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Analysis of the *TSC1* and *TSC2* genes in sporadic renal cell carcinomas

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Summary The genetic events involved in the aetiology of non-clear-cell renal cell carcinoma (RCC) and a proportion of clear cell RCC remain to be defined. Germline mutations of the *TSC1* and *TSC2* genes cause tuberous sclerosis (TSC), a multi-system hamartoma syndrome that is also associated with RCC. We assessed 17 sporadic clear cell RCCs with a previously identified *VHL* mutation, 15 clear-cell RCCs without an identified *VHL* mutation and 15 non-clear-cell RCCs for loss of heterozygosity (LOH) at chromosomes 9q34 and 16p13.3, the chromosomal locations of *TSC1* and *TSC2*. LOH was detected in 4/9, 1/11 and 3/13 cases informative at both loci. SSCP analysis of the whole coding region of the retained allele did not reveal any cases with a detectable intragenic second somatic mutation. Furthermore, RT-PCR analysis of *TSC1* and *TSC2* on total RNA from 8 clear-cell RCC cell lines confirmed expression of both TSC genes. These data indicate that biallelic inactivation of *TSC1* or *TSC2* is not frequent in sporadic RCC and suggests that the molecular mechanisms of renal carcinogenesis in TSC are likely to be distinct. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: *TSC1; TSC2*; sporadic renal cell carcinoma

The molecular genetic events leading to renal cell carcinoma (RCC) are not fully understood. Recurrent regions of deletion on chromosomes 3p, 4q, 6q, 8p, 9p and amplifications on 17q and Xq have been revealed by comparative genomic hybridisation (CGH) (Verdorfer et al, 1998; Bissig et al, 1999) and loss of heterozygosity (LOH) studies (Thrash-Bingham et al, 1995), as have alterations on 2, 3, 9–12, 16, 17 and 18 by restriction landmark genomic scanning (RLGS) (Cho et al, 1998a,b). The molecular pathology of RCC varies between histopathological subtypes. Thus chromosome 3p allele loss is the most frequent alteration in clear cell RCC (which accounts for ~80% of tumours) but is rare in non-clear-cell RCC. Several known and putative tumour suppressor genes (TSGs) map to 3p and both the von Hippel–Lindau (*VHL*) (Latif et al, 1993) TSG and further gene(s) at 3p12–p21 have been implicated in clear cell RCC. *VHL* is mutated, deleted or hypermethylated in up to 70% of sporadic clear cell RCCs in addition to von Hippel–Lindau disease associated renal cell carcinoma (Prowse et al, 1997), but does not appear to play a significant role in papillary or other non-clear-cell cancers (Foster et al, 1994; Gnarra et al, 1994; Herman et al, 1994; Shuin et al, 1994; Clifford et al, 1998). Inactivation of 3p12–p21 TSG(s) has been implicated in most clear-cell RCC irrespective of VHL status, and to date no differences in molecular pathology have been identified between clear-cell RCC with and without VHL inactivation (Clifford et al, 1998, 1999).

A role for the *cMET* gene that encodes the receptor for hepatocyte growth factor has been demonstrated in type 1 papillary RCC, since constitutionally activating germline missense mutations occur in a rare hereditary form of papillary RCC as do somatic mutations in some sporadic cancers (Schmidt et al, 1997). However the genes involved in the majority of non-clear-cell RCC remain to be defined. *TSC1* (van Slegtenhorst et al, 1997) and *TSC2* (European Chromosome 16 Tuberous Sclerosis Consortium, 1993) are Knudson-type TSGs that are constitutionally mutated in the hereditary disorder tuberous sclerosis (TSC). Renal angiomyolipomas are found in most affected individuals, but there also appears to be a less frequent association with RCC and several reports have described multifocal and bilateral disease in unusually young patients, suggesting a possible role for the TSC genes in RCC (Sampson et al, 1995; Bjornsson et al, 1996; Al-Saleem et al, 1998). The identification of LOH or intragenic mutation affecting the wild-type allele of *TSC1* or *TSC2* in TSC-associated RCC has further supported this hypothesis (Bjornsson et al, 1996; van Slegtenhorst et al, 1997; Al-Saleem et al, 1998). In rodent models there is already direct evidence for a role of the *TSC1* and *TSC2* orthologues in RCC. The Eker rat develops multifocal renal cystadenoma and carcinoma and carries a truncating germline mutation of the *Tsc2* gene (Kobayashi et al, 1995). Heterozygous engineered *Tsc1* and *Tsc2* knockout mice develop a similar renal cystadenoma/carcinoma phenotype (Kobayashi et al, 1999; Onda et al, 1999; Kwiatowski DJ, personal communication). Tumours from the mutant animals show somatic second hit mutations of the corresponding *Tsc1* or *Tsc2* wild-type allele (Yeung et al, 1995; Kobayashi et al, 1997, 1999; Onda et al, 1999). Bi-allelic somatic mutations of *Tsc1* and *Tsc2* have also been reported in chemically induced RCCs in non-Eker rats (Urakami et al, 1997; Satake et al, 1999). However, a comprehensive study of *TSC1* and *TSC2* in sporadic human RCC has not yet been reported. We therefore undertook a systematic molecular genetic study of the *TSC1* and *TSC2* genes in different types of sporadic human RCC.

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MATERIALS AND METHODS

Tumour and constitutional DNA samples

47 paired sporadic renal cell carcinomas and constitutional DNA samples were studied. These comprised 17 clear-cell RCCs known to harbour *VHL* mutations, 15 clear-cell RCCs in which no *VHL* mutation had been detected (Foster et al, 1994; Clifford et al, 1998) and 15 non-clear-cell RCCs. All patient samples were obtained with consent for molecular genetic analysis.

RNA from clear cell-RCC cell lines

Total RNA was extracted from 8 clear-cell RCC cell lines, CAKI1, KTCL26, SKRC18, SKRC39, SKRC45, SKRC47, SKRC52 and SKRC54 using the Qiagen RNeasy RNA extraction kit.

LOH analysis

7 polymorphisms at the *TSC1* locus and 7 at the *TSC2* locus that we have described previously (Parry et al, 2000) were genotyped to assay for LOH in paired tumour and constitutional DNA samples. The *TSC1* markers PM4 and PM2 are situated 50 kb and 5 kb telomeric to the gene respectively, PM1 is located in exon 9, markers 'exon 14' and 'exon 22' are RFLPs, the intron 21 polymorphism is a mononucleotide repeat and PM5 is 50 kb centromeric to the gene. The *TSC2* marker LP1 is 95 kb telomeric to *TSC2*, IVS8 is in intron 8, LP10 is in intron 10, exon 40 contains an RFLP, Kg8 lies within the 3′ UTR of *PKD1* with EJ1 and LP7 1.5 kb and 150 kb centromeric, respectively. PCR amplification of tumour and constitutional DNA samples was carried out in parallel in 96-well microtitre plates (Hybaid). Each 50 µl reaction contained 100 ng DNA, 25 pmoles primer (supplied by Oswel DNA Services), 0.2 mM dNTP (Boehringer Mannheim), 5 µl reaction buffer (100 mM Tris pH 8.3, 500 mM KCI, 15 mM $MgCl_2$, 0.01% gelatin (Cetus)) and 1 U AmpliTaq Gold Polymerase (Cetus). Cycling conditions were 94˚C 10 min, followed by 37 cycles of annealing temperature (55–60˚C) 1 min, 72˚C 1 min, 94˚C 30 s, and a final step of 72˚C 10 min. For autoradiography reverse primers were end labelled with γ^{33} dATP (Amersham) using T4 polynucleotide kinase (Life Technologies) according to manufacturers instructions and the products were electrophoresed on 6% polyacrylamide gels (National Diagnostics). The *TSC1* exon 14 1556 A/G polymorphism, the *TSC1* exon 22 3050 C/T polymorphism and the *TSC2* exon 40 1734 T/C polymorphism were assayed by digestion of 10 µl amplified product with the enzymes NIaIV, HaeIII and EcoRV respectively and visualisation on 3% agarose gels stained with ethidium bromide. LOH was determined by visual inspection of alleles from normal and tumour DNA samples by 3 independent observers and samples scored positive by all observers were reamplified and assessed again.

SSCP analysis and sequencing

In cases exhibiting LOH at the *TSC1* or *TSC2* locus, all known coding exons and exon flanking sequences of the corresponding retained allele were screened by SSCP for evidence of intragenic somatic mutations. Primer sequences and annealing temperatures used are available at the Cardiff-Rotterdam Tuberous Sclerosis Mutation Database Website (www.uwcm.ac.uk/uwcm/mg/tsc_db/pcrpub.html). Amplification reactions were carried out as previously described (Jones et al, 1997). SSCP was performed on

Table 1 Informativity and LOH of RCCs

	No. informative and No. showing LOH in parenthesis			
Tumour type	**No.**	***TSC1***	***TSC2***	***TSC1* and *TSC2***
Clear cell carcinoma with a *VHL* mutation	17	12 (3)	14 (1)	9 (4)
Clear cell carcinoma without a *VHL* mutation	15	12 (1)	14 (0)	11 (1)
Non clear cell carcinoma	15	14 (3)	14 (0)	13 (3)
Total	47	38 (7)	42 (1)	33 (8)

Table 2 RCCs showing LOH in the *TSC1* and *TSC2* regions

TSC1

Tumour	Patient	PM4	PM2	PM1	Exon 14	Intron 21	Exon 22	PM5
CC-*VHL* mut	8	+	NI	NI	NI	NI	NI	+
CC-*VHL* mut	10	+	+	NI	NI	NI	NI	NI
CC-*VHL* mut	180	–	–	NI	NI	–	NI	+
CC-*VHL* no mut	6	NI	+	NI	NI	NI	NI	NI
Non-CC	128	+	NI	NI	+	NI	NI	+
Non-CC	287	+	NI	NI	NI	+	NI	NI
Non-CC	297	+	+	NI	NI	NI	NI	NI

TSC2

Tumour	Patient	LP1	IVS8	LP10	EXON 40	EJ1	Kg8	LP7
CC-*VHL* mut	239	–	NI	NI	NI	NI	+	NI

CC-*VHL* mut – clear cell carcinoma with a mutation in the *VHL* gene; CC-*VHL* no mut – clear cell carcinoma with no identified mutation in the *VHL* gene; Non-CC – non clear cell renal carcinoma; + LOH detected; – No LOH and NI not informative. Shaded boxes indicate intragenic markers. Markers orientated from telomere towards centromere (left to right).

4 µl PCR product diluted 1:10 with gel loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol). Samples were denatured at 94˚C for 2 min and immediately loaded (5 h intervals) on a 0.8 mm MDE gel (Flowgen). Electrophoresis was performed in 0.6% TBE at 20 W for 18 h at room temperature. Products were visualised by standard silver staining (Jones et al, 1997). PCR products of samples displaying variant band patterns were sequenced using either the Sequenase PCR Product Sequencing kit (Amersham) or the Thermosequenase cycle sequencing kit (Amersham).

Reverse-transcription (RT)-PCR

Synthesis of the first strand cDNA, was performed on 50 ng of total RNA using the Superscript™II kit (Life Technologies). PCR was performed using 1 µl of the first strand cDNA product as a template. To assess expression of *TSC1*, the primers TS1RTX14F (5′-TGGATTCTGCAAGACCATGT-3′) and TS1RTX14R (5′-CTGCTGTGGTGATCTCAGAAA-3′) from exon 14 were used, for *TSC2* the primers TS2RTX14F (5′-TGCTCATCAACAGGC AGTTC-3′) and TS2RTX14R (5′-GCCACATCCCTTTCTTCCA-3′) from exon 14 and TS2RTX41F (5′-CACCGATATCTACCC-CTCCA-3′) and TS2RTX41R (5′-GACAGGCAATACCGTCCA AG-3′) from exon 40 were used.

RESULTS

38 of the 47 RCC's were informative for at least one marker in the *TSC1* region and seven showed LOH (Tables 1 and 2). 42 tumours were informative for one marker or more in the *TSC2* region and

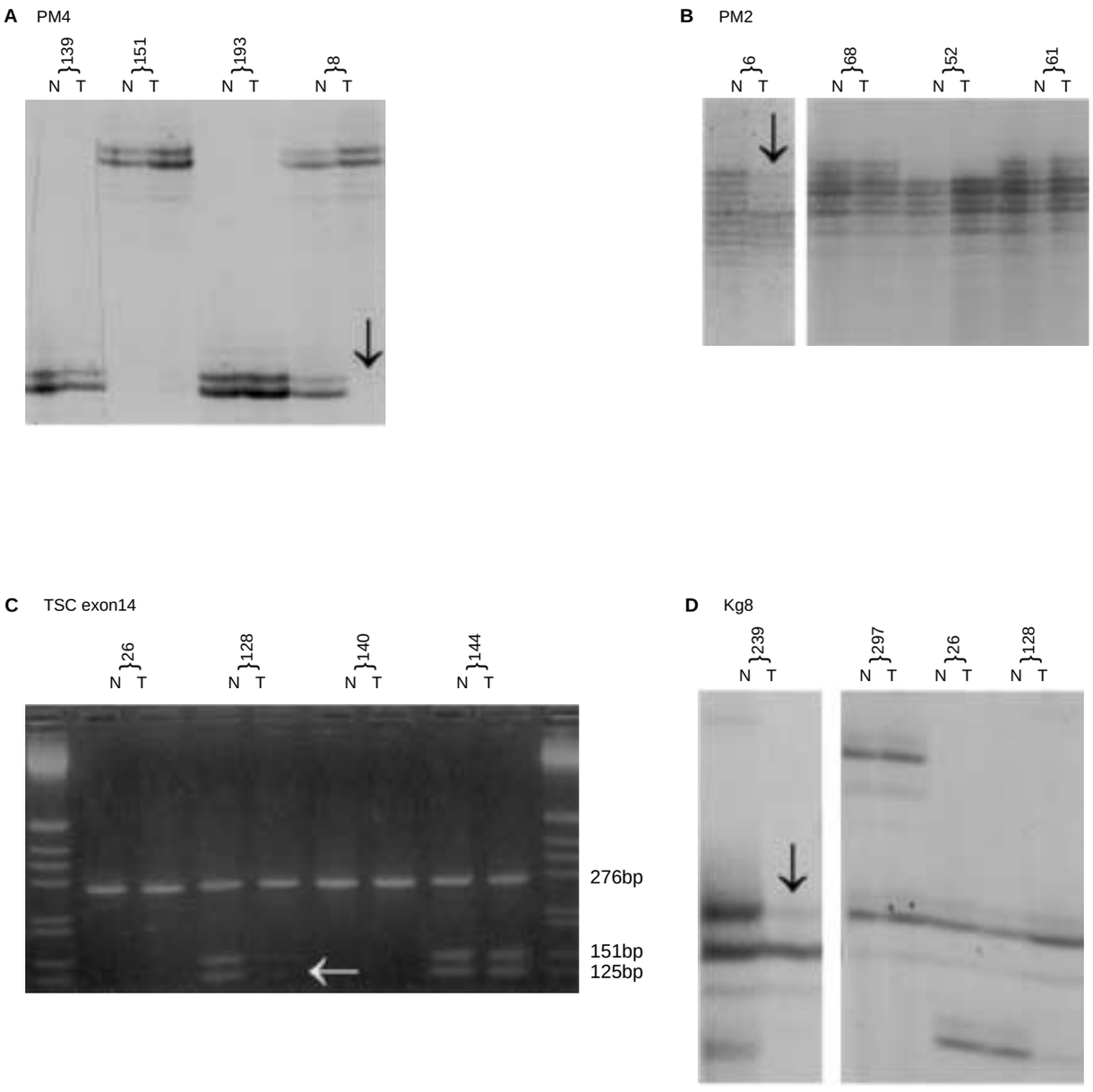

Figure 1 Representative genotypes and examples of loss of heterozygosity. (**A**) autoradiograph showing LOH at marker PM4 (*TSC1* locus) in tumour 8. (**B**) autoradiograph showing LOH at marker PM2 (*TSC1* locus) in tumour 6. (**C**) ethidium bromide stained agarose gel showing LOH at the *TSC1* exon 14 polymorphism, E445 (1556 A >G) in tumour 128. (**D**) autoradiograph showing LOH at Kg8 at the *TSC2* locus in tumour 239

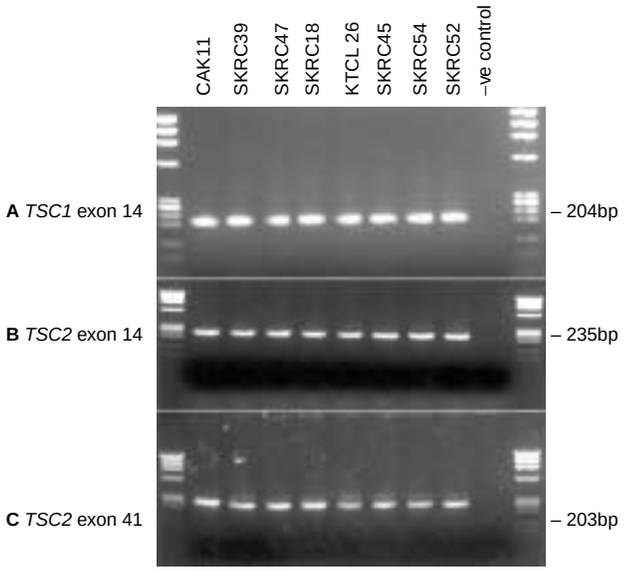

Figure 2 RT-PCR amplification of *TSC1* and *TSC2* transcripts from 8 clear cell RCC cell lines. Expected product sizes are indicated on the right hand side. No RNA was added to the RT-PCR for the negative control lane

one showed LOH (Tables 1 and 2). Of the 33 tumours informative for markers in both regions 8 showed LOH at one locus (Tables 1 and 2, Figure 1). SSCP analysis of all known coding exons (exons 3–23) and flanking intronic DNA of *TSC1* in the 7 RCCs that displayed LOH in the *TSC1* region revealed an aberrant conformer in one case, 297. However, sequencing showed this to result from a constitutional 2 bp deletion in intron 15 at bp 2218 + 71 that was considered likely to represent a non-pathogenic polymorphism. SSCP analysis in tumour 128 confirmed LOH, since one allele at a constitutionally heterozygous polymorphism in exon 14 was lost but no other mutations were detected. SSCP analysis of the coding exons 1–41 of *TSC2* in tumour 239, that showed LOH at the *TSC2* locus, did not reveal any aberrant conformers.

RT-PCR of total RNA from each of 8 independent clear-cell RCC cell lines confirmed expression of both *TSC1* and *TSC2* (Figure 2).

DISCUSSION

Our data do not support a frequent role for *TSC1* or *TSC2* inactivation in sporadic clear-cell (with or without VHL gene inactivation) or non-clear-cell RCC. Although LOH was observed in 5 of 20 clear-cell tumours and 3 of 13 non-clear-cell tumours informative at both the *TSC1* and *TSC2* loci, apparently random allelic loss at similar frequencies has been reported in malignant tumours of the colon, breast and pancreas (Vogelstein et al, 1989; Seymour et al, 1994; Radford et al, 1995). The lack of detectable 'second hits' affecting the retained *TSC1* or *TSC2* allele makes the biological relevance of LOH difficult to assess, as this frequently involves large genomic regions that include many genes of potential importance in tumourigenesis. It remains possible that the retained *TSC1* or *TSC2* alleles in some of the RCCs studied may have been inactivated by mutations that escaped detection, such as whole exon deletions or by epigenetic mechanisms such as promoter methylation. However, expression of both *TSC1* and *TSC2* was confirmed by RT-PCR analysis in each of 8 clear-cell RCC cell lines, ruling out biallelic inactivation of either gene by such mechanisms in these cases.

Although RCC does occur in TSC it may be overdiagnosed. The histological appearances of angiomyolipoma are very variable and some lesions could be mistaken for atypical RCC (Pea et al, 1998). However, careful histopathological assessment in a number of cases showing clear cell, granular, papillary and anaplastic morphology indicate that the association between TSC and RCC is real (Robertson et al, 1996; Henske et al, 1998). Detailed immunohistochemical analysis of 6 TSC-associated RCCs has pointed to immunophenotypic differences from the majority of sporadic RCC, since 4 tumours (all showing regions of anaplastic 'spindle-cell' morphology) displayed immunoreactivity with HMB-45, a marker that also stains TSC-associated angiomyolipomatous and lymphangioleiomyomatomatous lesions, but did not show immunoreactivity for cytokeratin antibodies that are characteristically strongly positive in RCC (Robertson et al, 1996). These differences may reflect alternative molecular mechanisms in renal carcinogenesis in TSC-associated and sporadic RCC. Further molecular characterisation of TSC-associated RCC should clarify whether this is indeed the case.

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