Mutations of K-ras oncogene in human adrenal tumours in Taiwan

S-R Lin¹, JH Tsai², YC Yang¹ and SC Lee¹

Departments of ¹Clinical Pathology, and ²Internal Medicine, Kaohsiung Medical College, Kaohsiung, Taiwan

Summary Recently, we have found a high frequency of p53 gene mutations in human functional adrenal tumours. As the tumorigenesis is a multigene defect, we believe that other oncogenes may also be involved in the initiation or progression of adrenal tumours. Using the single-strand conformational polymorphism (SSCP) method, we chose the ras oncogenes as the target in this screening procedure because their high mutation rates were detected in thyroid tumours. For the ras oncogenes analysed, exon 1 to exon 2 of H-ras and K-ras genes in the tumour tissues of 13 Conn's syndrome, two adrenal Cushing's syndrome, two non-functional adrenal tumours, one adrenocortical hyperplasia and eight phaeochromocytomas and its paired adjacent normal adrenal tissues were amplified and sequenced. No mutations were detected in the H-ras gene. But mutations of the K-ras gene were detected in 46% (6 of 13) of Conn's syndrome; the hot spots were located at codon 15, 16, 18 and 31, which were different from those previously found in other tumours (codon 12, 13 and 61). Northern blot analysis with 1.1 kb K-ras cDNA revealed that K-ras mRNA was more than tenfold over-expressed in four of Conn's syndrome, one case of Cushing's syndrome and one case of adrenocortical hyperplasia. The mutation sites and mutation type were not found in other tissues, which conferred that this was highly related to adrenocortical tumours. Yet, the correlation between K-ras oncogene and adrenocortical tumours needs to be clarified by further studies.

Keywords: K-ras; adrenal tumour; mutation; Taiwan

Many proto-oncogenes encode proteins that transmit signals that regulate normal cell growth (Cantly et al, 1991). Specific mutations convert these genes into oncogenes (Mark, 1989). Although some frequently targeted oncogenes are common to many different tumour cell types, others are uniquely mutated in particular forms of neoplasm (Barbacid, 1987).

Endocrine neoplasms are the major causes of endocrine disease in human beings. All endocrine organs, such as pituitary gland, thyroid gland, parathyroid gland, adrenal gland, pancreas and gonads, are known to possess the potential to develop neoplasms. Little is known about genetic changes that confer endocrine neoplasms. In 1989, Landis et al (1989) found that 43% pituitary GH-secreting tumours had G-protein mutations. Mutations of ras oncogenes, FTO oncogenes, G-protein and p53 tumour-suppressor genes had been reported in the human thyroid tumours (Eng et al, 1995). There were few studies concerning the oncogenes and the tumour-suppressor genes in the tumorigenesis of functional adrenal tumours. In 1990, Lyon et al found that 3 of 11 adrenal tumours had G-protein mutations. In 1989, Lemoine et al had reported that activation of ras oncogenes occurred at a very high frequency (80%) in a small series of human thyroid follicular carcinomas. A variety of human tumours has been studied for ras gene mutations to date (Bos, 1989). However, little is known about the prevalence or significance of activated ras oncogene in adrenal tumours. Recently, we found a high rate of p53 gene mutation in the functional adrenal tumours.

Table 1 Sex, age, clinical diagnosis, pathology and tumour size of patients studied

| Patient number | Sex | Age | Diagnosis | Pathology | Tumour size |
|----------------|-----|-----|-----------|-----------|-------------|
| 1              | F   | 38  | PA        | CA        | 1.5 x 1.2 x 0.5 |
| 2              | M   | 59  | PA        | CA        | 2.3 x 2.3 x 2.7 |
| 3              | F   | 43  | PA        | CA        | 2.5 x 2.0 x 0.7 |
| 4              | M   | 27  | PA        | CA        | 2.7 x 1.8 x 2.0 |
| 5              | M   | 36  | PA        | CA        | 1.1 x 1.0 x 1.0 |
| 6              | M   | 63  | PA        | CA        | 5.5 x 4.7 x 3.4 |
| 7              | F   | 42  | PA        | CA        | 1.6 x 1.5 x 0.9 |
| 8              | F   | 33  | PA        | CA        | 2.0 x 2.0 x 0.5 |
| 9              | M   | 41  | PA        | CA        | 1.8 x 1.5 x 0.9 |
| 10             | M   | 44  | PA        | CA        | 6.5 x 3.5 x 3.6 |
| 11             | M   | 43  | PA        | CA        | 2.0 x 1.5 x 1.2 |
| 12             | M   | 59  | PA        | CA        | 2.2 x 1.2 x 1.2 |
| 13             | F   | 37  | PA        | CA        | 2.2 x 1.9 x 0.6 |
| 14             | F   | 21  | CS        | CA        | 3.0 x 2.7 x 2.7 |
| 15             | F   | 35  | CS        | CA        | 4.0 x 3.5 x 2.5 |
| 16             | M   | 59  | NFA       | CA        | 4.5 x 4.0 x 4.0 |
| 17             | F   | 49  | NFA       | CA        | 4.2 x 3.6 x 4.0 |
| 18             | F   | 40  | PA        | CH        |             |
| 19             | M   | 53  | Phaeochromocytoma | 5.2 x 3.3 x 5.0 |
| 20             | F   | 36  | Phaeochromocytoma | 8.6 x 5.4 x 3.7 |
| 21             | F   | 30  | Phaeochromocytoma | 5.6 x 4.0 x 5.0 |
| 22             | F   | 49  | Phaeochromocytoma | 9.8 x 9.0 x 9.0 |
| 23             | M   | 36  | Phaeochromocytoma | 6.0 x 4.0 x 4.0 |
| 24             | F   | 41  | Phaeochromocytoma | 5.0 x 5.0 x 3.5 |
| 25             | F   | 47  | Phaeochromocytoma | 6.4 x 5.5 x 7.0 |
| 26             | F   | 49  | Phaeochromocytoma | 8.4 x 5.5 x 4.0 |

PA, primary aldosteronism; CS, Cushing's syndrome; NFA, non-functioning adrenal tumour; CA, adrenocortical adenoma; CH, adrenocortical hyperplasia; *Malignant, with liver metastases.
(Lin et al, 1994; 1996). As the tumorigenesis is a multigene defect (Knudson, 1989), we believe that other oncogenes may also be involved in the initiation or progression of adrenal tumours, especially the ras oncogenes. As the ras p21 protein is involved in signal transduction, activated ras oncogenes would likely be involved in simultaneous stimulation of cell growth as well as hormone synthesis and secretion. Hence, we took the H-ras and K-ras genes of 26 collected cases with functional adrenal tumours, including adrenocortical tumours and adrenomedullary phaeochromocytoma as the first target genes analysed. We also collected two non-functional adrenal tumours as a control, which were not easy to get because they were not easy to find. The control specimens were too few in number to be of any significance; however, they can provide a foundation for functional adrenal tumour study. To clarify the role of the ras oncogene in the tumorigenesis of human functional adrenal tumours, we performed molecular studies in 26 adrenal tumour tissues. The Northern blot analysis with K-ras cDNA demonstrated that four cases of Conn’s syndrome, one case of Cushing’s syndrome and one case of phaeochromocytoma had K-ras mRNA overexpression in the tumour cells. Using polymerase chain reaction single-strand conformational polymorphism (PCR-SSCP), cloning and sequencing, a high frequency of K-ras gene mutations was found in adrenocortical tumours.

**MATERIALS AND METHODS**

**Patients and tissues**

Twenty-six adrenal tumours and their remnant non-tumorous adrenal tissues were obtained from patients who underwent surgery for adrenal lesions. These included 13 patients with primary aldosteronism due to unilateral cortical adenoma, two patients with adrenal Cushing’s syndrome with a single adrenocortical adenoma, one patient with adrenocortical hyperplasia, eight patients with adrenal phaeochromocytoma, and two patients with non-functional adrenal tumour. The clinical data and the tumour sizes are listed in the Table 1.

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**Figure 1** PCR-SSCP analysis of K-ras mutations in human adrenal neoplasms. Representative samples including cases 1–6 and 10–22 are shown for a 128-bp fragment of exon 1 containing codon 12 and 13 (A), and cases 3–11 are shown for a 111-bp fragment of exon 2 containing codon 61 (B). An electrophoretic mobility shift of the bands differs between the tumour (T) and its paired normal tissues (N), representing a different conformer of the fragment and suggesting the presence of mutations in this fragment. No electrophoretic mobility shift over the samples tested suggests normal conformations over these samples. P is the positive control of K-ras codon 12 mutant derived from SW480 human colon adenocarcinoma cell line
DNA extraction

Genomic DNA was extracted from adrenal tumours and the paired adjacent normal adrenal gland tissues by proteinase-K (Stratagene, La Jolla, CA, USA) digestion and then phenol–chloroform extraction according to Sambrook’s method (Sambrook et al, 1989).

PCR-SSCP analysis

To search the mutations of the K-ras gene using PCR-SSCP analysis, two sets of primers including codon 12, 13 and 61 were used and are described below: 5'-CTGGTTCCGCGCCACTGACG- GAATATAGCCTGGT-3' (forward) and 5'-CTCGCTCGCC- CACGCCAGCTCACCCTATC-3' (reverse) for codon 12 and 13 of H-ras gene, which amplify a 148-bp PCR product of codon 1–7; 5'-CTGGTTCCGCGCCACTGACG- GAATATAGCCTGGT-3' (forward) and 5'-CTCGCTCGCC- CACGCCAGCTCACCCTATC-3' (reverse) for codon 12 of the K-ras gene, which amplify a 127-bp PCR product of codon 38–73; 5'-ATGACTGAA- TATACATCTGT-3' (forward) and 5'-CTCTATTTGATGTCG- TATC-3' (reverse) for codon 12 and 13 of the K-ras gene, which amplify a 128-bp PCR product of codon 1–37; 5'-TTCC- TACAGGAAGCAGTTAG-3' (forward) and 5'-CACAAA- GAAAGCCTCCCA-3' (reverse) for codon 61 of K-ras gene, which amplify a 111-bp PCR product of codon 38–81. The reaction mixture contained 50 pmol of each primer, 2.5 U of Taq DNA polymerase (Boehringer Mannheim), 100 mmol L⁻¹ each of

Figure 3. DNA blot analysis of the K-ras transcripts in adrenal tumours and paired normal adrenal tissues. Twenty micrograms of total RNA were electrophoresed, blotted and hybridized to a 32P-labelled 0.4 kb S1–EcoRI fragment of K-ras cDNA and rehybridized with a 700-bp PstI fragment of β-actin probe to correct for differences in loading (A). The signals on the autoradiographs were scanned with a Molecular Dynamic computing laser densitometer and MD ImageQuant software release version 3.0 (B)
deoxy-NTTP, [α-32P] deoxy-CTP (3000 Ci mmol⁻¹; 10 mCi ml⁻¹; New England Nuclear Research Products, Boston, MA, USA), 1.5 mmol l⁻¹ magnesium chloride, 50 mmol l⁻¹ potassium chloride, 10 mmol l⁻¹ Tris-HCl (pH 8.3), and gelatin at 10 µg ml⁻¹. A programmable thermal cycler (PTC-100, MJ Research, Watertown, MA, USA) was used to perform 40 cycles of denaturation for 30 s each at 94°C and annealing for 30 s at 55°C with an extension for an additional 1 min at 72°C. The final extension time was 7 min at 72°C. The PCR products were analysed in 8% denatured polyacrylamide gels.

**Cloning and sequencing analysis**

Amplified DNA was desalted and primers were removed by gel filtration with CL-6B Sepharose spin column (Pharmacia LKB Biotechnology, Sweden) and ethanol precipitation. The purified DNA was inserted into pDIRECT vector (Clontech Laboratories, Palo Alto, CA, USA). This material was the template for the dideoxy sequencing using MultiPol DNA Sequencing System (Clontech).

[α-32P] Deoxy-ATP (New England Nuclear Research Products) was used to label sequencing reactions. Plasmid DNA was prepared from isolated colonies using the alkaline lysis method. Double-stranded plasmid DNA was sequenced using T7 and T3 promoter sequences as sequencing primers. The accuracy of our sequencing data was confirmed by analysis of ten independent clones.

**Northern blotting**

Twenty micrograms of total RNA was denatured with 6.5% formaldehyde. The gels were blotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The filters were hybridized with a random primed, 32P-labelled, 0.4 kb *Stul–EcoRI* fragment of K-ras cDNA (Capon et al, 1983). The hybridized filters were then washed in 30 mmol l⁻¹ sodium chloride, 3 mmol l⁻¹ sodium citrate, and 0.1% sodium dodecyl sulphate (at 65°C) and autoradiographed. The membrane was rehybridized with a 700-bp PsiI fragment of β-actin probe to correct for differences in loading. The signals on the autoradiography were scanned with a Molecular Dynamics (Sunnyvale, CA, USA) computing laser densitometer and MD ImageQuant software release version 3.22.

**RESULTS**

A total of 13 primary aldosteronism, two Cushing’s syndrome, one adrenocortical hyperplasia, two non-functional adrenal tumours and eight adrenal medullary pheochromocytomas were selected for analysis. Age, sex and tumour size of all patients analysed are summarized in Table 1. There were 12 men and 14 women, with an age range of 27–63 years. Twenty-four of 26 patients with tumours clinically classified as functioning had detectable hormonal abnormalities; two had no detectable hormonal abnormality classified as non-functioning.

Twenty-six adrenal neoplasms were screened for the presence of activated H-ras and K-ras genes. Seven out of 16 functional adrenocortical adenomas showed an apparent electrophoretic mobility shift of the K-ras gene analysed between the tumour and its paired adjacent normal tissue (Figure 1); no electrophoretic mobility shift was found in the eight pheochromocytomas and two non-functioning adrenal adenomas. An electrophoretic mobility shift between the tumour and its paired normal tissue is characteristic of a mutation. Six out of seven such differences were detected in exon 1 of the K-ras gene containing codon 12 and codon 13 (Figure 1A) and one was detected in exon 2 of the K-ras gene containing codon 61 (Figure 1B). Furthermore, the results from SSCP analysis demonstrated that the K-ras gene mutant types were monoallelic in all cases that showed a normal band and

![Figure 4](image-url) The K-ras mRNA expression in tumour tissues and normal adrenal gland tissues were compared after RNA blot analysis. The results were analysed by detecting signal strength using densitometer scan autoradiographs. The bars represent the signal strength in tumour tissues/the signal strength in normal tissues. The numbers represent the case number.
Table 2  Results of H-ras and K-ras alterations in 26 cases with adrenal tumour

| Patient | SSCP | Codon base | Amino acid | Overexpression (ratio) | SSCP base | Codon | Amino-acid |
|---------|------|------------|------------|------------------------|-----------|-------|------------|
| 1       | +    | 15GCG→ACA | Gly→Thr   | + (4.0)                | –         | N     | –          |
|         |      | 16AAG→GAG | Lys→Glu   |                        | –         | N     | –          |
| 2       | +    | 19TGT→TGG | Leu→Ser   | + (5.6)                | –         | N     | –          |
| 3       | +    | 15GCG→AGC | Gly→Ser   | – (1.8)                | –         | N     | –          |
|         |      | 16AAG→AAA | Lys→Lys   |                        | –         | N     | –          |
| 4       | –    | N         |            | – (1.4)                | –         | N     | –          |
| 5       | –    | N         |            | – (1.2)                | –         | N     | –          |
| 6       | –    | N         |            | – (0.9)                | –         | N     | –          |
| 7       | –    | N         |            | – (0.9)                | –         | N     | –          |
| 8       | +    | 60GCT→TGT | Gly→Cys   | – (1.1)                | –         | N     | –          |
| 9       | –    | N         |            | – (1.1)                | –         | N     | –          |
| 10      | +    | 15GCG→ACG | Gly→Thr   | + (7.5)                | –         | N     | –          |
|         |      | 16AAG→AAA | Lys→Lys   |                        | –         | N     | –          |
|         |      | 18GCC→GTC | Ala→Val   |                        | –         | N     | –          |
|         |      | 31GAA→CAA | Glu→Gln   |                        | –         | N     | –          |
| 11      | –    | N         |            | – (0.9)                | –         | N     | –          |
| 12      | +    | 15GCG→ACG | Gly→Thr   | + (12.0)               | –         | N     | –          |
|         |      | 16AAG→AAA | Lys→Lys   |                        | –         | N     | –          |
|         |      | 18GCC→GTC | Ala→Val   |                        | –         | N     | –          |
|         |      | 31GAA→CAA | Glu→Gln   |                        | –         | N     | –          |
| 13      | –    | N         |            | – (0.8)                | –         | N     | –          |
| 14      | –    | N         |            | – (1.2)                | –         | N     | –          |
| 15      | +    | 15GCG→ACA | Gly→Thr   | + (13.0)               | –         | N     | –          |
|         |      | 16AAG→GAG | Lys→Glu   |                        | –         | N     | –          |
| 16      | –    | N         |            | – (0.9)                | –         | N     | –          |
| 17      | –    | N         |            | – (1.2)                | –         | N     | –          |
| 18      | –    | N         |            | + (6.2)                | –         | N     | –          |
| 19      | –    | N         |            | – (1.3)                | –         | N     | –          |
| 20      | –    | N         |            | – (1.2)                | –         | N     | –          |
| 21      | –    | N         |            | – (1.3)                | –         | N     | –          |
| 22      | –    | N         |            | – (1.1)                | –         | N     | –          |
| 23      | –    | N         |            | – (0.9)                | –         | N     | –          |
| 24      | –    | N         |            | – (0.9)                | –         | N     | –          |
| 25      | –    | N         |            | – (1.2)                | –         | N     | –          |
| 26      | –    | N         |            | – (1.1)                | –         | N     | –          |

+, Mobility shift; –, negative; N, normal.

a mobility shift band, except for case 8. With the positive control of SW480 cell line, any mutations at K-ras codon 12 would be detected. None of the 26 adrenal tumours was found to have DNA movements and distance change in the H-ras gene SSCP analysis (data not shown). To detect the type of mutations, a 128-bp fragment of exon 1 region and a 111-bp fragment of the exon 2 region of the K-ras gene were cloned from tumour specimens and sequenced. For accuracy, we performed bidirectional sequencing for ten individual clones using T3 and T7 primers. Comparison of the nucleotide sequences of these tumour specimens with their paired adjacent normal tissues and the wild-type sequence of human K-ras genes revealed a substitution from leucine to serine at codon 19 in case 2 (Figure 2A), and from glycine to cysteine at codon 60 in case 8. Cases 1, 3 and 15 contained two K-ras gene mutations: one at codon 15 and the other at codon 16. Four point mutations of the K-ras gene were identified in case 10 and case 12, including substitution from glycine to threonine at codon 15; silent mutation at codon 16; substitution from alanine to valine at codon 18; and substitution from glutamate to glutamine at codon 31 (Figure 2B and C). Northern blot analysis with a K-ras cDNA provided evidence of quantitative K-ras mRNA overexpression in 6 of 16 adrenocortical adenomas (Figures 3 and 4). In the six cases, there were two cases (case 12 and 15) that showed a 10–20 times increase in K-ras mRNA overexpression compared with that in the paired remnant adrenal gland. These results are summarized in Table 2.

DISCUSSION

The activation of ras genes has been implicated in transformation in vitro and tumorigenesis in vivo (Barbacid, 1987; Vogel et al, 1988; Mikako et al, 1992), but the role of these genes in the sequential events leading to the acquisition of the transformed phenotype is unclear. The ras-encoded proteins in mammalian cells are approximately 21 000 daltons (p21), bind guanine nucleotides and are localized to the inner face of the plasma membrane (Sigal et al, 1986). Three members of the cellular ras gene family have been identified: H-ras, K-ras and N-ras (Hall, 1990). Oncogenic ras proteins differ from their normal homologues by a single amino acid substitution, usually at positions 12, 13 and 61. It has been proposed that the deficiency in GTPase activity of the ras oncogenic protein could result in the derangement of normal regulatory mechanisms that control cell proliferation (Gibbs et al, 1985; Der et al, 1986; Bos, 1989). In 1991, Moley et al had analysed ten adrenal tumours from patients with
phaeochromocytoma and Moul et al (1993) had examined eight adrenal carcinomas, six phaeochromocytomas, two adrenal tumours, one aldosteronomas, two fresh phaeochromocytomas and one fresh benign adrenal gland (1993) for activating mutations at the 12, 13 and 61 codons of N-ras, H-ras and K-ras. There were no definite mutations detected at codon 12, 13 or 61 of the N-, H- and K-ras genes. A few cases of adrenal cortical tumours have been analysed and the functional character of the tumours have never been discussed. As the N-ras gene was most frequently activated in human myeloid leukaemia (Barbacid, 1990), we chose the H-ras and K-ras genes as the first target analysed. In the present study, no H-ras gene mutations were found in the 26 cases with adrenal tumours; we deduced that the H-ras oncogene has little to do with the tumorigenesis of functional adrenal tumours. But in the K-ras oncogene investigation it was confirmed that 6 out of 13 cases with Conni’s syndrome had obvious K-ras oncogene mutations, and the mutation rate was high at 46%. The mutation sites were not located on the hot spots on codon 12, 13 and 61 as has been established already, but they were, however, accumulated on codon 15, codon 16, codon 18 and codon 31. The mutation types in those cases were found to be monoallelic. Their mutation sites have not been reported in previous studies of K-ras gene mutation. The results of bidirectional sequencing of ten individual clones confirmed that these sites were mutated in the adrenal tumour specimens we collected. However, Sigal et al (1986) found p21 protein Lys16 was the decision site of the GTTP/GDP-binding site. If Lys-16 is replaced by Asn, the affinity between GDP and GDP will decrease 100 times, without affecting GTTP/GDP-binding specificity. In 1989, Power et al (1989) designed a mutant p21 protein with Ala instead of Gly-15 for studying the characters of the p21 protein. The results showed that this alteration could also affect the normal functions of the p21 protein. In addition, in 1992 Shirouzu et al (1992), in further studies of the mutation of the p21 protein Glu-31 replaced by Lys, found that this mutation could interfere in the signal transduction activity of the p21 protein by interfering the co-operation of the p21 protein and GAP (GTPase activating protein). Over-expression of K-ras mRNA occurs in approximately 37.5% (6 of 16 cases tested) of functional adrenocortical adenomas and predominantly occurred with the presence of mutations in the K-ras gene. This phenomenon was also found in mouse adrenocortical tumour cells (Schwab et al, 1983; George et al 1984; George et al, 1985). The phenomenon of over-expression of K-ras mRNA has been investigated by Schwab et al. (1993) in mouse models. They suggested that the overexpression of K-ras mRNA might be caused by K-ras gene amplification. We believe that the overexpression of K-ras mRNA found in our samples of human adrenal tumours may be for the same reason.

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