Carcinogenesis vol.23 no.4 pp.645–655, 2002

Genetic status of cell cycle regulators in squamous cell carcinoma of the oesophagus: the *CDKN2A* (*p16^{INK4a}* and *p14^{ARF}*) and *p53* genes are major targets for inactivation

Johanna Smeds[1], Petra Berggren[1], Xin Ma[1], Zhijian Xu[2], Kari Hemminki[1] and Rajiv Kumar[1,3]

[1]Department of Biosciences, Karolinska Institute, Novum, 141 57 Huddinge, Sweden and [2]Division of Head and Neck Surgery, Cancer Institute, Chinese Academy of Medical, Science, Beijing, China

[3]To whom correspondence should be addressed
Email: rajiv.kumar@cnt.ki.se

We determined inactivation of the *CDKN2A* (*p16^{INK4a}* and *p14^{ARF}*) gene in 21 cases of oesophageal squamous cell carcinoma (OSCC). The tumours were also analysed for mutations in exons 5–8 and allelic losses in the *p53* gene. In addition, we screened the *CDKN2B* (*p15^{INK4b}*), *CDKN2C* (*p18^{INK4c}*), *CDK4* and *p53R2* genes for mutations in the tumour tissues. Besides concomitant alterations in the *CDKN2A* and *p53* loci in more than half of the cases, our results showed that in 18 OSCC (86%) the *CDKN2A* (*p16^{INK4a}* and *p14^{ARF}*) gene was affected through mutations, homozygous/hemizygous deletions and promoter hypermethylation. Eight out of 10 tumours with mutations or promoter hypermethylation specific to the *CDKN2A/p16^{INK4a}* gene showed loss of the wild-type allele. One tumour with a single base deletion in the N-terminus (codon 8) of the *CDKN2A/p16^{INK4a}* gene carried a novel germ-line mutation or a rare polymorphism (Ile51Met) in exon 2 of the *CDK4* gene. Promoter hypermethylation in the *CDKN2A/p14^{ARF}* gene was detected in 11 tumours. In the *p53* gene 15 mutations were detected in 14 tumours. We detected an inverse relationship between *CDKN2A/p16^{INK4a}* inactivation and frequency of loss of heterozygosity at the *p53* locus (OR 0.09, 95% CI 0.01–0.98; Fisher exact test, *P*-value ~0.03). Screening of nine exons of the *p53R2* [Human Genome Organisation (HUGO) official name RRM2B] gene resulted in identification of a novel polymorphism in the 5′ untranslated region, which was detected in four cases. Our results suggest that the *CDKN2A* (*p16^{INK4a}* and *p14^{ARF}*) and *p53* genes involved in the two cell cycle pathways are major and independent targets of inactivation in OSCC.

Introduction

Oesophageal squamous cell carcinoma (OSCC) is one of the most common cancers in the world with extremely poor prognosis due to late presentation and rapid progression. This cancer shows a wide geographical variation in distribution with marked high and low-risk regions. The variation in the incidence reflects the strong influence of environmental factors in the cancer development. But many of these factors are not completely understood. In low-risk areas OSCC is associated with alcohol and tobacco intake, while, in high-risk areas a diet low in nutrition and contamination with *N*-nitroso compounds are often regarded as the major aetiological factors (1). Some reports also associate familial clustering of oesophageal cancer in high-risk areas indicating genetic predisposition (2). The genetic changes identified with oesophageal cancer show considerable heterogeneity (3). Several chromosomal regions with allelic losses at a high frequency reported include 3p, 5q, 9p, 9q and 13q (4). Some of the genetic changes seen in oesophageal carcinoma with consistency include mutations in the *p53* tumour suppressor gene and perturbation of the Rb pathway of cell cycle control (2,5).

Mutations in the *p53* gene are quite frequent in oesophageal carcinoma with a distinct pattern reported in tumours from high-risk areas compared with low-risk areas (6–9). Inactivation of the *CDKN2A* gene, which includes mutations, homozygous deletion and promoter methylation, have also been reported at varying frequencies (10–12). The well characterized *CDKN2A* locus at 9p21 encodes two unrelated cell cycle inhibitors, p16^{INK4a} (referred to as p16 throughout) and p14^{ARF} (referred to as ARF throughout) from a partially shared genomic sequence, which function upstream of Rb and p53, respectively (13). Somatic alterations in the *CDKN2A* gene occur in many cancer types and germ-line mutation carriers, besides melanoma, are predisposed to a high risk of pancreatic and breast cancers (14,15). Human p53 mutational data on OSCC and inactivation of CDKN2A/p16 in *N*-nitrosomethylbenzylamine induced OSCC in an animal model with zinc deficient background supports the major role of the *p53* and *CDKN2A* genes in genesis/progression of oesophageal cancer (5,16,17). Alterations in several other genes have also been reported occasionally (18). In addition, several oncogenic alterations detected include amplification of genes like *MYC*, *EGFR* and *GASC1* that lead to the deregulation of signal transduction.

The *CDKN2A* and *p53* gene products perform functions that are central to the maintenance of cellular integrity by protecting against uncontrolled proliferation and by keeping surveillance against induced DNA damage (Figure 1) (19,20). The p16 prevents cell cycle progression by disrupting the cyclin D/CDK4 kinase complex and in turn preventing the phopsphorylation of Rb. The hypophosphorylated Rb does not release transcription factors necessary for progression of a cell from G_1 to S phase, whereas nucleolar ARF inhibits MDM2 function through one of the several proposed mechanisms and stabilizes p53 (21). The stabilized p53 can induce temporary and permanent growth arrest, DNA repair, terminal differentiation or apoptosis in response to oncogenic signals and DNA damage (22). Understandably, the loss of both the *CDKN2A* and *p53* genes in pre-tumour and tumour cells are perhaps the key events that provide considerable growth stimuli leading to uncontrolled proliferation and destabilization. Concurrent disruption of the p16-Rb and ARF-p53 pathways in several cancer types is associated with poor prognosis and the dual inactivation is also shown to have an obligate role in tumour suppression in animal models (23,24).

In order to understand the mechanism of inactivation of the genes in the Rb and p53 pathways and their relative status in

Abbreviations: ARF, p14^{ARF}; LOH, loss of heterozygosity; OSCC, oesophageal squamous cell carcinoma; p16, p16^{INK4a}.

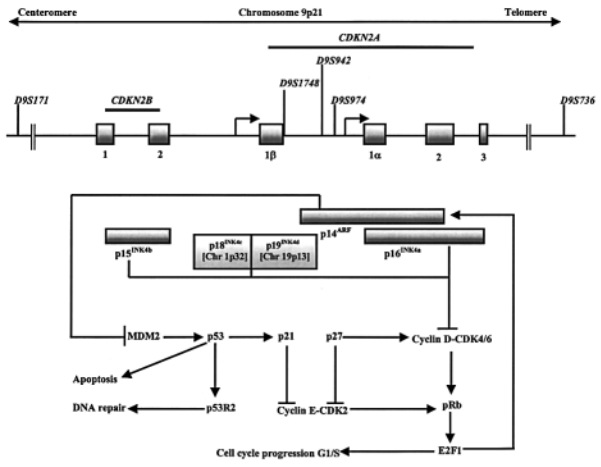

Fig. 1. Genomic organisation of the *CDKN2A* and *CDKN2B* genes on 9p21 (adapted from ref. 11). The *CDKN2A* gene from partially shared sequence encodes two cell cycle regulators p16 and ARF, which are transcribed from separate promoters located at 5′ side of unique exons 1α and 1β. Three microsatellite markers *D9S1748*, *D9S942* and *D9S974* are located between exon 1α and exon 1β (38). The p16, along with homologous p15 (encoded by *CDKN2B*), p18 (encoded by *CDKN2C* on chromosome 1p32) and p19 (encoded by *CDKN2D* on chromosome 19p13) inhibit the cyclin D-CDK4/6 complex and prevent phosphorylation of Rb. ARF inhibits MDM2 mediated degradation of p53 and is induced by different oncogenic signals including E2F1, which is a transcription factor released upon the hyperphosphorylation of the Rb. ARF functions in overlapping pathways and links Rb and p53. The stabilization of p53 mediated by ARF can lead to growth arrest via p21, apoptosis and DNA repair.

OSCC we carried out mutational analysis of various genes which include the *CDKN2A* (*p16* and *ARF*), *CDKN2B* ($p15^{INK4b}$), *CDKN2C* ($p18^{INK4c}$), *CDK4*, *p53* and *p53R2* in 21 cases of OSCC from a high-risk area in northern China including Linxian. The *p53R2*, a p53 downstream gene involved in DNA repair, is induced in response to DNA damage and therefore is a potential target of inactivation in different cancers (25). For the *CDKN2A* (*p16* and *ARF*) gene we also determined homozygous deletion and promoter methylation. In addition, we have determined frequency of loss of heterozygosity (LOH) within and outside the *CDKN2A* locus and at the *p53* locus.

Materials and methods

OSCC cases, tissues and DNA isolation

OSCC patients (age 35–81 years; median 55 years) were from northern China, including the Linxian province, a high-risk area for oesophageal cancer. Ten male cases out of 14 had a known history of smoking, whereas only one female case out of seven was a smoker (Table II). However, in five cases (four males, one female) smoking history was unknown. Case 2 in addition to oesophageal carcinoma also suffered from hepatitis and had liver cancer. Fresh biopsies containing tumour and benign tissues were taken from oesophageal cancer patients during the surgical procedures and snap frozen. The tumour and benign tissues were identified and separated by a pathologist by gross analysis. The majority of tumours analysed were of I–II grade (15 cases) and only four cases belonged to grade III, for two cases grades were not listed. DNA was isolated separately from tumour and benign tissues by isolating nuclear fractions followed by phenol–chloroform extraction.

Mutation detection

For mutation detection in different genes the PCR–SSCP technique was used. Exons 1(α and β)–3 of the *CDKN2A* gene, exons 1–2 of the *CDKN2B* gene, exons 2–3 of the *CDKN2C* gene, exon 2 of the *CDK4* gene and exons 1–9 of the *p53R2* gene were amplified by PCR using primers and annealing temperatures as described and given in Table I (26,27). Amplifications were carried out in 10 µl reactions containing 50 mM KCl, 1–2 mM $MgCl_2$, 0.11 mM of each dNTP, 1 µCi [α-^{32}P]dCTP, 0.15–0.3 µM of each primer and 0.3 U of *Taq* DNA polymerase. The temperature conditions were denaturation at 95°C 45 s, annealing (temperatures given in Table I) 45 s and polymerization at 72°C 45s for three cycles followed by 32 cycles at same temperatures with the segment time of 20 s each and a final extension at 72°C for 7 min. The amplified products were electrophoresed on a 0.5 MDE gel in three different conditions as reported previously.

Mutations in exons 5–8 of the *p53* gene were determined by a multiplex PCR and fluorescent SSCP method (28). The primers used for amplification were labelled with fluorescent dyes (Table I). Exons 5–8 were amplified in a single 10 µl vol reaction containing 20 ng genomic DNA, 0.2–0.4 µM of four primer pairs, 5 mM $MgCl_2$, 0.11 mM dNTPs, 10% glycerol and 2 U platinum *Taq* DNA polymerase (Life Technologies, Paisley, UK). PCR was carried out for 36 cycles with annealing temperatures of 61/60°C. PCR products were analysed by electrophoresis on 0.5–0.6× MDE gels in four different conditions using an ABI 377 (Applied Biosystems, Foster City, CA) automated sequencer attached with an external cooling system. The results were analysed using GeneScan 3.1 software.

Sequence analysis

Mutations and polymorphisms detected by SSCP in different genes were identified and confirmed by direct sequencing using rhodamine dye terminator cycle sequencing kit (Big Dye, Applied Biosystems). Individual exons containing mutations detected by aberrant band shifts in SSCP were amplified by PCR. The amplified products were purified by centrifugation through Sephadex micro-spin columns (Amersham-Pharmacia, Uppsala, Sweden) and subjected to 26 cycles of sequencing reactions with reverse and forward primers separately. The precipitated sequencing reaction products were electrophoresed on a 4% denaturing polyacrylamide gel in an automated sequencer (ABI 377, Applied Biosystems) and analysed using Edit View 1.0.1 software (Applied Biosystems). The sequence data were analysed using Align software in DNA star package.

Homozygous deletion

Homozygous deletion in the *CDKN2A* gene was determined by a real time PCR method using ABI PRISM 7700 Sequence Detection System (Applied Biosystems), which is based on the 5′ exonuclease property of the DNA polymerase (Taq Gold, Applied Biosystems). Primers and probes were designed to amplify a 92 bp fragment of exon 1β of the *CDKN2A* (target) gene and a 97 bp intronic fragment of the *GAPDH* (reference) gene (P.Berggren, R.Kumar, S.Sakano *et al.*, submitted for publication). Probes for the *CDKN2A* and *GAPDH* fragments were labelled with Vic and FAM dyes at the 5′ ends, respectively, and at the 3′ end with TAMRA dye, which functions as a quencher. Gene dosages were measured by calculating Ct values, which is the threshold for fluorescence detection, for amplification of an exon 1β fragment of the *CDKN2A* gene (target) and compared with Ct values for the *GAPDH* gene (reference). The initial copy numbers of DNA in both target as well as reference gene in tumour samples were calculated from linear curves generated by using decreasing amounts of starting DNA (20–1.25 ng). The ratio between the initial copy number of DNA in the target and reference gene was determined to ascertain homozygous deletion of the *CDKN2A* gene at exon 1β locus.

Frequency of LOH at microsatellite markers at the CDKN2A and near the p53 loci

Frequency of LOH was determined in 19 out of 21 tumours for which corresponding benign tissues were available. In two cases benign tissues were not available. Allelic losses were determined at five polymorphic dinucleotide repeat microsatellite markers on chromosome 9p21 that included *D9S736*, *D9S974*, *D9S942*, *D9S1748* and *D9S171*. These markers were amplified using primer sequences given in the Genome Data Base (http://www.gdb.org). The frequency of LOH at the *p53* locus on chromosome 17p13.1 was determined by analysing a closely located microsatellite marker *p53CA* (29). For the amplification of the microsatellite markers PCR reaction and analyses were carried out as described previously (27).

Bisulphite treatment of DNA and methylation-specific PCR

Two micrograms of each DNA was denatured in 25 µl 0.3 M NaOH at 37°C for 15 min. Freshly prepared (208 µl) 3 M sodium bisulphite (pH 5.0) and 12 µl fresh 10 mM hydroquinone solutions were added. The samples were incubated under mineral oil at 55°C for 16 h. DNA was purified using the Wizard DNA clean-up system (Promega, Madison, WI) and eluted in 50 µl of water. Purified DNA was treated with NaOH at a final concentration of 0.3 M at room temperature for 5 min and precipitated by ethanol and resuspended in water. Concentration of the recovered DNA was measured

Table I. Primers used in PCR amplifications of the *CDKN2A, CDKN2B*, *CDKN2C*, *CDK4*, *p53* and *p53R2* genes

Exon	Primer	Sequence	Ann. temp. (°C)	Product size (bp)
CDKN2A gene:				
1α	Forward	5′CGG CTG CGG AGA GGG GGA GAC	66	246
	Reverse	5′CAG CGC CCG CAC CTC CTC TA		
1β	Forward	5′GGA GGC GGC GAG AAC AT	63	221
	Reverse	5′GGG CCT TTC CTA CCT GGT CTT		
2 (5′ end)	Forward	5′GGG CTC TAC ACA AGC TTC CTT	63	277
	Reverse	5′AGC CAG GTC CAC GGG CAG AC		
2 (3′ end)	Forward	5′GGG AGG GCT TCC TGG ACA C	63	243
	Reverse	5′TTT GGA AGC TCT CAG GGT ACA		
3	Forward	5′GCC TGT TTT CTT TCT GCC CTC TG	57	144
	Reverse	5′CGA AAG CGG GGT GGG TTG T		
CDKN2B gene:				
1	Forward	5′GCG TCT GGG GGC TGC GGA ATG	63	293
	Reverse	5′CCT CGC CAA CGT AGA CTC CTG TA		
2	Forward	5′CCC GGC CGG CAT CTC CCA TAC	65	350
	Reverse	5′GTT GTG GGC TGG GGA ACC		
CDKN2C gene:				
2	Forward	5′CAA AGG AAA GGG GAA AAA GA	65	250
	Reverse	5′GGG TCA CGT AGG CAA CAT TA		
3 (5′ end)	Forward	5′AAT GGA AGG ACA GGC AGA TA	55	266
	Reverse	5′GCA GGT TCC CTT CAT TAT CC		
3 (3′ end)	Forward	5′CGA GGA TAA TGA AGG GAA CC	58	226
	Reverse	5′CCC TCC CCA CGT TTA TTG AA		
CDK4 gene:				
2	Forward	5′CTG TAA GCG ACT TTT GGT GAT AG	59	274
	Reverse	5′ GAC AAC ATT GGG ATG CTC A		
p53 gene:				
5[a]	Forward	5′TCT GTC TCC TTC CTC TTC CTA CA	61[b]	242
	Reverse	5′CAG CC TGT CGT CTC TCC		
6[a]	Forward	5′CCA GGC CTC TGA TTC CTC ACT GAT T	61	185
	Reverse	5′TTA ACC CCT CCT CCC AGA GAC CC		
7[c]	Forward	5′CTT GGG CCT GTG TTA TCT CCT A	61	231
	Reverse	5′GTA TGG AAG AAA TCG GTA AGA GGT G		
8[d]	Forward	5′CCT GAT TTC CTT ACT GCC TCT TGC T	61	207
	Reverse	5′CGC TTC TTG TCC TGC TTG CTT AC		
p53R2 gene:				
1	Forward	5′GGA CAG GCG AGA AAG CAG GAC	65	249
	Reverse	5′TGA GGG GGA AGA CGC AC AG		
2	Forward	5′GCG TGT TTG TAC TTT ATT TTT	53	192
	Reverse	5′TGC CAC GTA CCT CTT CT		
3	Forward	5′TTT ATT CCA GGT CGA CTT ATC	50	148
	Reverse	5′TAA ATC AAG CAA AAA TCT TAC		
4	Forward	5′CGC TTT AGT CAG GAG GTG	54	153
	Reverse	5′TTT GTA AAT AAA ATC CCA ACA AT		
5	Forward	5′TGT TTT TAC AGG GAA TTT TTA	50	125
	Reverse	5′ATA AAT TAG AGC CAC ATA CCA		
6	Forward	5′TCT CCC AGG GGA AAG AGT	54	164
	Reverse	5′AGA AAA ACA TTC CAT TCC TTA CT		
7	Forward	5′GAC ATT TTT CTT CCC TTT TAG	52	156
	Reverse	5′TGT GCT AAT TAC ACA AAC TTC		
8	Forward	5′TCA TCC CAG TTT TTA ACA	50	152
	Reverse	5′TAA CAC ATT TTT AAA CAC ATT AC		
9	Forward	5′TCC TTT TTA GGT TTT TCT G	50	206
	Reverse	5′TTT ACC AAT GAC AAG TTT ATA		

[a]Primers labelled with TET fluorescent dye.
[b]Exons 5–8 of the *p53* gene were amplified in a multiplex PCR.
[c]Primers labelled with HEX fluorescent dye.
[d]Primers labelled with 6-FAM fluorescent dye.

using spectrophotometer. Bisulphite-treated DNA was used for amplification of both *p16* and *ARF* promoter fragments of the *CDKN2A* gene. Each fragment was amplified by PCR using primers specific for both methylated and unmethylated sequences (30,31). DNA isolated from breast cancer cell line T47D and colon cancer cell line DLD-1 were used as positive controls for the methylated *p16* and *ARF* promoters, respectively. DNA isolated from lymphocytes donated by a healthy donor was used as a negative control.

HPLC determination of efficiency of bisulphite conversion

The efficiency of the conversion of unmethylated cytosine to uracil by bisulphite was determined by enzymatic hydrolysis of both treated and untreated DNA. The released deoxyuridine and deoxycytidine were separated by HPLC and detected by online diode array detector. Briefly, 5 µg sodium bisulphite-treated and untreated control DNA samples were incubated at 37°C with nuclease P1 (5 µg) in 20 mM NaOAc buffer (pH 5.0) in the presence of $ZnCl_2$. Incubation was continued for another 60 min in the presence of 1 µl (0.28 U) alkaline phosphotase in 100 mM Tris–HCl (pH 8.4). The samples were injected into HPLC (Beckman system Gold) connected to a 168-diode array detector for separation on a 4 µ Genesis 4.6 + 250 mm C_{18} reversed-phase column (Jones Chromatography, Hengoed, UK). The elution was with a linear gradient from 98 to 60% 50 mM ammonium formate (pH 4.6) in methanol over 30 min. The known amounts of uracil and cytosine

Table II. General information about the OSCC patients included in the study

Case	Age	Gender	Grade	Smoking status	Alcohol consumption
1	38	M	I–II	1 Pack/day; 15 years	Yes; 15 years
2[a]	45	M	I	5 Cig/day; 25 years	No
3	60	F	III	No	No
4	48	M	II	1 Pack/day; 30 years	Yes; 30 years
5	71	M	I	1 Pack/day; 30 years	Yes; 30 years
6	81	M	II	Not known	Not known
7	51	M	II	1 Pack/day; 40 years	Yes; 15 years
8	75	M	II	Not known	Not known
9	53	F	II	No	No
10	55	M	III	1 Pack/day; 30 years	No
11	59	F	III	No	No
12	35	F	–	No	No
13	55	F	II	No	No
14	65	F	I	1 Pack/day; 28 years	No
15	61	M	I–II	Not known	Not known
16	50	F	I	Not known	Not known
17	64	M	I	7 Cig/day; 30 years	No
18	54	M	I	2 Packs/day; 30 years	No
19	68	M	III	Not known	Not known
20	50	M	II	3 Packs/day; 10 years	Yes; 10 years
21	52	M	-	1 Pack/day; 30 years	No

[a]Case 2 in addition to oesophageal cancer also had liver cancer. Grade information for cases 12 and 21 not known.

deoxynucleosides were injected as standards for retention time determination and quantification.

Results

In this study we analysed DNA from tumour and benign tissues from 21 OSCC cases from a high-risk area of China for various alterations in different cell cycle regulatory genes. DNA samples were analysed for mutations in six genes, which included the *CDKN2A* (exon 1β, exon 1α–exon 3), *CDKN2B* (exons 1 and 2), *CDKN2C* (exons 2 and 3), *CDK4* (exon 2), *p53* (exons 5–8) and *p53R2* genes (exons 1–9). In an extended study we determined homozygous deletion at the *CDKN2A* (exon 1β) locus and hypermethylation in two separate promoters that transcribe p16 and ARF. In addition, we determined the frequency of LOH at the *CDKN2A* and *p53* loci using polymorphic dinucleotide microsatellite markers.

Mutations and polymorphisms: CDKN2A, CDKN2B, CDKN2C and CDK4 genes

In the *CDKN2A/p16* gene we detected five mutations in exon 2 and one mutation in exon 1α (Table III and Figure 2). No mutation was found in exon 1β of the *CDKN2A/ARF* in any tumour. One mutation each in exon 1α (case 8) and exon 2 (case 3) was a frame-shift causing single base deletion at codons 8 and 77 of the *CDKN2A/p16* gene, respectively (Tables III and IV). The frame-shift mutation at codon 77 in the tumour from case 3 also caused a frame-shift in the ARF transcript of the *CDKN2A* gene at codon 92, whereas the codon 8 frame-shift (case 8) being in exon 1α was unique to the p16 transcript. Incidentally, this tumour (case 8) also carried a novel C > G transversion in exon 2 of the *CDK4* gene at codon 51 causing an Ile to Met change in the amino acid residue. This base change was detected in the corresponding benign tissue as well, indicating that it could be a rare polymorphism in the *CDK4* gene. The other mutations detected in four OSCC (cases 2, 11, 19 and 20) were nonsense mutations in exon 2 of the *CDKN2A/p16* gene. In two tumours (cases 11 and 20) the nonsense mutations introduced a stop codon in place of an arginine residue at codon 58 and in the other two tumours (cases 19 and 2) stop codons were introduced at residues 80 and 110 of the *CDKN2A/p16* gene, respectively. The nonsense mutations affecting the p16 transcript (in cases 2, 11, 19 and 20) also caused missense changes in amino acid residues in the ARF transcript (Table IV). Three of the four single-base change mutations in the *CDKN2A* gene were at CpG sites. No mutation was detected in either the *CDKN2B* or *CDKN2C* genes.

Previously reported polymorphisms in the CDKN2A, CDKN2B, and CDKN2C genes were detected in the OSCC cases

The 500 C > G and 540 C > T polymorphisms in the 3′ untranslated region of the CDKN2A gene were identified in two cases each (Table III). The 500 C > G polymorphism was in linkage disequilibrium with the 74 C > A polymorphism ~50 kb apart in intron 1 of the CDKN2B gene as seen in our earlier studies (32,33). In addition, a silent T > C polymorphism at codon 114 in exon 3 of the CDKN2C gene was detected in three cases (3, 9 and 10).

The p53 and p53R2 genes

We detected 15 mutations in exons 5–8 of the *p53* gene in 14 OSCC cases (Tables III and IV, Figure 2). The majority of the mutations detected (nine mutations in eight cases) were in exon 5 with one tumour (case 5) containing two nonsense mutations. In addition, in two tumours mutations were in exon 6 (cases 3 and 12) and in four tumours (cases 2, 6, 10 and 21) mutations were in exon 8. We did not find any mutation in exon 7. Nine of the 15 mutations detected were transitions (cases 3, 4, 6, 8, 10–12, 17 and 21) and the rest were transversions (cases 2, 5, 9, 15 and 19). Four C > T transitions (cases 3, 11, 17 and 21) were at CpG sites. Of the detected mutations four were nonsense with two in exon 5 being in the same tumour (case 5) at codons 180 and 182. The other two nonsense mutations were at codons 165 (case 4) and 213 (case 3). The rest of the mutations were missense. We also detected a known C > T polymorphism in intron 7 in 14 cases (34,35).

We screened nine exons of the newly cloned *p53R2* gene, located on chromosome 8q23.1, for mutations and polymorphisms by the SSCP technique (25,36). The *p53R2* gene, which encodes the ribonucleotide reductase small subunit R2 homologue, is induced by p53. No mutation was detected in any of the nine exons. In the 5′ untranslated region of the gene we detected a novel polymorphism (37) that was present in four cases. One sample (case 16) with the polymorphism showed a specific loss of the wild-type allele in the tumour compared with the corresponding benign tissue that contained both alleles, while the rest of the polymorphic cases had both alleles intact (Table III).

Promoter methylation in the CDKN2A (p16 and ARF) gene

Hypermethylation was determined in the p16 and ARF promoters of the *CDKN2A* gene using the methylation-specific PCR method based on bisulphite conversion of unmethylated cytosines to uracils in DNA (30). Initially, we validated the bisulphite-based conversion method by separating and quantifying uracil and cytosine deoxynucleosides in the treated and non-treated DNA after enzymatic hydrolysis of DNA using nuclease P1 and alkaline phosphatase. The separation and quantification of uracil and cytosine deoxynucleosides was carried out using HPLC with an on-line diode array UV

Table III. Details of the alterations detected in various genes and marker loci in the squamous cell carcinoma of oesophagus

Case	*P16^{INK4a}*	*p14^{ARF}*	*p15^{INK4b}*	*D9S736*	*D9S974*	*D9S942*	*D9S1748*	*D9S171*	*CDK4*	*p53*	*p53CA*	*p53R2*
1	540 C > T[a]	Homozygous deletion		NI[b]	I	I	I	LOH			LOH	
2	W110ter[c]	G125R/Methylation		NI	NI	LOH	NI	NI		F270L	I	
3[d]	231 T deletion[e]/500 C > G	274 T deletion/ Methylation	74 C > A[f]	NI	LOH	LOH	LOH	NI		R213ter	I	
4				NI	NI	I	I	NI		Q165ter	LOH	
5				I	NI	I	NI	I		E180ter/C182ter	I	
6		Methylation		NI	NI	I	NI	NI		P278S	LOH	
7		Methylation		LOH	I	I	I	NI			I	
8	22 A deletion[e]	Methylation		NI	NI	LOH	LOH	NI	I51M[g]	IVS5 + 1G > A	I	
9				NI	I	I	I	NI		C176F	NI	
10		Homozygous deletion		NI	I	I	I	NI		E286L	LOH	
11	R58ter	P72L		NI	LOH	LOH	LOH	LOH		R175H	LOH	
12		Methylation		NI	I	I	I	NI		V216M	LOH	–88 C > A
13	Methylation	Methylation		NI	LOH	LOH	NI	NI			I	
14	Methylation	Methylation		NI	NI	I	NI	LOH			LOH	
15		Methylation		LOH	I	I	I	NI		C135W	LOH	
16	Methylation			LOH	LOH	LOH	LOH	NI			I	–88 C > A/LOH
17	Methylation/500 C > G	Methylation	74 C > A	NI	I	I	NI	NI		C135Y	I	–88 C > A
18	540 C > T			ND	ND	ND	ND	ND			I	
19	R80ter	P94L		LOH	LOH	LOH	LOH	NI		A138P	I	
20	R58ter	P72L/Methylation		ND	ND	ND	ND	ND			I	–88 C > A
21		Homozygous deletion		I	I	I	NI	I		R282W	LOH	

[a]500 C > G and 540 C > T polymorphisms are located in the 3′ UTR of the *CDKN2A* gene and the number refers to its position from the first base of the start codon of the p16 transcript.
[b]NI is non-informative; ND is not-done; I is informative; and LOH is loss of heterozygosity.
[c]ter indicates introduction of a stop codon due to a single base change.
[d]Cases 3, 9 and 10 carried T > C silent polymorphism in codon 114 of the *CDKN2C* (*p18^{INK4c}*) gene.
[e]For a single base deletion the number before nucleotides refers to the base number starting from A of ATG start codon.
[f]Intronic polymorphism located in intron 1 of the *CDKN2B* (*p15^{INK4b}*) gene and is located 27 bp upstream of exon 2 and the number is according to the position in GDB sequence accession number S69805. This polymorphism is in linkage disequilibrium with 500C > G polymorphism in the 3′ UTR of the *CDKN2A* gene.
[g]The base change in codon 51 of the *CDK4* gene was also detected in the corresponding benign tissue.

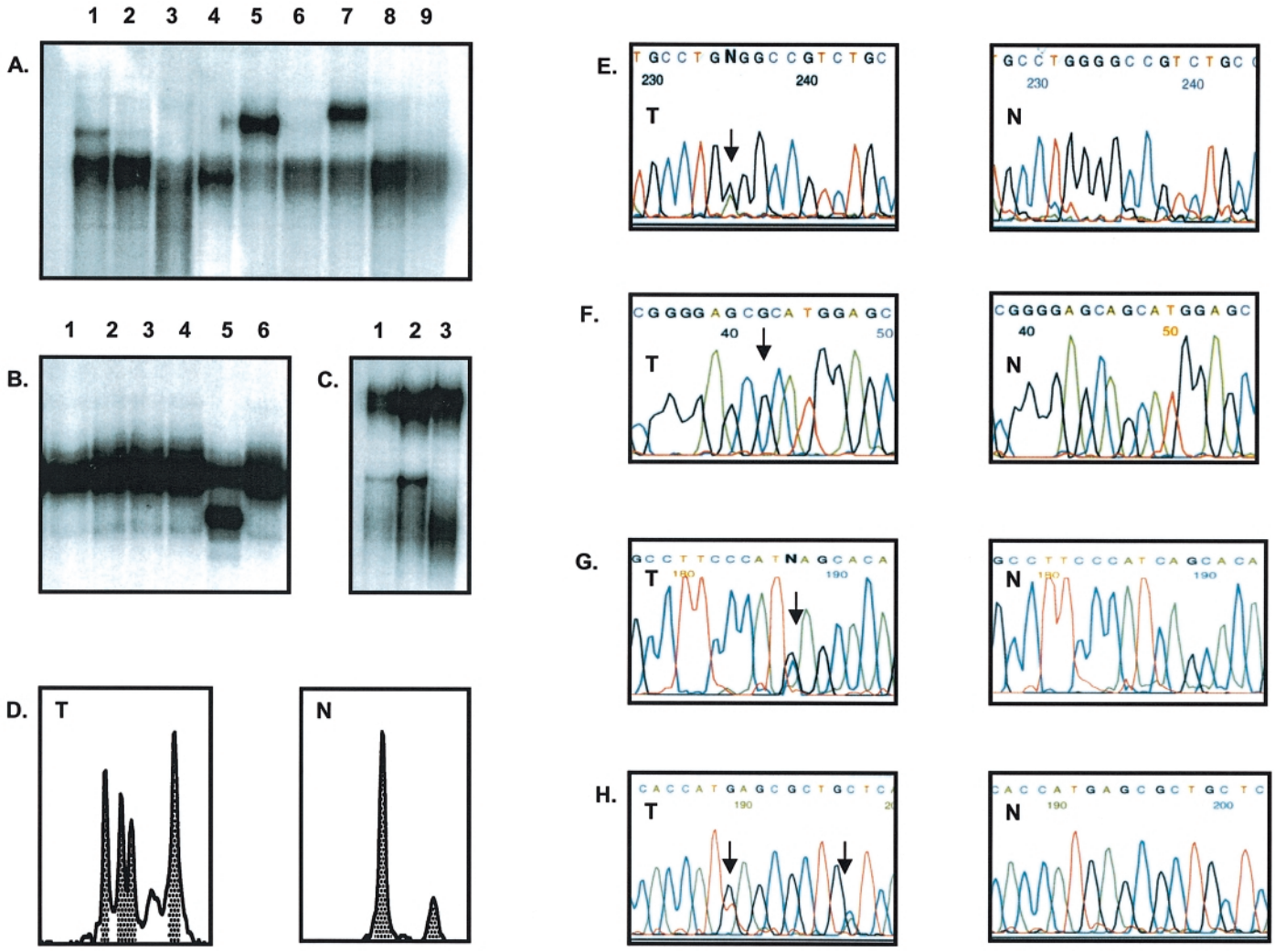

Fig. 2. (**A**) SSCP analysis of 5′ exon 2 (277 bp) of the *CDKN2A* gene with shifted bands in fragments amplified from tumour DNA in lanes 1 (case 2), 3 (case 3), 5 (case 11) and 7 (case 19). Lanes 2 (case 2), 4 (case 3), 6 (case 11) and 8 (case 19) show amplified fragments from corresponding benign tissues. Lane 9 contained the fragment amplified from a control DNA isolated from lymphocytes of a healthy donor. (**B**) SSCP analysis of exon 1α (246 bp) of the *CDKN2A* gene. Lanes 1–6 contain fragments amplified from DNA isolated from tumour tissues from cases 4–9. The shifted band in lane 5 (case 8) represented 22A single base deletion in codon 8 of the p16 transcript. (**C**) SSCP analysis of exon 2 (274 bp) of the *CDK4* gene with shifted bands in lanes 1 and 2 representing C to G change in codon 51 in both tumour as well as corresponding benign tissue in case 8. Lane 3 contains control DNA. (**D**) Electropherograph representing SSCP of exon 5 (242 bp) of the *p53* gene in tumour (T) with mutation in case 5 and from normal DNA (N). (**E**) Sequence analysis of part of exon 2 of the *CDKN2A* gene in tumour (T) in case 2 with G to A mutation that introduced a stop codon in place of Trp in codon 110 of $p16^{INK4a}$ and causes Gly to Arg change in codon 125 of *ARF*. The corresponding sequence from control DNA (N) did not show any change. (**F**) Sequence analysis of part of exon 1 of the *CDKN2A* gene in tumour (T) in case 8 with 22A deletion in codon 8 and corresponding normal sequence in a control DNA. (**G**) Sequence analysis of part of exon 2 of the *CDK4* gene in case 8 (T) showing C to G mutation in codon 51 causing Ile to Met change in the amino acid residue and corresponding normal sequence (N) from a control DNA. This base change was detected and confirmed in both tumour as well as benign tissue from the same patient. (**H**) Sequence analysis of part of exon 5 of the *p53* gene showing two mutations in the tumour DNA from case 5 introducing stop codons at 180 and 182 residues in place of Glu and Cys, respectively.

detector. In bisulphite-treated DNA we detected only uracil deoxynucleoside and no cytosine deoxynucleoside, indicating the complete conversion of cytosine to uracil during the procedure (Figure 3). Analysis of OSCC tumour samples resulted in the detection of methylation in the promoter specific to *CDKN2A/p16* in four tumours (cases 13, 14, 16 and 17) whereas *CDKN2A/ARF* promoter-specific methylation was detected in 11 cases (Table III).

Homozygous deletion at the CDKN2A (exon 1β) locus

Real time PCR was used for the detection of homozygous deletion at exon 1β of the *CDKN2A* gene. A 92 bp fragment located at exon 1β of the *CDKN2A* gene was co-amplified along with a 97 bp intronic fragment of the *GAPDH* gene. Ct-values were used to calculate the initial copy number of the target and the reference sequence and a ratio of the two was used to determine homozygous deletion at the *CDKN2A* (exon 1β) locus. Of all the OSCC analysed, homozygous deletion at the *CDKN2A* gene (exon 1β locus) was detected in three tumours (cases 1, 10 and 21; Table III). None of these tumours contained either mutation or methylation nor did these tumours show mono-allelic loss at the markers located within the *CDKN2A* locus.

LOH at the CDKN2A and p53 loci

Allelic losses were determined at the *CDKN2A* locus at 9p21 and at the *p53* locus at 17p13.1 by analysing polymorphic dinucleotide microsatellite markers in tumours and corresponding benign tissues. At the *CDKN2A* locus, allelic losses were determined at five microsatellite markers. Three of these

Table IV. DNA and amino acid sequence changes in the *p53* and *CDKN2A* (p16 and $p14^{ARF}$) genes in oesophageal squamous cell carcinoma

Case	Changes in the *CDKN2A gene*						Changes in the *p53 gene*			
	Sequence change	Amino acid		Codon		Exon	Sequence change	Amino acid	Codon	Exon
		p16	ARF	p16	ARF					
1										
2	TGG > TGA	Trp > Ter	Gly > Arg	110	125	2	TTT > TTA	Phe > Leu	270	8
3	CTC > C*C	Frame-shift	Frame-shift	77	92	2	CGA > TGA	Arg > Ter	213	6
4							CAG > TAG	Gln > Ter	165	5
5							GAG > TAG	Glu > Ter	180	5
							TGC > TGA	Cys > Ter	182	5
6							CCT > TCT	Pro > Ser	278	8
7										
8	AGC > *GC	Frame-shift	NE	8	–	1	IVS + 1 G > A			
9							TGC > TTC	Cys > Phe	176	5
10							GAA > AAA	Glu > leu	286	8
11	CGA > TGA	Arg > Ter	Pro > Leu	58	72	2	CGC > CAC	Arg > His	175	5
12							GTG > ATG	Val > Met	216	6
13										
14										
15							TGC > TGG	Cys > Trp	135	5
16										
17							TGC > TAC	Cys > Tyr	135	5
18										
19	CGA > TGA	Arg > Ter	Pro > Leu	80	94	2	GCC > CCC	Ala > Pro	138	5
20	CGA > TGA	Arg > Ter	Pro > Leu	58	72	2				
21							CGG > TGG	Arg > Trp	282	8

NE, no effect.

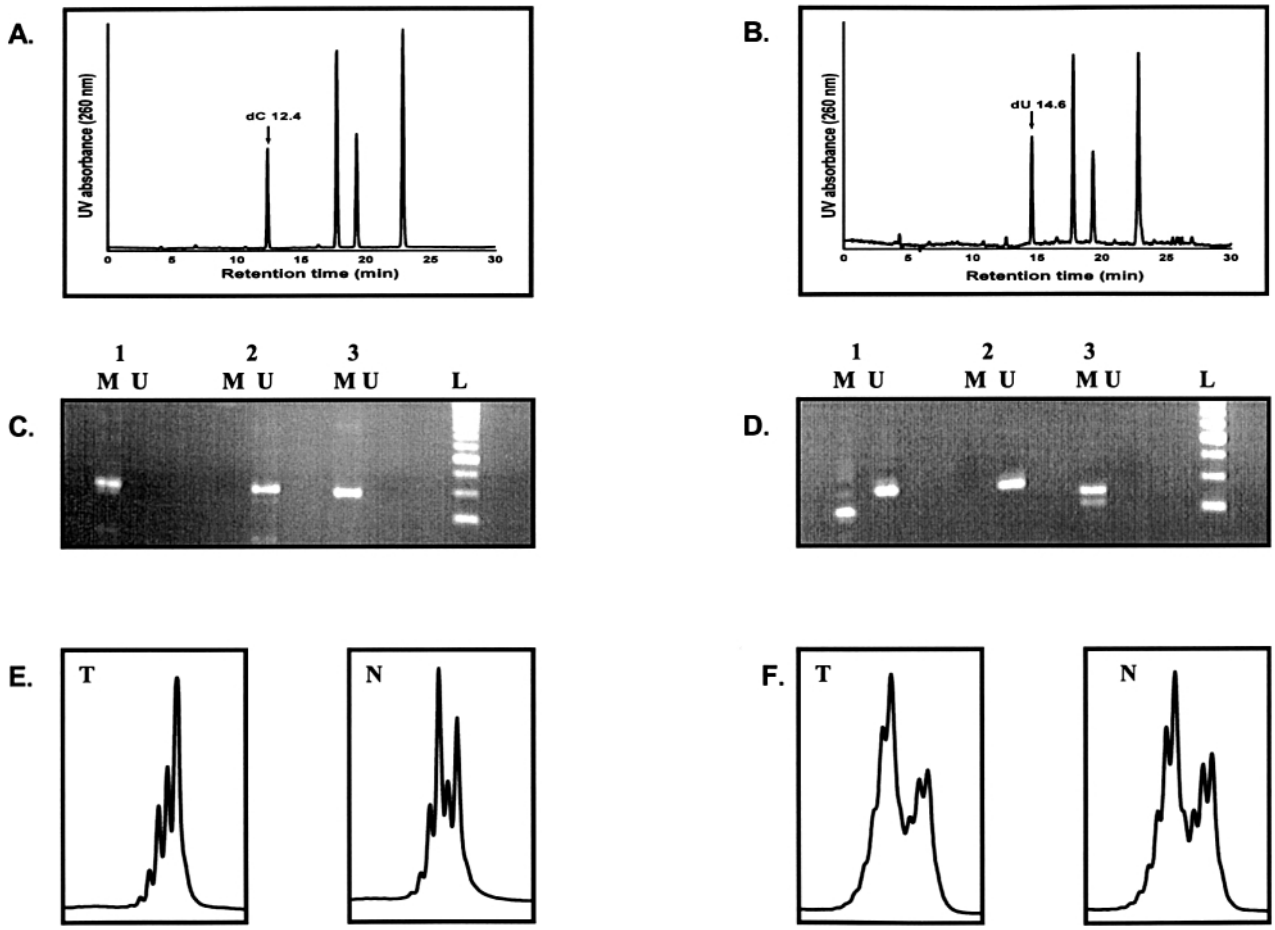

Fig. 3. (**A**) HPLC chromatogram showing separation of deoxynucleosides in untreated DNA after enzymatic hydrolysis with nuclease P1 and alkaline phosphotase. Cytosine deoxynucleoside eluted with a retention time of 12.4 min. (**B**) HPLC separation of deoxynucleosides in DNA treated with sodium bisulphite followed by enzymatic hydrolysis. The chromatograph shows complete conversion of cytosine to uracil as the uracil deoxynucleoside eluted with a retention time of 14.6 min. (**C**) Methylation-specific PCR analysis of promoter region of $p16^{INK4a}$ by treatment of DNA with bisulphite followed by PCR amplification with primers specific for methylated and unmethylated $p16^{INK4a}$ promoter. PCR products (150 bp) are seen in methylated lane in 1 (case 16) and in 3 in which DNA from breast cancer cell line T47D has been used as a positive control for methylation. PCR product (151 bp) is seen only in unmethylated lane in 2 (case 10) in which homozygous deletion was detected at exon 1β locus. Lane marked 'L' represents 50 bp DNA marker ladder. (**D**) In MSP analysis of the *CDKN2/ARF* promoter, PCR products are seen in 1 (case 12) in both methylated (122 bp) and unmethylated lanes (132 bp) and only in unmethylated lane in 2 (case 4). In 3, PCR product is seen only in methylated lane where DNA from colorectal cancer cell line DLD-1 was used as a positive control for the *ARF* promoter hypermethylation. (**E**) Loss of allele in tumour (T) with promoter hypermethylation in case 16 compared with corresponding benign tissue (N) determined at dinucleotide repeat microsatellite marker *D9S1748*. (**F**) Retention of both alleles in tumour (T) with promoter ARF hypermethylation in case 7 when compared with corresponding benign tissue (N) determined at the dinucleotide repeat microsatellite marker *D9S942*.

markers, *D9S1748*, *D9S942* and *D9S974* are located between exon 1β and exon 1α (38), whereas *D9S736* and *D9S171* are located at a distance >300 kb telomeric and centromeric to the *CDKN2A* locus, respectively (Figure 1). All the tumours that carried mutations in the *CDKN2A* gene (cases 2, 3, 8, 11, 19 and 20) also showed loss of wild-type allele at all the informative markers within the locus (Table III). Out of the four tumours with methylation specific to the *CDKN2A/p16* promoter, two (cases 13 and 16) showed a loss of the non-methylated allele at all informative loci, the other two tumours (cases 14 and 17) showed retention of both alleles (Figure 3). The correlation between methylation specific to the *CDKN2A/ARF* promoter and allelic loss was not clear. LOH was seen only in those tumours with the *ARF* methylation that also carried the *p16*-specific methylation/mutation (cases 8 and 13). One tumour (case 3) in addition to carrying a truncating mutation and LOH in the *CDKN2A* gene also showed ARF-specific promoter methylation. Three of the tumours with the *CDKN2A/ARF*-specific methylation showed allelic losses at outside loci, two at *D9S736* (cases 7 and 15) and one at *D9S171* (case 14). The latter was also hypermethylated at the *p16* promoter. None of the tumours without any mutation or promoter methylation showed allelic loss at the three markers *D9S1748*, *D9S942* and *D9S974*, which are located within the *CDKN2A* gene locus (Figure 1). One tumour (case 1) with homozygous deletion at exon 1β showed LOH at the *D9S171* marker, which is located outside the *CDKN2A* locus towards the centromeric site.

The frequency of LOH was also determined at the *p53* locus on chromosome 17p13.1 using a closely located dinucleotide repeat microsatellite. Seven tumours (cases 4, 6, 10–12, 15 and 21) with mutations in the *p53* gene showed loss of wild-type allele whereas five tumours (cases 2, 3, 8 17 and 19) with mutations had retained the wild-type allele (Fisher exact test *P*-value ~0.65) (Table III). One tumour (case 5) with two nonsense mutations in exon 5 did not show, as expected, loss of an allele; whereas case 9 with a mutation at codon 176 was non-informative at the marker studied. Two tumour samples

Table V. Correlations between different types of inactivation detected at the *CDKN2A* and *p53* loci in OSCC

	Cases with $p16^{INK4a}$ inactivation		Cases with $p14^{ARF}$ inactivation	
	Yes	No	Yes	No
Cases with LOH at *p53* locus				
Yes	2	8	8	2
No	8	3	8	3
	OR 0.09, 95% CI 0.01–0.98 *P*-value ~0.03 (Fisher exact test)		OR 1.50, 95% CI 0.14–18.1 *P*-value ~1.0 (Fisher exact test)	
Cases with *p53* mutations				
Yes	6	8	11	3
No	4	3	5	2
	OR 0.56, 95% CI 0.06–4.90 *P*-value ~0.65 (Fisher exact test)		OR 1.47, 95% CI 0.12–17.74 *P*-value ~1.0 (Fisher exact test)	

showed allelic loss at the locus with no mutation in any of the *p53* exons analysed.

Correlations between various genetic, epigenetic alterations and tumour grade

Out of 10 OSCC tumours with mutation/methylation/loss of wild-type allele in the *CDKN2A*/*p16* gene, six carried mutations in the *p53* gene of which only two also had LOH at the locus, whereas, eight OSCC tumours without mutation/methylation/allelic loss in the *CDKN2A/p16* gene carried LOH at the *p53* locus (Table V; two-tailed Fisher exact test, *P*-value ~0.03; OR 0.09 95% CI 0.01–0.98). However, no inverse correlation was detected between mutation/methylation in the *CDKN2A/p16* gene and mutations in the *p53* gene (two-tailed Fisher exact test, *P*-value ~0.65; OR 0.56 95% CI 0.06–4.90). Similarly, no correlation was found between the *CDKN2A/ARF* inactivation through methylation, homozygous deletion/mutation and mutations in the *p53* gene (Table V; two-tailed Fisher exact test, *P*-value ~1.0; OR 1.47 95% CI 0.12–17.74).

All the four cases (cases 3, 10, 11 and 19) with grade III tumours showed inactivation of the *CDKN2A* gene and mutation in the *p53* gene compared with five tumours (cases 2, 6, 8, 15 and 17) out of 15 with grade I–II (two-tailed Fisher exact, *P*-value ~0.03). Six patients (cases 1, 7, 13, 14, 16 and 20) with grade I–II tumours showed one or the other inactivation in the *CDKN2A* gene, whereas, three patients (cases 4, 5 and 9) with grade I–II tumours had only *p53* mutation. One grade I tumour from case 18 did not have any inactivation in either the *CDKN2A* or *p53*.

Discussion

The genetic studies on OSCC have resulted in a consistent detection of alterations in the *p53* and *CDKN2A* genes, albeit, at varying frequencies and inconsistent patterns (10–12). In the present study, adopting a comprehensive approach, we determined the genetic and epigenetic alterations in the six genes, which encode various components of the Rb and p53 pathways of cell cycle regulation in OSCC from a high-risk area in China. One of the main features of our results was the detection of genetic and epigenetic alterations concomitantly in both the *CDKN2A* and *p53* genes in more than half of the OSCC tumours studied. The other major highlights of our results were (i) that 86% of the OSCC tumours harboured one or the other genetic/epigenetic alteration in the *CDKN2A* (*p16* and *ARF*) gene and in more than half of the tumours alterations concurrently affected both p16 and ARF and (ii) alterations in the *p53* locus were found in 76% of the cases. The inactivation of either the *CDKN2A* or *p53* in a majority of low-grade tumours in this study suggests the possible important and independent role of these genes in initiation process of OSCC, whereas tumour progression probably involves acquisition of the loss of both genes.

The alterations detected in the *CDKN2A* gene in this study, besides hypermethylation and homozygous deletions, included six truncating mutations affecting the p16 transcript, of which five also caused missense or truncating alterations in the ARF transcript. Interestingly, the only mutation found exclusive to p16 (in exon 1α) was a single base deletion (22A del) in codon 8. Not many alterations are reported in the N-terminus of the p16, as the N-terminus encoded by the initial 10 codons does not form a part of the ankyrin repeat structure (39). Moreover, the ATG sequence in codon 9 creates an additional start codon, which due to an earlier discrepancy was designated as the 'real' start codon (40). Earlier *in vitro* studies on the cDNA lacking the initial eight codons showed no bearing on the functional properties (41,42). Nevertheless, we found that the tumour with the deletion mutation in codon 8 (of the p16 transcript) like five other tumours with mutations in different codons in exon 2 had lost wild-type allele. Intriguingly, this particular case (with the mutation in codon 8 of p16 transcript) carried a novel germ-line polymorphism (Ile51Met) in the *CDK4* gene along with ARF promoter hypermethylation. The activating alterations in the *CDK4* gene and inactivating alterations in the *p16* gene are usually thought to be mutually exclusive as the proteins function in the same Rb pathways (43). It is probable that the rare polymorphic change has little or no affect on the function of the CDK4 and the frame-shift mutation results in the loss of p16 protein. The hypermethylation in ARF, obviously, would rather target the MDM2-p53 pathway.

Promoter hypermethylation of the *CDKN2A* gene was another alteration that we detected in the present study at a high frequency in OSCC. Interestingly, the ARF promoter was found to be hypermethylated in more than half of the tumours studied. Hypermethylation, besides homozygous deletion, has emerged as the prime mechanism of ARF inactivation as mutations exclusive to the transcript are enigmatically rare (31,44,45). Moreover, the effect of mutations in the part of the transcript derived from the sequence shared with p16 is not fully resolved. The cell growth inhibition properties are attributed exclusively to the N-terminus encoded by exon 1β, although nucleolus localization signals, at least in human ARF, are suggested to be located in exon 2 (46–48). As in other studies on human tumours, we did not find any correlation

between ARF promoter hypermethylation (or any inactivation) and mutations in the *p53* gene. This non-correlation supports the hypothesis that ARF in addition to its involvement in p53-dependent pathways also functions independent of p53 (31,49,50). Methylation of p16 promoter in non-small cell lung carcinoma has been causally associated with exposure to tobacco smoke; a similar exposure-related methylation in p16 and ARF promoters in OSCC could not be ruled out (51). However, due to a limited number of cases and restricted information available in this study we could not deduce any relation between smoking and methylation status.

The *p53* mutational data in our study conforms to that reported in earlier studies on OSCC from high-risk areas as Iran and China (5,9). Within the limitations of the small number of tumours in our study, the majority of mutations we found were transitions with more than one-third being at the so-called CpG sites and a majority of the total mutations were located in exon 5. Despite the majority of the OSCC patients in this study being smokers, the p53 mutational spectra reflect the aetiology typical of a high-risk area of oesophageal cancer. The CpG transitions can arise either spontaneously or could be consequences of various mechanisms triggered by dietary or life style factors peculiar to the regions associated with a high risk of oesophageal carcinoma, which include exposure to dietary nitrosamines, fermented and moldy foods and nutritional deficiencies (9, 52). Unlike in the *CDKN2A* gene, not all mutations detected in the *p53* gene in OSCC tumours were accompanied by loss of wild-type allele and *vice versa*, although the number of the latter cases was less. Non-conformity of *p53* mutations with Knudson's two-hit hypothesis, as observed in this and other studies also, could be due to acquisition of dominant oncogenic properties by some missense mutants, which suppress wild-type p53 function by heteromerization (53,54). An interesting correlation that emerged from our results is an inverse relationship between cases with the *p16* inactivation (mutations and promoter methylation) and allelic loss (LOH) at the p53 locus. It is also possible that complete bi-allelic *p16* inactivation or reduced p53 dosage, due to loss of one allele, is sufficient for tumourigenesis (55). However, this hypothesis needs to be substantiated with an investigation of a larger number of tumours than in the present study.

Although in some of the reports, progression of OSCC is associated with genetic heterogeneity, our results clearly underscore the specific, unambiguous and independent inactivation of the *CDKN2A* and *p53* genes as major genetic alterations. Even in the *p53R2* gene, which is p53 induced and involved in DNA repair (56), we did not find any tumour-associated mutation. Our observation, in this study on OSCC, of a high frequency of alterations in the *CDKN2A* gene that affect both p16 and ARF is in conformation with animal models that show overlapping and cooperating functions of p16 and ARF in tumourigenesis (57,58). Further, the role of these genes in genesis of squamous cell carcinoma of the oesophagus is supported by the human data on detection of p53 mutations in pre-cancerous lesions and specific inactivation of the *CDKN2A* locus in exposure-simulated animal models. The inactivation of these genes can probably be associated with less than well-understood environmental exposure in the high-risk areas.

Acknowledgement

This study was supported by a grant from the Swedish Cancer Society.

References

1. Cheng,K.K. and Day,N.E. (1996) Nutrition and esophageal cancer. *Cancer Causes Control*, **7**, 33–40.
2. Mandard,A.M., Hainaut,P. and Hollstein,M. (2000) Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mutat. Res.*, **462**, 335–342.
3. Roth,M.J., Hu,N., Emmert-Buck,M.R., Wang,Q.H., Dawsey,S.M., Li,G., Guo,W.J., Zhang,Y.Z. and Taylor,P.R. (2001) Genetic progression and heterogeneity associated with the development of esophageal squamous cell carcinoma. *Cancer Res.*, **61**, 4098–4104.
4. Hu,N., Roth,M.J., Polymeropolous,M., Tang,Z.Z. *et al.* (2000) Identification of novel regions of allelic loss from a genomewide scan of esophageal squamous-cell carcinoma in a high-risk Chinese population. *Genes Chromosomes Cancer*, **27**, 217–228.
5. Hu,N., Huang,J., Emmert-Buck,M.R. *et al.* (2001) Frequent inactivation of the TP53 gene in esophageal squamous cell carcinoma from a high-risk population in China. *Clin. Cancer Res.*, **7**, 883–891.
6. Sepehr,A., Taniere,P., Martel-Planche,G., Zia'ee,A.A., Rastgar-Jazii,F., Yazdanbod,M., Etemad-Moghadam,G., Kamangar,F., Saidi,F. and Hainaut,P. (2001) Distinct pattern of TP53 mutations in squamous cell carcinoma of the esophagus in Iran. *Oncogene*, **20**, 7368–7374.
7. Taniere,P., Martel-Planche,G., Saurin,J.C., Lombard-Bohas,C., Berger,F., Scoazec,J.Y. and Hainaut,P. (2001) TP53 mutations, amplification of P63 and expression of cell cycle proteins in squamous cell carcinoma of the oesophagus from a low incidence area in Western Europe. *Br. J. Cancer*, **85**, 721–726.
8. Hernandez-Boussard,T., Rodriguez-Tome,P., Montesano,R. and Hainaut,P. (1999) IARC p53 mutation database: a relational database to compile and analyze p53 mutations in human tumors and cell lines. International Agency for Research on Cancer. *Hum. Mutat.*, **14**, 1–8.
9. Biramijamal,F., Allameh,A., Mirbod,P., Groene,H.-J., Koomagi,R. and Hollstein,M. (2001) Unusual profile and high prevalence of p53 mutations in esophageal squamous cell carcinomas from northern Iran. *Cancer Res.*, **61**, 3119–3123.
10. Esteve,A., Martel-Planche,G., Sylla,B.S., Hollstein,M., Hainaut,P. and Montesano,R. (1996) Low frequency of p16/CDKN2 gene mutations in esophageal carcinomas. *Int. J. Cancer*, **66**, 301–304.
11. Xing,E.P., Nie,Y., Song,Y., Yang,G.Y., Cai,Y.C., Wang,L.D. and Yang,C.S. (1999) Mechanisms of inactivation of p14ARF, p15INK4b and p16INK4a genes in human esophageal squamous cell carcinoma. *Clin. Cancer Res.*, **5**, 2704–2713.
12. Gamieldien,W., Victor,T.C., Mugwanya,D., Stepien,A., Gelderblom,W.C., Marasas,W.F., Geiger,D.H. and van Helden,P.D. (1998) p53 and p16/CDKN2 gene mutations in esophageal tumors from a high-incidence area in South Africa. *Int. J. Cancer*, **78**, 544–549.
13. Kumar,R., Sauroja,I., Punnonen,K., Jansen,C. and Hemminki,K. (1998) Selective deletion of exon 1beta of the p19ARF gene in metastatic melanoma cell lines. *Genes Chromosomes Cancer*, **23**, 273–277.
14. Rocco,J.W. and Sidransky,D. (2001) p16 (MTS-1/CDKN2/INK4a) in cancer progression. *Exp. Cell Res.*, **264**, 42–55.
15. Borg,A., Sandberg,T., Nilsson,K., Johannsson,O., Klinker,M., Masback,A., Westerdahl,J., Olsson,H. and Ingvar,C. (2000) High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. *J. Natl Cancer Inst.*, **92**, 1260–1266.
16. Shi,S.T., Yang,G.Y., Wang,L.D., Xue,Z., Feng,B., Ding,W., Xing,E.P. and Yang,C.S. (1999) Role of p53 gene mutations in human esophageal carcinogenesis: results from immunohistochemical and mutation analyses of carcinomas and nearby non-cancerous lesions. *Carcinogenesis*, **20**, 591–597.
17. Fong,L.Y., Nguyen,V.T., Farber,J.L., Huebner,K. and Magee,P.N. (2000) Early deregulation of the the p16ink4a-cyclin D1/cyclin-dependent kinase 4-retinoblastoma pathway in cell proliferation-driven esophageal tumorigenesis in zinc-deficient rats. *Cancer Res.*, **60**, 4589–4595.
18. Chen,L., Matsubara,N., Yoshino,T., Nagasaka,T., Hoshizima,N., Shirakawa,Y., Naomoto,Y., Isozaki,H., Riabowol,K. and Tanaka,N. (2001) Genetic alterations of candidate tumor suppressor ING1 in human esophageal squamous cell cancer. *Cancer Res.*, **61**, 4345–4349.
19. Sherr,C.J. (1998) Tumor surveillance via the ARF-p53 pathway. *Genes Dev.*, **12**, 2984–2991.
20. Sherr,C.J. (2000) The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res.*, **60**, 3689–3695.
21. Zhang,Y. and Xiong (2001) Control of p53 ubiquitination and nuclear export by MDM2 and ARF. *Cell Growth Differ.*, **12**, 175–186.
22. Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

23. Gronbaek,K., de Nully Brown,P., Moller,M.B., Nedergaard,T., Ralfkiaer,E., Moller,P., Zeuthen,J. and Guldberg,P. (2000) Concurrent disruption of p16INK4a and the ARF-p53 pathway predicts poor prognosis in aggressive non-Hodgkin's lymphoma. *Leukemia*, **14**, 1727–1735.
24. Bardeesy,N., Morgan,J., Sinha,M., Signoretti,S., Srivastava,S., Loda,M., Merlino,G. and DePinho,R.A. (2002) Obligate roles for p16 (Ink4a) and p19 (Arf)-p53 in the suppression of murine pancreatic neoplasia. *Mol. Cell. Biol.*, **22**, 635–643.
25. Tanaka,H., Arakawa,H., Yamaguchi,T., Shiraishi,K., Fukuda,S., Matsui,K., Takei,Y. and Nakamura,Y. (2000) A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage [see comments]. *Nature*, **404**, 42–49.
26. Kumar,R., Lundh Rozell,B., Louhelainen,J. and Hemminki,K. (1998) Mutations in the CDKN2A (p16INK4a) gene in microdissected sporadic primary melanomas. *Int. J. Cancer*, **75**, 193–198.
27. Kumar,R., Smeds,J., Lundh Rozell,B. and Hemminki,K. (1999) Loss of heterozygosity at chromosome 9p21 (INK4-p14ARF locus): homozygous deletions and mutations in the p16 and p14ARF genes in sporadic primary melanomas. *Melanoma Res.*, **9**, 138–147.
28. Berggren,P., Steineck,G. and Hemminki,K. (2000) A rapid fluorescence based multiplex polymerase chain reaction—single-strand conformation polymorphism method for p53 mutation detection. *Electrophoresis*, **21**, 2335–2342.
29. Jones,M.H. and Nakamura,Y. (1992) Detection of loss of heterozygosity at the human TP53 locus using a dinucleotide repeat polymorphism. *Genes Chromosomes Cancer*, **5**, 89–90.
30. Herman,J.G., Graff,J.R., Myohanen,S., Nelkin,B.D. and Baylin,S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA*, **93**, 9821–9826.
31. Esteller,M., Tortola,S., Toyota,M., Capella,G., Peinado,M.A., Baylin,S.B. and Herman,J.G. (2000) Hypermethylation-associated inactivation of p14 (ARF) is independent of p16 (INK4a) methylation and p53 mutational status. *Cancer Res.*, **60**, 129–133.
32. Sauroja,I., Smeds,J., Vlaykova,T., Kumar,R., Talve,L., Hahka-Kemppinen,M., Punnonen,K., Jansen,C.T., Hemminki,K. and Pyrhonen,S. (2000) Analysis of G(1)/S checkpoint regulators in metastatic melanoma. *Genes Chromosomes Cancer*, **28**, 404–414.
33. Kumar,R., Smeds,J., Berggren,P., Straume,O., Rozell,B.L., Akslen,L.A. and Hemminki,K. (2001) A single nucleotide polymorphism in the 3′ untranslated region of the CDKN2A gene is common in sporadic primary melanomas but mutations in the CDKN2B, CDKN2C, CDK4 and p53 genes are rare. *Int. J. Cancer*, **95**, 388–393.
34. Berggren,P., Hemminki,K. and Steineck,G. (2000) p53 intron 7 polymorphisms in urinary bladder cancer patients and controls. Stockholm Bladder Cancer Group. *Mutagenesis*, **15**, 57–60.
35. Berggren,P., Kumar,R., Steineck,G., Ichiba,M. and Hemminki,K. (2001) Ethnic variation in genotype frequencies of a p53 intron 7 polymorphism. *Mutagenesis*, **16**, 475–478.
36. Lozano,G. and Elledge,S.J. (2000) p53 sends nucleotides to repair DNA. *Nature*, **404**, 24–25.
37. Smeds,J., Nava,M., Kumar,R. and Hemminki,K. (2001) A novel polymorphism (–88 C>A) in the 5′ UTR of the p53R2 gene. *Hum. Mutat.*, **17**, 82.
38. Randerson-Moor,J.A., Harland,M., Williams,S. *et al.* (2001) A germline deletion of p14 (ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. *Hum. Mol. Genet.*, **10**, 55–62.
39. Ruas,M. and Peters,G. (1998) The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim. Biophys. Acta*, **1378**, F115–177.
40. Serrano,M., Hannon,G.J. and Beach,D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704–707.
41. Parry,D. and Peters,G. (1996) Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma. *Mol. Cell. Biol.*, **16**, 3844–3852.
42. Ranade,K., Hussussian,C.J., Sikorski,R.S., Varmus,H.E., Goldstein,A.M., Tucker,M.A., Serrano,M., Hannon,G.J., Beach,D. and Dracopoli,N.C. (1995) Mutations associated with familial melanoma impair p16INK4 function. *Nature Genet.*, **10**, 114–116.
43. Goldstein,A.M., Struewing,J.P., Chidambaram,A., Fraser,M.C. and Tucker,M.A. (2000) Genotype–phenotype relationships in U.S. melanoma-prone families with CDKN2A and CDK4 mutations. *J. Natl Cancer Inst.*, **92**, 1006–1010.
44. Burri,N., Shaw,P., Bouzourene,H., Sordat,I., Sordat,B., Gillet,M., Schorderet,D., Bosman,F.T. and Chaubert,P. (2001) Methylation silencing and mutations of the p14ARF and p16INK4a genes in colon cancer. *Lab. Invest.*, **81**, 217–229.
45. Esteller,M., Cordon-Cardo,C., Corn,P.G. *et al.* (2001) p14ARF silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. *Cancer Res.*, **61**, 2816–2821.
46. Rizos,H., Darmanian,A.P., Holland,E.A., Mann,G.J. and Kefford,R.F. (2001) Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF. *J. Biol. Chem.*, **276**, 41424–41434.
47. Rizos,H., Darmanian,A.P., Mann,G.J. and Kefford,R.F. (2000) Two arginine rich domains in the p14ARF tumour suppressor mediate nucleolar localization. *Oncogene*, **19**, 2978–2985.
48. Zhang,Y., Xiong,Y. and Yarbrough,W.G. (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, **92**, 725–734.
49. Sanchez-Cespedes,M., Reed,A.L., Buta,M., Wu,L., Westra,W.H., Herman,J.G., Yang,S.C., Jen,J. and Sidransky,D. (1999) Inactivation of the INK4A/ARF locus frequently coexists with TP53 mutations in non-small cell lung cancer. *Oncogene*, **18**, 5843–5849.
50. Eymin,B., Karayan,L., Seite,P., Brambilla,C., Brambilla,E., Larsen,C.J. and Gazzeri,S. (2001) Human ARF binds E2F1 and inhibits its transcriptional activity. *Oncogene*, **10**, 1033–1041.
51. Kim,D.H., Nelson,H.H., Wiencke,J.K., Zheng,S., Christiani,D.C., Wain,J.C., Mark,E.J. and Kelsey,K.T. (2001) p16 (INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. *Cancer Res.*, **61**, 3419–3424.
52. Yang,C.S. (1980) Research on esophageal cancer in China: a review. *Cancer Res.*, **40**, 2633–2644.
53. van Oijen,M.G. and Slootweg,P.J. (2000) Gain of function mutations in the tumor suppressor gene p53. *Clin Cancer Res.*, **6**, 2138–2145.
54. Sigal,A. and Rotter,V. (2000) Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res.*, **60**, 6788–6793.
55. Venkatachalam,S., Shi,Y.P., Jones,S.N., Vogel,H., Bradley,A., Pinkel,D. and Donehower,L.A. (1998) Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J.*, **17**, 4657–4667.
56. Guittet,O., Hakansson,P., Voevodskaya,N., Graslund,A., Arakawa,H., Nakamura,Y. and Thelander,L. (2001) Mammalian p53R2 protein forms an active ribonucleotide reductase *in vitro* with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *J. Biol. Chem.*, **276**, 40647–40651.
57. Krimpenfort,P., Quon,K.C., Mool,W.J., Loonstra,A. and Berns,A. (2001) Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature*, **413**, 83–86.
58. Sharpless,N.E., Bardeesy,N., Lee,K.-H., Carrasco,D., Castrillon,D.H., Agulrre,A.J., Wu,E.A., Horner,J.W. and DePinho,R.A. (2001) Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature*, **413**, 86–91.

Received October 31, 2001; revised January 11, 2002;
accepted January 14, 2002