

## Molecular analysis of ras oncogenes in CIN III and in stage I and II invasive squamous cell carcinoma of the uterine cervix

J J O'Leary, R J Landers, I Silva, V Uhlmann, M Crowley, I Healy, K Lutlich

### Abstract

**Aim**—To examine the prevalence of genital type human papilloma virus (HPV) and mutations at codons 12, 13, and 61 in H, Ki, and N-ras in CIN III and early invasive squamous cell carcinomas of the cervix.

**Methods**—Prevalence of HPV was examined in 20 CIN III and 20 stage I and II cervical carcinomas, using non-isotopic in situ hybridisation (NISH) and solution phase polymerase chain reaction (PCR). In addition, mutations at codons 12, 13, and 61 were examined in H, Ki, and N-ras in these CIN III and early invasive squamous cell carcinomas, to assess the prevalence of ras gene point mutations and to define where in the pathobiology of squamous cell carcinoma such events occur. A non-isotopic PCR/RFLP assay was used to define these mutations.

**Results**—Of the 20 CIN IIIs examined, 19 contained HPV 16 DNA sequences by PCR and NISH. Dual infection was not uncovered. The 20 early (stage I and II) invasive squamous cell carcinomas showed predominant HPV 16 positivity (17/20), with one case HPV 18 positive, confirmed on PCR and NISH. Activating mutations were not identified in any of the CIN III cases. Only one stage I, HPV 16 positive carcinoma showed an activating mutation in H-ras codon 12, which was not present in adjacent normal ectocervical mucosa from the same patient.

**Conclusions**—ras Activation does not appear to occur in conjunction with HPV infection, particularly of HPV 16 infected high grade cervical intraepithelial neoplasia, or to occur commonly in early cervical squamous cell carcinoma. The postulated model of HPV linked carcinogenesis suggests malfunctioning control of viral transcription as a necessary component of neoplastic progression. It is also clear that host gene alterations are equally necessary for HPV linked carcinogenesis to occur.

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There are three members of the ras oncogene family. N-ras is located on chromosome 1 (1p21p32), H-ras on chromosome 11 (11p15.1-p15.5), and K-ras on chromosome 12 (12p12.1-pter).<sup>1</sup> Each of these three genes

contains four coding exons which specify homologous proteins of 21 kDa and a 5' non-coding exon.<sup>2</sup> In addition, two pseudogenes, H-ras-2 and K-ras-1, have been identified and characterised in rats and humans<sup>3</sup>; these are mapped to the X chromosome to the 6p12-p23 locus.<sup>3</sup>

The protein encoded by the ras genes binds guanosine triphosphate (GTP) and guanosine diphosphate (GDP) with high affinity and are known to possess GTPase activity.<sup>2</sup> Such ras proteins share biochemical properties with "G proteins," which are known to play a role in signal transduction pathways from membrane bound receptors to adenylate cyclase.<sup>4</sup> In fact, certain domains of the ras proteins show significant sequence homology with the  $\alpha$  subunit of G proteins such as  $G_s$ , which is known to activate adenylate cyclase directly in response to  $\beta$  adrenergic stimuli, making ras proteins involved in signal transduction. ras Proteins probably exist in equilibrium in the cell between an active and an inactive stage. Inactive forms bind GDP. ras Proteins will remain inactive until they receive a stimulus from another protein/receptor upstream of a putative pathway of signal transduction. Such a stimulus results in the exchange of GDP for GTP, followed by a conformational alteration of inactive ras to active ras. Once interaction between the active ras proteins and the effector has taken place, immediate deactivation would occur. This is achieved by the intrinsic GTPase activity catalysing the hydrolysis of GTP and thus returning the ras proteins to the inactive GDP bound state. Mutation occurring in the ras genes shifts the normal equilibrium to the active form. Such stabilisation phenomena would result in continuous signal transduction and subsequently malignant transformation.

Theoretically, ras stabilisation can be achieved by mutations that inhibit the intrinsic GTPase activity of ras proteins, increase the exchange rate between GDP and GTP, or induce an active conformational change that does not require binding of guanine nucleotides. Indeed, normal ras genes if overexpressed also induce malignant transformation by producing many molecules in the GTP bound active state. Experimental evidence now indicates that inhibition of GTPase activity is the preferred mechanism of activation of ras oncogenes.<sup>5,6</sup>

Activation of ras genes may occur through (1) mutations in the coding regions and (2) enhanced expression. Mutations in the three ras oncogenes (H-ras, K-ras, and N-ras) are

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known to occur in the codons for amino acids 12, 13, and 61. Additionally, when H-ras is exposed to bisulphite particular mutations in codons 12, 13, 59, and 63 result, which shows that codons 59 and 63 are also potential targets for activating mutations.<sup>2</sup> Besides these two sites, mutations in codons 116 and 119 can also activate the ras gene.<sup>7</sup>

Enhanced expression can be obtained by the insertion of a strong promoter or enhancer in the vicinity of the ras gene,<sup>2-8</sup> by amplification of the normal proto-oncogene,<sup>9</sup> or by deletion of the first (non-coding) exon.<sup>10</sup> Mutations at codons 12, 13, and 61 are likely to lead to loss of GTPase activity which results in a constitutively activated ras oncogene.

Somatic deletions and mutations of H-ras gene in human cervical cancers have been described by Riou *et al*,<sup>11</sup> who examined the c-H-ras-1 locus in cervical cancers and described loss of one allele at this locus in 36% of heterozygous tumours and a mutation on codon 12 in 24% of tumours at an advanced stage.

These investigators also demonstrated amplification of c-myc and H-ras.<sup>12</sup> In addition, increased expression of the ras p21 gene has been found in cervical carcinoma, using the Y13-295 monoclonal antibody,<sup>13</sup> showing a higher staining intensity in malignant tumour cells than in benign or premalignant lesions. It has been suggested that serological testing for p21 may have potential for monitoring disease activity in patients with cervical carcinoma.<sup>14</sup>

H-ras point mutations have been implicated in the pathogenesis of cervical neoplasia. In contrast, a large study of K-ras activation in neoplasms of the human female reproductive tract did not show any point mutational activity in cervical carcinomas.<sup>15</sup> Interestingly, a low incidence of ras oncogene activation in human squamous cell carcinomas from other sites has been reported by Rumsby *et al*.<sup>16</sup>

In anogenital neoplasia, mutations of K-ras at codon 12 have been shown in anal carcinomas not infected with human papilloma virus (HPV). Hiorns *et al* suggest that ras activation does not appear to be a common event

in the genesis of such tumours and if it does occur it does not appear to cooperate with HPV.<sup>17</sup>

Another common HPV associated tumour, oesophageal carcinoma, has also been examined for point mutations at codons 12, 13, and 61 of the ras oncogenes. Victor *et al* failed to show any evidence of mutations in these codons in squamous cell carcinomas of the oesophagus in South Africa.<sup>18</sup>

In this study, we examine mutations in these codons in H, Ki, and N-ras in cervical intraepithelial neoplasia (CIN) grade III and early invasive squamous cell carcinomas of the cervix, to assess the prevalence of such mutations and to examine where in the pathobiology of squamous cell carcinoma such events occur.

## Methods

### MATERIALS

Archival paraffin wax embedded tissues of 20 CIN III and 20 stage I and II invasive squamous cell carcinomas were retrieved from the files of the department of pathology, University College Cork, Ireland. Six of the carcinoma group had coexistent CIN III in the resection specimen. Control histologically normal ectocervical tissue was examined in lesions (where possible) in order to assess the prevalence of genotypic geographical change in adjacent epithelium.

### HPV DNA IN SITU HYBRIDISATION

Three 5 µm sections (selected from three different areas of the tumour) were cut onto APES coated glass slides (PH106, C A Hendley, Essex, UK). In addition, two areas from the included cervix were also examined.

Tissue dewaxing was carried out using xylene and rehydration through a graded alcohol series. Proteolytic digestion was carried out using 0.5 mg/ml proteinase K in proteinase K buffer (50 mM Tris HCl, pH 7.6 + 5 mM EDTA) at 37°C for 10 minutes. For alkaline phosphatase detection, slides were immersed in 20% (vol/vol) aqueous acetic acid at 4°C for 15 seconds. For peroxidase detection, slides were immersed in 3% sodium azide/hydrogen peroxide to abolish endogenous peroxidase activity. Slides were then washed in phosphate buffered saline for five minutes. A postfixation step was not carried out. Dehydration of the sections then took place through graded alcohols to water.

Cloned HPV probes (6, 11, 16, 18, 31, 33) (genital types; gifts of Harald Zur Hausen) were labelled with biotin or digoxigenin using a standard nick translation protocol.<sup>19</sup> Probes were initially prepared at a concentration of 200 ng/ml.

The hybridisation buffer contained (2 × SSC (NaCl/sodium citrate), 5% dextran sulphate, 0.2% dried milk powder containing no vegetable extracts, and 50% formamide). Approximately 10–20 ng of the appropriate probe in the hybridisation buffer was applied to the centre of each well on the PH106 slides. Hybridisation was carried out as previously described.<sup>12</sup>

Table 1 Summary of ras gene assays for H-ras

Gene	Enzyme	Fragment size		
		Undigested	Mutant	Normal
<b>H-ras-12</b>	MspI (CCGG) <sup>a</sup>	312	291	236
H5': 5'GAGACCCTGTAGGAGGACCC3'				
H3': 5'GGGTGCTGAGACGAGGACT3'				
<b>H-ras-13</b>	HphI (GGTGA) <sup>b</sup>	107	107	97+(10)
H12S: 5'GCAGGAGACCCTGTAGGAGGAC3'				
H13A Hph: 5'GTCAGCGCACTCTTGCCTCA3'				
<b>H-ras-61</b>	AlwNI <sup>c</sup> (CAGNNCTG)	177	177	160+(17)
Nested PCR:				
H-61S: 5'ATGAGAGGTACCAGGGAGAG3'				
H-61A: 5'TCACGGGGTTACCTGTACT3'				
H-61AAIw: 5'CGCATGGCGCTGTACAGCTC3'*				

\*Mismatched nucleotides in the primers are underlined.

<sup>a</sup>Kahn *et al* (reference 22).

<sup>b</sup>Mitsudomi *et al* (reference 23).

MspI from Gibco BRL; HphI from New England Biolabs; AlwNI from Sigma.

Table 2 Summary of ras gene assays for K-ras

Gene	Enzyme	Fragment size		
		Undigested	Mutant	Normal
<b>K-ras-12</b>	T Bst NI (CC GG) <sup>b</sup>	157	143	114
K12S1:	5'ACTGAATATAAACTTGTGGTAGTTGGACCT3'			
K12A1:	5'TCAAAGAATGGTCTCGGAAC3'			
<b>K13 (asp)</b>	Hph I (GGTGA) <sup>c</sup>	157	114	157
K12S1:	5'ACTGAATATAAACTTGTGGTAGTTGGACCT3'			
K12A1:	5'TCAAAGAATGGTCTCGGAAC3'			
<b>K-ras 13</b>	Hae III (GGCC) <sup>e</sup>	116	116	91+(25)
K12S2:	5'CATGTTCTAATATAGTCACA3'			
K13AHae:	5'TATCGTCAAGGCACTCTTGCCTAGG3'			
<b>K-ras 61</b>	Bcl I (TGATCA) <sup>e</sup>	176	176 <sup>a</sup>	157+(19) <sup>a</sup>
K61SBcl:	5'GGATATTCTCGACACAGCTGAT3'			
K61A:	5'AATTACTCCTTAATGTCAGC3'			
<b>K-ras 61</b>	Ear I (GAAGAG) <sup>e</sup>	176	176 <sup>a</sup>	151+(25) <sup>a</sup>
K61SEar:	5'GGATATTCTCGACACAGCAGGTGA3'			
K61A:	5'AATTACTCCTTAATGTCAGC3'			

\*Mismatched nucleotides in the primers are underlined.

<sup>a</sup>See text for details (not all mutants are uncut).<sup>b</sup>Kahn *et al* (reference 22).<sup>c</sup>Mitsudomi *et al* (reference 23).

Bst NI, Hae III, Bcl I from Sigma; Hph I, Ear I from New England Biolabs.

Table 3 Summary of ras gene assays for N-ras

Gene	Enzyme	Fragment size		
		Undigested	Mutant	Normal
<b>N-ras 12 (method 1)</b>	Hph I (GGTGA) <sup>a</sup>	70	70	59+(11)
N12S1:	5'CTGGTGTGAAATGACTGAG3'			
N12AHph:	5'TGTCACTGCGCTTTTCCCAACATC3'			
<b>N-ras 12 (method 2)</b>	BspMI (GCAGGT) <sup>b</sup>	128	113	94
N12S2:	5'ACTGAGTACAACTGGTGGTTCAGCA3'			
N12A2:	5'GGGCTACCTGGCGCCCTCCACTCTATGG3'			
<b>N-ras 13</b>	Hph I (GGTGA) <sup>c</sup>	82	82	59+(23)
N12S1:	5'CTGGTGTGAAATGACTGAG3'			
N13AHph:	5'GATTAGCTGGATTGTCAGTGCCTTTTCCCAATC3'			
<b>N-ras 61</b>	MscI (TGGCCA) <sup>a</sup>	126	126	103+(23)
N61SBal:	5'TTGACATACTGGATACAGCTGGC3'			
N61A:	5'GTTAATATCCGCAATGACTTGC3'			

\*Mismatched nucleotides in the primers are underlined.

<sup>a</sup>Mitsudomi *et al* (reference 23).<sup>b</sup>O'Leary *et al*.<sup>c</sup>O'Leary, and Mitsudomi *et al* (reference 22).

Hph I, BspMI from New England Biolabs; MscI from Sigma.

Following hybridisation, medium stringency washings were initially applied using  $2 \times$  SSC at 60°C for 20 minutes, followed by  $0.2 \times$  SSC for 42°C at 20 minutes, then  $0.1 \times$  SSC at room temperature for five minutes and  $2 \times$  SSC at room temperature for five minutes. Higher stringency washes included  $0.2 \times$  SSC at 55°C and 60°C for 10 minutes and  $0.1 \times$  SSC at room temperature, 42°C, and 55°C.

The hybridisation signal was detected using one step, two step, or three step techniques as described previously.<sup>12</sup> Colorimetric detection was achieved using an NBT/BCIP substrate for alkaline phosphatase or AEC (Zymed kit for peroxidase; Zymed, California, USA).<sup>12</sup> Sections were counterstained with 2% methyl green for alkaline phosphatase detection or haematoxylin for the peroxidase detection system.

Tissue controls for non-isotopic in situ hybridisation (NISH) included HPV positive cervical warts and myocardium (negative for HPV). Reaction controls included hybridisation buffer on its own, biotin/digoxigenin

labelled plasmid sequences (pBR322), and an irrelevant probe (*Herpes zoster virus*, HZV). Labelled human placental DNA was used to check hybridisation efficiency.

Detection controls included omitting primary or secondary antibody steps and addition of the colorimetric substrate only.

**SOLUTION PHASE POLYMERASE CHAIN REACTION**  
HPV DNA sequences were derived from the EMBL genetic sequence database. HPV E6 sequences which remain intact following viral DNA integration were chosen. During oligo synthesis, a biotin reporter molecule with a 15 carbon atom linker arm was added to the 5' end of the oligonucleotide probe and subsequently used as an internal probe to confirm product specificity. The nucleotide sequence of the primers are as previously described.<sup>20</sup>

General HPV primers (which identify sequenced and unsequenced HPV) were also used for a pan HPV screen, unrestricted by the type specific HPV chosen above, and are as previously described.<sup>21</sup>

Sections 10 µm thick were cut from the tissue blocks and placed in sterile Eppendorf tubes. Strict anticontamination protocols were adopted throughout. Nucleic acid extraction was performed using proteinase K (0.1–0.5 mg/ml) in proteinase K buffer (100 mM NaCl, 10 mM Tris HCl, 25 mM EDTA, 0.5% SDS, pH 8.4). Proteinase K incubation was carried out for three to five days at 37°C with adequate mixing of samples. Proteinase K inactivation was then carried out at 94°C for 10 minutes. DNA was purified using a standard phenol chloroform isoamyl alcohol technique. Nucleic acid was precipitated using 3 M sodium acetate and ice cold ethanol.

For type specific HPV polymerase chain reaction (PCR), the PCR solution consisted of PCR buffer (50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatine, 200 µM of each deoxynucleotide triphosphate (dNTP), 1.0 µM of each primer, 2.5 units of AmpliTaq DNA polymerase, and 100 ng of DNA template). Samples were then subjected to 40 cycles of PCR in a Perkin Elmer 480 DNA thermocycler. Cycling parameters were as follows: 94°C for one minute, followed by 94°C for one minute, 55°C for two minutes, 72°C for three minutes, by 40 cycles with a final extension set for 72°C for five minutes.

For general primer PCR, amplification conditions were similar except 3.5 mM MgCl<sub>2</sub> was used and an annealing temperature of 40°C was applied.

Type specific HPV PCR products were confirmed by dot blot hybridisation, as previously described.<sup>20</sup>

#### ANALYSIS OF ras ONCOGENE POINT MUTATIONS

Nucleic acid was extracted as above. The prevalence of ras point mutations was assessed by a PCR/RFLP analysis. Primers used for amplification of ras genes are shown in tables 1–6.

Cell lines shown to have all six possible mutations at codon 12 of Ki-ras and at H-ras or N-ras codons 12 and 61 were used as positive

Table 4 Designed restriction fragment length polymorphisms for the detection of point mutations at codon 12 of the ras genes

Gene	Sequence (codon)				Mismatch	Enzyme	Restriction site
	10	11	12	13			
H-ras	—	GCC	GGC	GGT	—	MspI <sup>a</sup>	CCGC
K-ras	GGA	GCT	GGT	GGC	<u>CCT</u> (11)	Bst NI <sup>b</sup>	T CC GG A
Nras	GGA	GCA	GGT	GGT	<u>GAT</u> (13) <u>CCA</u> (10)	Hph I <sup>b</sup> Bsp MI <sup>b</sup>	GGTGA GCAGGT

Sequence around codon 12 is shown; mutations are underlined. Mismatched nucleotide and its position in the primer is shown.

<sup>a</sup>Kahn *et al* (reference 22).

<sup>b</sup>Mitsudomi *et al* (reference 23).

MspI from Gibco BRL; Bst NI from Sigma; Hph I and BspMI from New England Biolabs.

Table 5 Designed restriction fragment length polymorphisms (RFLP) for the detection of point mutations at codon 13 of ras genes

Gene	Sequence (codon)			Mismatch	Enzyme	Restriction site
	12	13	14			
H-ras	GGC	GGT	GTG	<u>GAG</u> (14)	Hph I	GGTGA
K-ras	GGT	GGC	GTA	<u>CTA</u> (14)	Hae III	GGCC
N-ras	GGT	GGT	GTT	<u>GAT</u> (14)	Hph I	GGTGA

Sequence around codon 12 is shown; mutations are underlined. Mismatched nucleotide and its position in the primer is shown.

Origin of RFLP, Mitsudomi *et al* (reference 23).

Hae III from Sigma; Hph I from New England Biolabs.

Table 6 Designed restriction fragment length polymorphisms (RFLP) for the detection of point mutations at codon 61 of the ras genes

Gene	Sequence (codon)					Mismatch	Enzyme	Restriction site
	59	60	61	62	63			
H-ras	GCC	GGC	CAG	GAG	GAG	<u>CTG</u> (63)	Alw NI	CAGN <sub>3</sub> CTG
K-ras	GCA	GGT	CAA	GAG	—	<u>GCT</u> <u>GAT</u> (59, 60)	Bcl I	TGATCA
			CGA CTA CAC CAT			<u>GAA</u> (61)	Ear I Taq I Xba I Mae III Nla III	GAAGAG TCGA TCTAGA GTCCAC CATG
N-ras	GCT	GGA	CAA	GAA	GAG	<u>GGC</u> (60)	Bal I	TGGCCA

Sequence around codon 12 is shown; mutations are underlined. Mismatched nucleotide and its position in the primer is shown.

Origin of RFLP: Bcl I, Ear I, AlwNI, Bal I, Mitsudomi *et al* (reference 23); Nla III, Kumar *et al* (reference 24); Taq I, Xba I, Mae III, Milici *et al* (reference 25).

AlwNI, Bcl I, Nla III, Taq I, and Xba I from Sigma; Ear I from New England Biolabs.

Table 7 Cell lines used in the study

Cell line	Activating mutation and codon
Calu I	TGT; K-ras codon 12
A549	<u>AGT</u> ; K-ras codon 12
SW480	GTT; K-ras codon 12
SW1116	<u>GCT</u> ; K-ras codon 12
A427	<u>GAT</u> ; K-ras codon 12
T24	Homozygous for valine at H-ras codon 12
HT1080	Homozygous for lysine at N-ras codon 61
NCL-H1915	<u>CTG</u> ; H-ras codon 61
NCL-H647	GAC; K-ras codon 13

Activating mutation underlined.

controls (Calu-1, SW 480, A427, SW1116, and T24) were purchased from ATCC, PHLS Centre for Applied Microbiology, Porton Down, Salisbury, UK) (table 7).

#### Codon 12 ras oncogenes

**H-ras 12**—Primers were used to amplify H-ras codon 12 sequences spanning two endogenous MspI (CCGG) sites, one of which included the

two target G residues at position 12 and the other of which is an MspI control site. Positive control cell line material used in this assay was the T24 cell line (homozygous for the valine codon 12 mutation). The sizes of the undigested, normal, and mutant fragments are given in table 1.

**K-ras 12**—This codon was amplified using a 5' end primer that contained a C substitution at the first position of codon 11. This created a Bst NI (CCTGG) site which overlaps the first two nucleotides of codon 12. The 3' primer also contained a substitution, creating a control Bst NI site. The cell line SW 480 was used as positive control material. The sizes of the undigested, normal, and mutant fragments are given in table 2.

**N-ras 12**—In this case, by introducing an A residue at codon 13 through the mismatched antisense primer N12AHph, a novel Hph site was created. Any mutation abolished the restriction site. The sizes of the undigested, normal, and mutant fragment are given in table 3. Details of the RFLPs at codon 12 are given in table 4.

#### Codon 13 ras oncogenes

By introducing an A residue at codon 14 for (H and N ras) and a C at codon 13 for K ras, screening for point mutations at codon 13 for each of the three ras oncogenes could be performed using the mismatched primers H13AHph, K13AHae, and N13AHph, which respectively created an HphI site for H-ras, an Hae III site for K-ras, and an HphI site for N-ras. Using the primers for K-ras codon 12 assay, the same amplified fragment can be digested with Hph I to diagnose aspartic acid mutations at the second position of codon 13. The particulars of the RFLPs are given in table 5.

#### Codon 61 ras oncogenes

**H-ras 61**—For this codon, AlwNI cut the fragment generated with primers H61AAlw and H61S (table 1) when no mutation was present. To maximise the efficiency of this assay, the entire codon was first amplified using H61S and H61A and a nested PCR for H61AAlw and H61S. The digestions are illustrated in tables 1 and 6.

**K-ras 61**—The enzyme Bcl I cut the PCR product amplified with K61SBcl (which has two mismatches at codon 59 and 60) and K61A when the first two nucleotides of codon 61 are CA. The third nucleotide of codon 61 affects the genetic code, and Ear I digestion was therefore necessary. EarI would only cut the fragment generated by primers K61SEar and K61A when the third nucleotide of codon 61 was A. By combining the digestions of Bcl I and Ear I, any mutation could be detected. These digestions are illustrated in tables 2 and 6.

**N-ras 61**—Bal I cut the fragment generated by N61SBal and N61A when the first two nucleotides were CA (table 6). Afl III will cut when codon 61 is CAT or CAC. Therefore when Bal I cuts and AflIII does not, then no activating mutation was present. The digestions are illustrated in tables 2 and 6.

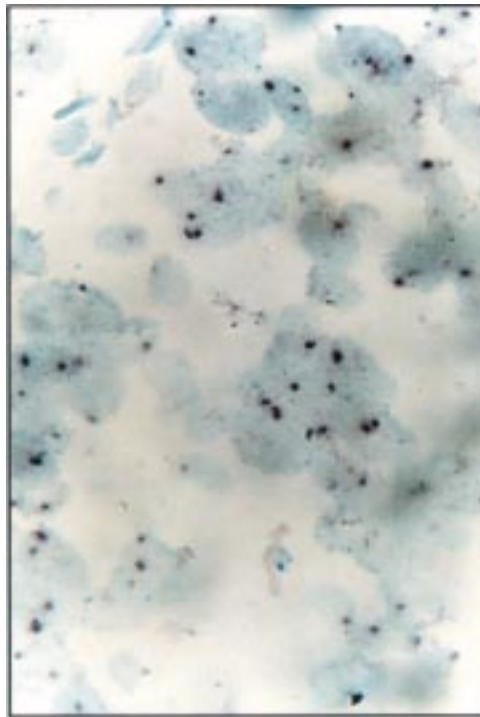


Figure 1 HPV 16 non-isotopic in situ hybridisation (NISH) assay in a cervical squamous cell carcinoma, showing punctate signals in the nuclei of many cells.

#### POLYMERASE CHAIN REACTION

Genomic DNA (100–500 ng) was amplified in a reaction volume of 50 or 100  $\mu$ l containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 0.01% (wt/vol) gelatin, 1.25 mM of each of four dNTP, 50 pmol of primers, and 2.5 units of Taq DNA polymerase (Ampli Taq, Perkin-Elmer, Warrington, Cheshire, UK).

The conditions for the PCR using a 480 thermal cycler (Perkin-Elmer) were as follows: 94°C for five minutes for initial denaturation, 40 cycles of 94°C for one minute, annealing for two minutes, and extension at 68°C for two minutes.

Annealing temperatures were as follows: K-ras codon 12, 45°C; H-ras codon 12, 62°C; N-ras codon 12, 55°C; K- and N-ras codons 13 and 61, 50°C; H-ras codon 13 and 61, 60°C.

Oligonucleotide primers were synthesised using a DNA synthesiser according to the published sequences data of the K-, H-, and N-ras genes. Primer sequences are shown in tables 1, 2, and 3. In case of the H-ras codon 61 screening, the efficiency of PCR was very low because of two mismatches in the primer H61AAIw. To overcome this, we employed heminested PCR using entire exon II PCR product as a template for second stage PCR.

#### RESTRICTION ENZYME DIGESTION AND GEL ELECTROPHORESIS

PCR product (16–20  $\mu$ l) was digested with the restriction enzymes shown in tables 1–3. The reaction conditions followed suppliers' recommendations. DNA was electrophoresed through 4–6% agarose gels, followed by ethidium bromide staining. Gels were photographed using an ultraviolet light transilluminator.

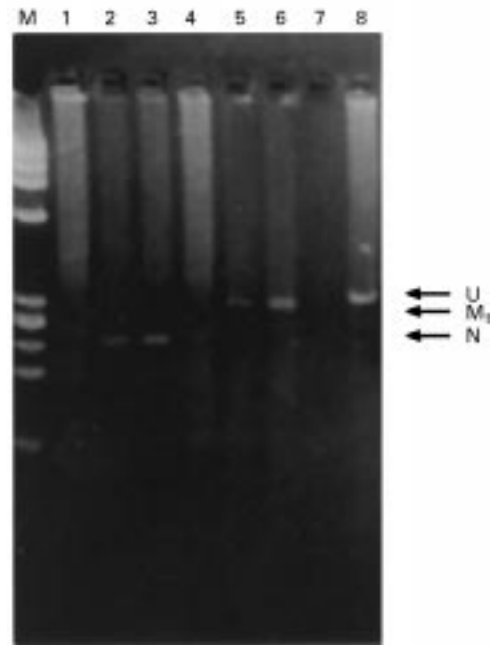


Figure 2 H-ras 12 mutation PCR/RFLP assay, using a 4% agarose gel. Marker ladder used is phi X174. U, uncut amplified product; Mt, mutant fragment (lane 6). Lane 5 shows mutated H-ras codon 12 in a tumour sample.

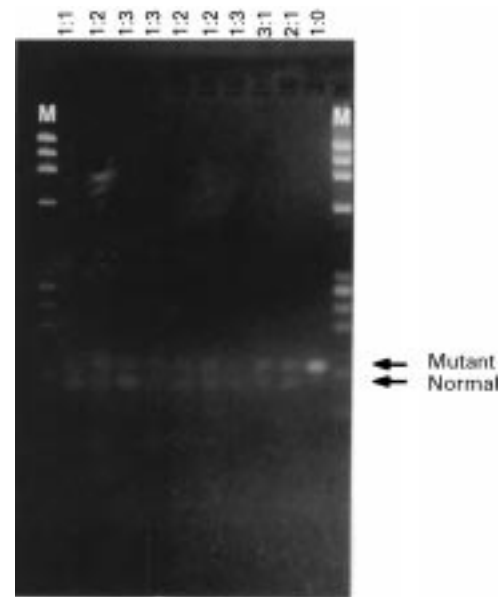


Figure 3 N-ras codon 61 assay to establish PCR/RFLP assay sensitivity: 25 ng of starting DNA template was used; genomic DNA was prepared from HT 1080 cells (homozygous for the mutant 61 lysine allele). This mutant DNA was mixed with normal genomic DNA obtained from human placenta and mixed in defined ratios. The amplified product was then digested with Bal I.

#### Results

Of the 20 CIN IIIs examined, 19 contained HPV 16 DNA sequences by PCR and NISH. Dual infection was not uncovered. The 20 early (stage I and II) invasive squamous cell carcinomas showed predominant HPV 16 positivity (17/20) with one case (1/20) being HPV 18 positive, again confirmed on PCR and NISH (fig 1).

Activating mutations were not identified in any of the CIN samples. Only one stage I HPV

16 positive carcinoma showed an activating mutation at codon 12 of H-ras. The adjacent normal ectocervical mucosa from the same patient was normal. All control cell lines yielded restriction patterns as predicted (fig 2). The sensitivity of the assay with loading contaminating non-mutated DNA was also established (fig 3) at a sensitivity of 1:3 (tumour to contaminating DNA sequences) using 25 ng of starting DNA template (fig 3), and 1:4 and 1:5 using higher DNA concentrations (100–500 ng). Control DNA from normal cervical margins did not reveal any activating mutations.

### Discussion

We examined 20 CIN III samples (19 HPV positive, one HPV negative) and 20 stage I and II cervical carcinomas (18 HPV positive, two HPV negative) to assess the prevalence of point mutations in H, K, and N-ras oncogenes, using a PCR/RFLP analysis. This assay offers a rapid, non-radioactive method of screening ras mutations in large study populations.

The optimal digestion conditions for RFLP analysis were first established using positive control cell lines containing defined activating mutations. We were confidently able to demonstrate mutant DNA in a 1:3 ratio in contaminating normal DNA, even using 25 ng of starting DNA template (fig 3). At higher starting DNA concentrations, ratios of 1:4 were easily achieved.

We identified an activating mutation at codon 12 H-ras in one HPV 16 positive stage I cervical squamous cell carcinoma. The surrounding normal ectocervical mucosa in this patient did not contain any activating mutation. No adjacent CIN III was identified in this case.

The findings are in keeping with those of Bos,<sup>3</sup> but are contrary to the findings of Riou *et al*,<sup>12</sup> in which mutations at codon 12 of H-ras were found in 24% of tumours at an advanced stage. Additionally 40% of tumours with mutations (in the Riou series), also contained an allelic deletion at that locus. Riou's data suggest that deletions and mutations of H-ras gene occur in advanced stages of cervical carcinoma. The tumours examined in our study were predominantly stage I and II—a different study group where different genetic events may be operational.

Previously, Rumsby *et al* found a low incidence of ras oncogene activation in a range of human squamous cell carcinomas.<sup>16</sup> Hiorns *et al* screened a series of anogenital tumours for activating mutations of the ras oncogene family using the polymerase chain reaction and a series of synthetic oligonucleotide probes<sup>17</sup> (an alternative method to the assay adopted in our study). Mutations were seen in only two cases (both K-ras codon 12), neither of which was HPV associated. Their findings readily suggest that ras activation does not appear to be a common event in the genesis of these tumours and when it did occur it did not appear to coexist with HPV infection. This supports the findings of our study, in which the majority of cases were HPV positive and activating ras

mutation negative. It seems likely, therefore, that in early anogenital neoplasia mutations at either codon 12 of H-ras and K-ras in particular are uncommon events.

Our data in relation to CIN III are in accordance with those of Le Van *et al*,<sup>26</sup> who were unable to identify H-ras 12 mutations in cases of CIN I, II, and III. However, Grendys *et al* have recently demonstrated mutations in Ha, Ki, and N-ras codon 61 in 24.2% of stage IB cervical carcinomas,<sup>27</sup> contrasting with 5% (1/20) in our study.

In conclusion, ras activation does not appear to occur in conjunction with HPV infection, particularly of HPV 16 infected high grade cervical intraepithelial neoplasia. In addition, ras activation is an infrequent event in early squamous cell carcinoma, although the interaction of HPV 16 and ras oncogenes in cellular transformation *in vitro* has been shown.<sup>28</sup> This leads us to believe that many different cell types differ in their susceptibility to transformation by such agents.

On current data, the postulated model of HPV linked carcinogenesis suggests malfunctioning control of HPV transcription as a necessary component for neoplastic progression. It is also clear, however, that host gene alterations are equally necessary for HPV linked carcinogenesis to occur.

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## Molecular analysis of ras oncogenes in CIN III and in stage I and II invasive squamous cell carcinoma of the uterine cervix.

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