LETTER TO JMG

STK11 genotyping and cancer risk in Peutz-Jeghers syndrome

V Schumacher, T Vogel, B Leube, C Driemel, T Goecke, G Möslein, B Royer-Pokora

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Peutz-Jeghers syndrome (PJS; OMIM #175200) is an autosomal dominant disorder characterised by mucocutaneous melanin pigmentation, gastrointestinal hamartomatous polyposis, and an increased risk for the development of various neoplasms.[1 2] Malignancies occur both in the gastrointestinal tract and in extraintestinal sites such as the pancreas, the breast, and reproductive organs. The estimated relative cancer risk may be 15 fold higher than in the general population[1] and appears to be particularly high in women (20 fold) because of an increased risk of development of breast cancer and gynaecological malignancies.[2]

Germline mutations in the *STK11/LKB1* gene on 19p13.3 are found in 30–70% of PJS cases, depending on the screening method, with considerable uncharacterised genetic heterogeneity remaining in this syndrome.[3 4] The disease causing gene has been identified by two independent groups.[5 6] Human *STK11* encodes a serine/threonine protein kinase that is highly homologous to the mouse protein Lkb1 and the *Xenopus* kinase XEEK1,[7 8] and is expressed in all human tissues.[9] The kinase domain of the human 433 amino acid protein is localised between residues 49 and 309,[7] and shows homology to the conserved catalytic core of the kinase domain common to both serine/threonine and tyrosine protein kinase family members.[10] Most mutations found in PJS patients are small deletions/insertions or single base substitutions leading to an abnormal truncated/kinase inactive protein.

Loss of the wild type allele in hamartomas and adenocarcinomas occurring in patients with PJS suggests that *STK11* is a tumour suppressor gene. Several studies have described a role in cell cycle arrest,[11] p53 mediated apoptosis,[12] Wnt signalling,[13 14] TGF-β signalling,[15] Ras induced cell transformation,[16] and cell polarity.[17–20] Growth suppression requires phosphorylation of STK11[21 22] and was found to be caused by activation of the CDK inhibitor p21.[23] Moreover, by associating with Brg1, an essential component of chromatin remodelling complexes, STK11 can induce growth arrest.[24] It was found that the lack of STK11 may support tumour cell growth through the induction of vascular endothelial growth factor.[25] Taken together, these data suggest that *STK11* mutations may contribute to tumorigenesis through various mechanisms such as induction of angiogenesis, suppression of growth arrest, apoptosis, and loss of cell polarity.

PJS is a cancer predisposing disorder; however, cancer risk may vary. Therefore, we studied whether specific *STK11* mutations may confer a lower or higher cancer risk in PJS patients by examining the site and type of mutations with regard to cancer frequency and cancer type.

Key points

- Peutz-Jeghers syndrome (PJS) is caused by germline mutations in the *STK11/LKB1* gene and is frequently associated with specific malignancies. However, clinical features vary, especially the risk of cancer.
- The aim of the study was to identify specific mutations associated with an increased or decreased cancer risk in PJS patients.
- *STK11* mutation analysis was performed in our 41 PJS patients by PCR-SSCP and DNA sequencing. By reviewing the literature, *STK11* mutations from 105 PJS patients were added to generate a combined dataset for genotype–phenotype correlation studies.
- *STK11* germline mutations were found in 27 of our 41 PJS patients (66%). Ten of the 27 mutations were associated with malignancies in the index patient and/or in affected relatives. The analysis of our data together with literature cases revealed that inframe deletions, splice site mutations, and missense mutations in the part of the gene encoding protein domains important for ATP binding and the site of catalysis (I–VIA) were rarely associated with cancer. However, missense mutations in the C terminus and in the part of the gene encoding protein domains, important for substrate recognition (VIB–VIII), were more frequently associated with malignancies. A comparison of mutation and tumour type revealed that PJS patients with breast carcinomas had predominantly truncating mutations.
- In the future, the determination of mutation type and site in PJS patients may be an important factor for patient management and tumour screening.

METHODS

A total of 24 familial and 13 apparently sporadic PJS cases without a family history were collected from a number of German institutions. In four cases, the family history could not be obtained. The patients fulfilled the diagnostic criteria suggested by Tomlinson and Houlston,[26] namely the presence of (*a*) two or more hamartomatous polyps of the PJS type, or (*b*) one PJS polyp along with classical PJS pigmentation or a family history of PJS. All cancer diagnoses were confirmed by tissue review or pathology reports. Patient data and family histories were documented according to a study protocol approved by the local ethics committee. Blood samples were collected for mutation analysis of *STK11* after informed consent was obtained.

STK11 mutation analysis

Genomic DNA was isolated from peripheral blood samples using the QIAmp Blood kit (Qiagen) as recommended by the manufacturer.

The nine coding exons of *STK11* were amplified from genomic DNA by PCR and analysed by single strand conformation polymorphism (SSCP). The sequences of the primers were those published by Dong *et al*,[27] and covered all exonic sequences, and splice acceptor and donor sites. Each PCR reaction contained 100 ng genomic DNA, 1× *Taq* DNA polymerase buffer (buffer J from the PCR Optimizer kit; Invitrogen), 5% dimethyl sulphoxide, 25 pmol of each primer, 200 µmol dNTPs and 1 U *Taq* polymerase in a total volume of 50 µl. PCR reactions were initiated by denaturing the DNA for 3 min at 94℃ in an MJ thermal cycler. PCR cycles were: 10 cycles at 94℃ for 1 minute, 60℃ for 2 min, and 72℃ for 1.5 minutes, followed by 20 cycles at 94℃ for 1 minute, 58℃ for 1 minute, and 72℃ for 1.5 minutes, with a final elongation at 72℃ for 10 minutes. Mutational analysis of PCR products by SSCP was performed as previously described.[28]

PCR products showing an abnormal SSCP pattern were directly sequenced in both directions after purification (PCR purification kit; Qiagen) using a DNA sequencing kit (SequiTherm Excel II; Epicentre Technologies) as recommended by the manufacturer. Reactions were run on a LICOR DNA sequencer (Long ReadIR 4200). When sequencing identified a mutation in the index case, all other relatives who fulfilled the clinical criteria of PJS were assumed to have the same *STK11* mutation.

Selection of patients by reviewing the literature

To augment the number of cases for genotype–phenotype correlation, a systematic Medline (National Library of Medicie, USA) search was carried out from January 1998 to June 2004 to identify all references under the key words ''STK11'' and ''LKB1'' in order to find articles describing mutations in sporadic or familial PJS patients. All cases for whom information about the presence or absence of a tumour and the specific mutation were available were included. This resulted in the addition of 66 PJS patients without cancer and 39 PJS patients with cancer from 17 references. Large deletions or other rearrangements were excluded from the evaluation because they were not detectable with our screening method (PCR-SSCP). All published mutations were re-evaluated using sequence information given in the respective publications and coded according to the gene nomenclature by den Dunnen.[29] The nucleotide numbering is derived from the cDNA sequence (GenBank Accession no. AF035625), where the A in the initiation codon ATG corresponds to base 1.

We used a family as unit genotype–phenotype analysis, for which mutations were counted once for each family. In a few specific cases we used an individual as unit analysis (cumulative cancer risk in our patient set, cancer risk for inframe deletions and splice site mutations).

RESULTS

In this study, 24 familial PJS cases, 13 apparently sporadic PJS cases, and 4 cases of unknown family history were included for DNA mutation analysis of the *STK11* gene. Table 1 details the clinical characteristics, family histories, and mutational data of the 41 index patients analyzed. We detected germline mutations in 27 of 41 (66%) patients. Of these, 17 were found in familial (71%) cases, 8 in sporadic (62%), and 2 were found in cases of unknown family history. We detected seven nonsense mutations, 10 deletions, five insertions, one splice site mutation, and four missense mutations. To our knowledge, nine of these are novel. Ten mutations were associated with cancer in the index patient and/or in relatives.

When affected relatives were included, our patient set consisted of 88 PJS patients in total. The overall cancer frequency in the collective was 19/88 (20%). A slightly higher incidence was seen in mutation carriers (15/63 = 24%) compared with non-carriers (4/25 = 16%).

For genotype–phenotype correlation analyses, we generated a combined dataset containing our 27 *STK11* mutations together with 105 mutations from the literature. Patients were subdivided into two groups: PJS cases without cancer in the index patient and/or relatives (group 1; n = 83), andPJS cases with cancer in the index patient and/or relatives (group 2; n = 49). Patient and mutational data of the combined dataset are given in fig 1 (A and B). The 15 splice site mutations are described separately.

STK11 mutation type and cancer

We evaluated whether the presence or absence of cancer is associated with a specific mutation type by comparing mutations in the tumour and the non-tumour groups (fig 2, table 2). Nonsense and frameshift mutations were evaluated together because both result in protein truncation. We found that inframe deletions and splice mutations were only rarely associated with cancer in PJS patients. None of the nine inframe deletions and only three of the 15 splice mutations were associated with malignancies in the index patient and/or affected relatives. In contrast, for nonsense and missense mutations no difference was seen regarding the tumour risk. Of the 79 nonsense mutations, 33 (42%) were associated with malignancies in the index patient and/or relatives, and 46 (58%) were not associated with malignancies. Of the 29 missense mutations, 13 (45%) were associated with malignancies in the index patient and/or relatives, and 16 (55%) were not associated with malignancies.

STK11 mutation site and cancer

To determine whether the mutation site may influence the tumour risk, we analysed the different mutation types separately for both patient groups.

Truncation mutations

No obvious differences were seen with regard to the position of the mutations in patients with or without cancer (fig 1). All but one mutation led to a complete or partial loss of the kinase domain localised between amino acids 49 and 309. Most mutations were unique.

Missense mutations

For the evaluation, we grouped the mutations according to the functional domains of the protein (fig 3). The distribution of missense mutations throughout the functional domains differed in PJS patients with and without cancer. Only one of the six missense mutations (Gln170Pro) in the part of the gene coding for the protein domain important for ATP binding and the site of catalysis (I–VIA) was associated with cancer in relatives of the analysed index patient. However, six of the eight mutations in the part of the gene coding for protein domains important in substrate recognition (VIB–VIII) and the C terminus were associated with malignancies in PJS patients. Two missense mutations in domain VIB–VIII were found in one sporadic and one familial case, and were not associated with cancer. One occurred in a PJS child who may yet develop a tumour, and the other in a patient whose age was not indicated in the reference. Mutations lying in domains IX–XI were found to be associated with cancer in 6/15 (40%) cases and without cancer in 9/15 (60%) cases. All mutations except the Phe157Ser, Asp194Asn, Gly242Glu, Arg304Trp, and Trp308Cys mutations were unique.

Inframe deletions

The nine inframe deletions were exclusively found in PJS patients without cancers. These were localised to exons 1, 2,

Table 1 Summary of clinical and mutational data from the 41 patients analysed in this study

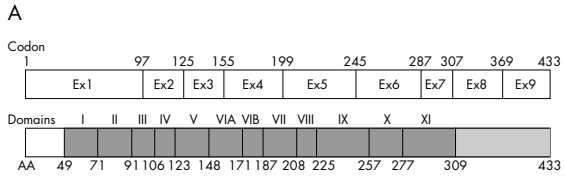
Index patient								Affected relatives			
Case no.	S/F	Age of index patient without cancer (years)	Cancer location	Cancer diagnosis/ death (in years)	Ex	Mutation (nucleotide)	Effect	PJS cases in family (incl. index patient)	Cancers in relatives	Cancer diagnosis/ death (in years)	Age of PJS affected relatives without cancer (years)
Cancer in index patient and/or relatives											
000/16/1	F		Breast	62	1	180C→G	Tyr60X	3	–	–	37, 38
000/10/1	F		Lung	29/33	2	354C→G	Tyr118X	2	Pancreas	32/33	–
000/7/1	F		Colon Pancreas	65 67/68	4	468C→G*	Tyr156X	5	–		28, 36, 40, 62
000/4/1	S		Breast bilateral	45	4	474_480del	158X	1	–		–
65/1/1	F	18	–	–	4	508C→T	Gln170X	6	Stomach CUP Lung	u/43 u/45 u/55	48, unknown
000/9/1	F		Breast bilateral	40	4	515_516insT	Ile172fs	3	Pancreas	u/55; u/55	–
000/14/1	F	26	–	–	4	580G→A	Asp194Asn	2	CUP	29/30	
57/1/1	F	53	–	–	5	642_644del G*	Gln214fs	3	CUP	u/45	61
77/3/1	F	41	–	–	5	666insGG*	Glu223fs	3	Colon	52/52	Unknown
39/1/1	S		AML	14	6	837_842insC	Gly279fs	1	–	–	–
29/1/1	F		Small intestine	42/44	–	–	–	2	–	–	24
59/1/1	F		Pancreas	63	–	–	–	2	–	–	Son died from car accident at 41 years
98/1/1	F	33	–	–	–	–	–	3	Pancreas	u/58	Unknown
00/23/1	F	41	–	–	–	–	–	3	CUP	78/78	44
No cancer in index patient and/or relatives											
1/2/1	S	18	–	–	1	147C→A*	Tyr49X	1	–	–	
11/1/1	F	33	–	–	1	153_157insG	Met51fs	3	–	–	39, 62
88/1/1	S	52	–	–	1	165_169del G	Leu55fs	1	–	–	
77/2/1	F	24	–	–	1	250A→T	Lys84X	2	–	–	Unknown
00/3/1	F	45	–	–	In1	291-1G→C	Not known	3	–	–	23, 25
83/1/1	F	22	–	–	4	493delG*	Glu165fs	2	–	–	Unknown
48/1/1	F	21	–	–	4	506insG*	Ser169fs	2	–	–	Unknown
00/22/1	U	36	–	–	5	640C→T*	Gln214X	1	–	–	
000/12/1	S	14	–	–	5	716_719del	Trp239fs	1	–	–	
56/2/1	S	38	–	–	5	734T→G*	Leu245Arg	1	–	–	
00/21/1	F	24	–	–	6	779del T*	Ile260fs	2	–	–	50
77/1/1	U	30	–	–	6	787_790del	Leu263fs	1	–	–	
18/1/1	F	21	–	–	6	790_793del	Phe264fs	3	–	–	47, uncle died bleeding age unknown
000/13/1	S	12	–	–	6	790_793del	Phe264fs	1	–	–	
78/1/1	F		Sertoli cell benign	5	7	907_915del	Ile303_Gln305 del	6	–	–	5, 9, 12, 14, 42
47/1/1	F	61	–	–	7	910C→T	Arg304Trp	3	–	–	33, 35
81/1/1	S	27	–	–	8	924G→T	Trp308Cys	1	–	–	
000/1/1 = 52/1/1	F	45	–	–	–	–	–	3	–	–	16, 20
000/2/1 = 13/1/1	S	57	–	–	–	–	–	1	–	–	
000/5/1	S	17	–	–	–	–	–	1	–	–	
000/6/1	F	32	–	–	–	–	–	3	–	–	10, unknown
00/18/1	S	59	–	–	–	–	–	1	–	–	
24/1/1	U	35	–	–	–	–	–	1	–	–	
54/1/1	F	35	–	–	–	–	–	2	–	–	Unknown
56/1/1	U	46	–	–	–	–	–	1	–	–	
89/1/1	S	64	–	–	–	–	–	1	–	–	
00/20/1	S	17	–	–	–	–	–	1	–	–	

*Novel mutations. S/F, sporadic/familial PJS; U, unknown; Ex/In, exon/intron; CUP, cancer of unknown primary.

3, 4, and 7, corresponding to the functional protein domains I, IV, V, VI, and XI, and ranged from deletions of six to 21 base pairs. Of the nine inframe deletions, two were found in sporadic and seven in familial cases with, in total, at least 29 affected members. It was assumed that all affected members carry the same mutation as the analysed index patient, but none of the 29 PJS patients with an inframe deletion has developed cancer by the publication date of each reference. However, the age of affected persons is unknown in most cases and a tumour may yet develop later in life.

Splice site mutations

Splice site mutations were found in 12 PJS cases without cancer and in three with cancer. The splice donor site of intron 3 and the acceptor sites of intron 3 and 7 were altered in PJS patients with pancreatic (patients PJS1[47] and 2472[32]) and colon cancer (patient P22[31]). In patients without malignancies, alterations of the splice donor sites of intron 1, 5, 6, and 7 and the splice acceptor sites of intron 1, 4, 5, and 8 were found (patient 4 and 6;[41] patient 2191, 2234, 442, 2653;[32] our patient 00/3/1; patient 4332;[34] patient PJS03;[37]



Germline mutations in PJS patients without cancer (group 1)	Mutation	Index patient	Age (yr)	S/F PJS	Affected	Cancer	LOH	Ref
	Tyr49X	1/2/1	18	S	1			*
	Tyr60X	PJ48	37	S	1			30
	Lys84X	77/2/1	24	F	2			*
	Arg86X	P28		F				31
	Tyr118X	419	child	S	1			32
	Gln137X	33		S	1			33
	Gln137X	26		S	1			33
	Gln152X	P21		F				31
	Gln152X	PJ52	34	F				30
	Gln170X	4350	48	F				34
	Gln214X	00/22/1	36	U				*
	Gln220X	BB	14	S	1			35
	Tyr272X	PJ47	31	F				30
	Met51fsX162	11/1/1	33	F	3			*
	Met51fsX162		34	F	3			36
	Gly52fsX63	54		S	1			33
	Leu55fsX63	88/1/1	52	S	1			*
	Leu55fsX63	418	child	F	> 3			32
	Gln112fsX129	PJ36	26	S	1			30
	Val116fsX161	1216	adult	F	2			32
	Met139fsX160	04		F				33
	Ser142fsX160	39		S	1			33
	Glu145fsX161	13		S	1			33
	Leu164fsX286	83/1/1	22	F	2			*
	His168fsX265	48/1/1	21	F	2			*
	Lys191fsX265	PJS02		S	1			37
	Lys191fsX265	56		F				33
	Arg211fsX264	919	adult	S	1			32
	Pro221fsX286	128	child	U				32
	Ile238fsX286	000/12/1	14	S	1			*
	Ser240fsX265	PJ24	48	S	1			30
	Asn247fsX286	2708	adult	S	1			32
	Thr250fsX286	749	child	F	2			32
	Asn259fsX286	00/21/1	24	F	2			*
	Leu262fsX286	77/1/1	30	U				*
	Leu263fsX286	PJE263		U				38
	Leu263fsX286	262	child	S	1			32
	Leu263fsX286	18/1/1	21	F	3			*
	Leu263fsX286	000/13/1	12	S	1			*
	Gly279fsX286	20		F				39
	Gly279fsX286	21		F				39
	Leu282fsX286	PJ51		F				30
	Ile300fsX335	PJS06		F	8			37
	Ala318fsX335	1002	child	F	2			32
	Pro319fsX359	45		F				33
	Pro323fsX359	3		S				40
	Leu50_Asp53del	PJS01		F	10			37
	Gly56_Val63del	27		F				33
	Val73_Val77del	21		F				33
	Lys108_Asn109del	2		S	1			39
	Gln137_Met139del	P32	27	F				31
	Leu167_Val173del	1		S	1			41
	Lys175_Asp176del	PJF512	16	F	2			38
	Ile303_Gln305del	78/1/1*	infant	F	6			*
	Ile303_Gln305del	229	infant	F	> 3			32
	Leu67Arg	118		F	> 2			32
	Lys108Arg	3	child	S	1			39
	Met136Arg	622		S	1			32
	Phe157Ser	P16	30	F				31
	Phe157Ser	PJ59		F				30
	Asn181Glu	32		F				33
	Leu182Pro	2733	child	S	1			32
	Thr230Pro	53		S	1			33
	Gly242Glu	69	child	F	3			32
	Gly242Trp	1475	child	S	1			32
	Leu245Arg	56/2/1	38	S	1			*
	Gly251Ser	PJ1						38
	Arg304Trp	47/1/1	61	F	3			*
	Arg304Trp	PJG42	39	S	1			38
	Trp308Cys	81/1/1	27	S	1			*
	Trp308Cys	PJS04		F	3			37

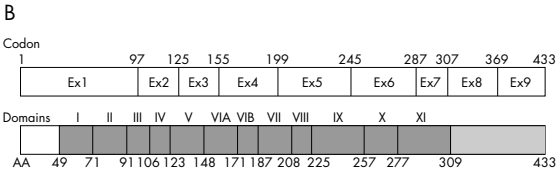
Figure 1 Clinical and mutational data from the combined dataset. The coding sequence of the *STK11* gene is shown on the top with the start codon as first codon and the functional domains of the protein are shown below. Dark grey box, kinase domains I-XI between residues 49 and 309; white box, aminoterminal region outside the kinase domain; light grey box, carboxyterminal region outside the kinase domain containing a phosphorylation and prenylation motive. Black region marks the amino acids changed by the frameshift and ends at the predicted stop codon. Age, age at publication; Yr, year; S/F PJS, sporadic or familial PJS; i, index patient; r, relative; Ref, Reference; dyspl., dysplastic; GI, gastrointestinal; CUP, cancer of unknown primary; IPMN, intraductal papillary mucinous neoplasm; SCTAT, ovarian sex cord tumour with annular tubules. *Our own study patients.

patient 4;[40] and patients PJ33, PJ61, and PJ69[30]). Assuming that all affected members of one family carry the same *STK11* mutation, 22 from at least 25 affected persons with splice site mutation had not developed a cancer by the publication date of each reference. However, as for the inframe deletions, the age of the affected persons is not known in most cases and a tumour may develop later in life.

The consequences of these mutations in the processing of the RNA transcript are not known, but it is likely that they result in abnormal splicing.

STK11 mutation type/site and cancer type

By comparing the type of *STK11* mutation with the cancer type, one preliminary observation could be made. Breast cancer in PJS was predominantly associated with truncation mutations. Of the 79 nonsense mutations, 11 (14%) were associated with breast cancer in the index patient and/or relatives. In contrast, only 2/29 (7%) missense mutations were associated with breast cancer in the index patient and/or relatives. The Trp239Cys mutation was found in family 2[41], and the Arg304Trp mutation was found in two cancer



Germline mutations in PJS patients with cancer (group 2)	Mutation	Index patient	Age (yr)	S/F PJS	Affected	Cancer	LOH	Ref
	Tyr60X	000/16/1	62	F	3	Breast (i)		*
	Tyr60X	277	adult	F	> 4	Breast male (1r)		32
	Glu70X	PJ77	23	F		Cervix (r)		30
	Lys84X	P25		F		Breast, Cervix, multiple myeloma (i, r)		31
	Tyr118X	000/10/1	38	F	2	Lung (i), Pancreas (1r)		*
	Gln123X	08		S	1	Pancreas (i)		33
	Gln123X	case 2		U		IPMN (i)	+	42
	Cys132X	2494	child	F	> 4	Breast (i, r), Duodenum (1r)		32
	Gln137X	11		F		Breast (r)		33
	Tyr156X	000/7/1	71	F	5	Colon (i), Pancreas (i)		*
	Cys158_Leu160delX	000/4/1	51	S	1	Breast bilateral (i)		*
	Gln170X	65/1/1	18	F	6	Stomach (1r), Lung (1r), CUP (1r)		*
	Gln265X	SCTAT1PJS		F		SCTAT (i)	+	43
	Lys416X	4		F		Endometrium (1r)		39
	Glu65fsX162			S	22	5 gastrointestinal cancer, 2 breast cancer		44
	Gln123fsX128	PJ60	61	S	1	Breast (i)		30
	Val143fsX286	PJ56	40	F		Stomach (r)		30
	Ile172fsX265	000/9/1	39	F	3	Breast bilateral (r), Pancreas (2r)		*
	Gln214fsX286	57/1/	53	F	3	CUP (1r)		*
	Ser216fsX264	PLK276-1P1				Polyp carcinomatous (i)	+	45
	Pro222fsX287	77/3/1	41	F	3	Colon (1r)		*
	Ile248fsX264	P27		F		Ovary (i), Thyroid (i)		31
	Gly257fsX264	P23		F		Colon (i, r)		31
	Leu263fsX286	P20		F		Pancreas (r)		31
	Gly279fsX284	39/1/1		F	1	AML (i)		*
	Gly279fsX286		34	S	1	Duodenum (i)	+	46
	Gly279fsX286	2295	child	S	> 4	Pancreas (1r), Kidney (1)		32
	Pro281fsX284	47		F		Colon (r)		33
	Pro281fsX284	49		S		Breast (r)		33
	Leu282fsX284	2		F	1	Prostate (i), Colon (i)		40
	Leu282fsX284	Ped5		S	3	Colon (r), Pancreas (r)		40
	Leu282fsX286	PJ42	35	F		Breast (r)		30
	Leu304fsX335	P29		F		Pancreas (r), Small Intestine (r)		31
	Gln170P	01		F		Colon (r), Gastric (r), Pancreas (r)		33
	Asp176Asn	PJS05		F	5	Pancreas and other GI neoplasia		37
	Asn181Thr	SCTAT2PJS		F		SCTAT (i)	+	43
	Asp194Asn	000/14/1	26	F	2	CUP (1r)		*
	Asp194Asn	PJ20	31	F		Stomach (r)		30
	Trp239Cys	2	45	F		Bowel (r), Breast (r)		41
	Gly242Glu	PJ45	31	F		Pancreas (r)		30
	His272Tyr	1		S	1	Thyroid (i), Cecal (i)		40
	Ala297Ser	Ped1		F	6	Colon (r), Endometrium (r)		40
	Arg304Trp	PJ35	39	F		Breast (i), Breast (2r)		30
	Arg304Trp	61		F		Pancreas (r), Ovary (r), Breast (r)		33
	Gly315Ser	7	18	F	3	Bowel (r)		41
	Phe354Leu	02		F		Colon (2r)		33

Figure 1 Continued.

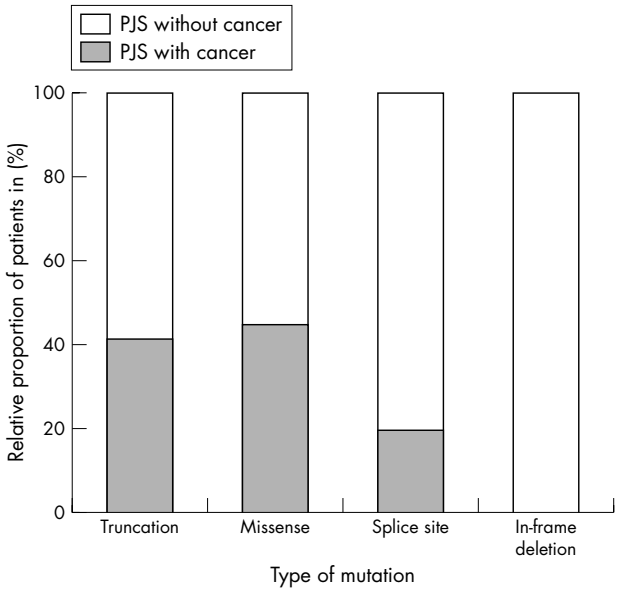


Figure 2 Type of mutations found in PJS patients with and without cancer.

families (PJ35[30] and 61[33]). Of the six patients who have developed malignancies in families PJ35 and 61, four had a breast tumour. Whether the Arg304Trp mutation contributes to a high breast cancer risk need to be confirmed in larger cohorts.

DISCUSSION

Mutational screening in our patient set

Mutation analysis of our 41 PJS patients revealed 27 mutations (66%), of which nine had not been described previously. This frequency is very similar to the 69% (22/32) found by Amos *et al*.[33] We could not find *STK11* mutations in 14 cases. This may be due to mutations in parts of the gene that were not analysed, such as introns and the promoter region. In addition, large genomic deletions as found by Le Meur *et al*,[48] and other rearrangements could not be detected by our analyses. However, Amos *et al* searched for larger deletions in 22 people without a detectable *STK11* mutation and found none.[33] This suggests that large deletions are unusual in PJS patients. As emphasised by Ballhausen and Guenther,[49] mutational screening should be performed on DNA and RNA in the future to detect other disease causing mutations in intronic areas. The lack of mutations may also simply suggest genetic heterogeneity of this disease as described by Olschwang *et al*[3] and Mehenni *et al*.[4]

Table 2 Type of mutations found in PJS patients with and without cancer

	PJS without cancer (n = 83)	PJS with cancer (n = 49)
Truncation (n = 79)	46/79 (58%)	33/79 (42%)
Missense (n = 29)	16/29 (55%)	13/29 (45%)
Splice site (n = 15)	12/15 (80%)	3/15 (20%)
Inframe deletion (n = 9)	9/9 (100%)	0/9 (0%)

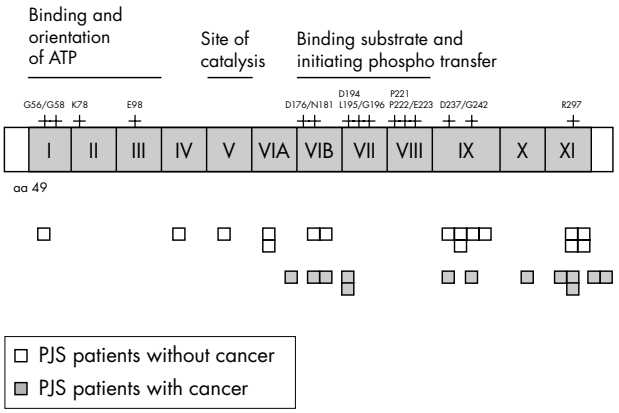


Figure 3 Distribution of missense mutations in PJS patients with and without cancers. Missense mutations are grouped according to the functional domains of the protein. A schematic drawing of the protein with the functional domains I–XI and their functions is shown on top. Invariant or near invariant residues throughout the protein kinase superfamily are marked with asterisks.[10]

In 21 of the 27 cases, mutations resulted in a premature stop codon and led to truncated proteins with incomplete catalytic domains. The truncated proteins tested so far by other groups did not show kinase activity, consistent with the notion that they disrupt STK11 enzymatic function (summarised in fig 4, additional online information). The change in the dinucleotide sequence ag to ac at the splice acceptor side from intron 1 in patient 00/3/1 probably results in aberrant splicing; however, we could not study the effect owing to lack of available RNA. An inframe deletion leading to the loss of Ile, Arg, and Lys at codon 303–305 in patient 78/1/1 has probably the same effect as the mutation Ile303_His306delinsAsn in patient SL26.[5] As described previously, this mutant protein showed kinase activity, but accumulation in the nucleus resulted in the loss of p21 activation and diminished growth suppression.[11 23 31 52] All four missense mutations target highly conserved residues. Two mutations, Arg304Trp and Trp308Cys, were shown to have no kinase activity in autophosphorylation studies.[37 52 54] The mutation Asp194Asn affects the conserved DLG triplet lying in the activation loop that helps to orientate the γ phosphate of ATP for transfer. No specific function is described for the conserved Leu245 residue localised in subdomain IX of the protein, which is changed to arginine in patient 56/2/1.

In a collaborative study, Lim *et al*[50] have described malignant tumours in 47/240 PJS patients with a *STK11* mutation (cumulative cancer risk 20%). When including the data from affected relatives for calculation of cancer risk in our patient samples we get a comparable proportion; 15/63 patients with a *STK11* mutation have developed a malignant tumour (cumulative cancer risk 24%). This is slightly higher than the cancer frequency found in our patients without *STK11* mutations (4/25 = 16%), and confirms the results from Lim *et al*.[30]

Correlation between type/site of mutation and cancer

To evaluate a correlation between *STK11* mutation type, mutation site, and cancer risk we generated a combined dataset composed of our 27 PJS cases with *STK11* mutations and 105 PJS cases from the literature.

When comparing the proportion of PJS patients with and without tumours having truncation or missense mutations, no obvious differences were seen, confirming the data from Lim *et al*.[50] However, inframe deletions and splice site mutations were only rarely associated with malignancies in PJS patients, suggesting that the effect of these mutations may play only a minor role in carcinogenesis. These observations are based on small patient numbers and need to be confirmed in larger cohorts.

The analysis of missense mutations in PJS from our combined dataset showed that 5/6 mutations in the region coding for the functional protein domains I–VIA were not associated with cancer in the index patient and/or relatives. The only mutation (Gln170Pro) associated with tumour development is known to induce a bend near the C terminal end of the α helix.[33] This may impact on substrate interaction and may thus have a similar effect as mutations in domain VIB–VIII, involved in substrate recognition. Mutations in this domain and mutations in the C terminus were associated with tumour development in 6/8 cases. Of the two patients with mutations in domains VIB–VIII but without tumours, one was a child with sporadic PJS who may yet develop a tumour later in life, while the other was a familial PJS case without indication of the age in the corresponding reference.[33] The data suggest that amino acid changes in domains VIB–VIII and the C terminus may have a stronger carcinogenic potential than mutations in domains I–VIA. Interestingly, the C terminus was found to be necessary for binding STRAD, an STK11 specific adaptor protein, which activates STK11 and is involved in translocation from the nucleus to the cytoplasm, resulting in complete polarisation of intestinal epithelial cells.[20 51]

Further genotype–phenotype correlation studies revealed that PJS patients with breast carcinoma had predominantly truncation mutations. This observation is in contrast to that described by Lim *et al*, who found no differences in breast cancer risk between nonsense and missense mutations.[50] For this reason, much larger datasets are needed to confirm our observations as this may reflect, at least in part, the higher overall incidence of nonsense compared with missense mutations.

Although functional in vitro assays have been previously performed by other groups to assess the effects of *STK11* mutations (summarised in fig 4; additional online information) the attempt to explain how mutation type and site may influence cancer risk is still unsatisfactory. Biological substrates of this serine/threonine kinase are: PAR1,[13] a positive regulator of the Wnt-βcatenin pathway; AMPK,[55 56] a key regulator of cellular metabolism; and STRAD,[20 51] possibly involved in MAPK signalling. However, the method by which STK11 inactivation contributes to tumour development is not yet completely understood. As mentioned in the introduction, STK11 is involved in growth suppression through various mechanisms. For this, the kinase activity, cytoplasmic localisation, and phosphorylation at Ser428 by $p90^{RSK}$ and cAMP dependent protein kinase are required. Fig 4 (additional online information) summarises in vitro experiments with mutant STK11, performed previously by other groups, showing a dramatic reduction in cytoplasmic and nuclear accumulation of the protein. Kinase activity assays, performed by autophosphorylation at Thr189, revealed that all truncation mutations tested in G361 melanoma cells had lost their growth suppression function, but some missense mutations and one inframe deletion allowed the protein to retaine kinase activity. Interestingly, three missense mutations within the functional domains VIB-VIII and one missense mutation in the C terminal domain allowed retention of kinase activity, but were nevertheless associated with cancer. Carcinogenesis based on missense mutations could therefore also be the result of a gain of function leading to the phosphorylation of a non-physiological target, due to structural changes in the protein.

The notion that mutations in one *STK11* allele are sufficient to cause polyps in PJS results from findings that

heterozygous *STK11*+/− mice develop hamartomatous polyps in the gastrointestinal tract similiar to those found in PJS patients.[57 58] These experiments suggest that the formation of polyps is not the result of loss of heterozygosity (LOH), but might be due to STK11 haploinsufficiency. However, where loss of the wild type allele was analysed in the tumour, carcinogenesis was linked to LOH (fig 1B).

Based on the analysis of our combined dataset, we propose two different mechanisms for tumour development. One is based on loss of STK11 function due to truncation mutations and subsequent LOH as a second hit. The other is based on missense mutations in the functional domains VIB–VIII and the C terminal domain. Whether these may act in a dominant negative fashion has to be determined in the future.

In summary, our results support the notion that the site and type of *STK11* mutations may influence the cancer risk in PJS patients. The findings reported here should be the basis for further larger studies in which a detailed clinical description of the patients is given with respect to age and tumour incidence. In the future, the early identification of mutation carriers with a higher or lower cancer risk will be an important factor for patient management and tumour screening.

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Authors' affiliations

V Schumacher, B Leube, T Goecke, B Royer-Pokora, Institute of Human Genetics and Anthropology, University of Düsseldorf, Universitaetsstrasse 1, 40225 Düsseldorf, Germany
T Vogel, Department of Traumatological Surgery, University of Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf , Germany
C Driemel, G Möslein, Department of General and Visceral Surgery, University of Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf , Germany

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This work is dedicated to Professor Dr med. h.c. F. Vogel

Correspondence to: Dr. Valérie Schumacher, Heinrich-Heine Universitaet Düsseldorf, Institut für Humangenetik und Anthropologie, Universitaetsstrasse 1, D-40225 Düsseldorf; schumacv@uni-duesseldorf.de

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