Allele loss and mutation screen at the Peutz-Jeghers (*LKB1*) locus (19p13.3) in sporadic ovarian tumours

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Summary Germline mutations in the *LKB1* (*STK11*) gene (chromosome sub-band 19p13.3) cause characteristic hamartomas and pigmentation to develop in patients with Peutz-Jeghers syndrome. Peutz-Jeghers syndrome carries an overall risk of cancer that may be up to 20 times that of the general population and Peutz-Jeghers patients are at increased risk of benign and malignant ovarian tumours, particularly granulosa cell tumours. Loss of heterozygosity (allele loss, LOH) has been reported in about 50% of ovarian cancers on 19p13.3. *LKB1* is therefore a candidate tumour suppressor gene for sporadic ovarian tumours. We found allele loss at the marker *D19S886* (19p13.3) in 12 of 49 (24%) sporadic ovarian adenocarcinomas. Using SSCP analysis, we screened ten ovarian cancers with LOH, 35 other ovarian cancers and 12 granulosa cell tumours of the ovary for somatic mutations in *LKB1*. No variants were detected in any of the adenocarcinomas. Two mutations were detected in one of the granulosa cell tumours: a mis-sense mutation affecting the putative 'start' codon (ATG \rightarrow ACG, M1T); and a silent change in exon 7 (CTT \rightarrow CTA, leucine). Like *BRCA1* and *BRCA2*, therefore, it appears that *LKB1* mutations can cause ovarian tumours when present in the germline, but occur rarely in the soma. The allele loss on 19p13.3 in ovarian cancers almost certainly targets a different gene from *LKB1*.

Keywords: Peutz-Jeghers; LKB1/STK11; ovary; adenocarcinoma; granulosa cell

Mendelian diseases which predispose to ovarian tumours include familial breast/ovarian cancer (resulting from germline *BRCA1* and *BRCA2* mutations), Gorlin syndrome (resulting from *PTCH* mutations), hereditary non-polyposis colon cancer (HNPCC, resulting from mismatch repair gene mutations) and Peutz-Jeghers syndrome (PJS, MIM175200). PJS is characterized by hamartomatous polyps of the gastrointestinal tract and other epithelia, and by freckling of the lips, buccal mucosa and other sites (Tomlinson and Houlston, 1997). PJS patients have an increased risk of neoplasia of multiple sites. This risk may approach a 20fold increase over the general population if all organs are considered, although the increased risk for any particular site is necessarily more modest (Murday and Slack, 1989).

The gene for PJS has recently been shown to be a serine/ threonine kinase, known as *LKB1* or *STK11* (Genbank U63333), which maps to chromosome sub-band 19p13.3 (Hemminki et al, 1997, 1998). This gene acts as a tumour suppressor in the hamartomatous polyps of PJS patients and probably also acts as a tumour suppressor in the other neoplasms that develop in PJS patients (Wang et al, 1999). It is not clear whether these neoplasms develop from hamartomas, or whether the *LKB1* locus plays a role in a different genetic pathway of tumour growth, although the former is more likely.

Received 13 July 1998 Revised 12 October 1998 Accepted 22 October 1998

Correspondence to: IPM Tomlinson, Molecular and Population Genetics Laboratory, 4th Floor, Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK Peutz-Jeghers patients are at increased risk of a number of gynaecological neoplasms. These include benign and malignant ovarian lesions, especially granulosa cell tumours (GCTs), in addition to adenoma malignum of the cervix and endometrial adenocarcinoma. Ovarian adenocarinomas show a relatively high frequency of loss of heterozygosity (allele loss, LOH) on chromosome 19p13.3 (Sato et al, 1991; Osborne and Leech, 1994; Amfo et al, 1995; Pejovic, 1995). *LKB1* is therefore a good candidate for involvement in the pathogenesis of sporadic tumours of the ovary. We have analysed a set of sporadic adenocarcinomas of the ovary for allele loss on chromosome 19p13.3 and then screened these tumours and a set of ovarian granulosa cell tumours for mutations in the *LKB1* gene.

METHODS

Using standard methods, DNA was extracted from 60 samples of unselected, fresh-frozen sporadic adenocarcinomas of the ovary and matched normal tissue or blood. After microdissection to enrich for tumour material, DNA was extracted from fixed, paraffin-embedded samples of 12 GCTs of the ovary using proteinase K digestion and the Qiagen tissue extraction kit. None of these cases had known clinical or familial features suggestive of PJS. Standard clinicopathological data (patient age, and tumour grade and stage) were available from hospital records.

For allele loss analysis at *LKB1*, the *D19S886* microsatellite marker was used; this maps within 500 kb of *LKB1* (http://www-bio.llnl.gov/). About 50 ng of DNA from paired tumour/normal samples from the ovarian adenocarcinoma patients were amplified using the polymerase chain reaction (PCR) under standard conditions. The forward primer had previously been end-labelled with

 γ^{-32} ATP using 3U of T4 polynucleotide kinase. Radio-labelled products were electrophoresed through 6% denaturing polyacrylamide gels, dried and exposed to X-ray film for 24 h. Quantitation of PCR products from tumours and the corresponding constitutional DNA was achieved using the Molecular Dynamics phoshorimager and software. Allele loss was scored if the area under an allelic peak was reduced to < 50% of its original value (relative to the other allele), thus making allowance for the presence of contaminating stromal tissue or inflammatory infiltrate in some of the tumours.

Single-strand conformational polymorphism (SSCP) analysis was performed on the tumour samples as the method of mutation screening at LKB1. For the adenocarcinomas, published oligonucleotides and reaction conditions were used for exon-by-exon amplification of LKB1 in the PCR (Wang et al, 1998). For the GCTs, new oligonucleotides were designed to produce shorter PCR target sequences which were more likely to amplify successfully from fixed archival material in the PCR (Table 1). A PCR protocol of 94°C (3 min) × 1, 94°C (1 min)/Ta °C (1 min)/72°C $(1 \text{ min}) \times 35$, 72°C $(5 \text{ min}) \times 1$ was used for the GCTs, with the addition of 0.02% dimethyl sulphoxide (DMSO) and 0.1% bovine serum albumin (BSA) to the reaction where necessary. PCR products were heated to 90°C for 5 min and subjected to electrophoresis on an 8% acrylamide gel (37.5:1 acrylamide: bisacrylamide, 10% glycerol) under non-denaturing conditions at 20 mA for about 16 h. DNA was detected by silver-staining of gels using standard methods. For all tumours with possible mutations according to SSCP analysis, the appropriate exon was reamplified from genomic DNA in the PCR, and purified PCR products were sequenced in forward and reverse orientation using the ABI Ready Reaction Dye Terminator Cycle Sequencing kit and the 373 sequencer. All sequencing reactions were performed in duplicate and alongside samples with wild-type genotypes, and patient samples with known germline mutations in LKB1.



Figure 1 M1T mutation in granulosa cell humour. Wild type sequence is not shown

RESULTS AND DISCUSSION

Allele loss was found at *D19S886* in 12 of 49 (24%) informative ovarian adenocarcinomas (out of the total of 60 studied). This is apparently a somewhat lower frequency of allele loss on 19p than found in some studies, but inspection of the data from these other studies shows that our results are in agreement if the previous analysis of multiple markers on 19p, the sample sizes used and the different tumour types studied are taken into account. Sato et al (1991) found allele loss at *D19S20* (4 cM from *D19S886*) in five of 19 (26%) adenocarcinomas of the ovary (map data from

Exon	Oligo (F,R)	Sequence	Та	Product length
1	GB1727	AGG GCT GGC GGC GGG ACT CC	58	211
	GB1936	TCC TTC ACC TTG CCG TAA GAG C		
	GB1920	GAC CTG CTG GGG GAA GGC TCT T	58	170
	GB2090	AAC CAT CAG CAC CGT GAC TGG		
2	GC1289	CTG ATA CAC CCC TGT CCT CTC TGT C	54	120
	GC1409	AGG CCC CGC GGT CCC AAC AC		
3	GD5531	CTC CAG AGC CCC TTT TCT G	59	255
	GD5786	TCA ATG ACT ATC AGG CCA CG		
4/5	GA826	GGC CCC AGG ACG GGT GTG TG	58	160
	GA986	CCC TAG CAC GTG CCT ACC TC		
	GA951	GTG GCA CCC TCA AAA TCT CC	57	183
	GA1134	TCC AGG CCG TTG GCA ATC TC		
	GA1071	ACC CGT TCG CGG CGG ACG	58	152
	GA1223	AGT GTG CGT GTG GTG AGT GC		
6	GA1659	TGA CTG ACC ACG CCT TTC TT	57	218
	GA1877	CCC CCA ACC CTA CAT TTC TG		
7	GA2412	CTC CTC GCC GGC TTC TCC TC	62	155
	GA2567	CCC CAC CAC GCC CTG CTC TA		
8	GA3439	GAC AGG CGC CAC TGC TTC TG	60	251
	GA3690	GGA CAT CCT GGC CGA GTC AG		
9	GE001	GTA AGT GCG TCC CCG TGG TG	59	337
	GE338	GTG GCA TCC AGG CGT TGT CC		

 Table 1
 Oligonucleotides used for study of paraffin-embedded archival tumour material

ftp://cedar.genetics.soton.ac.uk/pub/chrom19/map). Osborne and Leech (1994) found allele loss at *D19S177* (20 cM proximal to *D19S886*) in four of ten (40%) ovarian adenocarcinomas of the ovary. Amfo et al (1995) detected allele loss (in addition to one putative rearrangement) in two of nine (22%) adenocarcinomas at the *INSR* locus (23 cM proximal to *D19S886*). Our results do not differ significantly from any of these other studies (χ^2 test, details not shown). *LKB1* therefore remained an excellent candidate gene for acting as a tumour suppressor in ovarian tumorigenesis.

In a set of ten adenocarcinomas with LOH, in 35 other adenocarcinomas selected at random, and in 12 GCTs, a small number of variant bands suggestive of somatic mutations at LKB1 was detected on SSCP analysis. In all the carcinomas, these putative bandshifts were shown on sequencing to be intronic polymorphisms or other intronic variants with no predicted effect on mRNA or protein. A commonly observed biallelic polymorphism (C/G, heterozygosity 44% in a sample of 34 individuals) was found in intron 7 at the +8 splice donor site. This polymorphism is found at approximately equal allele frequencies (details not shown) in normal individuals, in PJS patients and in tumours, and it almost certainly has no effect on mRNA splicing; it may, however, be useful for future allele loss studies at the LKB1 locus. No LKB1 mutations were found in the adenocarcinomas. Two mutations were, however, detected in one of the granulosa cell tumours (Figure 1): a mis-sense mutation affecting the putative 'start' codon (ATG \rightarrow ACG, M1T); and a silent change in exon 7 (CTT \rightarrow CTA, leucine). These variants were not present in the germline. Analysis of the sequence showed that this tumour did not exhibit allele loss at LKB1. No particular clinicopathological features distinguished this granulosa cell tumour from any of the others.

It is not clear whether or not the M1T mutation in one of the granulosa cell tumours has any pathogenic effect. Certainly, it occurs outside the kinase core (codons 50–337) in which most germline mutations have been found (Hemminki et al, 1998). Although codon 1 provides the best candidate 'start' codon for *LKB1* translation, there are several alternative 'start' codons just downstream (at codons 11, 18 and 22) which may allow near-normal function of the LKB1 protein. Codon 22, in particular, is flanked by sequences which suggest that it could function efficiently as an alternative initiator of translation. It is even possible that the methionine at codon 1 is not the usual 'start' site for *LKB1*. The M1T mutation has not, however, been observed as a variant in over 50 other PJS patients, tumours and normal individuals sequenced for exon 1 of *LKB1*.

SSCP only detects about 80% of mutations (Sheffield et al, 1993; Ravnik et al, 1994; Vidal and Moller, 1994), and this figure may be somewhat lower for some types of point mutation. We have not excluded further possibilities for the involvement of LKB1 in ovarian tumorigenesis, such as gene silencing by promoter methylation, or hemi- or homozygous deletion of either locus (whether the entire gene or whole exons). Thus, it remains possible that mutations or transcriptional inactivation at LKB1 occur in a larger proportion of ovarian tumours than we have reported. It remains most likely, however, that - similar to that with the BRCA1 and BRCA2 genes in familial breast/ovarian cancer - inherited variation in LKB1 predisposes to ovarian tumours in PJS, but somatic mutations in the same gene are rarely important for the pathogenesis of sporadic tumours of this site. Occasional LKB1 mutations with pathogenic effects have been found in cancers of the colon, testis, lung and skin (Dong et al, 1998; Wanger et al, 1998; Rowan et al, 1999; Wang et al, 1999; Ylikorkala et al, 1999).

Although somatic *LKB1* mutations may be important in the pathogenesis of a minority of sporadic granulosa cell tumours, it is

most likely that *LKB1* is not mutated in ovarian adenocarcinomas and that the allele loss observed on chromosome 19p13.3 in these cancers targets a different locus from *LKB1*. Whether or not *INSR* is actually the gene involved, the combined data from previous work and our study suggest that allele loss on 19p13.3 in ovarian cancer targets a different locus from *LKB1*. Candidate genes in this region of chromosome 19p include basigin, *CDC34*, *PTPRS*, *AMH*, *ICAM1*, *ICAM3* and *CDKN2D*.

ACKNOWLEDGEMENTS

We are grateful to the following bodies for support: Imperial Cancer Research Fund (IT, Z-JW); Jane Ashley Trust (IT); Cancer Research Campaign MC; Henry Lester Trust (Z-JW); China Scolarship Council (Z-JW); MGH 175th Anniversary Scholarship (WF).

REFERENCES

- Amfo K, Neyns B, Teugels E, Lissens W, Bourgain C, De Sutter P, Vandamme B, Vamos E and De Greve J (1995) Frequent deletion of chromosome 19 and a rare rearrangement of 19p13.3 involving the insulin receptor gene in human ovarian cancer. Oncogene 11: 351–358
- Dong SM, Kim KM, Kim SY, Shin MS, Na EY, Lee SH, Park WS, Yoo NJ, Jang JJ, Yoon CY, Kim JW, Kim SY, Yang YM, Kim SH, Kim CS and Lee JY (1998) Frequent somatic mutations in serine/threonine kinase 11/Peutz-Jeghers syndrome gene in left-sided colon cancer. *Cancer Res* 58: 3787–3790
- Hemminki A, Tomlinson I, Markie D, Jarvinen H, Sistonen P, Bjorkqvist AM, Knuutila S, Salovaara R, Bodmer W, Shibata D, de la Chapelle A and Aaltonen LA (1997) Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. *Nat Genet* 15: 87–90
- Hemminki A, Markie D, Tomlinson IPM, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Järvinen H, Aminoff M, Höglund P, Pelin K, Ridanpää M, Salovaara R, Olschwang S, Bodmer WF, Olsen A, Stratton MR, de la Chapelle A and Aaltonen LA (1998) A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* 391: 184–187
- Murday V and Slack J (1989) Inherited disorders associated with colorectal cancer. Cancer Surv 8: 139–157
- Osborne RJ and Leech V (1994) Polymerase chain reaction allelotyping of human ovarian cancer. *Br J Cancer* **69**: 429–438
- Pejovic T (1995) Genetic changes in ovarian cancer. Ann Med 27: 73-78
- Ravnik GM, Glavac D and Dean M (1994) Sensitivity of single-strand conformation polymorphism and heteroduplex method for mutation detection in the cystic fibrosis gene. *Hum Mol Genet* 3: 801–807
- Rowan A, Bataille V, MacKie R, Healy E, Bicknell D, Bodmer WF and Tomlinson IPM (1999) Somatic mutations in the Peutz-Jeghers (*LKB1/STK11*) gene in sporadic malignant melanomas. *J Invest Dermatol* (in press)
- Sato T, Saito H, Morita R, Koi S, Lee JH and Nakamura Y (1991) Allelotype of human ovarian cancer. *Cancer Res* **51**: 5118–5122
- Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW and Stone EM (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 16: 325–332
- Tomlinson I and Houlston R (1997) Peutz-Jeghers syndrome. J Med Genet 34: 1007–1011
- Vidal PA and Moller DE (1994) Comparative sensitivity of alternative single-strand conformation polymorphism (SSCP) methods. *Biotechniques* 17: 490–492
- Wang Z-J, Taylor F, Churchman M, Norbury CG and Tomlinson IPM (1998) Genetic pathways of colorectal carcinogenesis rarely involve the PTEN and LKBI genes outside the inherited hamartoma syndromes. Am J Pathol 153: 363–366
- Wang Z-J, Ellis I, Zauber P, Iwama T, Marchese C, Talbot IC, Xue W-H, Yan Z-Y and Tomlinson IPM (1999) Allelic imbalance at the *LKB1* (*STK11*) locus on 19p13.3 in hamartomas, adenomas and carcinomas from patients with Peutz-Jeghers syndrome provides evidence for a hamartoma-(adenoma)-carcinoma sequence. J Pathol (in press)
- Ylikorkala A, Avizienyte E, Tomlinson IPM, Tiainen M, Roth S, Loukola A, Hemminki A, Johansson M, Sistonen P, Markie D, Neale K, Phillips RKS, Zauber P, Iwama T, Sampson J, Järvinen H, Mäkelä TP, Aaltonen LA (1999) Mutations and impaired function of LKB1 in familial and non-familial Peutz-Jeghers syndrome and a sporadic testicular cancer. *Hum Mol Genet* 8: 45–51