$p16^{INK4a}$ and $p14^{ARF}$ Tumor Suppressor Genes Are Commonly Inactivated in Cutaneous Squamous Cell Carcinoma

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The $p16^{INK4a}$ and $p14^{ARF}$ tumor suppressor genes (TSGs) are encoded within the CDKN2A locus on chromosome 9p21 and function as cell cycle regulatory proteins in the p53 and RB pathways. Inactivation of these genes by genetic and epigenetic changes has been described in some human cancers, but their importance in cutaneous squamous cell carcinoma (SCC) has not been established. Our detailed examination of 40 cutaneous SCC revealed loss of heterozygosity of 9p21 markers in 32.5% of cases. Mutational analysis confirmed five point mutations in four of 40 SCCs. These mutations changed the amino acid sequence of $p16^{INK4a}$ in four tumors and $p14^{ARF}$ in three tumors. Promoter methylation of $p16^{INK4a}$ and $p14^{ARF}$ was detected in 13 of 36 (36%) and 16 of 38 (42%) cases, respectively. Absent protein expression was confirmed by immunohistochemistry in 13 of 16 (82%) of the tumors with biallelic inactivating events. Overall, the frequency of 9p21 alterations was 76% and for both $p16^{INK4a}$ and $p14^{ARF}$, promoter methylation is the commonest mechanism of gene inactivation. Alterations at this locus were significantly more common in tumors from immunocompetent compared with immunosuppressed individuals. These data confirm the importance of inactivation of $p16^{INK4a}$ and $p14^{ARF}$ TSGs in the pathogenesis of cutaneous SCCs.

Key words: $p16^{INK4a}$/$p14^{ARF}$/tumor supressor genes/CDKN2A/cutaneous squamous cell carcinoma/gene hyper methylation

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Cutaneous squamous cell carcinoma (SCC) is the second most common cancer in Caucasians (Johnson *et al*, 1992). The incidence of the disease is rising rapidly in Europe, North America, and Australia (Holme *et al*, 2000; Alam and Ratner, 2001; Ramsay *et al*, 2002). Almost all cases of this carcinoma arise on sun-exposed areas of skin, implicating chronic exposure to ultraviolet radiation as the major etiological factor (Grossman and Leffell, 1997). Organ transplant recipients have a 100-fold increased risk of developing SCC, implying that immunosuppression may significantly alter individual susceptibility to the disease (Lindelof *et al*, 2000). In these patients cutaneous SCC has a more aggressive clinical course, a greater tendency to metastasize and a higher mortality rate when compared with the general population (Martinez *et al*, 2003). Cutaneous SCC can also arise from within chronic wounds, scars, burns, or ulcers (Alam and Ratner, 2001).

The molecular events associated with the initiation and progression of cutaneous SCC remain poorly understood. The cyclin-dependent kinase inhibitor 2A (CDKN2A) locus on human chromosome 9p21 is the second most commonly altered gene locus in human cancer after p53 (Liggett and Sidransky, 1998).

Abbreviations: Bp, base pairs; CDKN2A, cyclin-dependent kinase inhibitor 2A; HPV, human papillomavirus; LOH, loss of heterozygosity; MSP, methylation specific PCR; SCC, squamous cell carcinoma; TSG, tumour suppressor gene

CDKN2A uniquely encodes for two candidate tumor suppressor genes (TSG): $p16^{INK4a}$ and $p14^{ARF}$. They share common exons 2 and 3 but have alternatively spliced first exons (exon 1α for $p16^{INK4a}$; exon 1β for $p14^{ARF}$). These first exons are under the control of distinct promoters and uniquely create two proteins that have no sequence homology at the amino acid level (Stone *et al*, 1995). Both genes function as crucial cell cycle regulators. $p16^{INK4a}$ binds to CDK4 and CDK6, which prevent pRB phosphorylation and G1–S phase progression (Serrano *et al*, 1993). By binding to MDM2, $p14^{ARF}$ prevents both MDM2-mediated p53 degradation and MDM2-mediated Rb inactivation, causing arrest of the G1 and G2 phases of the cell cycle (Bates *et al*, 1998; Stott *et al*, 1998). Therefore loss of function of either $p16^{INK4a}$ or $p14^{ARF}$ may lead to unrestrained cell cycling and uncontrolled cell growth, a common alteration in the carcinogenic process.

Inactivation of the two TSG at the CDKN2A locus can occur by a variety of genetic mechanisms including mutations and deletions (Liggett and Sidransky, 1998). Due to the unique arrangement of the exons, a mutation in exon 2 has the potential to alter the amino acid sequence of either or both of the genes. In addition to genetic mechanisms, hypermethylation of the CpG islands of certain gene promoters, including $p16^{INK4a}$ and $p14^{ARF}$, is a significant means of transcriptional silencing in a variety of tumor types (Esteller *et al*, 2001b). The importance of this epigenetic mechanism of gene silencing in almost every stage of the

initiation and progression of human cancer has only recently begun to be fully appreciated (Jones and Baylin, 2002).

Of the two genes, p16^{INK4a} has been more intensively studied and has been proven conclusively to play an important role in carcinogenesis, particularly head and neck SCC and familial melanoma (Reed *et al*, 1996; Liggett and Sidransky, 1998). The role of p14^{ARF} inactivation in human cancers is less well established, but is supported by studies of transgenic mice carrying a disrupted exon 1β which are cancer-prone at an early age (Kamijo *et al*, 1997). In addition, both p14^{ARF} mutations and methylation can have a deleterious functional effect (Zhang and Xiong, 1999; Rizos *et al*, 2001; Esteller *et al*, 2001a). In cutaneous SCC there has been relatively little published concerning the inactivation of p16^{INK4a} and p14^{ARF} genes. Knockout mice experiments suggest a possible role for p14^{ARF} in the initiation of cutaneous SCC development (Serrano *et al*, 1996; Kamijo *et al*, 1997) and for p16^{INK4a} in determining the differentiation status of skin squamous tumors (Sharpless *et al*, 2001). In primary cutaneous SCC, loss of heterozygosity (LOH) at the 9p21 locus and CDKN2A mutations have been reported but with disparate results (Quinn *et al*, 1994a, b; Kubo *et al*, 1997; Kushida *et al*, 1999; Soufir *et al*, 1999; Saridaki *et al*, 2003). Only one study has addressed the important mechanism of gene promoter hypermethylation in this tumor type, looking at p16^{INK4a} alone (Soufir *et al*, 1999) and no previous work has examined p14^{ARF} promoter methylation.

We have performed a comprehensive study of the CDKN2A locus in cutaneous SCC to establish the importance of p16^{INK4a} and p14^{ARF} inactivation in the pathogenesis of this tumor. Using highly specific and sensitive techniques, a variety of possible mechanisms for gene silencing have been examined, including correlation of the results with protein expression.

Results

LOH analysis A total of 13 out of the 40 tumors (32.5%) exhibited allelic loss/LOH at one or more markers for 9p21. Eleven samples showed loss at all informative markers. This data is summarized in Table I and representative examples are given in Fig 1. No samples showed microsatellite instability at this locus.

Mutational analysis Five different point mutations were found in four of 40 SCC (Table II and Fig 2). All mutations were in exon 2 and consisted of four single C to T nucleotide changes and one double substitution CC to TT. Since these all occurred in dipyrimidine sites, they are consistent with a UVB fingerprint type of mutation. Corresponding genomic DNA did not show these mutations confirming tumor specificity.

Sample 31T had two different independent point mutations in exon 2. LOH analysis had previously shown loss of one allele in this tumor specimen implying that both mutations must have occurred in the same allele. This was confirmed by subsequent sub-cloning (data not shown).

Sample 49T had a double CC to TT mutation, which would alter the amino acid sequence in two codons of p16^{INK4a} but only one of p14^{ARF}.

Table I. LOH results

Case no.	D9S171	D9S1748	D9S974
1	○	○	–
3	–	○	–
5	○	○	○
8	○	–	○
11	○	○	○
14	○	○	○
17	○	○	○
18	●	–	●
19	○	○	○
20	●	●	●
21	–	–	●
24	●	●	–
25	○	–	○
26	○	○	○
27	○	○	–
28	–	○	○
29	–	–	○
31	●	●	–
32	–	●	–
33	–	○	–
34	○	○	○
35	○	●	●
36	●	●	–
37	○	○	×
38	●	●	–
39	○	–	●
40	●	●	●
41	○	○	○
42	○	○	–
44	–	○	○
45	–	○	–
46	○	–	○
47	○	○	×
48	○	○	–
49	○	○	–
50	–	○	○
51	●	●	●
52	○	○	×
53	○	○	–
54	●	●	×
N	31	33	22
LOH	9	10	7
% LOH ÷ *N*	29	30	32

● , loss of heterozygosity (LOH); ○ , retention of heterozygosity; × , homozygous/not informative; –, no result/not done.

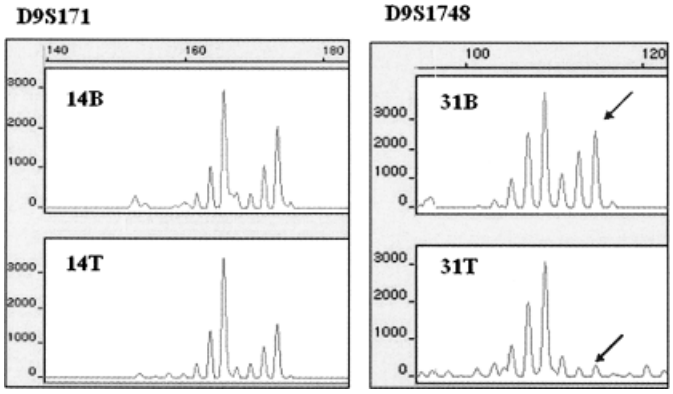

Figure 1
Loss of heterozygosity (LOH) analysis. Units on the *y*-axis represent intensity of fluorescent signal; on the *x*-axis is allele length in base pairs. The *y*-axis scaling differs between samples because quantities of PCR products vary in intensity. Examples show retention of heterozygosity (LOH index 0.64) for sample 14 at marker D9S171 and complete loss of the upper allele (LOH) for sample 31T compared to matched blood, 31B (arrows) at marker D9S1748. Note stutter bands are evident prior to the allele (highest peak) signal.

Two other samples 45T and 48T (and their corresponding genomic DNA) showed a G to A nucleotide polymorphism that is seen in 4%–6% of the general population (Bertram *et al*, 2002).

Methylation analysis Using MSP, evidence of methylation was observed for the $p16^{INK4a}$ promoter in 13 of 36 (36%) samples and for the $p14^{ARF}$ promoter in 16 of 38 (42%) samples (Fig 3). When initial bisulfite modification of DNA was successful, all samples produced a product with the primers specific for unmethylated DNA. This is due to amplification of the normal stromal tissue present in the initial biopsy specimen. In addition, in those samples with positive methylation, it is possible that the unmethylated band could also represent the partial methylation status of the tumor cells or genetic diversity of the tumor cell population. Four samples were methylated for both the $p16^{INK4a}$ and $p14^{ARF}$ promoters: 32T, 35T, 44T, and 53T.

Biallelic events Combining all the results from LOH studies, mutation screening and methylation analysis, biallelic changes (i.e. two of these three possible events) were present in ten of 38 samples for $p16^{INK4a}$ and six of 39 samples for $p14^{ARF}$ (Table III).

Immunohistochemical results $p16^{INK4a}$ expression was detected in 20 of 40 cutaneous SCC, of which 75% were graded + (Fig 4). Neither the amount nor the intensity of staining correlated with tumor differentiation status. In 23 of 36 (64%) cases there was concordance between the presence/absence of protein expression and genetic/epigenetic results. In seven of ten (70%) of tumors with biallelic genetic/epigenetic events, there was no protein expression evident.

$p14^{ARF}$ expression was seen in 17 of 40 (42.5%) of the samples; 14 of these being classified as +. In 23 of 37 (62%) cases there was concordance between the presence/absence of protein expression and genetic/epigenetic results. In all of the six tumors with biallelic genetic/epigenetic results, there was no protein expression evident.

Comparison of immune status with mechanistic data For $p16^{INK4a}$, eight of ten (80%) immunocompetent patients had genetic/epigenetic events compared with 12 of 28 (43%) immunosuppressed individuals (Table IV). Results were similar for $p14^{ARF}$ with 9 of 10 (90%) *vs* 16 of 29 (55%) respectively ($p = 0.05$ for both $p16^{INK4a}$ and $p14^{ARF}$, Fisher's exact probability test).

Table V summarizes the data on immune status, $p14^{ARF}$ and $p16^{INK4a}$ events (mutation, methylation, and immunohistochemical expression) and highlights those tumors in which bilallelic events were identified.

Discussion

In this study we report the systematic analysis of the CDKN2A locus in a well-characterized series of cutaneous SCC. We demonstrate that alterations at the CDKN2A locus are common in these cancers and we show inactivation of the two TSG at this locus via both genetic and epigenetic mechanisms.

Our results show that LOH at 9p21 is frequent in cutaneous SCC. The frequency of 9p21 allelic loss observed in this study (32.5%) is consistent with the largest previous study by Quinn *et al* (1994b) who found LOH in 31% of samples. But in two smaller series of cancers, Kushida *et al* (1999) and Soufir *et al* (1999) were unable to detect LOH. These three studies all used fewer markers at 9p21 in mostly non-microdissected tumor DNA, with a non-quantitative method of assessing LOH. Saridaki *et al*

Table II. Mutations found in CDKN2A exon 2 and the consequent changes in amino acid sequence for $p16^{INK4a}$ and $p14^{ARF}$ proteins

Sample	$p16^{INK4a}$ codon change	$p16^{INK4a}$ amino acid change	$p14^{ARF}$ codon change	$p14^{ARF}$ amino acid change
31T	ccc ⇒ ctc	Pro81Leu	acc ⇒ act	Thr142Thr
31T	ttc ⇒ ttt	Phe 90 Phe	cct ⇒ tct	Pro152Ser
41T	ccc ⇒ ctc	Pro114Leu	gcc ⇒ gct	Ala175Ala
49T	gcc ⇒ gct	Ala57Ala	ccg ⇒ ttg	Pro118Leu
	cga ⇒ tga	Arg58STOP	ccg ⇒ ttg	Pro118Leu
54T	cga ⇒ tga	Arg80STOP	ccg ⇒ ctg	Pro141Leu

All nucleotide changes C → T substitutions except for 49T that had a tandem CC → TT mutation.

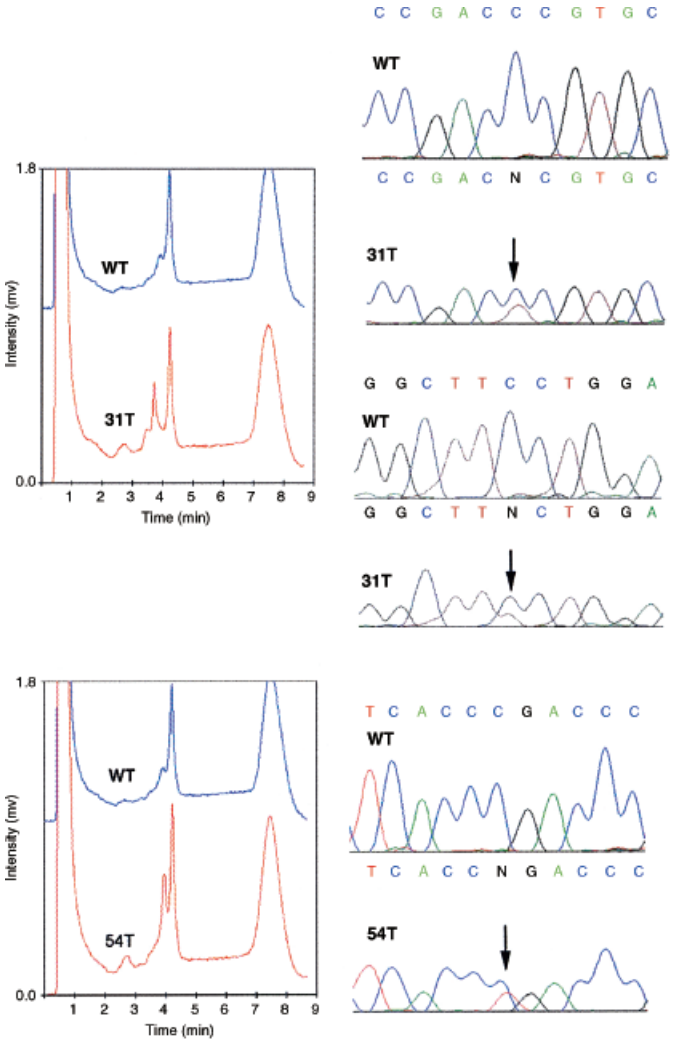

Figure 2
Examples of mutations found in two cutaneous SCCs. Denaturing high-performance liquid chromatography traces (*left*) and corresponding sequencing results (right) for samples 31 (*upper panel*) and 54 (*lower panel*). Sequencing traces show heterozygous C → T base changes (arrowed) in exon 2 at nucleotide (nt) positions 242 and 270 for 31T; 238 for 54T.

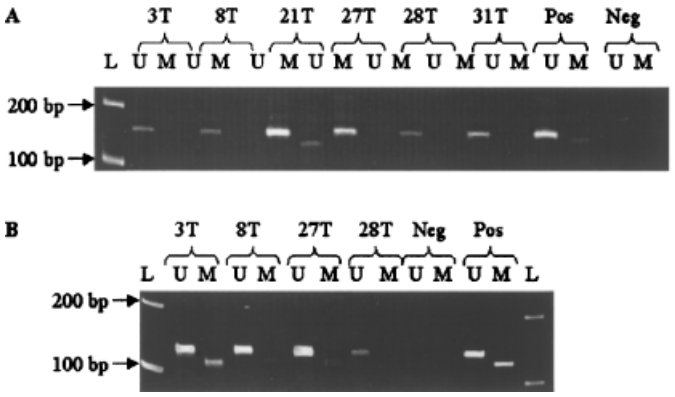

Figure 3
Representative examples of 10% acrylamide gels of methylation specific PCR products. The presence of a visible band in "U" lanes indicates unmethylated genes (normal stromal tissue); in "M" lanes indicates methylated genes. (*A*) p16INK4a is methylated in sample 21. (*B*) p14ARF is methylated in samples 3, 8, 27. Positive (Pos) controls were universally methylated or unmethylated DNA for the M and U PCR, respectively. H_2O served as a negative (Neg) control for both reactions. Controls were included in every run. L = marker ladder.

Table III. Summary of monoallelic and biallelic molecular abnormalities in cutaneous SCC

Molecular abnormalities	No. of SCC with p16INK4a alterations	No. of SCC with p14ARF alterations
None	18	14
LOH alone	4	8
Mutation alone	1	0
Methylation alone	5	11
LOH + mutation	2	1
LOH + methylation	7	3
Mutation + methylation	1	1
LOH + mutation + methylation	0	1

SCC, squamous cell carcinoma; LOH, loss of heterozygosity.

(2003) has recently shown a higher rate of LOH of 52% in a smaller series, which combined Bowen's disease (carcinoma *in situ*) and SCC. The samples in the present work were all histologically confirmed invasive SCC. Furthermore, microdissected DNA was tested and a quantitative method was used to calculate LOH. As such, our results are likely to be a more accurate estimate of the true prevalence of LOH at this locus in cutaneous SCC.

Using DHPLC, point mutations were detected in only four of 40 samples (10%). The sensitivity of our methodology is validated by detection of the Ala148Thr polymorphism that is found in 4%–6% of the general population (Bertram *et al*, 2002) and was detected in two of 40 of our samples (both tumor and genomic DNA from samples 45 and 48). All the mutations carried a UV signature (Brash *et al*, 1991), reflecting the importance of UV in the etiology of these tumors, which all arose on sun-exposed sites. This finding also provides evidence of a role for mutagenesis of the p16INK4a and p14ARF genes in the pathogenesis of UV-induced SCC.

Each of the mutations occurred in exon 2, the exon most commonly mutated in other human cancers. For p16INK4a, two of the mutations result in amino acid substitutions (Pro81Leu and Pro114Leu), whereas the other two mutations result in premature termination of the protein (Arg58-STOP and Arg80STOP). Each of these mutations has been previously described in other human cancer types (Pollock *et al*, 1996; Kubo *et al*, 1997; Fargnoli *et al*, 1998; Soufir *et al*, 1999; Bazan *et al*, 2002).

The two non-truncating mutations, Pro81Leu and Pro114Leu, which both occur in the key ankyrin repeat domains, result in functional impairment of the p16INK4a protein, (Koh *et al*, 1995; Ranade *et al*, 1995). Both of the premature termination p16INK4a mutations result in expression of truncated proteins lacking CDK binding activity (Parry and Peters, 1996). Three of the p16INK4a mutations also affect p14ARF. Of these, one (Pro118Leu) has previously been described in a cutaneous SCC (Soufir *et al*, 1999). The

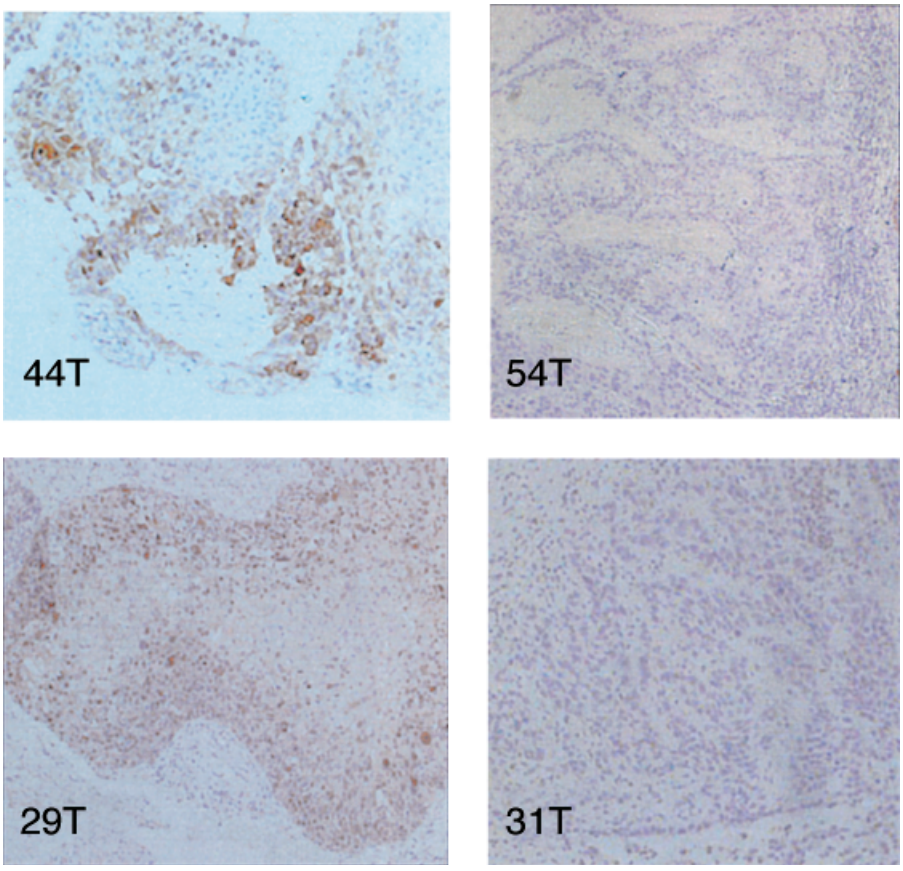

Figure 4
Immunohistochemistry slides showing predominant nuclear staining for $p16^{INK4a}$ antibody in tumor 44 and $p14^{ARF}$ antibody in tumor 29. Absence of expression is noted for $p16^{INK4a}$ in tumor 54 and $p14^{ARF}$ in tumor 31. Both these tumors had biallelic inactivating events detectable.

Table IV. Comparison of genetic events in immunosuppressed *versus* immunocompetent patients

	$p16^{INK4a}$ alterations		$p14^{ARF}$ alterations	
	Present	Absent	Present	Absent
Immunosuppressed	12	16	16	13
Immunocompetent	8	2	9	1
	$p = 0.048$		$p = 0.050$	

effect of these mutations on the function of $p14^{ARF}$ awaits formal characterization; however, other human cancer-associated point mutations have been shown to functionally impair $p14^{ARF}$ by diminishing its ability to activate and stabilize p53 (Zhang and Xiong, 1999; Rizos *et al*, 2001).

As described above, the mutations in CDKN2A target both $p16^{INK4a}$ and $p14^{ARF}$ in three cases, implying selective pressure to abrogate the function of both proteins during tumorigenesis in squamous epithelium. Unusually, 31T in which there was unequivocal evidence of LOH, contained two independent exon 2 missense mutations on the retained allele. One of these affects the amino acid sequence of $p16^{INK4a}$, the other $p14^{ARF}$. The presence of distinct mutations targeting each gene in 31T, 49T, and 54T, strongly argues that loss of function in both proteins is a key event in carcinogenesis in cutaneous SCC.

In this study, we did not examine exon 3 (which contributes coding sequence to only the four most c-terminal amino acids of $p16^{INK4a}$) nor did we analyze homozygous deletions. Homozygous deletions, while relatively common in carcinoma cell lines are rare in primary carcinomas (e.g. primary gastric carcinomas, Lee *et al*, 1997). Similarly, biallelic deletion is very rare in primary carcinomas of prostate and liver (Herman *et al*, 1995; Peng *et al*, 2002).

Our results show that methylation of the normally unmethylated promoter regions of both genes is the most common mechanism of inactivation in cutaneous SCC. $p14^{ARF}$ promoter methylation has never previously been examined in cutaneous SCC and was the commonest abnormality found in our series. In addition, this is the first formal demonstration of $p16^{INK4a}$ promoter methylation in cutaneous SCC. In a previous study using Southern blotting, it was reported that the $p16^{INK4a}$ promoter was not methylated in cutaneous SCC (Soufir *et al*, 1999). It is well recognized that the sensitivity of this method is substantially lower than MSP for detection of methylation (Herman *et al*, 1996). Moreover, our study analyzed 40 cases in contrast to the eight SCC analyzed by Soufir and co-workers. These factors may well account for the differences between the two studies. Our results accord well with studies using MSP in other squamous cancers (Reed *et al*, 1996; Gasco *et al*, 2002; Huang *et al*, 2002). Further analysis of our results reveals that $p16^{INK4a}$ was hypermethylated in the presence of an unmethylated $p14^{ARF}$ in nine of 13 (69%) of the cases; $p16^{INK4a}$ was unmethylated with $p14^{ARF}$ hypermethylation in 12 of 16 (75%) of tumors and four (10%) cases demonstrated methylation of both gene promoters. Thus, consistent with previous studies of colorectal carcinomas, methylation of the $p16^{INK4a}$ and $p14^{ARF}$ promoters are independent events (Esteller *et al*, 2000).

Table V. Summary table of all results for both p16^{INK4a} and p14^{ARF} genes

			p16^{INK4a} alterations			p14^{ARF} alterations			
Sample no.	IC/IS	LOH[a]	Mutation[b]	Methylation	Expression	Mutation[b]	Methylation	Expression	Biallelic events
1	IS	Ret		nr	+		−	−	
3	IS	Ret		−	+		+	+	
5	IS	Ret		−	−		−	−	
8	IS	Ret		−	−		+	−	
11	IS	Ret		−	+		+	−	
14	IS	Ret		+	−		−	−	
17	IS	Ret		−	+ +		−	+	
18	IS	LOH		−	+		−	−	
19	IS	Ret		nr	−		nr	+	
20	IS	LOH		nr	−		nr	−	
21	IS	LOH		+	+		−	±	[c]
24	IC	LOH		nr	+		−	+	
25	IS	Ret		−	+		−	+	
26	IS	Ret		−	−		−	−	
27	IS	Ret		−	+		+	−	
28	IS	Ret		−	−		−	+	
29	IS	Ret		−	+ +		+	+ +	
31	IC	LOH	Y	−	−	Y	−	−	[c, d]
32	IC	LOH		+	−		+	−	[c, d]
33	IS	Ret		−	−		+	−	
34	IS	Ret		−	+		+	−	
35	IS	LOH		+	−		+	−	[c, d]
36	IC	LOH		−	−		+	−	[d]
37	IC	Ret		−	−		+	+	
38	IS	LOH		+	−		−	−	[c]
39	IS	LOH		+	−		−	+	[c]
40	IC	LOH		+	+		−	+	[c]
41	IC	Ret	Y	+	+	N	−	−	[c]
42	IS	Ret		+	+		−	−	
44	IS	Ret		+	+ +		+	+ +	
45	IS	Ret		−	+		−	+ +	
46	IS	Ret		−	+ +		−	+	
47	IS	Ret		−	−		−	−	
48	IC	Ret		−	+		+	+	
49	IC	Ret	Y	−	−	Y	+	−	[d]
50	IS	Ret		+	+ +		−	+	
51	IC	LOH		+	−		−	+	[c]
52	IS	Ret		−	±		−	+	
53	IS	Ret		+	+		+	−	
54	IS	LOH	Y	−	−	Y	+	−	[c, d]

IC, immunocompetent; IS, immunosuppressed; LOH, loss of heterozygosity; Ret, retention of heterozygosity; nr, no result.
[a]See Table I for details.
[b]See Table II for details.
[c]Biallelic events for p16^{INK4a}.
[d]Biallelic events for p14^{ARF}.

Epigenetic changes may predate genetic events in the natural history of some carcinomas. For example, $p14^{ARF}$ hypermethylation is present in 32% of colorectal adenomas (Esteller *et al*, 2000) and $p16^{INK4a}$ hypermethylation occurs in pre-malignant, dysplastic oral squamous lesions (Gasco *et al*, 2002). It would therefore be of interest to test the pre-malignant forms of cutaneous SCC, namely Bowen's disease and actinic keratoses, and chronically UV-exposed skin, for this epigenetic abnormality.

Inactivation of the $p16^{INK4a}$ gene was associated with absent protein expression in almost all tumor cells in a study of head and neck SCC (Reed *et al*, 1996) and non-small cell lung cancer (Sanchez-Cespedes *et al*, 1999). Absent mRNA was also found to correlate with lack of $p16^{INK4a}$ protein expression in a study of mycosis fungoides (Peris *et al*, 1999). In our study concordance with genetic events was only found in 64% and 62% of samples for $p16^{INK4a}$ and $p14^{ARF}$, respectively. The most likely explanation for this is that methylation is not homogeneous in the SCC under study, but, rather, is restricted to a sub-population of tumor cells. This latter suggestion is supported by the immunohistochemical findings, which in most positive cases was patchy and heterogeneous throughout the tumor areas. This resembles the expression pattern seen in $p16^{INK4a}$ methylated oral and cervical SCC (Nuovo *et al*, 1999; Huang *et al*, 2002).

Tumors with biallelic inactivating events would be expected to lack functional protein. Immunohistochemistry confirmed that this was indeed the case in 13 of 16 of the studied cases (all six of the $p14^{ARF}$ cases and seven of ten of the $p16^{INK4a}$ cases). In each of the three tumors that retained some $p16^{INK4a}$ expression, one of the events was methylation of the $p16^{INK4a}$ promoter. The likely explanation for these observations is that methylation-dependent silencing occurred only in specific parts of the tumor, and that residual areas of unmethylated $p16^{INK4a}$ were present in each case in which $p16^{INK4a}$ was expressed. Use of *in situ* MSP has shown considerable heterogeneity in squamous carcinomas methylation, consistent with this explanation (Nuovo *et al*, 1999). Three tumors, 31T, 32T, and 54T, had biallelic events in both genes and all had no expression of either protein on immunohistochemistry.

For both $p16^{INK4a}$ and $p14^{ARF}$ significantly more tumors from immunocompetent individuals had genetic/epigenetic inactivating events compared with cutaneous SCC from immunosuppressed individuals. These results, however, are in keeping with a previous study which reported less allelic loss at chromosome 9p in skin tumors from immunosuppressed compared with immunocompetent patients (Rehman *et al*, 1997). The explanation for these findings remain elusive, but one could speculate that the significantly higher prevalence of human papillomavirus found in immunosuppressed cutaneous SCC obviates the need for direct genetic/epigenetic TSG alterations, similar to the mechanism proven in anogenital carcinomas (Harwood and Proby, 2002).

In conclusion, this is the largest and most comprehensive study of the CDKN2A locus in cutaneous SCC reported to date. Overall, the total frequency of 9p21 alterations was 76%, with abnormalities of $p16^{INK4a}$ detected in 53% of cases studied and $p14^{ARF}$ changes present in 64%. Promoter methylation in cutaneous SCC has never previously been demonstrated at this locus, but was the predominant mechanism of inactivation for both genes in our series. Biallelic events were common. These findings emphasize the importance of $p16^{INK4a}$ and $p14^{ARF}$ TSG in the pathogenesis of cutaneous SCC.

Materials and Methods

Patient characteristics Forty cutaneous, invasive SCC were obtained from 39 patients, following informed consent. These comprised 30 samples from 29 iatrogenically immunosuppressed patients and 10 samples from 10 immunocompetent patients. Twenty-nine patients were male and 10 female. All specimens were primary lesions and arose on sun-exposed areas of skin; namely face, scalp, upper limbs, and upper trunk.

Specimen preparation A small portion of tumor tissue was snap frozen immediately at operation and stored at $-70^{\circ}C$. The remainder was formalin-fixed, processed routinely and embedded in paraffin. The diagnosis was confirmed histologically by H&E staining of all samples. Venous blood was obtained from all but one patient.

DNA extraction Genomic DNA was extracted from the frozen tissue and venous blood using the Nucleon Genomic DNA Extraction Kit (Tepnel Life Sciences, Manchester, UK) according to the supplied protocol.

Microdissection and DNA isolation For the allelic imbalance studies tumor-enriched DNA was required. This was obtained by microdissection of a majority tumor cell population from up to six 8 μm sections cut from the paraffin blocks from each case. The tumor area on each slide was identified by reference to an adjacent hematoxylin and eosin-stained slide. The tumor tissue was obtained by scraping off unstained, waxed slides, with a single-edged razor blade. The tissue was placed in an eppendorf microfuge tube and dewaxed in 1 mL xylene on a rotating wheel for 1 h. After all the xylene had been removed, 4 μL Proteinase K solution (14 mg per mL; Roche Diagnostics Ltd., East Sussex, UK) and 100 μL 10% Chelex (Bio-Rad Laboratories, Hercules, CA) solution were added, and the mixture incubated in a rotating oven at $56^{\circ}C$ for 24 h. The tubes were then boiled at $100^{\circ}C$ for 8 min to inactivate the Proteinase K. After centrifugation, 5 μL aliquots of the supernatant were used in the subsequent allelotyping PCR. The samples were stored at $4^{\circ}C$ until used. From the one patient for whom venous blood was not obtained, normal skin was microdissected and genomic DNA extracted as described above.

Allelotype/LOH analysis Microdissected tumor DNA and matched genomic DNA from each patient was analyzed for allelic loss using three highly polymorphic microsatellite (dinucleotide repeat) markers, D9S171, D9S1748, and D9S974, which span the CDKN2A locus at 9p21. These markers were chosen because of their location with respect to the CDKN2A exons (Fig 5). In addition they all have a high informativity rate (heterozygosity score) and produce a PCR product of a small enough size for successful amplification of DNA from paraffin-embedded samples. All primer oligonucleotide sequences are available through The Genome Database (http://www.gdb.org). For each primer pair, the 5′ end of the forward primer was synthesized with a fluorescent label (6FAM or HEX) using amidite-labelling chemistry. Amplification was carried out using a standard PCR protocol (PCR reagents including Taq polymerase supplied by Bioline, London, UK) on a Hybaid Cycler (Hybaid Ltd, Middlesex, UK) using a $60^{\circ}C$ annealing temperature for D9S171 and $65^{\circ}C$ for D9S1748 and D9S974. The PCR products produced for each sample were pooled in a 1:1 ratio in the presence of Hi-Di formamide (Applied Biosystems Warrington, UK) and a 500-ROX internal size standard (Applied Biosys-

tems). Genotyping was performed on an ABI 3100 automated DNA Analyzer (Applied Biosystems), which is capillary based and laser activated to detect fluorescent PCR products. The resultant allele profiles for each sample were analyzed using ABI Genescan 3.1 software (Applied Biosystems), which yields quantification of results in terms of trace peak size and height.

Assessment of allele loss Different marker traces were distinguishable by size and fluorescent tag/color. Heterozygous individuals were identified i.e. those with two PCR products of different size in normal DNA. The sizes of the two alleles were assigned to the peaks of the greatest height; smaller peaks were interpreted as stutter bands caused by slippage of the polymerase. LOH was assessed for each blood/tumor pair by a quantitative method calculating the LOH index (Okabe *et al*, 2000). The LOH index is defined as the allele ratio in normal DNA divided by the allele ratio in the corresponding tumor DNA. The allele ratio is the peak height of the smaller length allele divided by the peak height of the larger length allele. Allelic loss was defined by an LOH index of less than 0.5 or more than 2.0 i.e. at least a 50% signal change in tumor allele profile compared to normal allele profile.

Mutational analysis by denaturing high-performance liquid chromatography (DHPLC) and direct sequencing Exons 1α, 1β, and 2 of CDKN2A were amplified for each sample using primers that spanned the entire exon and intron–exon boundary. The PCR products for exons 1β and 2 were split to provide two overlapping PCR products for ease of subsequent analysis. The primer sequences used were as follows (PCR product sizes in base pairs (bp) in brackets): exon 1α forward: GAAGAAAGAGGA-GGGGCTG; reverse: GCGCTACCTGATTCCAATTC (340 bp); exons 1β-1 forward: TCAGGGAAGGCGGGTGCG; reverse: GCCGCGGGATGT-GAACCA (245 bp); Exons 1β-2 forward: GCCGCGAGTGAGGGTTTT; reverse: CACCGGCGGTTATCTCCTC (263 bp); Exon 2A forward: AGCTTCC-TTTCCGTCATGC; reverse: GCAGCACCACCAGCGTG (203 bp); Exon 2B forward: GACCCCGCCACTCTCACC; reverse: GTGCTGGAAAATGAATGC-TCTG (310 bp). PCR amplification was carried out using 50–100 ng of the frozen tissue genomic DNA in a standard PCR protocol (PCR reagents including Taq polymerase supplied by Bioline). Five percent dimethyl sulfoxide (BDH Laboratories, Poole, UK) was added to the reaction mixture for optimal PCR amplification of GC-rich regions. The annealing temperature for exons 1β and 2-2 was 60°C and for exon 1α and 2A was 55°C. The PCR products obtained were heated to 94°C PCR for 5 min followed by cooling to 40°C at a rate of 0.03°C per s to ensure heteroduplex formation. Mutational analysis was then carried out using DHPLC with the Transgenomic WAVE System (Transgenomic, UK). The optimum temperatures for the analysis of each PCR fragment were calculated using the WAVE-Maker software (Transgenomic). Mutations in samples showing DHPLC traces that varied from the wild-type profile were identified by sequencing an independent PCR product. These PCR products were first purified using a PCR purification kit (QIAquick; Qiagen, Crawley, UK), followed by direct sequencing using ABI Prism Big-Dye terminator chemistry (Applied Biosystems) in accordance with the manufacturer's instructions. Each sample was sequenced in both forward and reverse directions in independent PCR reactions and the products were analyzed on an ABI 377 automated sequencer. Sequence electropherograms were aligned using Sequence Navigator software (Applied Biosystems).

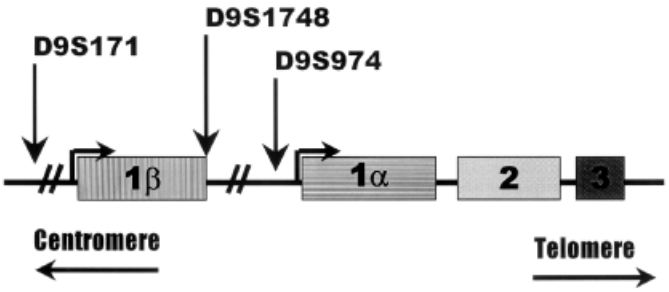

Figure 5
Location of microsatellite markers used in allelotyping, relative to CDKN2A exons.

Methylation analysis For methylation specific PCR (MSP), genomic DNA was bisulfite modified using the reagents supplied in the CpGenome Modification Kit (Intergen Company, Oxford, UK), according to the manufacturer's instructions. To analyze the methylation status of both the p16^{INK4a} and the p14^{ARF} gene promoter regions, MSP was carried out with separate reaction mixtures using unmethylated (U) and methylated primers (M). Control samples were included in every run: positive controls were bisulfite modified universally methylated or unmethylated DNA (Intergen) in the M and U PCR, respectively. Negative controls substituted distilled water for DNA in the PCR. A "hot-start" PCR was employed using AmpliTaq Gold polymerase (Roche). For p16^{INK4a} MSP was carried out using the CpG WIZ Amplification Kit (Intergen). For p14^{ARF} MSP, primers were synthesized as previously described (Esteller *et al*, 2000) and used in a 25 μL reaction mixture composed of: 2.5 μL 10 $\times$ GeneAmp PCR Buffer II, 2.0 μL $MgCl_2$ solution 25 mM, 4.0 μL 5 mM dNTP mix, U or M primer 20 pmol, 1 U AmpliTaq Gold polymerase and 5 μL (50 ng) bisulfite modified DNA. The annealing temperature for both the U and M reactions was 60°C. PCR products were resolved on 10% non-denaturing acrylamide gels. The sizes of the PCR fragments for the p16^{INK4a} U and M forms are 154 and 145 bp, respectively, and for the p14^{ARF} U and M forms are 132 and 122 bp, respectively.

Immunohistochemical analysis Serial 4 μm sections of formalin-fixed, paraffin-embedded biopsy samples were cut and one stained with H&E to confirm the histological diagnosis. Further sections were stained for both p16^{INK4a} and p14^{ARF} proteins using commercially available primary antibodies: p16^{INK4a}/MTS1 Ab-7 (16P07) mouse monoclonal antibody (NeoMarkers Inc., Fremont, California) and p14 ARF (C-18) (sc-8613) goat polyclonal antibody (Santa Cruz Biotechnology, Inc.) Sections were dewaxed in xylene and rehydrated through graded alcohol. Endogenous peroxidase activity was inhibited by incubating in 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was performed by boiling in 10 mM citrate buffer pH 6.0, for 4 min in a microwave. After blocking of non-specific binding by pre-incubation with donor horse/rabbit serum for 30 min, the primary antibody at a concentration of 1:100 was added for overnight incubation at 4°C. For detection, biotinylated horse anti-mouse (for p16^{INK4a}) or biotinylated rabbit anti-goat (for p14^{ARF}) sera (Vectastain ABC Kit, Vector Laboratories, Burlingame, California) were applied as secondary antibodies for 30 min at room temperature, followed by incubation with the avidin–biotin complex chromogen (Vectastain) for 20 min at room temperature. The reaction was developed using DAB (3,3′-diaminobenzidine) chromogen solution (Biogenex Liquid DAB, Biogenex, California) and counterstained with hematoxylin. Appropriate positive and negative control slides were included in every run.

Interpretation of immunostaining Slides were assessed by two independent observers (VB and CP). CP had no prior knowledge of the clinical details or molecular studies. p16^{INK4a} and p14^{ARF} are both nuclear proteins and only nuclear reactivity was considered positive for protein expression. Internal positive controls included infiltrating inflammatory cells and basal keratinocytes for p16^{INK4a} and appendageal structures, such as hair follicles and glandular structures; for p14^{ARF}. Staining was graded as negative (−), less than 5% of tumor nuclei positive or cytoplasmic staining only ($\pm$), less than 25% of tumor cells positive (+), 25%–50% of tumor cells positive (++) or more than 50% of tumor cells positive (+++).

Ethical approval This study was approved by the East London and City Health Authority Research Ethics Committee.

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