Urol Res (1992) 20:121–126

Urological Research

Detection of a rare point mutation in Ki-*ras* of a human bladder cancer xenograft by polymerase chain reaction and direct sequencing

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Accepted: July 1, 1991

Summary. This paper represents the first report of a codon 59 mutation in Ki-*ras* from a spontaneous human transitional cell carcinoma of the bladder. Point mutations have the potential to activate the *ras* genes if they occur in critical coding regions. These include the sequences of codons 12, 13, 59, 61 and 63. Mutations in codons 12, 13 and 61 have been reported in a wide variety of human cancers, including transitional cell carcinoma of the bladder. However mutations in codon 59 have been reported only in retroviral Ki-*ras* and as a result of in vitro mutagenesis experiments. We have used the polymerase chain reaction and direct sequencing to detect mutations of Ki-*ras*, and allele-specific restriction analysis to detect mutations of N-*ras* in xenografts and continuous cell lines established from bladder cancer biopsies of ten different patients as well as in direct biopsy specimens from five human bladder tumours. For studies of Ki-*ras,* a 139 bp fragment which spanned the critical codons 12 and 13 and a 128 bp fragment that spanned the sequences of codon 59, 61 and 63 were enzymatically amplified and then sequenced. No N-*ras* mutations were detected. A heterozygous mutation of Ki-*ras* at codon 59 GCA → G/ACA was detected in one line. This mutation is being expressed and appears stable as it was detected over several xenograft passages and was present in paraffin-embedded tissue from the primary tumour of the patient. The biological significance of the mutation in bladder cancer is currently under study.

Key words: Transitional cell carcinoma – Xenografts – Human bladder cancer – *ras* mutations – Polymerase chain reaction – Direct sequencing

Mutations of N-*ras,* Ki-*ras* and H-*ras* genes have been detected in different bladder cancers [1]. In this project, the role of N-*ras* and Ki-*ras* oncogenes in a series of xenografts and cell lines derived from human bladder cancers has been investigated. Both genes are members of the *ras* proto-oncogene family, which produce a 21 kDa protein that functions as a signal transducer of mitogenic signals [4]. Mutations within critical regions of the coding regions, including codons 12 and 13 in exon 1 and codons 59, 61 and 63 in exon 2 [1, 26], can lead to oncogene activation. While mutations in codons 12, 13 and 61 have been reported in a wide variety of human malignancies [13], the occurence of codon 59 mutations has been limited to retroviruses and in vitro mutagenesis experiments [1].

The recent development of sensitive methods for the detection of point mutations, such as the polymerase chain reaction (PCR) and the (ASRA) allele-specific restriction analysis technique [27], has made it possible to screen large numbers of samples. Direct sequencing of PCR products allows rapid screening of several critical sequences in one experiment.

We report a rare mutation of codon 59 found in a human transitional cell carcinoma (TCC) xenograft line UCRU-BL-14 and in the primary human tumour tissue from which the cell line was derived, which is stable and is being expressed.

Materials and methods

Cell lines

The ten xenografted cell lines used were all established and characterized in the Urological Cancer Research Unit of Royal Prince Alfred Hospital, Sydney, from different human bladder TCCs. The lines, designated UCRU-BL-13 [22], -17 [20], -23, and -28 (P. J. Russell et al., unpublished) were also established as long-term tissue culture cell lines, while the lines UCRU-BL-14, -15, -19, -21, and -22 were maintained as xenografts in BALB/c nude mice [19, 21]. The features of the original tumours and of the additional patient biopsy samples studied are shown in Table 1.

RNA and DNA preparation

Total RNA or high molecular weight DNA was isolated from finely dissected tumours or tissue culture cells by GITC (Guanidinium isothiocyanate) extraction and $CsCl_2$ purification [29] or by phenol/

Table 1. Pathology of bladder cancer biopsies including those from which xenografts and cell lines were established

Cell line	Pathology	Grade	Stage
UCRU-BL-12	TCC	II	Ta
UCRU-BL-13	TCC	II	T3
UCRU-BL-14[a]	TCC plus signet ring carcinoma	II	T1
UCRU-BL-15	TCC	III	T4
UCRU-BL-17	TCC + Sq + Ad	III	T4
UCRU-BL-19	TCC + Sq	III	T2
UCRU-BL-21	TCC	II	T2
UCRU-BL-22	TCC	II	T1
UCRU-BL-23	TCC	II-III	Tx
UCRU-BL-28[a]	TCC + Ad	II-III	T4
BIOPSY 1	TCC	II	Tx
BIOPSY 2	TCC	II	Tx
BIOPSY 3	TCC	III	Tx
BIOPSY 4	Adenocarcinoma		T3
BIOPSY 5	TCC	II	T3 and Tis

TCC, Transitional cell carcinoma; Sq, squamous carcinoma; Ad, adenocarcinomatous differentiation

[a] Both tumours derived from same patient

chloroform extraction [3] respectively. Integrity and quality of the RNA and DNA were checked by formaldehyde denaturing gel or agarose gel electrophoresis respectively.

Northern analysis

Total RNA (20 µg) was electrophoresed on a formaldehyde denaturing gel, and transferred by capillary action in 20× SSC (175.3 g NaCl, 88.2 g Sodium citrate, in 1 l H_2O, pH 7.0) to hybond N^+ nylon membrane (Amersham). After alkali fixation, the membrane was prehybridized in 5× SSPE (175.3 g NaCl, 27.6 g $NaH_2PO_4 \cdot H_2O$, 7.4 g EDTA in 1 l H_2O, pH 7.4), 0.5% sodium dodecyl sulphate (w/v), 5× Denhardt's solution, 20 µg/ml denatured Herring sperm DNA and 10% dextran sulphate (w/v) and probed with 50 ng of purified Ki-*ras*-2 exon 2 PCR product prepared by random priming (sp. act. $= 2 \times 10^9$ cpm/µg) using "prima gene" kit (Promega Corp., Madison, Wis., USA). Hybridized filters were washed in 2× SSPE, 0.1% SDS at room temperature for 10 min, 1× SSPE, 0.1% SDS 65° C for 10 min, 0.5× SSPE, 0.1% SDS 65°C for 10 min and finally a 0.1% SSPE, 0.1% SDS 65°C for 10 min before autoradiography (on Kodak X-Omat film). RNA dot blots were made by dotting serial dilutions of total RNA in 20× SSC onto Hybond N^+ in a vacuum dot blot apparatus. RNA was fixed to the membrane by alkali and hybridized under conditions described above. Densitometric analyses were performed to determine the relative expression of Ki-*ras* by the different tumour samples.

Polymerase chain reaction

A modified standard PCR reaction [22] was used. Genomic DNA (0.5–1.0 µg) was added to 100 µl of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin (w/v) 200 µM dATP, dCTP, dGTP and dTTP, 100 pmol of each specific primer (amplimer) and 2.5 units of DNA *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn., USA). For exon 1, the primers used were: 5′ TTA TTA TAA GGC CTG CTG AA; 3′ CAA GAT TTA CCT CTA TTG TT, to obtain a PCR product of 138 bp spanning codons 1–42. For exon 2, primers were: 5′ GGA GAA ACC TGT CTC TTG G, and 3′ primer C TTC AAA TGA TTT AGT ATT ATT TAT GGC, for a 128 bp product spanning codons 48–91. White mineral oil (50 µl) was layered on top of the reaction mix to prevent evaporation during cycling. All reactions were denatured for 3 min at 94°C before 35 cycles of annealing/extension (49°C for 50 s, exon 1 and 62°C for 50 s, exon 2) and denaturation (94°C for 30 s, exons 1 and 2) and were carried out using a Hybaid Intelligent Heat Block (Integrated Sciences, Sidney, Australia). "No DNA" control reactions were run concurrently with all amplifications.

Formalin-fixed tissue (30-µm section) was used as a template for PCR [24] after deparaffinization by sequential extraction with 1 ml of xylene followed by centrifugation,then 500 µl of 95% ethanol followed by repelleting, and finally dessication in a dessicating centrifuge to remove residual solvent. The remaining material was added to a standard 100 µl PCR reaction.

Amplification of RNA required addition of total RNA (100 ng) to a standard PCR reaction mix minus the *Taq* polymerase and the amplimers. Digestion of possible contaminating genomic DNA was carried out with 20 units of RNasin and 0.5 µl of DNase (Boehringer Mannheim) at 37°C for 30 min. DNase was heat inactivated by incubation at 95°C for 5 min before adding 100 pmol of each amplimer. After cooling, the reaction was incubated at 37°C for 30 min with 10 units of reverse transcriptase (New England Biolabs, Beverly, Mass., USA), the activity of which was stopped by heat denaturation (95°C for 5 min). DNA *Taq* polymerase (2.5 units) was then added and the reaction mix was treated as a normal PCR reaction. After one PCR reaction, a partially non-specific PCR product was visible. Then 0.5 µl of the first PCR product was used in a second reaction to amplify a specific product.

Direct sequencing

PCR products were purified by centricon 30 micro-concentrators (Amicon, Danvers, Mass., USA) to remove unused components (NTPs and amplimers), dried down in a desiccating centrifuge and stored at −20°C until required. Approximately half the PCR product was used in the sequencing reaction. Template DNA diluted to 5 µl was mixed with 2.5 pmol of ^{32}P-radiolabelled amplimer previously end-labelled using T_4 polynucleotide kinase and ^{32}P dATP. This mixture was then heated to 95°C for 5 min and was then snap chilled in a dry ice/ethanol bath for 10 s. Then 2 µl of 5× Sequenase buffer was added to the annealed mixture and the recommended sequencing procedure for Sequenase (USB, United States Biochemicals, San Diego, Calif., USA) was used except that no labelling step was included. The sequencing reactions were then electrophoresed on 8% denaturing acrylamide gels (7 M urea) and finally autoradiographed on Kodak X-Omat film.

Allele-specific restriction analysis

DNA samples from each of the bladder cancer specimens listed in Table 1 were screened for mutations in codons 12, 13 and 61 on N-*ras* by the ASRA technique [27]. This method involves PCR amplifications using a series of mismatched primers, each of which introduces appropriately positioned base substitutions in N-*ras* to create a restriction site provided the adjacent sequence is normal. Resistance of the amplified product to digestion indicates the presence of a mutation in the original template. The primer sequences and the conditions used were identical to those described by Todd and Iland [27].

Results

PCR and direct sequencing analysis for Ki-*ras* mutations in exons 1 and 2 was carried out on DNA samples of human bladder xenografts and cell lines derived from 10 patient tumours and on 5 biopsy specimens derived from other patients' tumours. One confirmed heterozygous

Table 2. Summary of mutations found at critical coding regions of Ki-*ras* in UCRU xenografts and cell lines: Codons with reported transforming potential are listed across the table

Line UCRU-BL-	Codon				
	12	13	59	61	63
12	GGT	GGC	GCA	CAA	GAG
13	---	---	---	---	---
14[a]	---	---	ACA	---	---
14/0	---	---	ACA	---	---
14/5	---	---	ACA	---	---
14 RNA	---	---	ACA	---	---
15	---	---	---	---	---
17	---	---	---	---	---
19	---	---	---	---	---
21	---	---	---	---	---
22	---	---	---	---	---
23	---	---	---	---	---
28	---	---	---	---	---
B1[b]	ND	ND	---	---	---
B2[b]	ND	ND	---	---	---
B3[b]	ND	ND	---	---	---
B4[b]	ND	ND	---	---	---
B5[b]	ND	ND	---	---	---

ND, Not done
[a] Paraffin-fixed original tumour, from which xenograft line was derived
[b] Human bladder cancer biopsy sample

mutation was detected in the tumours studied. UCRU-BL-14 contained a single base substitution in the first position of codon 59 (GCA → G/ACA) resulting in an amino acid substitution of alanine to threonine, and this mutation was maintained through several xenograft passages (Table 2). It was not homozygous as both an A and G band were present on sequencing autoradiographs (Fig. 1).

Analysis of paraffin-embedded samples showed that the codon 59 mutation was also present in the original human tumour tissue from which the UCRU-BL-14 line was established (Fig. 1C). As these experiments required a secondary PCR, which is vulnerable to minute levels of contamination, a "no DNA" control was used to confirm the absence of contamination.

Expression of the mutation was determined from PCR off cesium chloride ($CsCl_2$) purified, DNased total RNA. Direct sequencing of a modified PCR product of UCRU-BL-14/5 RNA showed that both mutant and normal Ki-*ras* were being expressed (Fig. 1D). The relative levels of mutant and normal transcripts cannot be quantified from sequencing autoradiographs and require further investigation.

Contamination with mutant sequences or homologues of the target gene can lead to false positives from PCR experiments. However, sequence data from every sample failed to show the presence of other Ki-*ras* homologues, including human pseudo Ki-*ras*-1 gene and viral Ki-*ras* (Table 3). Moreover, since the pseudogene, but not functional human Ki-*ras*-2 gene contains a restriction site for *Mbo*II, the PCR product of UCRU-BL-14/0 was digested with *Mbo*II, and electrophoresed on a 5% Nusieve agarose gel. When a gel stab of the undigested band was used as a template for a second PCR, the mutation was still present upon sequencing.

Southern analysis of the ten lines failed to show any gross rearrangements, deletions or amplifications of the Ki-*ras* gene (data not shown). Northern and RNA dot blot analysis, however, showed that Ki-*ras* was grossly overexpressed in UCRU-BL-14 (Fig. 2). Estimates from RNA dot blots suggest that there were approximately 350 copies of Ki-*ras* message per cell as compared to the reported 20–50 transcripts per cell in other bladder lines [10].

The lines, UCRU-BL-14 and -28, were derived from different tumours obtained 4 years apart from the same patient. UCRU-BL-14 was grown from a biopsy specimen obtained prior to chemotherapy which contained two tumours in juxtaposition, a TCC grade II and a signet ring carcinoma. Only the TCC grew in the nude mice as described previously [19]. The UCRU-BL-28 line was established from a highly invasive tumour (T4) containing elements of adenocarcinoma which had recurred in the same patient after previously successful treatment with cisplatin and radiotherapy. Despite the similar origin of

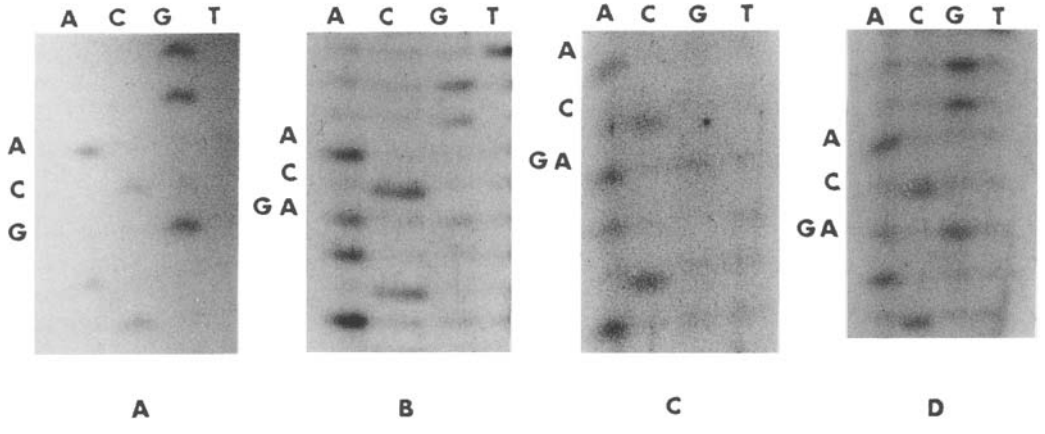

Fig. 1A–D. Autoradiographs of sequencing gels of polymerase chain reaction (PCR) products off DNA (**A–C**) or reverse transcribed RNA (**D**) loaded from left to right, ACGT. **A** Wild-type sequence (GCA) of codon 59 (UCRU-BL-29); **B** codon 59 Ki-*ras* mutation (G/ACA) in UCRU-BL-14 xenograft; **C** codon 59 Ki-*ras* mutation in DNA derived from deparaffinized original tumour tissue; **D** codon 59 Ki-*ras* mutation expressed in RNA of UCRU-BL-14/0 xenograft

Table 3. Nucleotide sequences for the second exon of HKi-*ras*-2 gene and homologues

hKi-*ras* 2:	GTA	ATT	GAT	GGA	GAA	ACC	TGT	CTC	TTG	GAT
hKi-*ras* 1:	---	---	---	---	---	---	---	---	---	---
mKi-*ras*:	---	---	---	---	---	---	---	---	---	---
vKi-*ras*:	---	---	---	---	---	---	---	---	---	---
					(59)		(61)		(63)	
hKi-*ras*2:	ATT	CTC	GAC	ACA	GCA	GGT	CAA	GAG	GAG	TAC
hKi-*ras*1:	---	--T	---	---	A--	---	---	--A	---	---
mKi-*ras*:	---	---	---	---	---	---	---	---	---	---
VKi-*ras*:	---	---	---	---	A--	---	---	---	---	---
hKi-*ras*2:	AGT	GCA	ATG	AGG	GAC	CAG	TAC	ATG	AGG	ACT
hKi-*ras*1:	-A-	---	---	---	*--	---	---	---	---	---
mKi-*ras*:	---	---	---	---	---	---	---	---	--A	---
vKi-*ras*:	---	---	---	---	*--	---	---	---	--A	---
hKi-*ras*2:	GGG	GAG	***	***	GGC	TTT	CTT	TGT	GTA	TTT
hKi-*ras*1:	---	-G-	GGG	GGG	---	---	---	---	---	---
mKi-*ras*:	---	---	***	***	---	---	---	---	---	---
vKi-*ras*:	---	---	***	***	---	---	---	---	---	---
hKi-*ras*2:	GGC	ATA	AAT	AAT	ACT	AAA	TCA	TTT	GAA	GAT
hKi-*ras*1:	---	---	---	---	-T-	---	---	---	---	---
mKi-*ras*:	---	---	---	---	---	---	---	---	---	---
vKi-*ras*:	---	---	---	---	---	---	---	---	---	---

Nucleotide sequences for the second exon of hKi-*ras*-2 and corresponding sequences from the Ki-*ras* homologues: human Ki-*ras* pseudogene (hKi-*ras*-1) [15], murine Ki-ras (mKi-ras) [11] and the Kirsten murine sarcoma viral Ki-ras (vKi-ras) [28]. Regions of homology are marked by dashes while deletions are marked by an asterisk

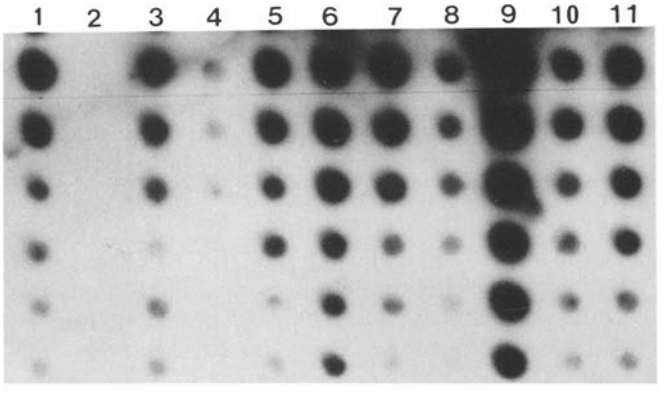

Fig. 2. Total RNA dot blot of UCRU-BL cell lines probed with random primed Ki-*ras* exon 2 PCR product; from left to right: *1* double serial dilution of PCR derived exon 2 of Ki-*ras* (10 pg – 0.3 pg); *2* herring sperm DNA, *3*, UCRU-BL-23; *4*, UCRU-BL-22; *5*, UCRU-BL-21; *6*, UCRU-BL-19; *7*, UCRU-BL-17; *8*, UCRU-BL-15; *9*, UCRU-BL-14; *10*, UCRU-BL-13; *11*, UCRU-BL-12

these two tumours, repeated PCR and sequencing of the second exon of Ki-*ras* in UCRU-BL-28 has failed to reveal any mutation of the Ki-*ras* gene.

No mutations in codons 12, 13 or 61 of N-*ras* were detected in the bladder cancer cell lines and biopsies listed in Table 1 when screened using the ASRA technique (results not shown).

Discussion

A point mutation in a *ras* gene was first detected in a human bladder cancer cell line, T24 [25]. Subsequent efforts to demonstrate *ras* gene mutations have shown that they occur relatively infrequently in spontaneous human bladder tumours ([13]; also reviewed in [23]). However, a recent study [6] has described mutations of codon 12 of the H-*ras* gene in tissue from 2 of 13 diploid and 10 of 20 aneuploid bladder tumours. These authors suggested that the presence of *ras* mutations may identify aggressive variants of urothelial carcinomas.

This paper describes the occurrence of a rare mutation in codon 59 of the Ki-*ras* gene in a spontaneous human tumour and in a xenografted line, UCRU-BL-14, derived from this tumour. Amplification of the Ki-*ras* pseudogene in these studies was ruled out by the stringent conditions of the reaction, and by studies using an *MBo*II restriction site present in the pseudogene. The codon 59 mutation is not likely to play a common role in bladder cancer, since it has been detected in only 1 of 15 tumours analysed (Table 2) including 10 bladder cancer xenograft or cell lines and 5 biopsies of patient material. Similarly, no N-*ras* mutations were detected by the ASRA technique in any of the tumours analysed.

The presence of a codon 59 Ki-*ras* mutation in a primary human tissue sample suggests that it is not a result of instability of the xenografted BL-14 line while being maintained in nude mice. In contrast, the mutation has been stably maintained over serial passages of the tumour through nude mice. Previous studies on the instability of

ras genes in tissue culture cell lines have shown that point mutations can be accumulated during serial passage in vitro [12]. The presence of the codon 59 Ki-*ras* mutation in primary tumour tissue (Fig. 1C) also refutes the possibility of murine or viral Ki-*ras* homologue contamination.

The absence of a codon 59 mutation in the cell line, UCRU-BL-28, may be explained in several ways. Firstly, it is possible that the UCRU-BL-28 line, which shows predominantly adenocarcinomatous differentiation, was derived from the signet ring carcinoma in the patient, which had failed to "take" in the nude mice when the line UCRU-BL-14 was established. Alternatively, the UCRU-BL-28 tumour may have been completely independent of the tumour from which UCRU-BL-14 was derived or UCRU-BL-28 may have arisen from a subpopulation of UCRU-BL-14 which was wild type with respect to Ki-*ras* and which survived radiotherapy and chemotherapy. The loss of putative transforming mutations in relapsed patients following clinically induced remission has been reported in studies of leukaemic patients [8]. It is possible that therapy is providing a selective pressure that leads to the emergence of smaller resistant subpopulations which do not bear this mutation. Our findings on the mutation in Ki-*ras* are in contrast to those in the H-*ras* gene described above [6], in which *ras* mutations appeared to be more likely to occur in aneuploid, and therefore aggressive tumours. The UCRU-BL-14 line (Ki-*ras* 59 mutation) was derived from a diploid tumour, whilst the UCRU-BL-28 line (no Ki-*ras* mutation) was from a multiply aneuploid, stage T4 tumour.

Recently, a codon 59 mutation in the N-*ras* gene has been described in leukaemia [18], but the role of this mutation has not yet been elucidated.

In vitro mutagenesis of Ha-*ras* at codon 59 (GCA to ACA) causes transformation of NIH3T3 cells (based on the NIH3T3 foci forming assay) [9]. In addition, *Thr 59* mutant p21 displays autophosphorylating abilities and this can affect GTP binding [16]. Studies are currently underway to determine whether the *Thr 59* mutant p21 expressed by UCRU-BL-14 xenografts is also autophosphorylating. Recent circular dichroism studies have shown that P21 protein bearing a Thr 59 mutation is less structured than the normal protein [30]. Finally, *Thr 59* mutants display a high level of nucleotide interchange [14]. While several functional abnormalities are present in *Thr 59* mutant p21, the origin of its transforming activity is unknown.

Given that tumorigenesis is a multi-step process requiring the steps of initiation and potentiation at a molecular level, the presence of a transforming codon 59 mutation and overexpression of Ki-*ras* (Fig. 2) in UCRU-BL-14 suggests that this may have been the activating event in this tumour. Experiments to investigate the tumorigenic effect of Ki-*ras* gene containing a Ki-*ras* 59 codon mutation in immortalized urothelial cells are now underway.

Direct sequencing of PCR products confers several advantages over other methods for the detection of mutations. Firstly, large regions of sequence can be analysed in one experiment. If RNA had been used as the template it would have been possible to screen the coding regions in exons 1 and 2 in Ki-*ras* from one reaction. While direct sequencing and oligonucleotide probing of PCR products have limits of detection of approximately 10% and 5% respectively [2, 17], direct sequencing shows whether a mutation is homozygous or heterozygous and is not limited by multiple mutations that are in close proximity [5]. It is also important to note that direct sequencing is necessary when studying PCR products as the sequencing of cloned PCR products is prone to artefacts caused by DNA polymerase error [7].

This is the first report of a codon 59 mutation in Ki-*ras* from a spontaneous human transitional cell carcinoma of the bladder. While this mutation does not appear to play a major role in the tumorigenesis of this disease, this work shows that analysis of codon 59 in *ras* genes is warranted. Since the majority of studies reported on tumour samples have been done by PCR and allele-specific oligonucleotide hybridization, it is likely that codon 59 mutations in the *ras* gene family may go undetectable. As the development of direct sequencing and PCR continues, it is possible that the reported occurrence of this mutation will increase.

Acknowledgements. We should like to thank Ms Alison Todd, Kanematsu Laboratories for providing us with the primers used for N-*ras* analysis by the ASRA technique, and for assistance with the use of this technique.

This work was supported by a grant from the National Health and Medical Research Council, Australia.

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