ORIGINAL ARTICLE

STRAD in Peutz-Jeghers syndrome and sporadic cancers

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Background/Aims: *LKB1* is a tumour suppressor gene that is associated with Peutz-Jeghers syndrome (PJS), a rare autosomal dominant cancer predisposition syndrome. However, germline mutations in the *LKB1* gene are found in only about 60% of patients with PJS, suggesting the existence of a second PJS gene. The *STRAD* gene, encoding an LKB1 interacting protein that activates LKB1, which subsequently leads to polarisation of cells, is an interesting candidate for a second PJS gene and a potential tumour suppressor gene in sporadic carcinomas.

Methods: The involvement of STRAD in 42 PJS associated tumours (sporadic lung, colon, gastric, and ovarian adenocarcinomas) was studied using loss of heterozygosity (LOH) analysis of eight microsatellite markers on chromosome 17, including *TP53*, *BRCA1*, and *STRAD* markers.

Results: Loss of the marker near the *STRAD* locus was seen in 13 of 29 informative cases, including all gastric adenocarcinomas. Specific LOH of the *STRAD* marker was found in four of 29 informative cases. For these patients all exons and exon–intron boundaries of the *STRAD* gene were sequenced, but no somatic mutations were identified. Furthermore, no germline *STRAD* mutations were found in 10 patients with PJS and family members without *LKB1* germline mutation.

Conclusions: Despite the frequent occurrence of LOH in the *STRAD* region, these results indicate that inactivation of the *STRAD* gene is not essential in the sporadic adenocarcinomas studied, although it is possible that *STRAD* may be inactivated in different ways. In addition, no evidence was found for the hypothesis that STRAD is a second PJS susceptibility gene.

Peutz-Jeghers Syndrome (PJS) is a rare autosomal dominant disorder characterised by hamartomatous polyposis and mucocutaneous melanin pigmentation.[1 2] Patients have an 18 times higher relative risk of developing cancer at several sites,[3] with the highest risk of developing cancers in the colon, breast, pancreas, stomach, and ovary.[4 5] Hemminki *et al* linked PJS to a locus on chromosome 19p13.3 where later the *LKB1* gene (serine/threonine kinase 11) was identified.[6] *LKB1* has been postulated as a tumour suppressor gene; however, its exact role has not been clarified yet, but nearly all germline mutations of *LKB1* lead to loss or impairment of the kinase activity of the protein. Loss of LKB1 has been studied in a variety of sporadic tumours, but so far few losses have been described except for lung adenocarcinomas, where approximately one third of the patients shows inactivation of *LKB1*,[7] and to a lesser extent in pancreatic (4%) and biliary (6%) cancers.[8] Somatic mutations of *LKB1* have also been reported in malignant melanoma (11%).[9]

> ''Because of the essential function of STRAD in activating LKB1 and inducing cell polarity, *STRAD* may also function as a tumour suppressor gene''

Several studies have been aimed at elucidating the function of LKB1. It has been implicated in p53 mediated apoptosis,[10] control of cell cycle arrest,[11] Wnt signalling,[12 13] transforming growth factor β signalling,[14] energy metabolism,[15] and cell polarity.[16] Baas *et al* identified STRAD (STE 20 related adapter) as an LKB1 interactor using yeast two hybrid analysis.[17] Upon STRAD expression, LKB1 was activated and translocated from the nucleus to the cytoplasm, resulting in complete polarisation of cultured intestinal epithelial cells. Because of the essential function of STRAD in activating LKB1 and inducing cell polarity, *STRAD* may also function as a tumour suppressor gene.

Although defects in *LKB1* were identified as causative for PJS, mutations in *LKB1* have been detected only in about 60% of patients,[18] leaving the underlying gene defect of the syndrome unclear in the remaining patients. It is possible that *LKB1* mutations have been missed because of shortcomings in the techniques used, but this is not likely to explain the relatively high proportion of patients without a germline *LKB1* mutation. A second disease locus has been proposed at chromosome segment 19q13.4, the distal region of 1p, and on chromosome 6.[19–21] Several genes located at 19q13.4 have been excluded as a second PJS gene, in addition to LIP1 (LKB1 interacting protein 1).[22] As a result of its interaction with LKB1 and its essential role in the activation of LKB1, *STRAD* is an interesting candidate for a second PJS susceptibility gene, or it might be an effector gene determining the possible Peutz-Jeghers phenotype.

In our study the direct involvement of *STRAD*, located at 17q23.3, in sporadic adenocarcinomas was studied using loss of heterozygosity (LOH) analysis of chromosome 17, in combination with mutation analysis. In addition, sequence analysis of *STRAD* was performed for 10 patients with PJS and their family members without an identified *LKB1* germline mutation.

MATERIALS AND METHODS

Patient material

Our research was carried out in accordance with the ethical guidelines of the research review committee of the Academic Medical Centre, Amsterdam, the Netherlands.

Abbreviations: IHC, immunohistochemistry; LOH, loss of heterozygosity; PJS, Peutz-Jeghers syndrome

A study population of 42 sporadic adenocarcinomas consisting of eight ovarian adenocarcinomas, 10 cardia adenocarcinomas, 12 sigmoid adenocarcinomas, and 12 lung adenocarcinomas was selected from the pathology archives of the Academic Medical Centre (Amsterdam, the Netherlands). Formalin fixed, paraffin wax embedded tissue was used, and for each case non-neoplastic tissue was obtained from a tumour free lymph node or, when not available, from normal tissue.

Cell pellets from five lymphoblast cell lines from three patients with PJS and both parents of another patient with PJS (as a result of too little tissue from the patient) without known *LKB1* germline mutations from the Erasmus University (Rotterdam, the Netherlands), in addition to genomic DNA from five patients with PJS from three families without *LKB1* germline mutations from the Free University (Amsterdam, the Netherlands), were available from a study population of patients with PJS fulfilling the clinical criteria.

Microdissection and DNA isolation

Tumour tissue was microdissected from dewaxed haematoxylin stained 5 µm tissue sections. The percentage of tumour cells ranged from 70% to 90%. DNA was isolated using the PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. DNA concentrations were measured using the PicoGreen double stranded DNA Quantitation kit (Molecular Probes, Leiden, the Netherlands).

Genomic DNA from cell pellets was isolated by proteinase K/sodium dodecyl sulfate precipitation, followed by phenol/chloroform extraction and ethanol precipitation.

Loss of heterozygosity

Eight markers were used for LOH analysis, two on chromosome 17p13.1 around the *TP53* locus (markers TP53 and p53Alu) and six on the 17q arm: D17S791, D17S1795, D17S795, markers D17S250 and D17S855 around *BRCA1*, and marker D17S944 near *STRAD*. Primer sequences of these markers can be found on The Genome Database website (www.gdb.org). The primer sequence of p53Alu is: forward, 5′-GAATCCGGGAGGAGGTTG and reverse, 5′-AACAGCTCCTTTAATGGCAG-3′. The forward primers were FAM (6-carboxy-fluorescein) or TET (6-carboxy-4,7,2′,7′-tetrachlorofluorescein) labelled.

The polymerase chain reaction was performed using 10mM dNTPs, 1.5mM $MgCl_2$, 0.5µM of both primers, 0.2 U Taq polymerase (Life Technologies Inc, Carlesbad, California, USA), and at least 10 ng of DNA in a 20 µl reaction. The reaction was started with a three minute denaturation at 96℃, followed by 30 cycles of 45 seconds of denaturation at 96℃, one minute of annealing at temperatures varying between 52℃ and 64℃, and a 45 second elongation step at 72℃; the programme ended with a five minute elongation step at 72℃ in a PTC-100 or PTC-200 cycler (MJ Research Inc, Waltham, Massachusetts, USA).

Analysis was carried out using an automated ABI 377 or ABI 3100 sequencer (Applied Biosystems, Foster City, California, USA) with a Genescan™ 350ROX size standard (Applied Biosystems) and the manufacturer's Genescan® 2.1 software.

Normal samples with two distinctly sized alleles of a particular marker were termed informative. For all informative markers, the allelic imbalance factor was calculated as described by Cawkwell *et al*.[23] LOH was assumed if the allelic imbalance factor was greater than 1.6 or less than 0.6. Observed losses were confirmed to exclude induced LOH.

Immunohistochemistry

Immunohistochemistry (IHC) for p53 and LKB1 was performed on dewaxed and rehydrated 4 µm sections. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol for 20 minutes. The slides were submerged in antigen retrieval solution (10mM Tris/1mM EDTA (pH 9.0) for p53 IHC and 0.01M citrate (pH 6.0) for LKB1 IHC) and heated for 10 minutes at 120℃. After cooling for 10 minutes, 5% normal goat serum in phosphate buffered saline was applied for 10 minutes. The sections were incubated for one hour with the primary antibody. Staining for p53 and LKB1 was carried out using the DO-7 and BP53-12 (Neomarkers, Freemont, California, USA; 1/2000 dilution) and anti-LKB1 (Cell Signaling Technology, Beverly, California, USA; 1/20 dilution) antibodies, respectively. The Powervision+poly–horseradish peroxidase detection system (ImmunoVision Technologies, Daly City, California, USA) was used to visualise the antibody binding sites, with 3,3-diaminobenzidine as chromogen. Sections were counterstained with haematoxylin.

Sequence analysis

Table 1 shows the primers used to amplify the *STRAD* exons and exon–intron boundaries.

Polymerase chain reaction products were purified using the Qiagen PCR purification kit (Qiagen Benelux BV, Venlo, the Netherlands) and the sequencing reaction was performed using the ABI Big Dye Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol. Samples were run on an ABI 3100 genetic analyser and analysed using Sequence Navigator.

RESULTS

To study the possibility that *STRAD* may function as a tumour suppressor gene, LOH analysis was performed on a panel of 42 sporadic adenocarcinomas that consisted of tumours described in patients with PJS or tumours of which variants occur in PJS. Adenocarcinomas of the lung, colon, and stomach have been described in patients with PJS.[3] Ovarian adenocarcinomas were selected because one third of patients with the rare ovarian sex cord tumour with annular tubules have PJS.[24] To identify losses of the entire chromosome arms and losses of the known tumour suppressor genes on chromosome 17, eight microsatellite markers located on chromosome 17 were used: markers flanking *TP53* (17p13.1), *BRCA1* (17q21), and *STRAD* (17q23.3), in addition to three other markers on 17q (fig 1). Furthermore, p53 IHC was performed to substantiate the LOH results for TP53.

As shown in fig 1, 13 of 29 patients showed LOH of the D17S944 marker near the *STRAD* locus. All informative gastric adenocarcinomas showed LOH of the *STRAD* marker, whereas only one of the 12 lung adenocarcinomas showed LOH. Four of the eight ovarian and seven of the 12 colon adenocarcinomas showed LOH. To investigate whether LOH near the *STRAD* gene was specific in these patients, the LOH status near the *TP53* and *BRCA1* loci was also assessed.

Three of eight ovarian adenocarcinomas showed retention of all informative markers on chromosome 17. In contrast, two cases showed LOH of all informative markers tested. In the other cases, *TP53*, *BRCA1*, or another gene might be affected except for ovarian adenocarcinoma 7 where *STRAD* seemed to be specifically lost.

Two of the 10 gastric adenocarcinomas showed retention of all chromosome 17 markers and another two showed LOH of all informative markers on chromosome 17. In most cases ($>$ 70%) LOH of the *TP53* markers was seen. The LOH results were supported by positive p53 immunohistochemistry suggestive of a *TP53* mutation and loss of functional p53. Although all informative cases showed LOH for the *STRAD*

Table 1 Primer sequences and polymerase chain reaction conditions for sequencing of 13 exons and exon–intron boundaries of *STRAD*

Exon	Forward primer	Reverse primer	Tm (℃)	$MgCl_2$ (mM)
1	ggtctggcggcggcgcggcagtaaaac	cagtccctcctggctctgccaccgattccatctcc	65	1.25
2	ataggtgaagtccagtgctttgaatgg	ctgaaaagcagtgtatttggca	52	1.25
3	gtctaaaggctgatagctaatttgttgcggc	caatctatagcaattggcaattctaagggcaagtgtaacc	65	2.5
4	ttgctgttctgaatgtgggtgtgtcttctg	ggctgttgcatggagatgacttcttgtggaagtttgtgtg	52	1.25
5	cctcgtgggtctgaattaccaccttcacccca	ccaggggctgctgcagctttctcagcag	65	1.25
6	ccttttgttgttcttgtccttgcgtatggc	gagtgtgtcacatggagggaggg	65	1.25
7+8	gctgccatctttgtgttgagcggc	ggcggcctttcccatgattacag	65	1.25
9	gccaggtggagtgtgtgaagacca	gggagccttcaggcattgcc	65	1.25
10	ctctctttagttctaccc	gagtagctctcacactcagg	52	2.5
11	ccgctacagcccaggtcc	cttcgggagagcagagggtg	65	2.5
12+13	ggctgaggcataagtggcagga	ggatgcagaggccacccagag	65	1.25

marker, only one gastric adenocarcinoma (stomach 8) might have specifically lost *STRAD*, because all other markers except for *TP53* showed retention in this adenocarcinoma. In this case, the negative IHC indicated that a remaining *TP53* wild-type allele might be present.

All colon adenocarcinomas showed LOH of one or more markers on chromosome 17. In three of the 12 cases all chromosome 17 markers showed LOH. Similar to the gastric adenocarcinomas, a high proportion (six of 12) of colon adenocarcinomas showed LOH of *TP53*. In three cases (colon 6, 7, and 11), the *STRAD* locus might be lost specifically because no LOH for *TP53* or *BRCA1* was identified in these cases. In cases 10 and 12, the LOH pattern suggested that genes other than *TP53* and *BRCA1* were affected.

The lung adenocarcinomas did not show LOH for the informative markers on chromosome 17. Retention of all markers was found in two of the 12 cases. Again *TP53* LOH was found frequently (seven of 12 informative cases). Lung cases 9 and 12 showed LOH of one of the markers around the *TP53* gene; however, no positive IHC indicative of a mutation in the remaining allele was found. None of the lung adenocarcinomas showed specific LOH near the *STRAD* locus; however, in lung adenocarcinoma case 1 either *BRCA1* or *STRAD* might be lost.

In total, LOH analysis revealed five of 29 informative cases with specific LOH near the *STRAD* locus (ovary 7, stomach 4, and colon 6, 7, and 11). Mutational analysis of *STRAD* was used to investigate whether the second *STRAD* allele was inactivated. No mutations were found in the 13 exons or intron–exon boundaries. However, a single nucleotide polymorphism 62279118 C/T was identified in exon 2, 18 base pairs upstream of the start codon. Cases ovary 7 and colon 6 were heterozygous and a homozygous T allele was found in cases stomach 4, colon 7, and colon 11, whereas none of the patients was homozygous for the wild-type C allele.

Although LOH analysis showed LOH near the *STRAD* locus in 13 of the 29 sporadic tumours, no *STRAD* mutations were found in cases where this region appeared to be specifically affected. Because *STRAD* could also be inactivated in other ways and thus affect the stability or localisation of LKB1, IHC was carried out on all cases studied.

LKB1 staining was in general relatively weak and varied between tumours. Tumours of the stomach and colon were on average more strongly positive than ovarian and lung adenocarcinomas. Staining was cytoplasmic and occasionally membranous. No nuclear staining, as would be expected in the case of dysfunctional STRAD, was observed.

Germline abnormalities of the *LKB1* gene have been reported in about 60% of patients with PJS, with the cause of the syndrome being unclear in the remaining patients. The presence of a second disease locus has been proposed, but no second PJS gene has been identified to date. Because of the essential function of STRAD in activating LKB1, the question was raised whether *STRAD* might be a second PJS gene.

Therefore, *STRAD* mutation analysis was carried out in nine patients with PJS and one family member where no *LKB1* germline mutation had been identified, suggesting that another gene may have caused the syndrome. Sequence analysis of exons and exon–intron boundaries revealed no mutations, although the above described single nucleotide polymorphism in exon 2 recurred in several patients. Of the 10 PJS family members, five were homozygous for the wild-type C allele, three were homozygous for the T allele, and two were heterozygous.

DISCUSSION

The association between loss of epithelial organisation and malignant progression in mammalian tumours has long been known, making disorganisation of cell architecture one of the primary diagnostic features of transformation. The function

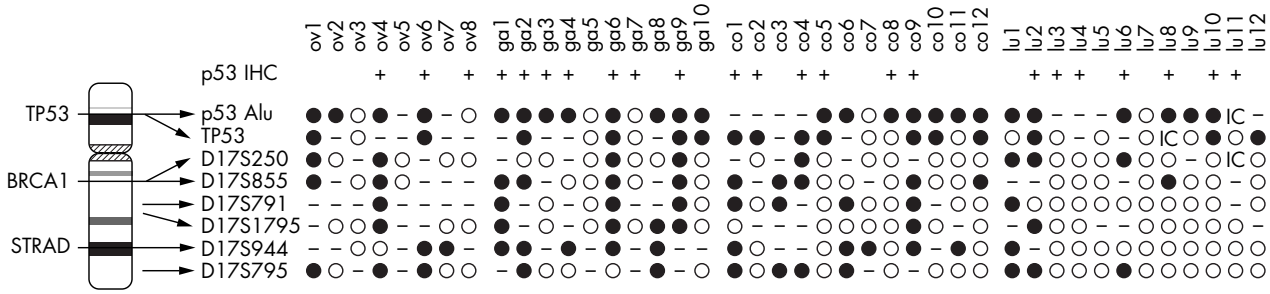

Figure 1 Loss of heterozygosity (LOH) results for eight microsatellite markers on chromosome 17, including those near the *STRAD*, *BRCA1*, and *TP53* loci, in addition to the results for p53 immunohistochemistry (IHC) for 42 sporadic ovarian, gastric, colon, and lung adenocarcinomas. Dark circles, LOH; open circles, retention; −, not informative; +, positive staining; and IC, inconclusive.

of LKB1 in regulating cell polarity should provide insight into a possible mechanism of tumour formation in patients with PJS. Loss of both LKB1 alleles in PJS tumours may lead to a disruption of polarity, ultimately resulting in transformation and tumour growth. *LKB1* functions as a tumour suppressor gene in PJS and *LKB1* aberrations have also been described in sporadic tumours. One third of sporadic lung adenocarcinomas showed inactivation of *LKB1*,[7] and this was also seen to a lesser extent in sporadic biliary and pancreatic carcinomas.[8] In addition, somatic mutations of *LKB1* have been reported in malignant melanoma.[9] The link between LKB1 and cell polarity through STRAD raised the question of whether STRAD might also be involved in sporadic tumour formation. Because this involvement may be tumour specific, we selected four sporadic tumour types of which subtypes occur in patients with PJS to study LOH of *STRAD* and LKB1 expression.

To eliminate cases that show LOH of the entire chromosome or chromosome arm, in addition to LOH of the known tumour suppressor genes on chromosome 17, *TP53* and *BRCA1*, the LOH status of eight microsatellite markers was assessed. LOH of the *STRAD* marker was found in 13 of 29 informative cases. Ten of the adenocarcinomas showed no LOH of the informative markers on chromosome 17 and another seven cases showed LOH of all informative markers on chromosome 17. LOH of chromosome 17 was most prominently present in the group of colon adenocarcinomas and least prominent in lung adenocarcinomas.

LOH near the *TP53* locus was found in most gastric, colon, and lung adenocarcinomas (50–70%), whereas only two of the eight ovarian adenocarcinomas showed LOH. These results indicate that loss of *TP53* is indeed a common event in tumour formation, as has been described previously for these tumours. LOH of *TP53* was reported in approximately 60% of gastric adenocarcinomas.[25] In lung cancer it has been shown that the short arm of chromosome 17 is one of the most frequently affected chromosomal regions, and there is solid evidence that the *TP53* gene is a target for these deletions.[26] In colon carcinomas loss of chromosome 17p, including the *TP53* locus, was described as a late event in the adenoma–carcinoma sequence.[27] In ovarian tumours, LOH at the *TP53* locus has been described in up to 81% of cases.[28 29] In our study, we found LOH of *TP53* in two of eight cases, indicating that in ovarian adenocarcinomas LOH of other genes may be more important than LOH at the *TP53* locus.

> ''There are no indications that expression of the STRAD protein is aberrant in these sporadic adenocarcinomas, despite the high percentage of loss of heterozygosity at 17q''

In ovarian carcinomas, inactivation of *BRCA1* may occur more frequently. LOH of *BRCA1* has been described in up to 50% of ovarian tumours.[28 30] In our study, only one of eight ovarian adenocarcinomas showed LOH of both *BRCA1* markers. In addition, only one case with LOH of *BRCA1* was identified in the colon and lung adenocarcinomas in our study. LOH of the 17q21 region was reported in approximately 50% of colon and lung adenocarcinomas.[31 32] Although in gastric adenocarcinomas a relatively high percentage of LOH at 17q21 was described, no *BRCA1* mutations were found, which is indicative of the presence of another tumour suppressor gene.[33]

According to Knudson's two hit theory, tumour suppressor genes are inactivated by a recessive mutation in one allele and loss of the other wild-type allele.[34] Frequent LOH is consequently considered to be an indicator of gene inactivation. Specific LOH of *STRAD* was found in five of our cases. Mutational analysis did not identify mutations in the exons or intron–exon boundaries. Because the possibility remains that *STRAD* may be inactivated in another way, and this could result in the aberrant localisation of LKB1, LKB1 immunohistochemical staining was performed. However, no nuclear staining indicative of dysfunctional STRAD was observed. Based on these results, we concluded that there are no indications that expression of the STRAD protein is aberrant in these sporadic adenocarcinomas, despite the high percentage of LOH at 17q. Several reports have already suggested that other tumour suppressor genes may be located at chromosome 17q in the tumours studied. Alterations in chromosome segment 17q23, and marker D17S794 in particular, tended to be associated with early stage lung adenocarcinoma and longer survival.[35] Furthermore, a putative tumour suppressor gene locus was identified distally from the *STRAD* locus at 17q25, with LOH ranging from 25% to 70% of studied ovarian adenocarcinomas.[36] At chromosomal location 17q21.3, the metastasis suppressor *NM23-H1* has been suggested to play a role in colon cancer progression and metastasis.[37] However, LOH of *NM23-H1* was found in only 14% of gastric carcinomas, indicating that inactivation is not essential in gastric cancer.[38]

Based on our results, STRAD does not seem to be essential for tumour formation in the sporadic tumour types studied. In patients with PJS this may be different, because a link with LKB1 has already been established. The essential role of STRAD in LKB1 activation in the establishment of cell polarity suggests that defects in the *STRAD* gene have the same effects as loss of LKB1 function. However, despite its important role for the function of LKB1, no germline mutations were detected in the patients with PJS studied, therefore excluding *STRAD* as a potential second PJS gene. Although alternative mechanisms of inactivation such as hypermethylation, exon deletions, or intronic mutations cannot be excluded, it does not seem likely that loss of STRAD function can cause PJS. A final possibility of germline hemi-allelic methylation, as seen for MLH1 in some patients with early onset colorectal cancer and high microsatellite instability,[39–41] seems to be an unlikely explanation for the involvement of STRAD in PJS and sporadic cancers. Even if this is the case in patients with PJS and known LKB1 germline mutations, it is unlikely that hemi-allelic methylation, which has been found only sporadically thus far, accounts for the patients with PJS without LKB1 germline mutation.

During the course of our study the search for mutations in the LKB 1 gene was continued using a special procedure for large deletions in unresolved Dutch families affected by PJS. This revealed new intron deletions that result in skipping of multiple exons in the *LKB1* mRNA. Thus, at present 15 of 19 Dutch families affected by PJS have a mutation in the *LKB1* gene (AM Westerman *et al,* unpublished data, 2005). Although the proportion of patients with PGS not linked to

Take home messages

- Although there was a high occurrence of loss of heterozygosity in the *STRAD* region at 17q in patients with Peutz-Jeghers syndrome (PJS), inactivation of the *STRAD* gene is not essential in the sporadic adenocarcinomas studied in these patients, although it is possible that *STRAD* may be inactivated in different ways
- In addition, no evidence was found for the hypothesis that STRAD is the second PJS susceptibility gene

the *LKB1* gene may be lower than previously estimated, other LKB1 interacting or activating proteins remain possible candidates for a second PJS gene.

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