characterized by a 100-amino acids, monomeric DNA binding domain (DBD) *(Kops & Burgering, 1999; Kops et al., 1999)*. These three

family members are directly phosphorylated and regulated by Akt. In cancer cell lines lacking functional PTEN, FKHRL1 and FKHR are constitutively phosphorylated by Akt and are hence constitutively cytoplasmic and unable to activate transcription. Thus, Forkhead is a critical effector of both cell-cycle progression and apoptosis downstream of PTEN *(Nakamura et al., 2000)*. In addition, other Forkhead family members have also been implicated in the induction of apoptosis both through the upregulation of FasL *(Brunet et al., 1999)* and through the regulation of the pro-apoptotic Bcl-2 interacting mediator (Bim1) *(Dijkers, et al. 2000)*. Human Caspase-9, a member of the protease family intimately associated with the initiation of apoptosis, is thought to be phosphorylated and inhibited by Akt. *(Cardone et al., 1998)*. However, the Akt phosphorylation site is not conserved in the Capase 9 proteins from other mammals making its *in vivo* importance unclear. In addition to the inhibition of pro-apoptotic factors, Akt can also activate the transcription of anti-apoptotic genes through the activation of the transcription factor NFkB. When bound to its inhibitor, termed IkB, NFkB localises to the cytoplasm. Akt associates and activate the IkB kinases (IKKs). Activated IKKs phoshorylate IkB targeting it for degradation by the proteosome. This allows NFkB to translocate to the nucleus and activate transcription of a variety of substrates including anti-apoptotic genes such as the inhibitors of apoptosis (IAP) c-IAP1 and 2 (*Kane, et al 1999; Romashkova & Makarov, 1999*).

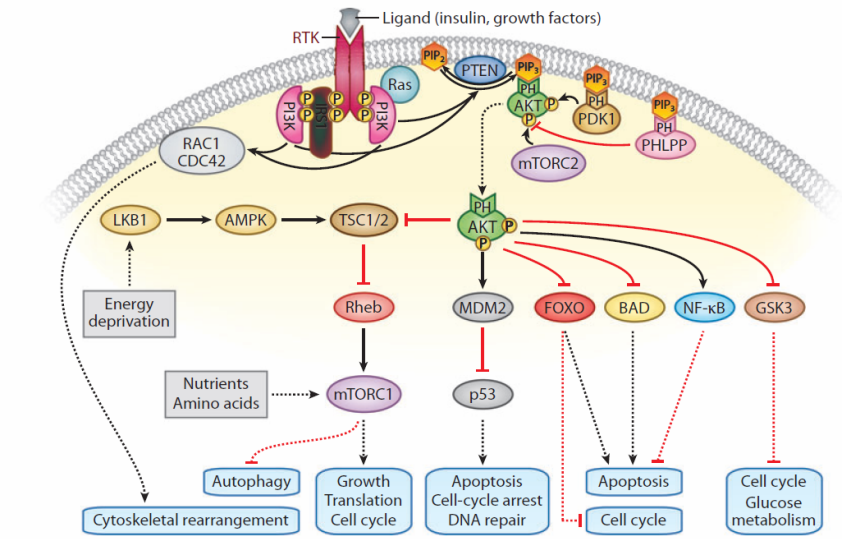


Figure 2:*PI3K/PTEN/AKT Pathway*

Recent data indicate that serine/threonine protein kinase Akt (Akt) signalling cooperates with Wingless (Wnt) to activate β-catenin in intestinal stem and progenitor cells through phosphorylation at Ser552 (P-β-catenin552). The phosphorylation at Ser552 was associated with the transcriptional activation. This activation impaires the β-catenin degradation translocates to the nucleus where it binds Tcell factor/lymphoid enhancing factor and initiates transcription of Wnt target genes such as *c-myc* and *cyclin- D1*. Nuclear accumulation of β-catenin is a hallmark of activated canonical Wnt/β-catenin signalling. PI3K-mediated generation of PI-3,4,5-triphosphate recruits Akt for activation of proliferation and survival signalling. He et al15 reported that Phosphatase and tensin homolog (PTEN) deficiency increased PI3K/Akt activation, resulting in excessive proliferation and crypt fissioning within small-bowel (SB) polyps.

Reduced β-catenin degradation and enhanced nuclear localization of stabilized β-catenin are key events in stem cell activation in a variety of systems including the intestine, Phosphatidylinositol 3-kinase (PI3K)/serine/threonine protein kinase Akt (Akt) signalling, in fact has been proposed to induce proliferative signals in intestinal epithelial cells (IECs). Recent studies suggest PI3K/Akt signalling is up-regulated in crypt IECs; in chronic ulcerative colitis (CUC) and active Crohn's disease (CD) indicating a probable involvement of this pathway in the inflammation. .

PI3K-induced and Akt-mediated β-catenin signalling are required for progenitor cell activation during the progression from CUC to colitis-associated cancer (CAC); these factors might be used as biomarkers of dysplastic transformation in the colon *(Lee G, et al. 2010)*

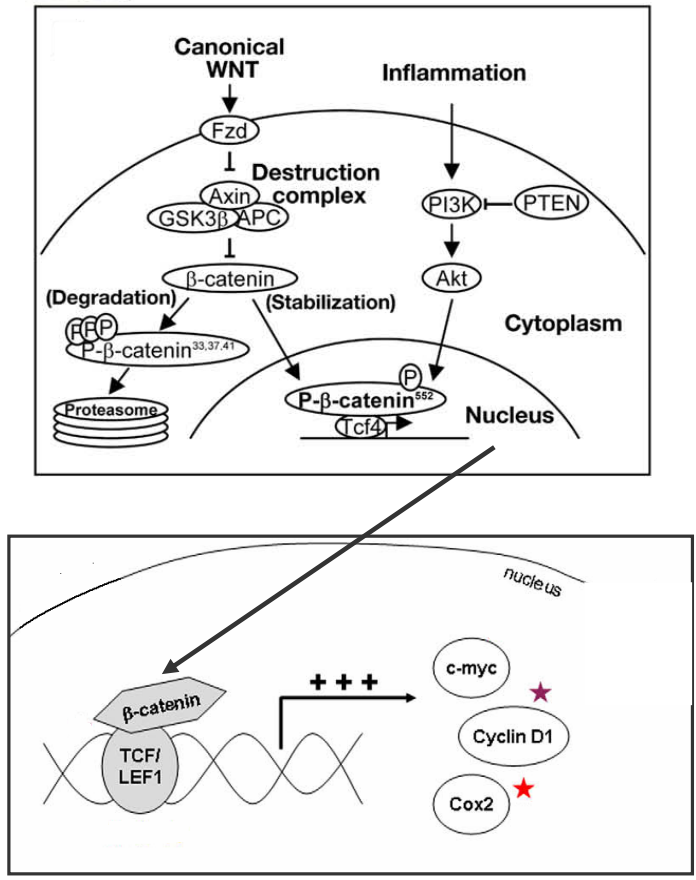


Figure 3: Link between PI3K/AKT and WNT pathways β-catenin-mediated.

The LKB1 Pathway:

LKB1 serves to activate AMPK by direct phosphorylation of Thr172 in its activation loop, which is essential for AMPK catalytic activity. AMPK, a cellular energy sensor, functions as a master regulator of cellular energy metabolism. Depletion of intracellular ATP levels due to either physiological stimuli such as exercise and muscle contraction or pathological stresses such as hypoxia, oxidative stress and glucose deprivation, activate AMPK (Fig. 4). AMPK functions to restore intracellular ATP levels by inhibiting ATP-consuming processes such as protein translation and cell growth; moreover promotes ATP-generating processes such as gluconeogenesis and lipogenesis. Thus, under energy starvation AMPK inhibits cell proliferation by directly phosphorylating TSC2 and enhancing its ability to switch off mTORC1 signalling. Mutational inactivation of LKB1 results in hyperactivation of mTORC1 signalling under low energy conditions, suggesting that LKB1 is required for repression of mTORC1 in a AMPK- and TSC2-dependent manner. *(Krymskaya1, V.P. and Goncharova E.A. 2009)*

In addition to the involvement in energy metabolism, LKB1 has the capacity to regulate multiple cellular processes, such as cell cycle arrest, Wnt signalling, transforming growth factor beta signalling and chromatin remodelling.

LKB1 forms a complex with pseudokinase STRAD and the scaffolding protein MO25*,(Baas AF,2003)* that activates at least 14 serine/threonine kinase by phosphorylation of the "T-Loop" threonine localized in their kinase domain. This implicates the involvement of LKB1 in several signalling pathways *(Shaw RJ, 2004; Lizcano JM 2004).* The first identified physiological substrate of LKB1was AMPK (AMP-activated protein kinase), which is a master regulator of cellular energy charge.21 Ten of these 14 AMPK related protein kinases possess an ubiquitin associated domain (UBA) immediately downstream the kinase catalytic domain. The UBA domains found in AMPK related kinases do not interact with polyubiquitin or other ubiquitin-like molecules. Whereas, the UBA domains appear to play an essential conformational role and are required for the LKB1-mediated phosphorylation and activation of AMPK-related kinases. Specifically, UBA domain directly interacts with the catalytic domain of these enzymes, allowing them to be in a conformation that can be readily phosphorylated and activated by the LKB1 complex *(Woods A, 2004; Jaleel M, 2006).* Thus, it could be intriguing to investigate whether mutations in the UBA domains might be present in families with PJS and/or in other cancer patients.

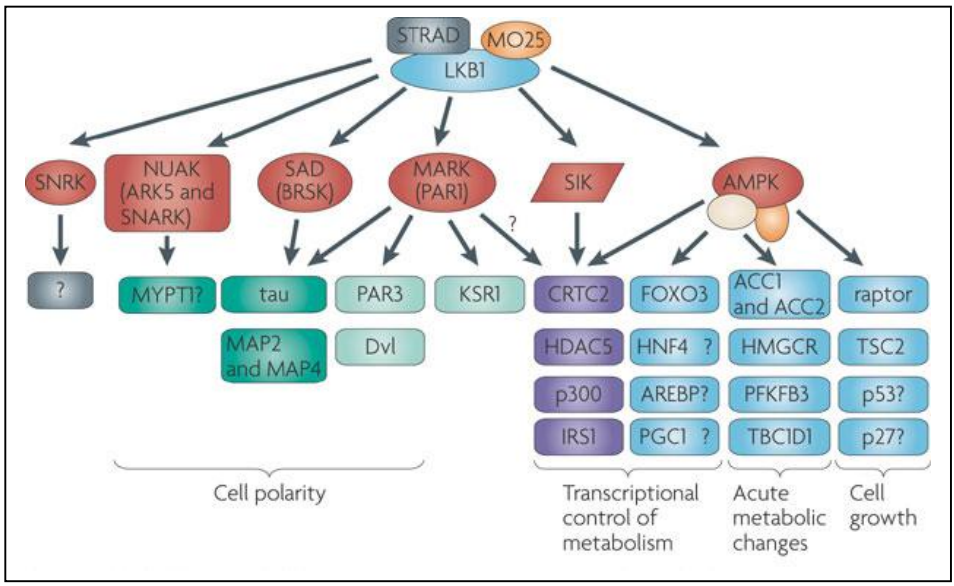


Figure 4: The LKB1 molecular Pathway

1.4 Molecular therapeutic target.

The increase of protein synthesis by mTOR has been implicated in the carcinogenesis of various human tumors by regulating the cell cycle, apoptosis and angiogenesis through downstream targets such as cyclooxygenase-2 (COX-2), p53, cyclin D1, c-Myc and hypoxia inducible factor-1a [5–7]. The signalling pathway that activates mTOR is altered in many human cancers, and treatment of cancer with mTOR inhibitors has shown responses in various cancers. [8,9]. (*No JH et al. 2009*)
Essentially PI3Ks, PDK1, AKT and mTOR are heavily targeted for therapy in different ways. These proteins are that could be drug target of cancer cells. In fact cancer cells with the activated pathway will be more dependent upon this pathway for their survival. *(No JH, et al. 2009)*
There are currently no approved therapies for PHTS and PJS in clinic. However, rapamycin, a specific inhibitor of mTORC1, discovered more then 30 years ago, attracts a renewed interest. Rapamycin (sirolimus is the official generic name) is a prototypical inhibitor of mTORC1 signalling. Rapamycin forms a cytosolic complex with FK506 binding protein 12 (FKBP12), which inhibits the catalytic activity of mTOR. *(Sabatini DM, 2006).* However, the precise mechanism of rapamycin-induced inhibition of mTORC1 signalling is not completely understood, and at least two mechanisms of rapamycin action have been proposed. It was shown that under some experimental conditions FKBP12-rapamycin destabilizes the interaction of mTOR with raptor, which is required for mTOR activity. Separate studies demonstrate that the FKBP12- rapamycin complex suppresses mTORC1 autophosphorylation, which also may inhibit mTORC1 activity76 suggesting the possibility that the rapamycin-dependent mTORC1 inhibition may involve more then one mechanism. Because mTORC1 signalling is a highly conserved pathway that regulates protein synthesis and cell growth in all eukaryotes and because of its activation not only in hamartoma syndromes, but in many types of cancer, there is a growing interest in rapamycin and its analogs. In 1999 rapamycin was approved by the Food and Drug Administration (FDA) for the prevention of renal allograft rejection. Currently, clinical studies demonstrate that rapamycin and its analogs have shown anti-cancer activity in variety of malignancies. Furthermore, a number of rapamycin analogs are in preclinical development. Numerous compounds as PI3K, Akt and mTORC1 inhibitors also have been filed. Currently, the phase II clinical trial of rapamycin in patients with Cowden syndrome is open for enrollment at the Warren Grant Magnuson Clinical Center, Maryland

(NCI, ClinicalTrials.gov Identifier NCT00722449). (*Krymskaya1, V.P. and Goncharova E. A, 2009)* .
PI3K-mTOR signalling also regulates chemokine (C-X-C motif) ligand 1 (CXCL-1), cyclooxygenase-2 (COX-2), and interleukin-8 (CXCL-8) that enhance tumor metastasis. PI3K and AKT regulate epithelial–mesenchymal transition (EMT), which is a change allowing tissue invasion and metastatic potential *(Cheng et al., 2008; Onoue et al.2006).* A large body of evidence support that it has a role in tumor promotion, and accordingly selective COX-2 inhibitors have been shown to be beneficial in colorectal cancer patients. These results encouraged to perform a short-term pilot clinical trial in colorectal cancer patients with celecoxib showing that a subset (2/6) of patients responded to the treatment with reduced polyposis *(Katajisto P, et al. 2006).*

1.5 Aim of the work

The inherited hamartomatous syndromes occur at approximately 1/10th the frequency of the adenomatous syndromes and account for <1% of colorectal cancer. However, proper identification of the specific syndrome has major importance for the affected individual and at-risk family members as the malignant potential in these autosomal dominant syndromes is quite high. Although the inherited hamartomatous polyposis syndromes are less common and less well characterized than the adenomatous polyposis syndromes, major advances in the molecular understanding and genetic basis of these syndromes have similarly needful. The identification of affected patients points to the need for genetic counseling prior to predictive gene testing for the individual and at-risk family members. Potentially, as our understanding of these novel genes accumulates, our ability to diagnose, classify, treat, and hopefully prevent polyp formation and malignant transformation will improve. (Schreibman IR 2005). All the evidence indicates that the PI3K/AKT pathway is a promising target for cancer chemotherapy in hamartomatous syndromes. Indeed, many companies and academic laboratories have initiated a variety of approaches to inhibit the pathway at different points. Therefore, proper diagnosis of tumours with an activated PI3K pathway is pre-requisite for the use of the targeted therapies *(Carnero A., 2010)* .
The aim of this study was the precise molecular characterization, of the genetic defect and the evaluation of involvement of the molecular pathway downstream, to better clarify the molecular mechanism and the potential targeted therapy in patients with hamartomatous poliposys syndromes.
To better clarify the molecular background of the PJS families, we have analyzed the entire coding sequence and splice junctions of the STK11/LKB1 gene in a series of PJS patients. All patients recruited in this study showed well-established clinical diagnostic criteria for PJS, including the characteristic PJS polyps in the gastrointestinal tract and classical PJS pigmentation; about half of which presented family history of PJS .
Finally in order to try to understand more specifically the molecular mechanisms that characterize the onset of PHTS, we have first screened the PTEN coding region in three italian patients with clinical diagnosis of PHTS and afterwards, in periferal blood cells of these patients, we have defined the expression profile of other genes directly related to PI3K/Akt phatway, as cMYC, COX2, CCND1 and TNFa, or involved in colorectal cancer onset, as APC.

MATERIALS AND METHODS

2.1 Patients

A total of 9 unrelated patients, exhibiting mucocutaneous pigmentation and hamartomatous polyposis, were referred by gastroenterologists and pediatric gastroenterologists to the laboratory, for genetic analysis. The histological aspects of polyps were unambiguous in all cases.

Characteristic features defining the clinical diagnosis were considered: presence of pigmentation, polyps localization, histological diagnosis (the specific phenotypes are listed in Table 2 (see "Results" section). When at least two patients belonged to the same family, they were considered as familial cases.

Samples from all families that participated in the study were collected after informed consent of the participants.

2.2 LKB1/STK11 and PTEN germline point mutations analysis

Samples of genomic DNA and RNA from affected individuals and at-risk family members individuals were isolated from peripheral blood lymphocytes by standard methods. RNA was obtained from peripheral white blood cells collected with EDTA. We used a combination of 4 techniques to analyze sequence variants within the LKB1 gene: the direct sequencing of amplified fragments, for point mutations DNA analysis of the nine exons and flanking intronic sequences; patients without LKB1/STK11 point mutations were further investigated by Southern blot analysis and genomic amplification of large fragments for detection of deletions of all or part of the gene. RT-PCR and quantitative Real-time RT-PCR techniques were used for the analysis of the LKB1/STK11 gene at the RNA level.

For PTEN analysis we have performed a qualitative RT-PCR, followed by the direct sequencing of amplified fragments, and also the direct sequencing of genomic DNA fragment in the region where we have found an alteration in cDNA sequence. Finally quantitative Real-time RT-PCR assay was performed to evaluate the RNA levels.

Genomic DNA from at least one affected individual per family was PCR-amplified in eight different genomic regions that covered the entire coding sequence of the LKB1 gene and all of the splicing junctions. The oligonucleotide primers used are described in Table 2 and the conditions for PCR amplification are the following: 94 °C for 5 min, initial denaturing phase; 33 cycles at 94 °C (30 sec), 60-67 °C (20 sec) 72 °C

(45 sec) and 72 °C for 3 min, final extension. Exons 3 and 9 were amplified as Nested PCR with the following oligonucleotides (the 3a couple for the first reaction and the 3b couple for the Nested amplification reaction of the exon 3 and the 9a couple for the first reaction and the 9b couple for the Nested amplification reaction of the exon 9, respectively, as reported in Table 2).

STK11/LKB1 genomic region (GeneBank N°: NC_000019.9)	Bp
1FP-AACACAAGGAAGGACCGCTCA-	
1RP-GTCACGGTGCTGATGGTTCTGTC-	421 bp
2FP-GAGGTACGCCACTTCCACAG-	
2RP-GCTCCTCTTCCCGTCTCCTT-	284 bp
3aFP-CCTCCAGAGCCCCTTTTCT-	
3aRP-GCCACACTGCTTGTCCTGAT-	264 bp
3bFP-CCCCCTGAGCTGTGTGTC-	
3bRP-TTTCCGTGAGGCCACACT-	201 bp
4FP-GTGTGCCTGGACTTCTGTGA-	
5RP-ACTCACCACACGCACACTC-	476 bp
6FP-AACCACCTTGACTGACCACGC-	
6RP-CAGAAATGTAGGGTTGGGGGTGTC-	250 bp
7FP-GCGGGGTCCCCCTTAGGAG-	
7RP-CTGGTTGAGCGGGCGCTAG-	264 bp
8FP-GGAGCTGGGTCGGAAAACTGGA-	
8RP-CAGGATGTCCCACGGGAGCA-	321 bp
9aFP-GTAAGTGCGTCCCCGTGGTG-	
9aRP-GCTAGTCAGTCATGGTGACCG-	400 bp
9bFP-CCTGTGGCTGGGGTTGC-	
9bRP-GATGCCACAGCCAGCCGTGA-	334 bp
10aFP-CTGACCTCTTCCGTCTTCCT-	
10arp-TGGAACCAACCAAACAAAAA-	560 bp
10bfp-GGAGACCAGGCTCCTGAC	
10brp-TGAGACGCGAACAAAACC-	593bp

Table 2: Oligonucleotides primer used for genomic sequences DNA analysis

Amplification and direct sequencing were also set up to screen the region coding for the UBA domains of AMPK related protein kinases, phosphorylated and activated by STK11/LKB1. Each region was PCR-amplified in one or two fragments; the oligonucleotide primers used are

described in Table 3 and the conditions for PCR amplification are the following: 94 °C for 5 min, initial denaturing phase; 33 cycles at 94 °C (30 sec), 60-67 °C (20 sec) 72 °C (45 sec) and 72 °C for 3 min, final extension.

Gene	Primer sequence	bp
Mark1	STK11 M1 10FP-TGTTGTGTGTGTCCCAAGTC-	460
	STK11 M1 10RP-AAATGACAATGCAAGTATTTTTCAA	
	STK11 M1 11FP-TGGTATCCGTAGGTTAATAGTCCAG-	340
	STK11 M1 11RP-TTGGTACCCAGAAATACAACCA-	
Mark2	STK11 M2 10FP-TGCCCTTTTCCTTCTCTGTG-	410
	STK11 M2 11RP-GATGAGCTTCTGCCCTCAAC	
Mark3	STK11 M3 10FP-TTTTTGAGTTTATGCTTTGAACGA	323
	STK11 M3 10RP-TGTGCTTAACTTTCCATTATTCACAT	
	STK11 M3 11FP-GGATGATTTCTTACCCAAACAAAA-	200
	STK11 M3 11RP-CAGGGTATTTCAGCTCTTTCTGAT	
Mark4	STK11 M4 10FP-GGCAGGGCAGAAGCTGTAT-	441
	STK11 M4 10RP-GAGGCTGAGGCACAAGAATC-	
	STK11 M4 11FP-AGCCTGGGTGACAGAGTGAC	313
	STK11 M4 11RP-CACCCCAAGTCCCTCCAC-	
Brsk1	STK11 B1 10FP-CCCGGTTCCCAATAATGTTT-	339
	STK11 B1 10RP-AAACCATGCCCCATGAGAG-	
	STK11 B1 11FP-ACCATGGGCAGAAATACAGG-	326
	STK11 B1 11RP-CCCTTCTTTCCTTGGGAATC-	
Brsk2	STK11 B2 10FP-ACAGGGAGAGTGGCAGGAT-	694
	STK11 B2 11RP-CTGAGTACAGACGAAGGTCAGG-	
Qsk	STK11 K 8FP-GGGTTGACAGCTTCCTCCTT-	605
	STK11 K 9RP-TGTCCTGAACTAGGTCACTGGA-	
Ampk α1	STK11 A 8FP-CCTGATCCTGCCATTTTTCT-	554
	STK11 A8RP-TTGCCAAATATGCTAATAATCAAAA-	
Qik	STK11 Q 7FP- CCCTGAAGTGCAAGGTGATT-	408
	STK11 Q 7 RP- CCTAGGCGCTGGAGATACAT-	
	STK11 Q 8FP- CAGTTCTTTGCCTTTACCACTTG	340
	STK11 Q8RP- GAAAAACGGAGTGTAGAGGATGA	
Sik	STK11 S8FP- TTGGGTGGCAGGACTGAG	536
	STK11 S8RP- TCCTTTAGTGGGGTGGACAA	
	STK11 S9FP- CCAAGAATAACCTGGGATCG	755
	STK11 S9RP- TCTGTCTGGAGTGCAAGCTG	
Snrk	STK11 S2 5FP- TATTCCCATCTGCCCCACT	477
	STK11 S2 5RP- AGGTGTCCAAAGGCTGTGTT	
	STK11 S2 6FP- CCTGATATGGCTGACGTTTG	234
	STK11 S2 6RP- CTCACCTAAACTGGGCCTTG	

Table 3: Oligonucleotides primers used for analysis of the UBA domains of AMPK related protein kinases

Amplification of genomic DNA from the PHTS1 patient was performed in different genomic region of exon 5 of the PTEN gene. The oligonucleotide primers used are described in Table 4 and the conditions for PCR amplification are the following: 94 °C for 5 min, initial denaturing phase; 33 cycles at 94 °C (30 sec), 60-67 °C (20 sec) 72 °C (45 sec) and 72 °C for 3 min, final extension.

PTEN region (GeneBank N°:NG_007466.1)	Bp
5cFP-TTGAAGACCATAACCCACCA-	214
5RP-CTCTGGTCCTTACTTCCCCAT-	

Table 4: Oligonucleotides primer used for PTEN mutation analysis

Sequencing analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of the STK11/LKB1 mRNA sequence (GenBank Accession number NM_000455.4); the GenBank Accession number of the genomic STK11/LKB1 region is: AC_000019.4 The GenBank Accession number of PTEN genomic sequence is: NG-007466.1. All oligonucleotides were obtained with primer 3 Software (http://www.genome.%20wi.mit.edu/cgi%20bin/primer/primer3) and checked with web Basic Local Alignment Search Tool program (BLAST, NCBI home page http://ncbi.nlm.nih.-%20gov/blast).
The novel mutations were searched for in 100 control samples to exclude the possibility of a rare polymorphism.

2.3 Qualitative RNA based analysis of patents.

For RNA analysis, we synthesized cDNA with 1 µg of total RNA, 500 ng of random hexamers, and 1 µl Superscript III reverse transcriptase (INVITROGEN), in the presence of 4 µl 5X RT buffer, 1 µl DTT (0.1 M) and 1 mM dNTPs. The reaction was run for 50 min at 42 °C in a 20 µl reaction volume, heated to 70 °C for 15 min and snapchilled on ice.
For STK11/LKB1 analysis 1 µl of the cDNA was amplified with primers described in Table 2, that produce three overlapping fragments (1FP–4RP, 2FP–8RP, 5FP–9RP) covering exons 1–9 of the cDNA. The reaction was performed as described above: 94 °C for 7 min, initial denaturing phase; 40 cycles at 94 °C (1 min), 60°C (45 sec) 72 °C (2 min) and 72 °C,

and a 5-min final extension.
For PTEN analysis 1 µl of the cDNA was amplified were amplified as Nested PCR with the following oligonucleotides (the PTEN 5'UTR-1FP/3'UTR-1RP couple for the first reaction and the PTEN 5'UTR-2FP/3'UTR-2RP couple for the Nested amplification reaction as reported in Table 5) that cover the entire coding sequence of the gene and all of the splicing junctions, and produce two fragments of 1815 bp and 1550 bp, respectively.
The PCR products were analyzed on a 1% agarose gel in trisacetic acid (TAE)–EDTA standard buffer and visualized by ethidium bromide staining.

STK11/LKB1 mRNA (GeneBank N°: NM 000455.4)	Bp
1cFP-TTTGGAGAAGGGAAGTCGG 4cRP-ACCCTCAAAATCCGACCT	671
2cFP-GAGGAGGTTACGGCACAAA 8cRP-GTACTTGGAGGACCTGCAC	729
5cFP-ACGGCCTGGACACCTTCT 9cRP-CAAGCAGCAGTGAGGCTGG	631
PTEN region (GeneBank N°:NG 007466.1)	**Bp**
PTEN 5'-UTR 1FP-TTCCATCCTGCAGAAGAAGC PTEN 3'UTR-1RP-TCTGAGCATTCCCTCCATTC	1815
PTEN 5'-UTR-2FP-GCAGCTTCTGCCATCTCTCT PTEN 3'UTR-2RP-TCACCACACACAGGTAACGG	1550

Table 5: Oligonucleotides primer used for RT-PCR analysis

2.4 Quantitative RNA based analysis of patents.

The quantitative RNA real-time assays was performed using the iCycler iQ Real-Time Detection System BIO-RAD. Amplification was carriedout in a total volume of 15 µl containing the SYBR Green PCRMaster Mix 1X (BIO-RAD), using 15 ng of cDNA synthesized from poly(A) mRNA. The real-time PCR reaction was optimized according to the manufacturer's instructions but scaled down to 15 µl per reaction. The PCR conditions were standard (iQ™ SYBR Green Supermix) and all reagents were contained in the standard iQ™ SYBR Green Supermix (BIORAD). The reactions were: 95 °C for 3 min initial denaturing phase;

95 °C for 15 s and 60 °C for 1 min. At the end of the PCR, the temperature was increased from 55 to 95 °C at a rate of 3 °C/min, and the fluorescence was measured every 10 s to construct the melting curve. A nontemplate control was run for each assay, and all determinations were performed at least in triplicate to ensure reproducibility. Synthesis of expected PCR product was confirmed by melting curve analysis. Oligonucleotides yielding 100–150-bp-long PCR products at an annealing temperature of 60 °C were obtained with Primer Express Software (Applied Biosystems, Foster City, USA) and primer 3 (http://wwwgenome.%20wi.mit.edu/cgi%20bin/primer/primer3), and checked with web Basic Local Alignment Search Tool program (BLAST, NCBI home page http://ncbi.nlm.nih.-%20gov/blast).

To measure STK11/LKB1 mRNA level, we selected two couples of oligonucleotides (reported in Table 2) within exons 1 - 2 and 7 - 8, to amplify the canonical messenger of the STK11/LKB1 gene.

To analyze PTEN gene mRNA, we used the couple of oligonucleotides (reported in Table 6) within exons 5 - 6, to amplify the canonical messenger of the STK11/LKB1 gene

Finally a relative quantization of APC,cMYC,CCNDI,COX-2 and TGFβ mRNA level was performed in CS patients (All primers usedare reported in Tab.6).

STK11/LKB1 mRNA (GeneBank N°: NM_000455.4)	Bp
C1QFP-GCCGTCAAGATCCTCAAGAA C2QRP-TTCGTTGTATAACACATCCAC	140
C7QFP-GCCAAGAGGTTCTCC C8QRP-GTGCAGGTCCTCCAAGTAC	153
PTEN region (GeneBank N°:NG_007466.1)	**Bp**
5c2FP-ATGGGAAGTAAGGACCAGAG 6cRP-TCTTGTGAAACAACAGTGCCA	129
APC mRNA (GeneBank N°: NM_000038.5)	**Bp**
13c2FP-TGCGAGAAGTTGGAAGTGTG 14cRP- TCAGTGCAATGTGCTGACAA	131
cMYC mRNA (GeneBank N°: NM_002467.3)	**Bp**
2FP- CACCACCAGCAGCGACTCT 3RP-GCCTGCCTCTTTTCCACAGA	113
CCND1 mRNA (GeneBank N°: NM_053056.2)	**Bp**
3cFP- GAACAAACAGATCATCCGCA 4cRP-GCGGATTGGAAATGAACTTC	86
COX-2 mRNA (GeneBank N°: NM_000963.2)	**Bp**
5FP-CAGCACTTCACGCATCAGTT 6RP-CGCTGTCTAGCCAGAGTTTCA	109
TNFα mRNA (GeneBank N°: NM_000594.2)	**Bp**
TNFA-bFP-CCCTGGTATGAGCCCATCTA TNFA-bRP-CGGCAAAGTCGAGATAGTCG	145

Table 6: Oligonucleotides primer used for real-time analysis

The relative expression of the target transcript was calculated with the comparative Ct method (Applied Biosystems User Manual) using a cDNA fragment from the glucuronidase (GUS) housekeeping gene as control.

2.5 Amplification analysis of genomic DNA

Genomic PCR reaction was performed for the analysis of region encompassing exons from 2 to 8 of the LKB1 gene (from nucleotide 12515 to nucleotide 17436). The first product consists of a 4200 bp long

fragment including the LKB1 genomic region from exon 2 to exon 8. The second and third products consist of two fragments, of 2160 bp (from exon 2 to exon 4) and of 2000 bp long (from exon 5 to exon 8), respectively. The oligonucleotide primers used are described in Table 5 and the conditions for PCR amplification are the following: 94 °C for 7 min, initial denaturing phase; 40 cycles at 94 °C (1 min), 60°C (45 sec) 72 °C (5 min) and 72 °C, and a 20-min final extension.
To analyze the genomic alteration of patient number 2, carrier of exons 2 and 3 skipping at the RNA level, we performed a set of PCR reactions encompassing genomic region between exons 1 to 4. This region was amplified in 13 PCR fragments, using the same reverse oligonucleotides in each reaction (IVS 3RP in Table 7) and different forward oligonucleotides spaced each other by about 1000 bp. The oligonucleotide primer sequences are described in Table 2 and the PCR conditions were the following: 95 °C for 2 min, initial denaturing phase; 10 cycles at 95 °C (1 min), 66°C to 64 °C with a decrement of 0.2°C each cicle (30 sec), 72 °C (3 min), 30 cycles at 95 °C (1 min), 64 °C (30 sec), 72 °C (3 min) and 72 °C 5-min, final extension. The PCR products were analyzed into a 1% agarose gel in trisacetic acid (TAE)–EDTA standard buffer and visualized by ethidium bromide staining.

STK11/LKB1 genomic region (Gene Bank n° NC_000019.9)
IVS1A - CCTGAGCTGGACCCGTCTG
IVS1B - CCTTATCGCAGCCAGACACG
IVS1C - GGCTGGGAAGGGTGTGTTTC
IVS1D - CAATTTCATCCTGGCCCTGAGT
IVS1E - CGTGGCCAACATGGTGAAAC
IVS1F - TCTGTTTGTGCCCCTCTCTGG
IVS1G - CTGGAGTGCAGTGGCGTGAT
IVS1H - AGCCCCACCTCTCTGTGAGC
IVS1I - GTCTTGCCTCCCAGGAATGG
IVS1L - TGTCGGGTTACAGGCGTGA
IVS1M - CCCTGGGTGGTTCCACATCT
IVS1N - AGGCTGAGGCAGGGAAATCA
IVS2FP - CCTGGGACCGTCCTGCAT
IVS3RP - AGCCTCCCAAACACCACAGG

Table 7: Oligonucleotides primer used to analyse genomic alteration of patient number 2

2.6 In silico analysis.

The nucleotide sequences of genomic LKB1 region (NCBI NC_000019.9) were analyzed with the RepeatMasker program using the default settings (exon.cshl.org/ESE/). This is a program that screens DNA sequences for interspersed repeats and low complexity DNA sequences. The output of the program is a detailed annotation of the repeats that are present in the query sequence as well as a modified version of the query sequence in which all the annotated repeats have been masked (default: replaced by Ns). Sequence comparisons in RepeatMasker are performed by the program cross-match, an efficient implementation of the Smith-Waterman-Gotoh algorithm developed by Phil Green (A.F.A. Smit, R. Hubley & P. Green RepeatMasker at http://repeatmasker.org).

2.7 Southern blot analysis

Genomic Southern blot was performed after endonuclease digestion of genomic DNA from PJS patients and healthy non affected controls with BamHI restriction enzyme; afterwards it was probed with a cloned cDNA fragment that comprised the entire coding region of LKB1/STK11 gene.

RESULTS

3.1 LKB1/STK11 and PTEN germline point mutations analysis

We have analyzed the disease-causing mutations in 9 unrelated PJS and 3 PHTS patients from Italy. All patients showed the classical phenotype associated to these two disease; multiple hamartomatous polyps in the gastrointestinal tract and mucocutanoeus pigmentation for PJS and hamartomatous gastric or colonic polyps, colon cancer, penis macules for PHTS. Sequencing of the 9 exons and the exon–intron junctions of LKB1/STK11 gene led to the identification of 6 unique pathologic germline mutations. (Table 8). According to the human gene mutation database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd/search.html), 2 are known and 3 are novel germline LKB1 point mutations. Of these one is located in exon 1, one in exon 4 and three in exon 6. The sixth mutation consists in an intragenic deletion that eliminates exons 2 and 3 of the gene.

Two of the point mutations are frameshift mutations: the c.169-170insG mutation, that consists in an insertion of a G nucleotide at position 169-170 and the c.842delC mutation, that consists in a C nucleotide deletion at position 842. Both mutations create a premature stop codon respectively 105 and 5 codons below.

The mutation named c.580G→A is a missense mutation in exon 4, that consists in a G to A substitution at position 580, causing the change of an Aspartic Acid in an Asparagine residue at position 194.

The last two germline point mutations identified, previously not described in literature, are two in frame micro-deletions localized in exon 6. The first, called c.747-749delCAC determines the deletion of the Threonine residue at position 250; the second, named c856-858delCTC determines the deletion of the Leucine residue at position 286. Both mutations modify the catalitic domain (kinase domain) of the LKB1/STK11 protein, characterized by highly conserved residues among different species (human, mouse, xenopus), likely altering the correct protein function.

These two in frame deletion are not common polymorphisms, since they were not detected in the DNA of the 100 normal Caucasians controls.

In the family of the patient carrier of the c.747-749delCAC mutation, the molecular diagnosis was performed in six relatives, two of whom were affected and four apparently healthy individuals. This mutation always segregates with the disease in this family; furthermore, we were be able to carry out the presymptomatic diagnosis in the two young proband's at-

risk sons (of eight and seven years old respectively), both resulted carriers of the deletion.
We found about the same prevalence of LKB1/STK11 mutations both in sporadic PJS patients (66.7%; 2 out of 3) and familial cases (60%; 3 out of 5).
No mutations were identified in the region coding for UBA domain of the AMPK related protein kinases. Moreover, two patients, negative for LKB1/STK11 gene mutation, proved to be carriers of two intronic single nucleotide substitutions not reported in database (NCBI: http://www.ncbi.nlm.nih.gov/snp) with unknown meaning (the c.983+55C→T in MARK2 gene and the c.968+32G→A in BRSK2 gene, in patient number 8; the c.1007+43C→A in MARK4 gene and the c.972+57C→T in SIK gene, in patient number 5).
In patient PHTS1 we found a missense mutation named c.406T→C in exon 5 of PTEN gene, this mutation just described in literature before that determines the aminoacidic change of cysteine residue136 in arginine *(Kubo Y, et al. 2000).*

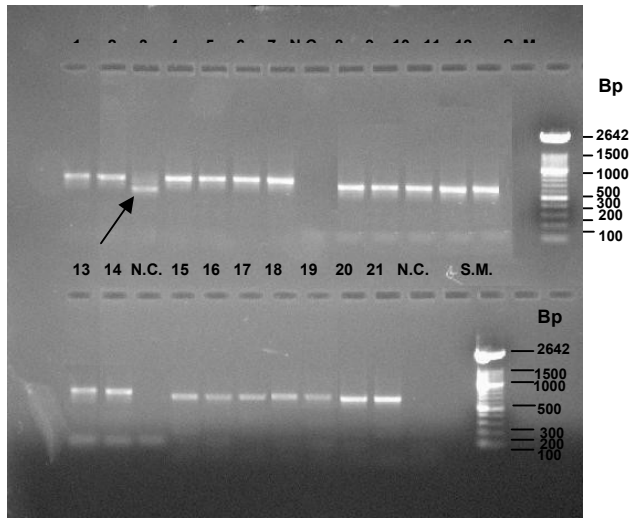
Patient n°	S/F	Age of Diagnosis	Mutation	Exon	Clinical manifestations
PJS1	F	16aa	842delC p.Leu281fs4X	6	hamartomatous duodenal polyposis and mucocutaneous pigmentation
PJS2	S	5aa	Deletion exons 2-3	Δ2-3	hamartomatous polyposis and oral mucocutaneous pigmentation
PJS3	N.I.	14aa	**169-170insG**	1	hamartomatous polyposis and oral mucocutaneous pigmentation
PJS4	F	13aa	580G>A p.D194N	4	hamartomatous duodenal and stomach polyposis and mucocutaneous pigmentation
PJS5	S	18aa	-----	----	hamartomatous gastric polyposis and mucocutaneous pigmentation
PJS6	S	13aa	**856-858delCTG** **p.286delL**	6	hamartomatous gastric polyposis and mucocutaneous pigmentation
PJS7	F	16aa	----	----	hamartomatous duodenal polyposis and mucocutaneous pigmentation
PJS8	F	10aa	----	----	hamartomatous gastric polyposis and mucocutaneous pigmentation
PJS9	F	17aa	**747-749delCAC** **p.250delT**	6	hamartomatous gastric polyposis and mucocutaneous pigmentation
PHTS1	F	53 yy	**c.406T→C** **C136R**	5	BRR macrocephaly, brain asymmetry arteriovenous malformations glycogenic acanthosis hamartomatous gastric polyps colon cancer, the penis macules keratoses of the hands and feet
PHTS2	S	45yy	↓ mRNA	----	CS hamartomatous gastric polyps colon cancer, glycogenic acanthosis keratosis of the hands and feet, a few freckles on penis
PHTS3	F	54yy	↓↓ mRNA	-----	CS hamartomatous colonic polyps colon cancer, kidney horseshoe.

Table 8: Clinical phenotype and mutation spectrum of Italian PJS and PTHS patients studied. F: familial cases; S: sporadic cases; N.F.: mutation not found; the bold character indicates the novel mutations.

3.2 Qualitative RNA based analysis of patents.

We examined LKB1/STK11 mRNAs from peripheral blood lymphocytes of four PJS patients negative for germline LKB1/STK11 point mutations and three healthy individuals, as controls. Using the RT-PCR technique we amplified three fragments encompassing exons 1–4, 2–8 and 5–9, respectively. In addition to the normal sized fragment, which was observed in all samples tested, a low-molecular-weight fragment was detected in one affected individual (Fig 5a line number 3). Sequence analyses showed in Fig. 5b of the expected and low-molecular-weight fragment revealed the skipping of exons 2 and 3 in the shorter fragment. The deletion of exons 2 and 3 results in a novel exon 1/4 connection, that maintains the reading frame and encodes for a protein lacking a region that includes part of the kinase domain. No other mRNA alterations were detected in all samples analysed.

a)



b)

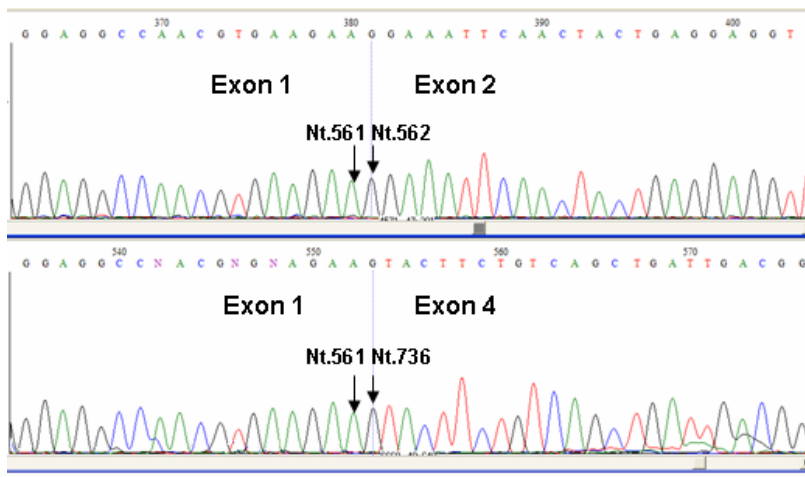


Figure 5: cDNA analysis of STK11/LKB1 coding region.
a) Gel electrophoresis of the amplified products by RT-PCR technique; numbers from 1 to 7 correspond to PJS patients as described in table 1. The arrow indicates a PCR product of molecular weight shorter than expected. N.C.: PCR negative control; S.M.: DNA molecular weight marker.
b) Sequence analysis of the normal sized (above) and low-molecular weight (below) RT-PCR products. The arrows indicate the nucleotides at the canonical junction between exon 1 and exon 2 (above) and at the novel junction between exon1 and exon 4 (below).

PTEN analysis was performed amplifying the whole coding region of the gene in a unique fragment, using RT-PCR reaction has revelled only the normal sized fragment, which was observed in all samples tested. This result exclude the presence of splicing alterations or intragenic deletions in these patients. Direct sequencing of amplified fragments reveded in patient PHTS1 a T→C transversion at position 406. These missense mutation, called c.406T→C determines the aminoacidic substitution of the C 136 with the R. The nucleotide sequence accession number for this messenger region is: NM_000314.4. No other sequence alterations were detected in all samples analysed.

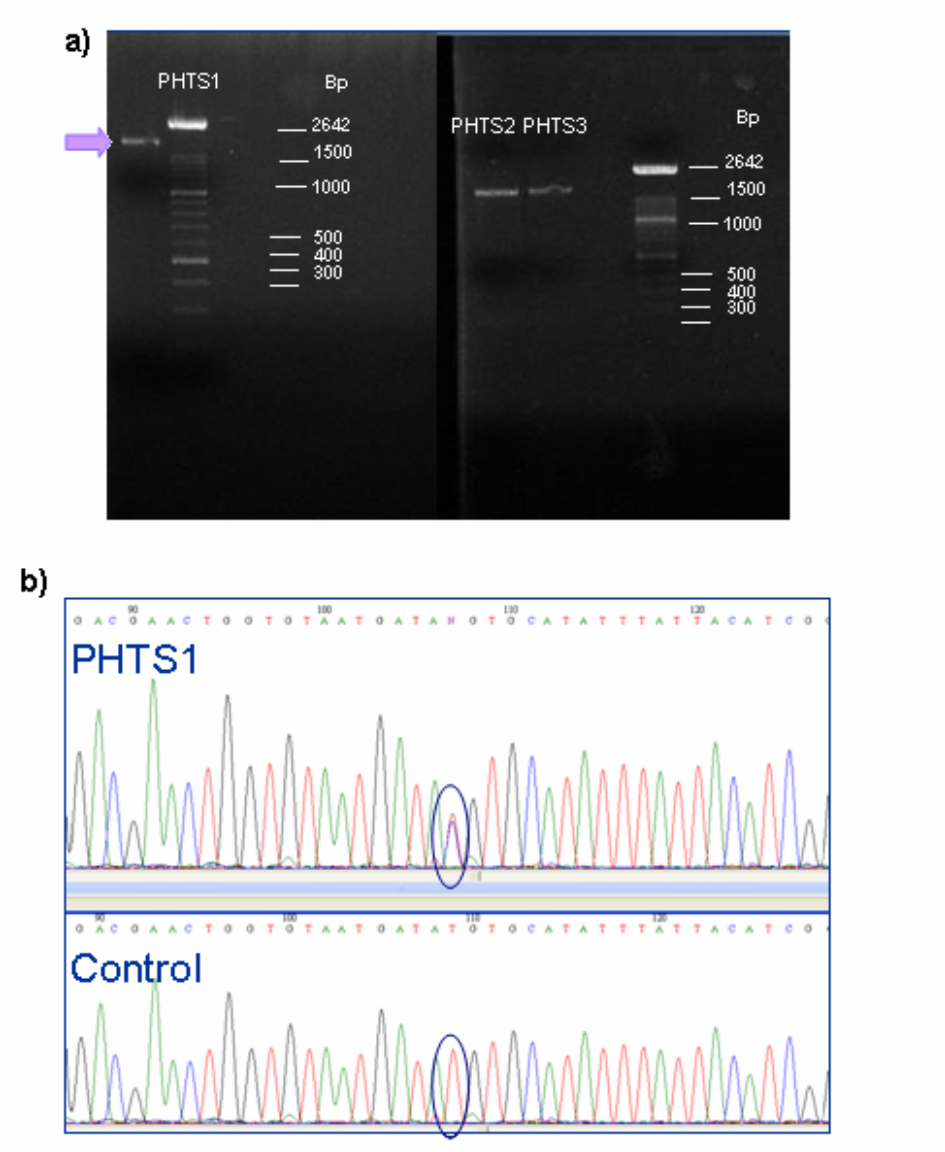
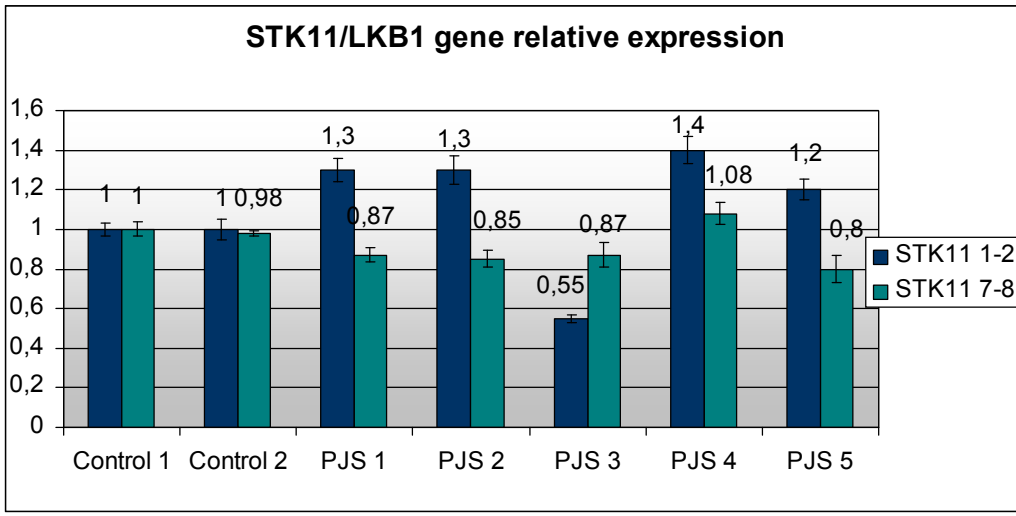


Figure 6: cDNA analysis of PTEN coding region: **a)** Gel electrophoresis of the amplified products by RT-PCR technique; **b)** Sequence analysis of the RT-PCR products.

3.3 ***Quantitative RNA based analysis of patients.***

Finally quantitative Real-time RT-PCR assay was performed to evaluate the RNA levels. To verify whether quantitative mRNA alterations were responsible for PJS and for (PHTS) in our patients, we performed quantitative Real-Time PCR analysis on cDNA from peripheral blood lymphocytes. Fig. 7a shows the relative expression of the LKB1 transcript containing exons 1–2 (columns 1) and of the LKB1 transcript containing exons 7-8 (columns 2), normalized versus the glucuronidase (GUS) transcript. We found a quantitative mRNA alteration in patient number 2, in whom the LKB1 1–2 transcript fragment, but not the LKB1 7-8 transcript fragment, was significantly down-expressed (the decrement was 0.5-fold compared to the healthy control).

Fig. 7b shows the relative expression of the PTEN transcript containing exons 5–6, normalized versus the glucuronidase (GUS) transcript. We found a quantitative mRNA alteration in both patients negative for the presence of mutation in the PTEN gene coding region, with a relative espression of 0.3 and 0.1 fold compared with the healty controls. As expected from previous results, it is not observed any change in the PTEN gene expression level.



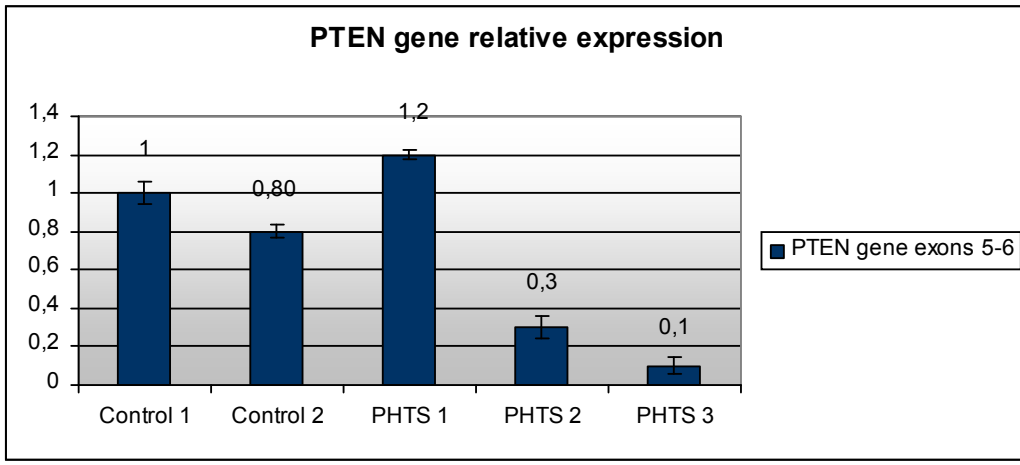
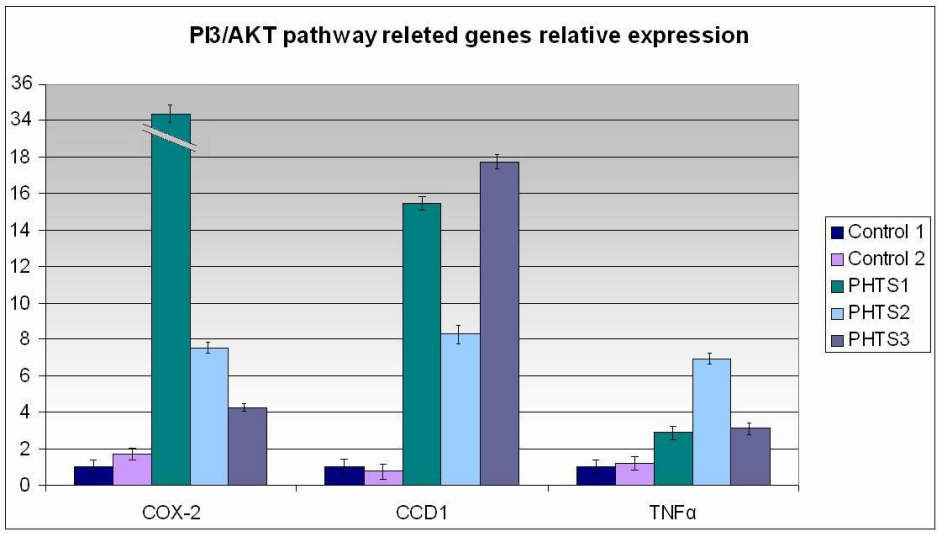


Figure 7: Real-time PCR quantification analysis. Relative expression, calculated with the comparative Ct method, of two *STK11* transcript fragments spanning the junctions between exons 1-2 and exons 7-8, respectively, b) of PTEN gene fragments spanning the junctions between exons 5-6,compared to glucuronidase transcript fragment. Patients numbering corresponds to that adopted in Table 2.

Relative expression analysis, performed in all the three PHTS patients,for the four genes related to the molecular pathway PI3K/AKT has shown an increment of expression for COX-2,CCD1 and TNFα genes, in all PHTS patients, whereas APC transcript fragment, was down-expressed (as show in fig.8)



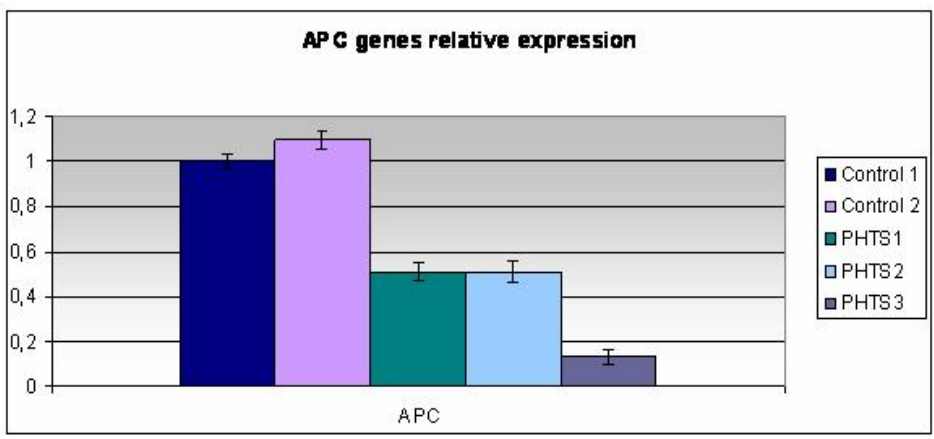


Figure 8: Relative expression of four PI3K/AKT pathway genes calculated with the comparative Ct method, compared to glucuronidase transcript fragment. Patients numbering corresponds to that adopted in Table 2.

3.4 Amplification analysis of genomic DNA

No qualitative DNA alterations were detected using genomic amplification analysis, as described the in method section. Gel electrophoresis of amplified fragments followed by etidium bromide staining evidences only products of expected size (data not shown).

According to the quantitative mRNA data, PJS patient number 2, carrier of exons 2 and 3 deletion at the RNA level, resulted heterozygous at polymorphic marker localized in exon 4 of the LKB1 gene, thus excluding a whole-gene deletion. However, we have been unable to detect point mutations at either the splice sites or the neighboring intronic sequences (up to 40 bases) of the two exons (2 and 3) at the genomic DNA level, suggesting the presence of an intragenic deletion encompassing exons 2 and 3. To verify this hypothesis we have amplified this region with specific oligonucleotides, as described in the methods section, and have characterized the precise breakpoints of this mutation. Abnormal fragment products were identified in patient number 2 but not in the healthy control subjects, of about 1000, 2000 and 3000 bp long respectively, using primer pairs IVS1D-FP/IVS3-RP, IVS1E-FP/IVS3-RP and IVS1F-FP/IVS3-RP. No amplified fragment was obtained by using several forward primers within IVS1 and IVS2 and reverse primer (IVS3-RP) (Fig. 9a).

As shown in Figure 9b, sequence analysis of the amplified fragment IVS1F-FP/IVS3-RP revealed the loss of a genomic region of about 7000 bp long. We have characterized the breakpoints of this region within two sequences of 12 bp that share 100% homology, located in intron 1 from nucleotide 6999 to nucleotide 7010 and in intron 3 from nucleotide 73987 to nucleotide 13998. The two 12 bp long sequences are located within two sequences of 26 bp that differ from each other only by one nucleotide (96% homology) (Fig. 9b). The nucleotide sequence accession number for this genomic region is: NC_000019.9.

To confirm the mutation c.406T→C detected in PHTS patient 1, we had performed a genomic amplification analysis for exon 5 of PTEN gene. The subsequently sequence analysis had confirmed the presence of the mutation also in the genomic region of gene.

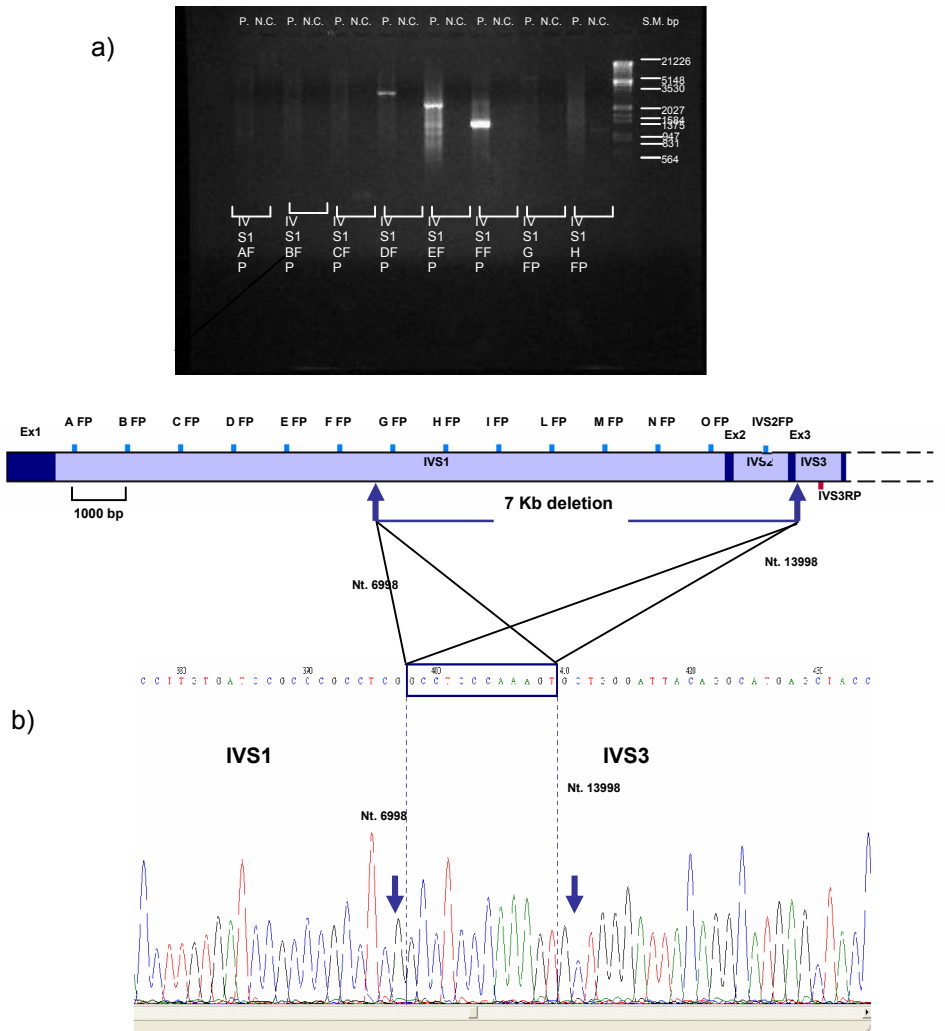
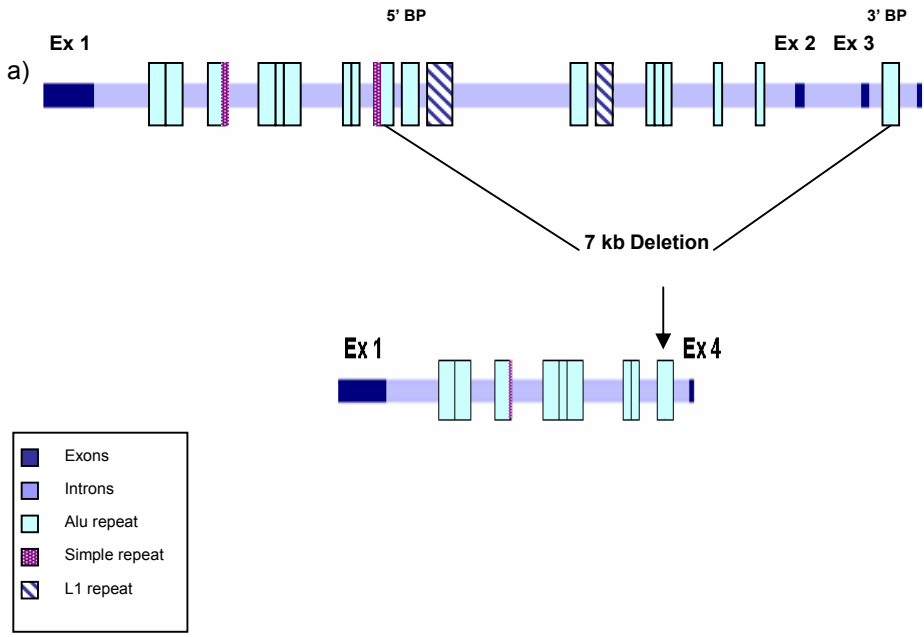


Figure 9: Molecular characterization of the STK11/LKB1 intragenic deletion.
a) Gel electrophoresis of the amplified products by genomic PCR performed on patient number 3. P.: patient; N.C.: PCR negative control; S.M.: DNA molecular weight marker. b) Sequence analysis of the amplified product obtained with primer pair IVS1-F-FP and IVS3-RP. Boxed sequence corresponds to the twelve nucleotides repeated both at the 5' and 3' breakpoints sharing 100 % of homology and representing the novel genomic connection produced by the deletion event. A schematic representation of the primer pairs position on the gene, used for the deletion breakpoints characterization, is shown above the electropherograms. Bold arrows indicate the genomic region involved in the deletion and the genomic nucleotides position involved in the novel genomic junction, referred to GenBank sequence with accession number: NC_000019.9.

3.5 In silico analysis.

Using the Repeat Masker program, the 5′ and 3′ breakpoints of this 7 kb deletion were found to lie within the core of two Alu sequences (26 bp) of the complete Alu elements (both AluY subfamily), (Fig. 10B, Table 9). Furthermore, a fragment of 400 bp, homologous to the 3′ region of long interspersed nuclear element L1 (L1MB5), is located immediately downstream of 5′ breakpoint of the deletion. A 266-bp fragment, homologous to the 3′ region of another L1 repeat (L1MB7), is located about 3100 bp downstream of the 5′ breakpoint. In addition, there are other 16 Alu repeat elements and 2 simple repeats interspersed within the genomic region including introns 1-3; the Repeat Masker program recognizes a total of 18 Alu sequences, 3 L1 sequences and 6 simple sequences (Table 9).



b)

```
5' Alu Y TCAGCCTCCCAAGTAAGCTGGAACTACAGGTGCCTGCCACCAAGCCTGGCTAATTTTGTT
         ||||| |||| ||| |||||| | |||||| ||| ||||||| ||| ||||||    | ||
3' Alu Y TCAGCTTCCCGAGT-AGCTGGGATTACAGGCGCCCGCCACCACGCCCGGCTAA---T-TT

5' Alu Y TTTGTATTTTTAGTAGAGACGGGGTTTCACCGTGTTAGCCAGTATGGTCTCGATCTCCTG
         |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||| | ||||||
3' Alu Y TTTGTATTTTTAGTAGAGACGGGGTTTCACCGTGTTAGCCAGGATGGTCTCAAACTCCTG

5' Alu Y ACCTTGTGATCCGCCCGCCTCGGCCTCCCAAAGTCCTGGGATTACAGGCTTGAG
         |||| |||||||||| ||||| |||||||||||| ||||||||||||| ||||
3' Alu Y ACCTCGTGATCCGCCTGCCTCAGCCTCCCAAAGTGCTGGGATTACAGGCATGAG
```

Figure 10: Schematic representation of the intragenic deletion
a) Repetitive elements present in the genomic region from exons 1–4 of the STK11/LKB1 gene and a diagram (below) of the deletion are shown. The arrow indicates the novel genomic junction.
b) Sequence of Alu element present at the 5' and 3' breakpoints (5' BP, 3' BP, of part a); boxed nucleotides represent the core sequence of Alu elements.

query sequence	-position in query- begin	end	(left)	C	matching repeat	repeat class/family	-position in repeat- (left) begin	end	begin (left)
NC_000019.9	662	707	(27093)	+	GA-rich	Low_complexity	2	47	(0)
NC_000019.9	862	1172	(26628)	C	AluSq2	SINE/Alu	(0)	312	1
NC_000019.9	1329	1377	(26423)	C	Charlie5	DNA/hAT-Charlie	(215)	2409	2361
NC_000019.9	1614	1656	(26144)	C	L1MD	LINE/L1	(2513)	3633	3591
NC_000019.9	1657	1807	(25993)	C	AluSp	SINE/Alu	(14)	299	149
NC_000019.9	1813	1924	(25876)	+	AluSx	SINE/Alu	113	224	(88)
NC_000019.9	2840	2977	(24823)	+	GC_rich	Low_complexity	1	138	(0)
NC_000019.9	3074	3123	(24677)	+	GC_rich	Low_complexity	1	50	(0)
NC_000019.9	4166	4203	(23597)	+	CT-rich	Low_complexity	140	177	(0)
NC_000019.9	5208	5508	(22292)	C	AluY	SINE/Alu	(11)	300	1
NC_000019.9	5509	5821	(21979)	C	AluSg4	SINE/Alu	(5)	307	1
NC_000019.9	6202	6528	(21272)	+	AluSx	SINE/Alu	1	312	(0)
NC_000019.9	7489	7774	(20026)	+	AluSp	SINE/Alu	1	294	(19)
NC_000019.9	7782	7915	(19885)	+	AluSg	SINE/Alu	1	134	(176)
NC_000019.9	7916	8211	(19589)	+	AluSp	SINE/Alu	2	308	(5)
NC_000019.9	9175	9444	(18356)	C	AluJo	SINE/Alu	(2)	310	9
NC_000019.9	9460	9744	(18056)	C	AluY	SINE/Alu	(0)	311	1
NC_000019.9	9752	9880	(17920)	C	FLAM_C	SINE/Alu	(10)	133	1
NC_000019.9	9892	10196	(17604)	C	AluY	SINE/Alu	(6)	305	2
NC_000019.9	10197	10626	(17174)	C	L1MB5	LINE/L1	(32)	6142	5700
NC_000019.9	12646	12933	(14867)	C	AluSx1	SINE/Alu	(20)	292	1
NC_000019.9	12988	13239	(14561)	C	L1MB7	LINE/L1	(5)	6179	5913
NC_000019.9	13245	13500	(14300)	+	AluSc	SINE/Alu	45	300	(9)
NC_000019.9	13507	13751	(14049)	+	AluJb	SINE/Alu	22	298	(14)
NC_000019.9	13782	14034	(13766)	C	L1MB7	LINE/L1	(302)	5876	5610
NC_000019.9	14103	14372	(13428)	C	AluJb	SINE/Alu	(0)	312	29
NC_000019.9	14800	15057	(12743)	+	AluSc8	SINE/Alu	37	295	(17)
NC_000019.9	16432	16745	(11055)	C	AluY	SINE/Alu	(0)	311	1
NC_000019.9	22167	22469	(5331)	C	AluSg	SINE/Alu	(5)	305	1
NC_000019.9	24083	24103	(3697)	+	(CCCCAG)n	Simple_repeat	2	22	(0)
NC_000019.9	25609	25634	(2166)	+	GC_rich	Low_complexity	1	26	(0)
NC_000019.9	25612	25666	(2134)	+	GC_rich	Low_complexity	1	55	(0)
NC_000019.9	26583	26637	(1163)	+	GC_rich	Low_complexity	1	55	(0)
NC_000019.9	26728	26780	(1020)	+	GC_rich	Low_complexity	1	53	(0)

Table 9: Repetitive sequences in the STK11/LKB1 genomic region.
LINE: long interspersed nuclear element; SINE: short interspersed nuclear element.
Modified from RepeatMasker program (exon.cshl.org/ESE/)

3.6 Southern blot analysis.

All LKB1/STK11 mutation-negative probands were analyzed by Southern blot to detect deletions that would not be revealed by a combination of genomic amplification and RNA based analysis. No deletions were detected, since all fragments were of expected size (data not shown).

DISCUSSION

Recognition and correct differential diagnosis of the hamartomatous polyposis syndromes, although they are quite rare, is essential because patients have a high lifetime risk of gastrointestinal and extraintestinal carcinoma and their first-degree relatives have high risk of recurrence of the sindrome.

Inactivating mutations in LKB1 have been detected in 18–70% of the PJS patients *(Lizcano JM et al. 2003)* and the absence of detectable LKB1 mutations could be explained by the existence of an alternative LKB1 gene inactivation mechanism that remains undiscovered by conventional screening methods or, alternatively, by genetic heterogeneity. Some families have been reported to be clearly unlinked to the LKB1 locus *(Woods A, et al. 2003; Jaleel M, et al. 2006)* and linkage analysis has previously suggested a second locus on chromosome 19q13.4. Furthermore, a t(11;19)(q13;q13.4) translocation in a small bowel PJS polyp has been found, although no mutations within the genes in this region have yet been reported.[21,24,25] Candidates for the second PJS locus might be genes known or suggested to interact with the LKB1 either by direct association with LKB1 or by their own function; three such genes, LIP1, MO25a, and STRAD have been screened with negative results.

The mutation screening strategy used in our study was targeted to detect *STK*11/LKB1 and PTEN genetic alterations as point mutations, large genomic rearrangements and mutations in the regulatory domains of the gene, leading to qualitative or quantitative alterations of its transcript. Despite a detailed molecular genetic analysis, we observed germline *STK*11/LKB1 mutations in 6 out of 9 patients (about 66.7%), thus indicating that such a novel mechanism affecting *STK*11 gene expression in PJS patients is yet to be investigated; however, genetic heterogeneity in PJS could not be excluded.

Using the strategy described above we have characterized the breakpoints of the *LKB1/STK11* intragenic deletion found in one PJS patient and have suggested the mechanism probably involved. This rearrangement is most likely an Alu-Alu homologous recombination event that deletes about 7 kb of the *LKB1* genomic region encompassing exons 2 and 3 (Fig. 3C). Two 26 bp core sequences of two Alu elements (both AluY sequences), showing a 96% homology, are localized at the 5' and 3' end of the breakpoints, respectively. This sequence, could itself act as a recombinase (Fig. 3C).

Alu-mediated homologous recombination is a mechanism well documented so far, (Kolomietz E et al. 2002, Grover D, 2004) however it is the first evidence that this mechanism is involved in the STK11/LKB1 gene rearrangements. Since the average frequency of Alu elements along the human genome is estimated to be one element every 4 kb, the region spanning the 7 kb deletion (including introns from 1 to 3 of the LKB1 gene), shows an over-representation of complete Alu elements (Fig. 3C). Seventeen Alu elements are detected in this region by using in *silico* analysis, which corresponds to more than one element every 1 kb in this 14.5-kb region (Table 3).

The over-representation of Alu elements indicates that this is a region of DNA instability; thus, it is not surprising that several intragenic rearrangements involving this region have been reported so far. Intriguingly, genomic sequence of chromosome 19 from GeneBank position 1203099 to 1230861 shows the presence of Alu elements at the genomic regions involved in the intragenic deletions in most of the cases so far described. The intragenic deletions described include the exon 1 (encompassing 5'UTR region and exon 1), exons from 1 to 10, exons 2 and 3, exons from 2 to 10, exons 4 and 5 and the exon 8; [6-8] in parallel Alu elements are localized at chromosomal region encompassing the 5'UTR, IVS1, IVS3, IVS8 and the 3'UTR regions of the STK11/LKB1 gene; furthermore, repeated sequences, as Simple-repeat and GC-rich Low-complexity sequences, are included in the region corresponding to 5'UTR, IVS1, IVS9 and 3'UTR. Genomic sequence also reveals two incomplete L1 elements near the deletion, about 490 bp and 3.3 kb downstream the 5' breakpoint (Fig. 3B, Table 3), but it is still unclear whether it could play an active role in the rearrangement, since the homologies are with the 3' untranslated region of L1 elements.[31]

The *LKB1* allele containing this intragenic deletion code for a LKB1 mRNA that lacks exons 2 and 3 and maintains the protein reading frame; the translated product should results in a protein lacking the portion of LKB1 catalytic domain. Noteworthy, the clinical manifestation is that of severe phenotype with very early onset age of the disease (5 years).

According to the literature data, our results suggest that large intragenic rearrangements could intervene at the *LKB1* locus and that their incidences have probably been underestimated by mutation screening strategies based on molecular biology techniques.

However, we observed germline *STK*11 mutations in about 66.7% of affected subjects, although it is not possible to exclude that genetic locus heterogeneity exists in PJS manifestations. Because of over-

representation of Alu elements involving this region, in our opinion is reasonably to hypothesize that Alu-mediated homologous recombination could give rise to several intragenic rearrangements, as translocations or inversions, not always detectable using classical molecular biology techniques. These rearrangements could in part explain the remaining PJS cases without identified molecular alterations.

Finally, we investigate the hypothesis that mutations in the ubiquitin-associated domain (UBA) of the AMP-related kinase genes, might be present in families with PJS. The AMPK related kinases are activated following phosphorylation of their T-loop threonine residue by the LKB1 complex[32] and nine of these contain an UBA domain immediately C-terminal to the kinase catalytic domain; this domain allows LKB1-induced phosphorylation and activation. No mutations were identified in the region coding for UBA domain of the AMPK related protein kinases; however, two of the analyzed patients resulted carrier each of two intronic single nucleotide substitutions not reported in database (described in result section). Additional studies are necessary to shed light on their meaning.

Concerning the molecular analysis PHTS patients, unfortunately we could analyze a lower series o f patients, so we have identified a missense mutation in one of the three PHTS patients screened. However, our casuistry have to related to the rarity of the disease, which appears to be underestimated, because the PHTS syndromes presents a incomplete and variable penetrance, that can complicate the clinical diagnosis.

In both of the PHTS patients negative for the presence of mutation in gene coding region, the quantitative analysis showed a marked decrease in the levels of messenger. We have excluded the possibility of a deletion of the entire gene in these patients due to the presence of heterozygous polymorphisms, and the DNA real-time quantitative analysis. So this decrease is probably due to other mechanisms of inactivation in the PTEN gene, as alterations in the promoter region or the involvement of modifier genes *(Zhou XP, et al. 2003)*.

The next step of our study was to test the effects of PTEN inactivation on the expression of several genes regulated by PI3K/AKT molecular pathway or otherwise involved in CRC development.

PTEN is a tumour-suppressor gene that has attracted significant interest given its high mutation frequency in human cancers and its roles in apoptosis/proliferation via negative regulation of AKT/PKB activity (Downward, 2004; Parsons, 2004). Consistent with the direct protein-protein interactions that regulate p53 function (Freeman et al., 2003; Lei

et al., 2006), PTEN mutation in individuals with Cowden Disease results in cancer predisposition (Liaw et al., 1997) associated with cutaneous hyperkeratosis (Fistarol et al., 2002), suggesting that roles in keratinocyte differentiation can be added to PTEN activities that are essential for normal development. In transgenic mice, Pten hetrozygotes (Stambolic et al., 2000) or conditional knockouts (Li et al., 2002; Suzuki et al., 2003) exhibit neoplasia associated with increased anti-apoptotic AKT activities, cell migration/adhesion anomalies (Subauste et al., 2005) and cell cycle control failure (Di Cristofano et al., 2001). In addition, recent models demonstrate that PTEN via PI3K–Akt signalling, cooperates with Wnt to increase β-catenin signaling during inflammation. Instead, further recent study has linked this interaction with the inflammatory process, in fact inhibition of PI3K signaling in interleukin (IL)-10 knockout (IL-10 β-/β-) mouse colitis abrogated β -catenin signaling, crypt proliferation, and dysplasia (Lee G, et al 2010). Furthermore evidence shows a connexion between AKT and TNFα-NF-kB pathway. In fact NF-κB increases Akt activity as evidenced by increased phosphorylated form of Akt and its downstream target GSK-3β. These data suggest that the increased Akt activity is facilitated by reduction in PTEN expression. NF-κB-induced activation of Akt also suggests an interesting positive feedback loop, where activated Akt further facilitates NF-κB activation. Such a feedback loop may augment Akt activity in tumors and increase tumor growth and invasion. The NF-κB inhibitor IκB-α is degraded rapidly upon TNF-α stimulation and then resynthesized after NF-κB stimulation. One implication of the role of IκB-α as a temporal regulatory switch to turn off NF-κB by resynthesis of IκB-α is the hypothesis that some NF-κB-responsive genes are activated with a short pulse of NF-κB, whereas other genes need longer exposure to activate transcription. The bimodal temporal signal activation of NF-κB/IκB-α to up-regulate the two classes of NF-κB target genes was demonstrated for NF-κB-induced activation of the chemokine interleukin-10. These findings underlying the interaction between PTEN/PI3K/Akt and NF-κB at the level of transcription and offer one possible explanation for increased tumorigenesis and inflammation in systems where NF-κB is chronically activated. (Kim S, et al. 2004).

The results of our analysis, performed on mRNA of the three CS patients and two healthy controls extracted from peripheral blood lymphocytes, showed in previous section, is according to this hypothesis. In fact we have found a sensible increase in mRNA levels of COX-2,CCD1 and TNFα genes whereas decrease expression of APC gene in all PHTS

patients.
Our data represent the first evidence of a PI3K/Akt pathway deregulation in periferal blood cells of PHTS patients that probably determine a pro inflammation activation. Knowledge of specific molecular pathways constitutively deregulated in this syndrome could be helpful in optimizing molecular targeted therapy and preventative care. Otherwise the data suggests the interesting possibility of use these PI3K/Akt pathway downstream genes as molecular markers that could support a more precise and rapid diagnosis of PHTS syndromes.

REFERENCES

Alhopuro P, Katajisto P, Lehtonen R, et al. "*Mutation analysis of three genes encoding novel LKB1-interacting proteins, BRG1, STRADalpha, and MO25alpha, in Peutz-Jeghers syndrome.*" Br J Cancer 2005;92(6):1126-9

Altiok S, Batt D, Altiok N, Papautsky A, Downward J, Roberts TM, Avraham H. "*Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells.*" J Biol Chem. 1999 Nov 5;274(45):32274-8

Amos CI, Keitheri-Cheteri MB, Sabripour M, Wei C, McGarrity TJ, Seldin MF, Nations L, Lynch PM, Fidder HH, Friedman E, Frazier ML "*Genotype–phenotype correlations in Peutz–Jeghers syndrome.*" J Med Genet 2004; 41(5):327–333

Aretz S, Stienen D, Uhlhaas S, et al. "*High proportion of large genomic STK11 deletions in Peutz-Jeghers syndrome.*" Hum Mutat 2005;26(6):513-9.10.

Baas AF, Boudeau J, Sapkota GP, et al. "*Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD.*" EMBO J 2003;22(12):3062-72.

Blumenthal GM and Dennis PA *"PTEN hamartoma tumor syndromes"* Eur J Hum Genet. 2008;16(11):1289-300.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. "*Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.*" Cell. 1999 Mar 19;96(6):857-68.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. "*Regulation of cell death protease*

caspase-9 by phosphorylation." Science. 1998 Nov 13;282(5392):1318-21.

Carnero A. "*The PKB/AKT pathway in cancer.*" Curr Pharm Des. 2010;16(1):34-44.

Chen HM, Fang JY."*Genetics of the hamartomatous polyposis syndromes: a molecular review.*" Int J Colorectal Dis. 2009;24(8):865-74.

Chow E, Macrae F. "*A review of juvenile polyposis syndrome.*" J Gastroenterol Hepatol. 2005;20:1634-1640.

De Rosa M, Dourisboure RJ, Morelli G, et al. "*First genotype characterization of Argentinean FAP patients: identification of 14 novel APC mutations.*" Hum Mutat 2004;23(5):523-4.

Denggao Yao, Claire L. Alexander, Jean A. Quinn, Weng-Chyn Chan, Hong Wu and David A. Greenhalgh, "*Fos cooperation with PTEN loss elicits keratoacanthoma not carcinoma, owing to p53/p21WAF-induced differentiation triggered by GSK3β inactivation and reduced AKT activity*" J Cell Sci. 2008; 15;121(Pt 10):1758-69.

Derynck R, Zhang YE. "*Smad-dependent and Smad-independent pathways in TGF-beta family signalling.*" Nature 2003;425:577–584.

Desai DC, Neale KF, Talbot IC, Hodgson SV, Phillips RK. "*Juvenile polyposis.*" Br J Surg. 1995;82:14-17.

Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C, Pandolfi PP. "Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse." Nat Genet. 2001 Feb;27(2):222-4.

Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW,

Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ. "*Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1).*" Mol Cell Biol. 2000 Dec;20(24):9138-48.

Downward J. *"PI 3-kinase, Akt and cell survival."* Semin Cell Dev Biol. 2004 Apr;15(2):177-82. Review.

Elliott RL, Blobe GC. "*Role of transforming growth factor beta in human cancer.*" J. Clin. Oncol 2005;23:2078–2093.

Eng C "*To be or not to BMP.*" Nat Genet 2001;28(2):105–107

Eng C *"Will the real Cowden syndrome please stand up: revised diagnostic criteria."* J Med Genet 2000;37(11):828–830

Eng C: *"PTEN: one gene, many syndromes."* Hum Mutat 2003;22:183– 198.

Fearon E.R., Vogelstein B., "*A genetic model for colorectal tumorigenesis*", Cell 1990(61) 759–767.

Fistarol SK, Anliker MD, Itin PH. *"Cowden disease or multiple hamartoma syndrome--cutaneous clue to internal malignancy."* Eur J Dermatol. 2002 Sep-Oct;12(5):411-21. Review.

Fonsatti E, Del Vecchio L, Altomonte M, Sigalotti L, Nicotra MR, et al "*Endoglin: an accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies.*" J Cell Physiol. 2001;188:1-7.

Fruman DA, Meyers RE, Cantley LC."*Phosphoinositide kinases.*" Annu Rev Biochem. 1998;67:481-507. Review.

Grady WM. "*Genetic testing for high-risk colon cancer patients*" Gastroenterology 2003;124:1574–1594

Grover D, Mukerji M, Bhatnagar P, et al. *"Alu repeat analysis in the complete human genome: trends and variations with respect to genomic composition."* Bioinformatics. 2004;20(6):813-7.

Hampel H, Frankel W, Panescu J, et al. "*Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients*" Cancer Res 2006;66:7810– 7817.

Hearle NC, Rudd MF, Lim W, et al. "*Exonic STK11 deletions are not a rare cause of Peutz-Jeghers syndrome.*" J Med Genet 2006;43(4):e15.

Heinen CD, "*Genotype to phenotype: Analyzing the effects of inherited mutations in colorectal cancer families.*" Mutat Res. 2009

Hemminki A, Tomlinson I, Markie D, et al. "*Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis.*" Nat Genet. 1997;15(1):87-90

Hobert JA, Eng C. "*PTEN hamartoma tumor syndrome: an overview.*" Genet Med. 2009;11(10):687-94. Review.

Howe JR, Bair JL, Sayed MG, Anderson ME, Mitros FA, Petersen GM, Velculescu VE, Traverso G, Vogelstein B. "*Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis.*" Nat Genet. 2001;28(2):184-7.

Hui-Min Chen & Jing-Yuan Fang "*Genetics of the hamartomatous polyposis syndromes: a molecular review*" Int J Colorectal Dis 2009;(24):865–874

Jae Hong No, Yong-Tark Jeon, In-Ae Park, Daehee Kang, Jae Weon Kim, Noh-Hyun Park, Soon-Beom Kang, Yong-Sang Song, "*Expression of mTOR protein and its clinical significance in endometrial cancer*" Med Sci Monit, 2009; 15(10): BR301-305

Jaleel M, Villa F, Deak M, et al. "*The ubiquitin-associated domain of AMPK-related kinases regulates conformation and LKB1-mediated phosphorylation and activation.*" Biochem J 2006;394(Pt 3):545-55.

Jasperson KW, Tuohy TM, Neklason DW, Burt RW. "*Hereditary and familial colon cancer.*" Gastroenterology. 2010 Jun;138(6):2044-58.

Katajisto P, Vallenius T, Vaahtomeri K, Ekman N, Udd L, Tiainen M, Mäkelä TP."*The LKB1 tumor suppressor kinase in human disease.*" Biochim Biophys Acta. 2007;1775(1):63-75.

Kane LP, Shapiro VS, Stokoe D, Weiss A. "*Induction of NF-kappaB by the Akt/PKB kinase.*" Curr Biol. 1999 Jun 3;9(11):601-4.

Katajisto P, Vallenius T, Vaahtomeri K, Ekman N, Udd L, Tiainen M, Mäkelä TP. "*The LKB1 tumor suppressor kinase in human disease.*" Biochim Biophys Acta. 2007 Jan;1775(1):63-75. Epub 2006 Aug 16. Review.

Kim S, Domon-Dell C, Kang J, Chung DH, Freund JN, Evers BM. "*Down-regulation of the tumor suppressor PTEN by the tumor necrosis factor-alpha/nuclear factor-kappaB (NF-kappaB)-inducing kinase/NF-kappaB pathway is linked to a default IkappaB-alpha autoregulatory loop.*" J Biol Chem. 2004 6;279(6):4285-91.

Kinzler KW, Vogelstein B. "*Landscaping the cancer terrain.*" Science. 1998;280:1036-1037.

Kolomietz E, Meyn MS, Pandita A, et al. "*The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors.*"

Genes Chromosomes Cancer 2002;35(2):97-112.

Kops GJ, Burgering BM. "*Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling.*" J Mol Med. 1999 Sep;77(9):656-65. Review.

Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM. "*Direct control of the Forkhead transcription factor AFX by protein kinase B.*" Nature. 1999 Apr 15;398(6728):630-4.

Krymskaya1, V.P. and Goncharova E. A, "*PI3K/mTORC1 activation in hamartoma syndromes.*" Cell Cycle 2009 (8):3, 403-413.

Kubo Y, Urano Y, Hida Y, Ikeuchi T, Nomoto M, Kunitomo K, Arase S. "*A novel PTEN mutation in a Japanese patient with Cowden disease.*" Br J Dermatol. 2000;142(6):1100-5.

Kuruvilla FG, Schreiber SL. "*The PIK-related kinases intercept conventional signaling pathways.*" Chem Biol. 1999 May;6(5):R129-36.

Lee G, Goretsky T, Managlia E, Dirisina R, Singh AP, Brown JB, May R, Yang GY, Ragheb JW, Evers BM, Weber CR, Turner JR, He XC, Katzman RB, Li L, Barrett TA."*Phosphoinositide 3-kinase signaling mediates beta-catenin activation in intestinal epithelial stem and progenitor cells in colitis.*" Gastroenterology. 2010;139(3):869-81, 881.e1-9.

Lei Q, Jiao J, Xin L, Chang CJ, Wang S, Gao J, Gleave ME, Witte ON, Liu X, Wu H. *"NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss."* Cancer Cell. 2006 May;9(5):367-78.

Ley T.J., E.R. Mardis, L. Ding, B. Fulton, M.D. McLellan, K. Chen, D. Dooling, B.H.Dunford-Shore, S. McGrath, M. Hickenbotham, L. Cook, R. Abbott, D.E. Larson, D.C. Koboldt, C. Pohl, S. Smith, A. Hawkins, S. Abbott, D. Locke, L.W. Hillier, T.Miner, L. Fulton, V. Magrini, T. Wylie, J. Glasscock, J. Conyers, N. Sander, X. Shi, J.R. Osborne, P. Minx, D. Gordon, A. Chinwalla, Y. Zhao, R.E. Ries, J.E. Payton, P. Westervelt, M.H. Tomasson, M. Watson, J. Baty, J. Ivanovich, S. Heath, W.D.Shannon, R. Nagarajan, M.J. Walter, D.C. Link, T.A. Graubert, J.F. DiPersio, R.K. Wilson, "*DNAsequencing of a cytogenetically normal acute myeloid leukaemia genome*", Nature 456 (2008) 66–72.

Lichtenstein P, Holm NV, Verkasalo PK, et al. "*Environmental and heritable factors in the causation of cancer – analyses of cohorts of twins from Sweden, Denmark, and Finland.*" N Engl J Med 2000;343:78–85.

Lizcano JM, Göransson O, Toth R, et al. *"LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1."* EMBO J 2004;23(4):833-43.

Manfredi M., *"Hereditary Hamartomatous Polyposis Syndromes: Understanding the Disease Risks As Children Reach Adulthood"* Gastroenterology & Hepatology 2010Volume 6, Issue 3.

Marra G, Armelao F, Vecchio FM, Percesepe A, Anti M. *"Cowden's disease with extensive gastrointestinal polyposis."* J Clin Gastroenterol. 1994;18:42-47.

Massague J, Blain SW, Lo RS. "*TGFβ signaling in growth control, cancer, and heritable disorders.*" Cell 2000;103:295–309.

McGarrity TJ, Amos C (2006) Peutz–Jeghers syndrome: clinicopathology and molecular alterations. Cell Mol Life Sci 63 (18):2135–2144

Montagnani M, Chen H, Barr VA, Quon MJ."*Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179).*" J Biol Chem. 2001 Aug 10;276(32):30392-8.

Myers MP, Tonks NK."*PTEN: sometimes taking it off can be better than putting it on.*" Am J Hum Genet. 1997 Dec;61(6):1234-8.

Nakamura N, Ramaswamy S, Vazquez F, Signoretti S, Loda M, Sellers WR. "*Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN.*" Mol Cell Biol. 2000 Dec;20(23):8969-82.

No JH, Jeon YT, Park IA, Kang D, Kim JW, Park NH, Kang SB, Song YS. "*Expression of mTOR protein and its clinical significance in endometrial cancer.*" Med Sci Monit. 2009 Oct;15(10):BR301-305.

Okano J, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H. "*Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation.*" J Biol Chem. 2000 Oct 6;275(40):30934-42.

Parsons R. *"Human cancer, PTEN and the PI-3 kinase pathway."* Semin Cell Dev Biol. 2004 Apr;15(2):171-6. Review.

Pilarski R, Eng C:" *Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome.*" J Med Genet 2004; 41: 323–326

Romashkova JA, Makarov SS. "*NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling.*" Nature. 1999 Sep 2;401(6748):86-90.

Sabatini DM. *"mTOR and cancer: insights into a complex relationship."* Nat Rev Cancer 2006; 6:729-34.

Schreibman IR, Baker M, Amos C, McGarrity TJ. "*The hamartomatous polyposis syndromes: a clinical and molecular review.*" Am J Gastroenterol. 2005 Feb;100(2):476-90.
Shaw RJ, Kosmatka M, Bardeesy N, et al. "*The tumour suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress*". Proc Natl Acad Sci USA 2004;101(10):3329-35.

Sheng H, Shao J, O'Mahony CA, Lamps L, Albo D, Isakson PC, Berger DH, DuBois RN, Beauchamp RD. "*Transformation of intestinal epithelial cells by chronic TGF-beta1 treatment results in downregulation of the type II TGF-beta receptor and induction of yclooxygenase-2.*" Oncogene 1999;18:855–867.

Simpson L, Parsons R: *"PTEN: life as a tumor suppressor."* Exp Cell Res 2001; 264: 29–41.

Sjoblom T., S. Jones, L.D. Wood, D.W. Parsons, J. Lin, T.D. Barber, D. Mandelker,bR.J. Leary, J. Ptak, N. Silliman, S. Szabo, P. Buckhaults, C. Farrell, P. Meeh, S.D.bMarkowitz, J. Willis, D. Dawson, J.K.V. Willson, A.F. Gazdar, J. Hartigan, L. Wu,bC. Liu, G. Parmigiani, B.H. Park, K.E. Bachman, N. Papadopoulos, B. Vogelstein,bK.W. Kinzler, V.E. Velculescu, "*The consensus coding sequences of human breast and colorectal cancers*", Science 314 2006;268–274.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. "*Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN.*" Cell. 1998 Oct 2;95(1):29-39.

Starink TM, van der Veen JP, Arwert F, de Waal LP, de Lange GG, et al. *"The Cowden syndrome: a clinical and genetic study in 21 patients."* Clin Genet. 1986;29:222-233.

Stoffel E, Mukherjee B, Raymond VM, et al. *"Calculation of risk of colorectal and endometrial cancer among patients with Lynch syndrome."* Gastroenterology 2009;137:1621–1627

Subauste MC, Nalbant P, Adamson ED, Hahn KM. "*Vinculin controls PTEN protein level by maintaining the interaction of the adherens junction protein beta-catenin with the scaffolding protein MAGI-2.*" J Biol Chem. 2005 Feb 18;280(7):5676-81.

Suzuki A, Kaisho T, Ohishi M, Tsukio-Yamaguchi M, Tsubata T, Koni PA, Sasaki T, Mak TW, Nakano T. *"Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination."* J Exp Med. 2003 Mar 3;197(5):657-67

Suzuki A, Itami S, Ohishi M, Hamada K, Inoue T, Komazawa N, Senoo H, Sasaki T, Takeda J, Manabe M, Mak TW, Nakano T. *"Keratinocyte-specific Pten deficiency results in epidermal hyperplasia, accelerated hair follicle morphogenesis and tumor formation."* Cancer Res. 2003 Feb 1;63(3):674-81.

Sweet K, Willis J, Zhou XP, Gallione C, Sawada T, Alhopuro P, Khoo SK, Patocs A, Martin C, Bridgeman S, Heinz J, Pilarski R, Lehtonen R, Prior TW, Frebourg T, Teh BT, Marchuk DA, Aaltonen LA, Eng C. "*Molecular classification of patients with unexplained hamartomatous and hyperplastic polyposis.*" JAMA. 2005 16;294(19):2465-73.

Tenesa A, Dunlop MG. "*New insights into the aetiology of colorectal cancer from genome-wide association studies.*" Nat Rev Genet 2009;10:353–358.

Tomlinson IPM, Houlston RS. *"Peutz–Jeghers syndrome."* J Med Genet 1997;34(12):1007–1011.

Vanhaesebroeck B, Waterfield MD. "*Signaling by distinct classes of phosphoinositide 3-kinases*"Exp Cell Res. 1999 Nov 25;253(1):239-54. Review.

Volikos E, Robinson J, Aittomäki K, et al. "*LKB1 exonic and whole gene deletions are a common cause of Peutz-Jeghers syndrome.*" J Med Genet 2006;43(5).

Wilkes MC, Mitchell H, Penheiter SG, Doré JJ, Suzuki K, Edens M, Sharma DK, Pagano RE, Leof EB, "*Transforming Growth Factor-B Activation of Phosphatidylinositol 3-Kinase Is Independent of Smad2 and Smad3 and Regulates Fibroblast Responses via p21-Activated Kinase-2*" Cancer Res. 2005 15;65(22):10431-40.

Winship IM, Dudding TE (2008) *"Lessons from the skin-cutaneous features of familial cancer."* Lancet Oncol 9(5):462–472

Wirtzfeld DA, Petrelli NJ, Rodriguez-Bigas MA. "*Hamartomatous polyposis syndromes: molecular genetics, neoplastic risk, and surveillance recommendations*" .Ann Surg Oncol. 2001;8:319-327.

Wood LD, D.W. Parsons, S. Jones, J. Lin, T. Sjoblom, R.J. Leary, D. Shen, S.M. Boca, T. Barber, J. Ptak, N. Silliman, S. Szabo, Z. Dezso, V.Ustyanksky, T. Nikolskaya,Y. Nikolsky, R. Karchin, P.A. Wilson, J.S. Kaminker, Z. Zhang, R. Croshaw, J. Willis, D. Dawson, M. Shipitsin, J.K.V. Willson, S. Sukumar, K. Polyak, B.H.Park, C.L. Pethiyagoda, P.V.K. Pant, D.G. Ballinger, A.B. Sparks, J. Hartigan, D.R. Smith, E. Suh, N. Papadopoulos, P. Buckhaults, S.D. Markowitz, G. Parmigiani, K.W. Kinzler, V.E. Velculescu, B. Vogelstein, "*The genomic landscapes of human breast and colorectal cancers*", Science 318 (2007) 1108–1113.

Woods A, Johnstone SR, Dickerson K, et al. *"LKB1 is the upstream kinase in the AMP-activated protein kinase cascade."*CurrBiol 2003;13(22):2004-8.

Woods A, Johnstone SR, Dickerson K, et al. *"LKB1 is the upstream kinase in the AMP-activated protein kinase cascade."*CurrBiol

2003;13(22):2004-8.

Xu Y, Pasche B. "*TGF-beta signaling alterations and susceptibility to colorectal cancer.*" Hum Mol Genet. 2007 Apr 15;16 Spec No 1:R14-20.

Yaffe MB, Leparc GG, Lai J, Obata T, Volinia S, Cantley LC. "*A motif-based profile scanning approach for genome-wide prediction of signaling pathways.*" Nat Biotechnol. 2001 Apr;19(4):348-53.

Zhou H, Li XM, Meinkoth J, Pittman RN. "*Akt regulates cell survival and apoptosis at a postmitochondrial level.*" J Cell Biol. 2000 Oct 30;151(3):483-94.

Zhou XP, Waite KA, Pilarski R, Hampel H, Fernandez MJ, Bos C,Dasouki M, Feldman GL, Greenberg LA, Ivanovich J, Matloff E, Patterson A, Pierpont ME, Russo D, Nassif NT, Eng C "*Germline PTEN promoter mutations and deletions in Cowden/Bannayan–Riley–Ruvalcaba syndrome result in aberrant PTEN protein and dysregulation of the phosphoinositol-3-kinase/Akt pathway.*" Am J Hum Genet 2003 73(2):404–411

Zimmermann S, Moelling K. "*Phosphorylation and regulation of Raf by Akt (protein kinase B).*" Science. 1999 Nov 26;286(5445):1741-4.

ORIGINAL CONTRIBUTION

Implication of Adenomatous Polyposis Coli and MUTYH Mutations in Familial Colorectal Polyposis

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PURPOSE: Familial adenomatous polyposis is an autosomal dominantly inherited syndrome characterized by hundreds or thousands of colorectal polyps and a high risk of colorectal cancer at a young age. Truncating germline mutations in the adenomatous polyposis coli gene are detected in approximately 80 percent of patients with classical familial adenomatous polyposis and in approximately 10 percent of the attenuated familial adenomatous polyposis patients.

METHODS: We investigated the adenomatous polyposis coli and MUTYH genes mutations in a well-characterized series of 25 unrelated Italian patients with familial adenomatous polyposis.

RESULTS: We characterized the specific adenomatous polyposis coli gene mutation in 10 probands, and identified eight truncating mutations (4 novel and 4 known mutations) and two splicing mutations. One of these, a novel missense mutation in exon 15, activates an exonic splicing enhancer control sequence. Moreover, 11 MUTYH gene mutations have been identified in 7 patients without a dominant family history of polyposis.

CONCLUSIONS: This study enlarges the genotype-phenotype correlations of familial adenomatous polyposis and suggests that messenger alterations could be responsible for a subset of familial adenomatous polyposis cases without germ-line adenomatous polyposis coli or MUTYH gene mutations. It also confirms that genotype-phenotype correlations in MUTYH-associated polyposis are very complex.

KEY WORDS: Adenomatous polyposis coli gene; Familial adenomatous polyposis; MUTYH: Human homolog of *E Coli MutY* gene.

Familial adenomatous polyposis (FAP) is an inherited autosomal dominant precancerous condition characterized by multiple adenomatous polyps in the colon and rectum. If not treated, these polyps invariably develop to colorectal cancer, typically by the age of 40 years.[1] This condition is primarily associated with germline mutations in the adenomatous polyposis coli (*APC*) gene (OMIM*175100),[2,3] an ubiquitously expressed tumor suppressor, which contains at least 21 exons.[4]

The clinical manifestations and severity of FAP vary greatly with the mutation site. The disorder is classically characterized by more than 100 colorectal adenomas, early onset of colorectal carcinoma, and specific extracolonic features.[5] Attenuated familial adenomatous polyposis (AFAP) is a milder form of the disease in which patients have fewer than 100 adenomas. AFAP patients with dominant inheritance harbor germ-line mutations in the 5′ or 3′ regions of the *APC* gene or in regions affected by alternative splicing events.[6–9] Another group of patients have the FAP or AFAP phenotype and recessive inheritance. These patients often harbor inherited biallelic mutations in the base-excision repair (*BER*) gene *MYH* (MUTYH; OMIM# 604933, Gene Bank NM_012222.1).[10,11]

Finally, a group of individuals have an AFAP-like phenotype, with 3 to 100 polyps throughout the colorectum and often with unclear or no family history of polyposis. In most cases, no germ-line *APC* or *MYH* mutations have been detected in these patients.

By using a combination of the polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, protein truncation test (PTT), single-strand conformation polymorphism

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(SSCP), DNA sequencing, and real-time polymerase chain reaction techniques, we analyzed the entire coding region of the *APC* gene at the DNA and RNA levels in 25 unrelated Italian probands affected by FAP. We also analyzed the coding region and intron-exon boundaries of the *MYH* gene for disease-causing mutations in patients with FAP negative for *APC* mutations.

PATIENTS AND METHODS

We studied 25 unrelated Italian probands affected by FAP. Their polyposis phenotypes are listed in Table 1. The inheritance for multiple polyposis is defined as dominant (D) when every affected individual has an affected parent and as recessive (R) when the disease appears at least in one affected sibling of the proband, but not in the ancestors or collateral relatives. No inheritance (NI) is indicated in families in which no affected relatives of the proband are present. All patients received clinical and genetic counseling and provided written, informed consent to the study.

DNA and RNA were obtained from peripheral white blood cells collected with EDTA. We used a combination of four techniques to analyze sequence variants in the *APC* gene: the PTT, SSCP, direct sequencing of amplified fragments, RT-PCR, and quantitative real-time analysis. For the PTT, overlapping fragments of exon 15 were amplified and the reaction was performed as described elsewhere.[12] PCR-SSCP analysis was performed as previously reported.[12] Sequencing analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of the *APC* sequence (GenBank Accession number: M74088.1) and *MYH* sequence (GenBank Accession number: NM_012222.1).

The quantitative RNA and DNA real-time assays are described elsewhere.[13] To measure mRNA, we selected oligonucleotides within exons 13 and 14 to amplify the canonical messenger of the *APC* gene. The relative

TABLE 1. Clinical phenotype and mutation spectrum of Italian FAP patients studied

Patient	Phenotype	Type of inheritance	Extracolonic manifestation	No. of colon polyps	APC mutation	MYH mutation
1	Classical FAP	R	NO	100–1000	NF.	c.494A->G (p.Y165C); c.692G->A (p.R231H)
2	Classical FAP	R	NO	100–1000	NF.	c.502C->T (p.R168C); c.1395–97delGGA (p.466delE)
3	Classical FAP	D	NO	100–1000	APC deletion	N.F.
4	AFAP	N.I.	NO	7	N.F.	c.1145G->A (p.G382D); c.270C->A (p.Y90X)
5	AFAP	D	Thyroid nodules	<100	c.697delC (p.Q233fs59X)	
6	Classical FAP	D	Thyroid nodules	100–1000	N.F.	N.F.
7	AFAP	N.I.	NO	25	NF.	c.972G->C (p.Q324H)
8	Classical FAP	D	NO	100–1000	c.4006G->T (p.R1336X)	
9	Classical FAP	D	NO	100–1000	IVS9+1G->A	
10	AFAP	R	Lung cancer (proband's father, 68 years)	15–20	N.F.	N.F.
11	AFAP	N.I.	Stomach cancer (proband's father, 81 yr)	7	N.F.	N.F.
12	AFAP	R	NO	25	RNA alteration	N.F.
13	Classical FAP	N.I.	NO	100–1000	c.3927–31delAAAGA (p.G1309Hi1311fs3X)	
14	AFAP	N.I.	NO	10	c.4909G->A (p.D1637N)	N.F.
15	AFAP	R	NO	<100	N.F.	N.F.
16	Classical FAP	D	NO	100–1000	c.3865delT (p.Cys1289fs16X)	
17	AFAP	N.I.	Lymphoma (proband's father, 53 yr)	24	N.F.	c.310C->T (p.R231H); c.494A->G (p.Y165C)
18	Classical FAP	D	NO	100–1000	c.3927–31delAAAGA (p.G1309Hi1311fs3X)	
19	Classical FAP	D	NO	100–1000	c.3161–62delAC (p.H1054fs1X)	
20	Classical FAP	D	NO	100–1000	c.694C->T (p.R232X)	
21	AFAP	N.I.	NO	11	N.F.	N.F.
22	AFAP	N.I.	NO	<100	N.F.	N.F.
23	Classical FAP	D	NO	100–1000	c.2522T->A (L841X)	
24	AFAP	N.I.	NO	50–100	NF.	c.1145G->A (p.G382D)
25	AFAP	N.I.	NO	50–100	NF.	c.1145G->A (p.G382D)

FAP = familial adenomatous polyposis; AFAP = attenuated familial adenomatous polyposis; classical FAP = >100 polyps; AFAP 3–4/99 polyps; R = recessive inheritance; D = dominant inheritance; NI = no inheritance; NF = mutation not found; the blood character indicates the novel mutations.

expression of the target transcript was calculated with the comparative Ct method (Applied Biosystems User Manual) by using a cDNA fragment from the glucuronidase (GUS) housekeeping gene as control.

To measure DNA, we selected oligonucleotides that amplify the entire *APC* gene exon 14, including the intron-exon boundaries, and oligonucleotides that amplify the entire *MYH* gene exon 15, including the intron-exon boundaries, used as control gene. The *APC* DNA target was quantified by using the ΔCt of a normal control as calibrator to calculate the relative quantification. As positive controls we measured, in each determination, the *APC* genomic DNA of two carriers of the *APC* locus deletion previously characterized.[14]

For RNA analysis, we synthesized cDNA. Half microliter of the cDNA was amplified with primer C29.1 and primer 15C3 as previously reported.[13] Finally, nested PCR amplifications were performed with 1 μl of the first PCR product with the following primers. The primers used to amplify the fragment encompassing the region from exon 8 to exon 12 of the cDNA were: *APC* 8cFP and *APC* 12cRP.[13] The primers used for PTT analysis of exons 1–14 were: 1T7: 5′-T7-GCT GCA GCT TCA TAT GAT C-3′, and 15B3b: 5′-GGG TAA CAC TGT AGT ATT CAA AT-3′ and the reaction was performed as described above.

To analyze exon 15 mRNA alterations in the carrier of the 4909G->A mutation (p.D1637N), we amplified 0.5 μl of the cDNA with primers 13cFP and 15KRP described by Groden *et al*.[15] Finally, seminested PCR amplifications were performed with 1 μl of the first PCR product with the same forward primer 13cFP and different reverse primers 15GRP, 15GIRP, and 15JRP.[15] The PCR products were analyzed on 1 to 2 percent agarose gels in tris-acetic acid (TAE) EDTA standard buffer and visualized by ethidium bromide staining. The missense mutation (4909G->A) was searched for in 50 control samples to exclude the possibility of a rare polymorphism. The nucleotide sequences of normal and mutated exon 15 were analyzed with the ESEfinder program using the default settings (exon.cshl.org/ESE/). This is a prediction program that evaluates the influence of base substitution on putative exonic splicing enhancers (ESE) sites. Exonic splicing enhancers (ESEs) act as binding sites for members of the Serine-Rich (SR) protein family; the program scores every sequence for the presence of ESE motifs recognized by the human SR proteins SF2/ASF, SRp40, SRp55 and SC35.[16] Exons 1–16 of the *MYH* gene were analyzed by PCR-SSCP and direct sequencing of the amplified fragments, as previously described.[17]

RESULTS

We have analyzed the disease-causing mutations in 25 unrelated patients with FAP from southern Italy. Of these patients, 9 showed the classical FAP phenotype with dominant inheritance, 1 showed the classical FAP phenotype without inheritance for the disease, 2 showed the classical FAP phenotype with recessive inheritance, and 13 showed the AFAP phenotype. Only one of the latter patients had a dominant inheritance, three had recessive inheritance, and the remaining nine patients had an unclear family history or did not report positive family history of FAP.

As shown in Table 1, we found the disease-causing mutation of the *APC* gene in nine of ten probands (90 percent) with a dominant syndrome (FAP or AFAP) and in one FAP patient with a classical phenotype but without a family history of this disease. Eight of these ten (80 percent) *APC* mutations are truncating mutations (4 novel mutations and 4 previously described; Patients 5, 8, 16, 23 and 13, 18, 19, 20, respectively; Table 1). The ninth is a splicing mutation (Patient 9) and the tenth is probably a deletion of the entire or a part of the gene (Patient 3). In fact, in Patient 3, quantitative PCR analysis performed on cDNA and genomic DNA showed significant down-expression of mRNA associated to genomic *APC* germline deletion encompassing exon 14 (Figs. 1A and B, sample P1). This patient resulted apparently homozygous (suggesting hemizygosity) at all polymorphic markers spanning the *APC* gene, consistent with a whole-gene deletion, thus confirming that haploinsufficiency can result in a classical polyposis phenotype. Also, another patient with FAP with an apparently recessive inheritance, showed significant down-expression of mRNA but not genomic deletion when analyzed with the same quantitative PCR technique (Figs. 1A and B, sample P2; Table 1, Patient 12). In this last case, the down-expression of the RNA could be probably caused by mutations in the promoter region or in other regulating elements. Therein, in both cases a limited amount of functional *APC* protein encoded could be most likely the pathogenetic molecular alteration. Finally, a splicing variant was identified in a patient with AFAP phenotype without inheritance for the disease (Patient 14).

Two of the mutations characterized are splicing mutations: one is a transition in the first nucleotide (+1) of the consensus donor splice-site of intron 9 (IVS9+1G>A), first described by Gavert *et al*.,[18] and the other is a novel missense mutation in exon 15 (4909G->A), which most likely generates a splicing enhancer motif that activates an upstream cryptic splice site.

Patient 9, who had the IVS9+1G>A mutation, is a 16-year-old boy affected by a very aggressive FAP phenotype with adenomatous colorectal polyps and rectal adenocarcinomas. We investigated the pathogenetic mechanism underlying the aggressive FAP phenotype in this patient. Protein truncation analysis of fragment 1T7–15B3b generated a polypeptide of the expected normal size and another of about 34 kDa from the mutant allele. RT-PCR analysis of exons 8–12 showed the expression of the *APC* chain that lacks exon 9 and 9a thereby creating a frame-shift and a premature stop codon (data not shown).

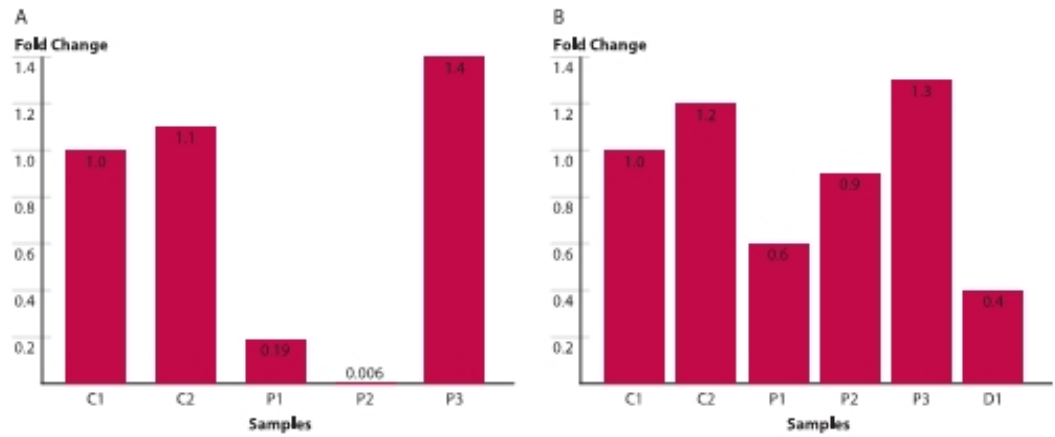

FIGURE 1. Real-time PCR quantification analysis. A. Relative expression, calculated with the comparative Ct method, of the adenomatous polyposis coli main transcript containing exon 14 compared with Glutathione-synthetase in healthy control subjects (C1 and C2) and patients with familial adenomatous polyposis (P1, P2, and P3). B. Relative genomic DNA quantification, calculated with the comparative Ct method, of the exon 14 adenomatous polyposis coli amplified fragment compared with exon 15 MUTYH amplified fragment in healthy control subjects (C1 and C2), patients with familial adenomatous polyposis (P1, P2, and P3), and patient with familial adenomatous polyposis carrying adenomatous polyposis coli gene deletion (D1).

It is likely that this overexpressed *APC* light-chain molecule could antagonize the tumor-suppressor effect of the *APC* protein causing the aggressive phenotype in this patient.

The other patient (Table 1; Patient 14), a 62-year-old man without a dominant family history of FAP, had an attenuated FAP (AFAP) phenotype with few colonic polyps (approximately 10) and without extracolonic manifestations. The family history was unclear; a cousin died from colorectal cancer at aged 65 years, but his parents died from causes other than colorectal cancer. Because this patient developed only few adenomas, colonoscopy is recommended every two years. This AFAP patient had a missense mutation in exon 15, c.4909G->A (p.D1637N), which consists in a G to A transition at position 4909 and causes a substitution of the aspargine 1637 to aspartic acid. PCR-SSCP analysis and DNA sequencing was performed on all 15 exons of the *APC* gene, including their exon-intron boundaries, and no other mutation was found. This novel missense mutation was not found in any of the 50 normal control samples analyzed.

Using the "ESEfinder" program, we made a computational analysis to look for ESE sequences in the region of exon 15 encompassing the mutation (from nucleotide 4896 to nucleotide 4945). Binding motifs for known splicing enhancer proteins (SR proteins: SF2/ASF, SC35, SRp40, and SRp55), were found in this region in both normal and mutated sequences. Remarkably, we identified an increase of the SC35 binding motif affinity in the mutated sequence (score 3.0) compared with the control sequence (score 2.5; Fig. 2A).

Using RNA extracted from lymphocytes of the patient and two healthy control subjects, we performed RT-PCR and nested PCR as described in *METHODS*. An abnormal fragment product was identified, in the patient and not in the healthy control subjects, for fragments 13cFP-15IRP and 13cFP-15JRP. No amplified fragment was obtained with the13cFP-15GRP primers, because it is internal to the mRNA deletion.

As shown in Figures 2D and E, sequence analysis of the cDNA amplified fragment revealed the lost of the exon 15 region from nucleotide 2214 to nucleotide 4820, probably caused by activation of a cryptic noncanonical GA-AG splice site pairs. This deleted messenger was produced only from the mutated allele; in fact, the sequence shows only the mutated nucleotide at position 4909 (Fig. 2D), whereas the genomic DNA sequence of the same region shows both normal and mutated nucleotides (Fig. 2C).

An unusual feature of mutation 4909G->A is that the change does not alter an authentic splice site nor does it generate a cryptic splice site, rather, the mutation probably activates an ESE control sequence that indirectly activates a cryptic splice junction upstream of the mutated site within the same exon.

We also analyzed coding region and intron-exon boundaries of the *MYH* gene for disease-causing mutations in patients negative for *APC* mutations, using PCR-SSCP and direct sequence techniques. We found 11 *MYH* mutations, all previously described,[10,19,20] in seven patients without a dominant family history of polyposis. Four patients had biallelic mutations of the *MYH* gene; one of these, a carrier of mutations Y90X and G382D

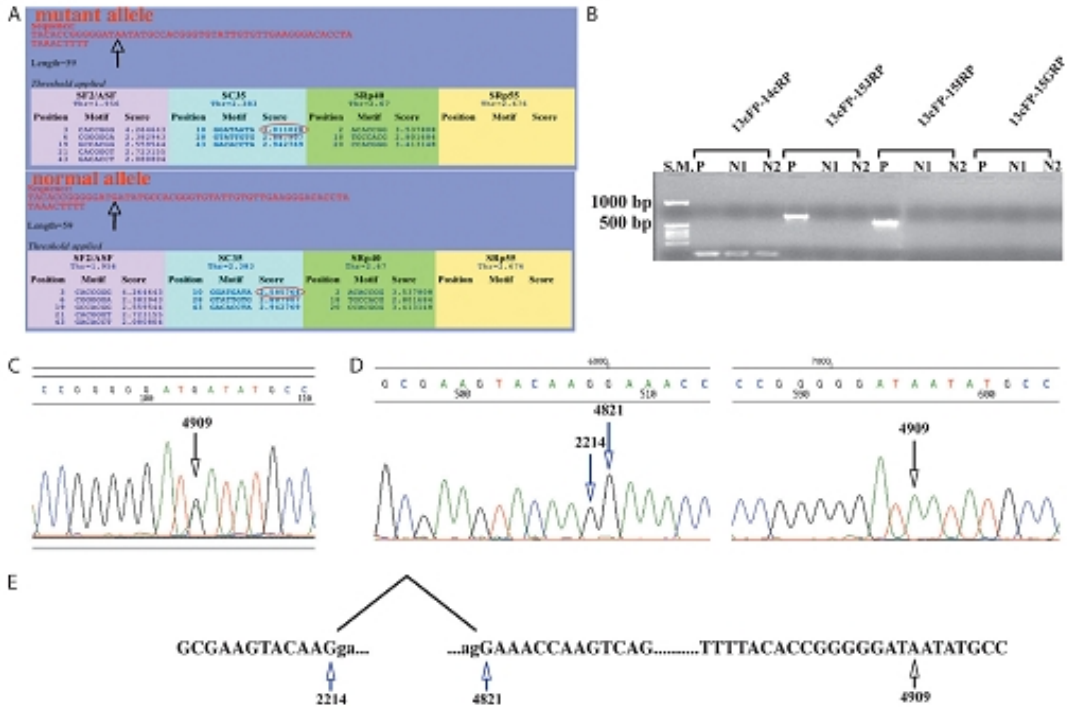

FIGURE 2. Molecular analysis of the 4909G->A (D1637N) mutation. A. Computational analysis performed, with ESEFinder program, of the genomic DNA encompassing the mutation. B. RT-PCR analysis of fragments 13cFP-14cRP (control fragment), 13cFP-15JRP, 13cFP-15IRP, and 13cFP-15GRP, performed on the messenger of patient carrier of this mutation (P) and two healthy control subjects (N1 and N2). S.M. = molecular weight marker. C. Sequence analysis of the genomic DNA region encompassing the mutation. D. Sequence analysis of the deleted messenger. Nucleotide position of the mutation (black arrows); novel exon junctions (blue arrows). E. Schematic representation of the alternative splicing event caused by the mutation. Nucleotide position of the mutation (black arrows); novel exon junctions (blue arrows).

(Table 1; Patient 4), had an attenuated phenotype without a family history of polyposis. Of the other three patients, two showed the classical polyposis phenotype with recessive inheritance and were carriers respectively of the R168C/466delE (Table 1; Patient 2) and Y165C/R231H (Patient 1) mutations. Interestingly, the third patient (Patient 17), also a carrier of the same Y165C/R231H mutations, showed an attenuated phenotype without a family history of disease. Finally, three patients, with attenuated phenotype and no family history of disease, were carriers of only one mutation: two of these had the G382D mutation (Patients 24 and 25) and the last one had the Q324H mutation (Patient 7).

DISCUSSION

Our data enlarge the spectrum of *APC* gene mutations and shed light on the correlations between the kind of *APC* germline mutations and the clinical manifestations of FAP. Furthermore, the data suggest that mRNA alterations could be responsible for a subset of FAP cases in which no germ-line *APC* or *MYH* mutations have been detected. Approximately 15 percent of inherited human diseases involve splicing errors caused by mutations in splice sites or in splicing control sequences.[21] Most splicing mutations are distributed within the coding exons or in the adjacent 5′- and 3′-splice sites, which frequently lead to exon skipping. In human disease genes, there are numerous mutations in ESE control sequences that have been shown to cause aberrant exon skipping.[22] According to the literature data, we suggest that exonic single-base substitutions may affect splicing when occurring at binding sites for splicing regulatory factors. However, disease-causing missense and silent mutations in tumor-suppressor genes are rarely reported because their functional consequence often remains uncertain.

In this context, *APC* molecular screening should be extended at the mRNA level to obtain a more precise molecular diagnosis and a better characterization of patients with FAP. It is our opinion that the molecular tecniques at the RNA level and computational analysis

could increase the mutation detection rate, particularly in nonclassical cases.

Interestingly, the patient carrier of the IVS9+1G>A mutation that leads to an *APC* messenger lacking exons 9 and 9a showed a very aggressive FAP phenotype. Usually, mutations in this region are associated with an attenuated phenotype. In fact, exon 9 undergoes alternative splicing in a fraction of mRNA molecules and mutations localized in the spliced-out portion of the exon are expected to result in the residual production of wild-type transcript.[23] However, variable phenotypes in the alternatively spliced region of exon 9 have been reported.[24] An AFAP phenotype has been described in a kindred bearing a mutation at the splice-donor site of exon 9a by Varesco *et al.*[25] Moreover, FAP families carrier of mutations in the same region showing a classical polyposis phenotype with hundreds of colorectal adenomas were described by Gavert *et al.*[18] and Curia *et al.*[26] Interestingly, in the family studied by Curia *et al.*[26] the polyps were detected in three patients who were aged 47, 12, and 8 years, respectively. The authors suggest that, in addition to the mutation site, the type of the mutation and the transcript dosage effect contribute to the phenotypic heterogeneity of the disease. Intriguingly, the mutation described by Varesco *et al.*[25] is localized at position +5 of the splice-donor site, whereas the mutations reported by Gavert *et al.*[18] and Curia *et al.*,[26] as those described in this study, occurred at the first base of the same splice-donor site. The two different sites of mutations could cause different efficiency of exon skipping. Moreover, the mechanism of splicing sites selection, as well as the mechanisms that regulate the stability of RNA and that contain premature termination codons (PTCs), also could differ significantly as a result of individual or tissue-specific differences, thus leading to the phenotypic variability.[13]

Our study confirmed that genotype-phenotype correlations in *MYH*-associated polyposis (MAP) are very complex; biallelic *MYH* mutations can result in classic or attenuated polyposis. Furthermore, patients with the same *MYH* biallelic mutations can show different phenotype.[11] Finally, in a relevant fraction of patients with colon polyposis, a mutation is found in a single *MYH* allele (monoallelic mutation).

This causes problems not only for the interpretation of the results but also for the clinical management (diagnosis and counseling) of patients and families in whom such variants of uncertain pathogenic significance are detected.

Glossary of Genetic Terms

Oligonucleotides: a polynucleotide of low molecular weight, consisting of approximately 20 nucleotide polymers, which anneals to a complementary sequence of deoxyribonucleic acid (DNA) and has a 3′-OH terminus at which a DNA polymerase begins the synthesis of a DNA chain.

Splice sites: sequences immediately surrounding the exon-intron boundaries recognized by the splicing machinery and used to connect two adjacent exons.

Cryptic splice site: a splice site generally not utilized by the splicing machinery for the splicing mechanism.

Exonic splicing enhancer (ESE) control sequence: pre-mRNA cis-acting element required for splice-site recognition.

Haploinsufficiency: genetic condition caused by a mutation causing loss of function of one allele that determines disease when the contribution of a normal allele is not sufficient to prevent disease.

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REFERENCES

1. Rustgi AK. Hereditary gastrointestinal polyposis and nonpolyposis syndromes. N Engl J Med 1994;331:1694–702.
2. Groden J, Gelbert L, Thliveris A, *et al.* Mutational analysis of patients with adenomatous polyposis: identical inactivating mutations in unrelated individuals. Am J Hum Genet 1993;52:263–72.
3. Kinzler KW, Nilbert MC, Su LK, *et al.* Identification of FAP locus genes from chromosome 5q21. Science 1991;253:661–5.
4. Santoro IM, Groden J. Alternative splicing of the *APC* gene and its association with terminal differentiation. Cancer Res 1997;57:488–94.
5. Fearnhead NS, Britton MP, Bodmer WF. The ABC of *APC*. Hum Mol Genet 2001;10:721–33.
6. Nagase H, Nakamura Y. Mutations of the *APC* (adenomatous polyposis coli) gene. Hum Mutat 1993;2:425–34.
7. van der Luijt RB, Meera Khan P, Vasen HF, *et al.* Germline mutations in the 3′ part of *APC* exon 15 do not result in truncated proteins and are associated with attenuated adenomatous polyposis coli. Hum Genet 1996;98:727–34.
8. Spirio L, Olschwang S, Groden J, *et al.* Alleles of the *APC* gene: an attenuated form of familial polyposis. Cell 1993;75:951–7.
9. Soravia C, Berk T, Madlensky L, *et al.* Genotype-phenotype correlations in attenuated adenomatous polyposis coli. Am J Hum Genet 1998;62:1290–301.
10. Al-Tassan N, Chmiel NH, Maynard J, *et al.* Inherited variants of *MYH*-associated with somatic G:C->T:A mutations in colorectal tumors. Nat Genet 2002;30:227–32.
11. Sieber OM, Lipton L, Crabtree M, *et al.* Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in *MYH*. N Engl J Med 2003;348:791–9.
12. De Rosa M, Scarano MI, Panarello L, *et al.* The mutation spectrum of the *APC* gene in FAP patients from southern Italy: detection of known and four novel mutation. Hum Mutat 2003;21:655–6.
13. De Rosa M, Morelli G, Cesaro E, *et al.* Alternative splicing and nonsense-mediated mRNA decay in the regulation of a new adenomatous polyposis coli transcript. Gene 2007;385:8–14.

14. De Rosa M, Scarano MI, Panariello L, *et al*. Three submicroscopic deletions at the *APC* locus and their rapid detection by quantitative-PCR analysis. Eur J Hum Genet 1999;7:695–703.
15. Groden J, Thliveris A, Samowitz W, *et al*. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 1991;66:589–600.
16. Carteggi L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: a web resource to identify exonic splicing enhancers. Nucleic Acids Res 2003;31:3568–71.
17. De Rosa M, Dourisboure RJ, Morelli G, *et al*. First genotype characterization of Argentinean FAP patients: identification of 14 novel *APC* mutations. Hum Mutat 2004;23:523–4.
18. Gavert N, Yaron Y, Naiman T, *et al*. Molecular analysis of the *APC* gene in 71 Israeli families: 17 novel mutations. Hum Mutat 2002;19:664.
19. Jones S, Emmerson P, Maynard J, *et al*. Biallelic germline mutations in *MYH* predispose to multiple colorectal adenoma and somatic G:C→T:A mutation. Hum Mol Genet 2002;11:2961–7.
20. Sampson JR, Dolwani S, Jones S, *et al*. Autosomal recessive colorectal adenomatous polyposis due to inherited mutations of *MYH*. Lancet 2003;362:39–41.
21. Krawczak M, Thomas NS, Hundrieser B, *et al*. Single base-pair substitutions in exon-intron junctions of human genes: nature, distribution, and consequences for mRNA splicing. Hum Mutat 2007;28:150–8.
22. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet 2002;3:285–98.
23. van der Luijt RB, Vasen HF, Tops CM, Breukel C, Fodde R, Meera Khan P. *APC* mutation in the alternately spliced region of exon 9 associated with late onset familial adenomatous polyposis. Hum Genet 1995;96:705–10.
24. Sieber OM, Segditsas S, Knudsen AL, *et al*. Disease severity and genetic pathways in attenuated familial adenomatous polyposis vary greatly but depend on the site of the germline mutation. Gut 2006;55:1440–8.
25. Varesco L, Gismondi V, Presciuttini S, *et al*. Mutation in a splice-donor site of the *APC* gene in a family with polyposis and late age of colonic cancer death. Hum Genet 1994;93:281–86.
26. Curia MC, Esposito DL, Aceto G, *et al*. Transcript dosage effect in familial adenomatous polyposis: model offered by two kindreds with exon 9 *APC* gene mutations. Hum Mutat 1998;11:197–201.

CORRESPONDENCE

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Alu-Mediated Genomic Deletion of the Serine/Threonine Protein Kinase 11 (*STK11*) Gene in Peutz–Jeghers Syndrome

Dear Sir:

The key feature of Peutz-Jeghers syndrome (PJS) is a greatly increased risk of developing malignant tumors in multiple tissues. In addition to an elevated risk of gastrointestinal malignancies, such as gastroesophageal, small bowel, and colorectal cancers, an increased risk of cancers at other sites, particularly in breast, pancreas, ovary, uterus, cervix, lung, and testicle, has been reported.[1,2] Thus, an appropriate diagnosis is crucial for cancer prevention.

PJS (MIM 175200), which has an incidence of 1 in 150,000 in North America and Western Europe, is a rare autosomal-dominant inherited precancerous condition characterized by multiple polyps in the gastrointestinal tract and distinctive mucocutaneous pigmentation.

Germline mutations in the serine/threonine kinase 11 gene (*STK11/LKB1*) (OMIM*602216) are documented in up to 70%–80% of the PJS patients; of these, about 15% have germline deletions of all or part of the gene.[3–5] In the remaining 20%–30% of PJS patients, defects in other genes or not yet identified ways of *LKB1* inactivation might be responsible for PJS.

The aim of this work is to clarify the molecular basis of the disease in Italian PJS patients. We investigated the *STK11/LKB1* gene mutations in a well-characterized series of 9 unrelated Italian PJS patients, by using a combination of polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, DNA sequencing, Southern blot analysis, and real-time PCR techniques.

As shown in Table 1, we have characterized the specific *STK11* mutation in 6 probands, consisting of: 2 truncating mutations (1 novel and 1 known mutation), 1 missense known mutation in the exon 4, and 2 novel small in-frame deletions in exon 6. Finally, we have found an intra-exonic in-frame deletion encompassing exons 2 and 3 and we have characterized the breakpoints of this *LKB1/STK11* intragenic deletion. This rearrangement, that deletes about 7 kb of the *LKB1* genomic region encompassing exons 2 and 3 (Figure 1*A*), is most likely an Alu–Alu homologous recombination event. Two 26-bp core sequences of 2 Alu elements (both AluY sequences), showing a 96% homology, are indeed localized at the 5′ and 3′ end of the breakpoints, respectively. This sequence, could itself act as a recombinase (Figure 1).

Alu-mediated homologous recombination is a mechanism well documented so far; however, it is the first evidence that this mechanism is involved in the *STK11/LKB1* gene rearrangements. Because the average frequency of Alu elements along the human genome is estimated to be 1 element every 4 kb,[6] the region spanning the 7-kb deletion (including introns from 1 to 3 of the *LKB1* gene), shows an over-representation of complete Alu elements. Seventeen Alu elements are detected in this 14.5 Kb region by using in silico analysis with the

Table 1. Clinical Phenotype and Mutation Spectrum of Italian PJS Patients Studied

Patient number	Type of inheritance	Onset age	STK11/LKB1 mutation	Exon	Clinical manifestation
1	F	18y	c.842delC p.L281fs4X	6	Hamartomatous duodenal polyposis and oral mucocutaneous pigmentation
2	S	14y	**c.169-170insG p.E57fs105X**	1	Hamartomatous intestinal polyposis and oral mucocutaneous pigmentation
3	S	5y	**NC_000019.8:g.6998_13998del (7 KB del. spanning exons 2–3) p.E98_G115del**	2-3	Hamartomatous intestinal polyposis and oral mucocutaneous pigmentation
4	F	13y	c.580G>A p.D194N	4	Hamartomatous duodenal and gastric polyposis and oral mucocutaneous pigmentation
5	S	18y	N.F.	—	Hamartomatous gastric polyposis and oral mucocutaneous pigmentation
6	S	13y	**c.856-858delCTG p.286delL**	6	Hamartomatous gastric polyposis and oral mucocutaneous pigmentation
7	F	16y	N.F.	—	Hamartomatous duodenal polyposis and oral mucocutaneous pigmentation
8	F	10y	N.F.	—	Hamartomatous gastric polyposis and oral mucocutaneous pigmentation
9	F	17y	**c.747-749delCAC p.250delT**	6	Hamartomatous gastric polyposis and oral mucocutaneous pigmentation

F, familial cases; S, sporadic cases; N.F., mutation not found; the bold character indicates the novel mutations.

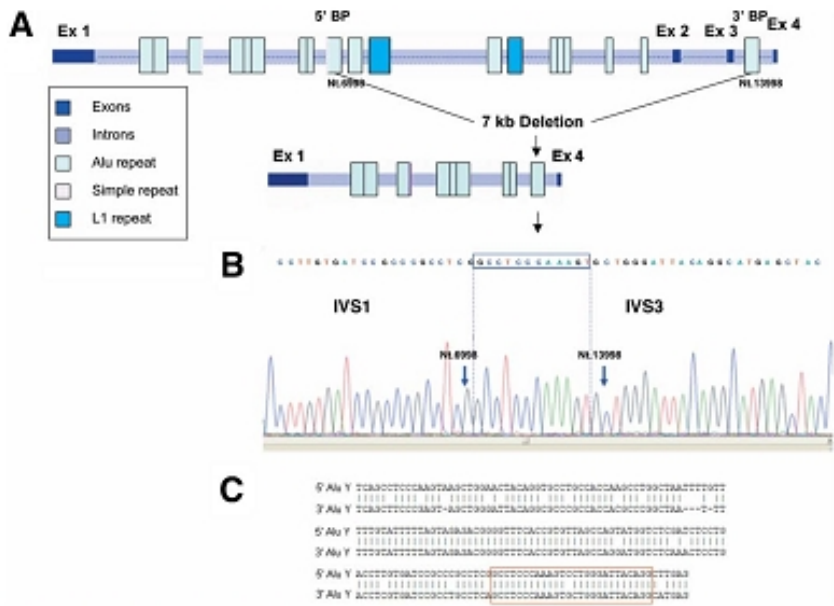

Figure 1. *Molecular characterization of the STK11/LKB1 intragenic deletion. (A)* Repetitive elements present in the genomic region from exons 1–4 of the STK11/LKB1 gene and a diagram (below) of the deletion are shown. The *arrow* indicates the novel genomic junction; 5′ BP and 3′ BP: 5′ and 3′ breakpoints. *(B)* Sequence analysis of the amplified product obtained from deleted allele. *Boxed* sequence corresponds to the twelve nucleotides repeated both at the 5′ and 3′ breakpoints sharing 100% of homology and representing the novel genomic connection produced by the deletion event. *Bold arrows* indicate the genomic region involved in the deletion and the genomic nucleotides position involved in the novel genomic junction, referred to GenBank sequence with accession number: NC_000019.9. (C) Sequence of Alu elements present at the 5′ and 3′ breakpoints (5′ BP, 3′ BP, of *A*); boxed nucleotides represent the core sequence of Alu elements.

RepeatMasker program, which corresponds to >1 element every 1 kb. The over-representation of Alu elements indicates that this is a region of DNA instability; thus, it is not surprising that several intragenic rearrangements involving this region have been reported so far. Intriguingly, genomic sequence of chromosome 19 from GeneBank position 1203099 to 1230861 shows the presence of Alu elements at the genomic regions involved in the intragenic deletions in most of the cases so far described.

Our strategy to screen the entire coding region of the *LKB1* gene, both at DNA and RNA level, has allowed the identification of the disease causing mutations in about 67% of PJS patients. Other gene inactivating mechanisms might be responsible for PJS in mutations negative population subset. However, the existence of genetic heterogeneity cannot be excluded.

Because of an over-representation of Alu elements in this region, in our opinion, is reasonable to hypothesize that Alu-mediated homologous recombination could give rise to several intragenic rearrangements, as translocations or inversions, not always detectable using classical molecular biology techniques. These rearrangements could in part explain the PJS cases without identified molecular alterations.

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1. Lim W, Olschwang S, Keller JJ, et al. Relative frequency and morphology of cancers in STK11 mutation carriers. Gastroenterology 2004;126:1788–1794.
2. Mehenni H, Resta N, Park JG, et al. Cancer risks in LKB1 germline mutation carriers. Gut 2006;55:984–990.
3. Volikos E, Robinson J, Aittomäki K, et al. LKB1 exonic and whol gene deletions are a common cause of Peutz-Jeghers syndrome J Med Genet 2006;43:e18.
4. Aretz S, Stienen D, Uhlhaas S, et al. High proportion of larg genomic STK11 deletions in Peutz-Jeghers syndrome. Hum Muta 2005;26:513–519.
5. Hearle NC, Rudd MF, Lim W, et al. Exonic STK11 deletions are no a rare cause of Peutz-Jeghers syndrome. J Med Genet 2006;43 e15.
6. Mighell AJ, Markham AF, Robinson PA. Alu sequences. FEBS Let 1997;417:1–5.

Conflicts of Interest
The authors disclose no conflicts.

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