Analysis of p16 expression and allelic imbalance / loss of heterozygosity of 9p21 in cutaneous squamous cell carcinomas

Sarah E. Gray a, Elaine Kay a, Mary Leader a, M. Mabruk a, b, *

a Molecular Oncology Laboratory, Pathology Department, Royal College of Surgeons in Ireland and Beaumont Hospital, Dublin, Ireland
b Advanced Medical and Dental Institute, University Sains Malaysia, Penang, Malaysia

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Abstract

Deletions of the short arm of chromosome 9 have been reported in different types of malignancies. This chromosomal region contains a number of known tumour suppressor genes, including the p16 INK4A (CDKN2A), p15 INK4B and MTAP tumour suppressor genes located at 9p21. In this study twenty-two paraffin embedded invasive cutaneous SCC were examined for allelic imbalance/loss of heterozygosity (AI/LOH) of the 9p region (in particular 9p21), and for p16 protein expression. DNA was isolated from microdissected sections of normal and tumour cells and analysed for AI/LOH by using six fluorescently labelled microsatellite markers that map to the 9p region. P16 protein expression was examined by immunohistochemistry. At each of the six microsatellite markers the majority of SCC analysed showed AI/LOH. Overall both AI/LOH within the CDKN2A locus and absence of p16 protein expression were frequent among the cutaneous SCC analysed, suggesting that p16 inactivation may play a role in cutaneous SCC development. The majority of the SCC analysed also had AI/LOH of the marker within the MTAP gene, and at markers flanking the CDKN2A gene; thus further investigation as to a possible role for these genes in the development of cutaneous SCC is warranted.

Keywords: cutaneous SCC • AI/LOH • p16 • p15 • MTAP

Introduction

P16 INK4A is a tumour suppressor protein that acts as a CDK 4/6-specific cyclin dependent kinase inhibitor.[1] P16 INK4A competes with Cyclin D for binding to CDK4/6 and thus prevents Rb from being hyperphosphorylated; resulting in Rb-dependent cell cycle arrest in the G1 phase of the cell cycle.[2,3]

The p16 INK4A tumour suppressor protein is encoded by the cyclin-dependent kinase inhibitor 2A gene (CDKN2A), which is located on chromosome 9p21.[4, 5] The CDKN2A locus also encodes another tumour suppressor protein known as p19ARF. P16 INK4A (P16) and p19ARF have separate promoters and a separate exon 1, but share the same

* Correspondence to: Professor Mohamed MABRUK
Advanced Medical and Dental Institute, University Sains Malaysia, USM11800, Penang, Malaysia.
Tel.: 006046532728
Fax: 006046532724
E-mail: mmabruk03@yahoo.co.uk

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exon 2 and exon 3, though the two proteins are translated in different reading frames. P19ARF binds and inhibits Mdm2, thus preventing Mdm2 mediated p53 degradation. [1,6]

Alterations of the p16 gene by point mutations, deletion or methylation have been found in a subset of many primary tumours.[7–15]

Enders et al. have reported that murine esophageal SCC cell lines lacking endogenous p16 expression show growth inhibition when exogenous p16 is expressed.[16]

A role for p16 inhibition in the development of cutaneous SCC has been suggested by the findings that mice with targeted disruption of exon 2 and 3 of the cdkn2A locus are susceptible to the development of cutaneous SCC after UV irradiation.[17] Also, mutations of the CDKN2A locus, some of which are signature mutations of UV, have been identified in up to 20% of sporadic cutaneous SCC and in 42% of cutaneous SCCs from PUVA treated Psoriasis patients.[18–21]

The 9p21 region also contains two other tumour suppressor genes, P15INK4B (CDKN2B) and Methylthioadenosine phosphorylase (MTAP). P15INK4B acts as an effector of TGF-β mediated cell cycle arrest.[22] The p15INK4B protein binds to the cdk4- Cyclin D complex, displacing p27, thus freeing p27 to bind to and inhibit the cdk2- Cyclin E complex, which is required for entry into the S phase of the cell cycle.[3, 22] Homozygous deletions of the p15INK4B gene have been found in approximately half of esophageal SCC cell lines and head and neck SCC (HNSCC) examined and also in a subset of ovarian tumours and bladder cancer cell lines [23–26]. Hypermethylation of the p15INK4B gene has also been found in the majority of HNSCC analysed, as well as in a subset of pulmonary SCC and oral SCC [12, 27–29].

The MTAP gene is a housekeeping gene that plays an important role in the salvage of both adenine and methionine. MTAP protein functions by catalysing the phosphorylation of methylthioadenosine (MTA), which leads to MTA (a by-product of the synthesis of polyamines) being broken down into adenine and methionine [30]. MTA acts as a potent inhibitor of both polyamine aminopropyltransferase and methyltransferases [31–32]. MTAP has been shown to act as a tumour suppressor gene in vitro [32].

An inverse correlation between MTAP protein expression and progression of melanocytic tumours has been observed by Behrmann et al., which suggests that inactivation of MTAP may play a role in the development of melanomas.[31] Homozygous deletions of the MTAP gene have been found in just over a third of bladder cancer cell lines, non-small cell lung carcinoma and osteosarcoma.[26, 33–34]

The aim of this study was to examine the integrity of the CDKN2A locus (encoding p16 protein), the p15INK4B and the MTAP gene regions in twenty-two histologically confirmed cutaneous SCC by analysing the presence of allelic imbalance/loss of heterozygosity (AI/LOH) at microsatellite markers within and/or flanking the genes of interest. P16 protein expression in these cutaneous SCC tissue samples was also examined.

**Materials and methods**

**Samples for analysis**

Twenty-two histologically confirmed formalin fixed paraffin-embedded cutaneous invasive squamous cell carcinoma (SCC) were randomly selected from the archives of Beaumont Hospital, Dublin, for analysis.

**Stereomicroscopic microdissection and DNA extraction**

Tumour and normal samples were microdissected from preselected areas on Haematoxylin stained sections and DNA isolated from the tumour and normal tissue cells by proteinase K digestion as described by Butler et al. [35]. For each SCC sample, the corresponding reticular dermal tissue was selected as a constitutional normal control as recommended previously by Zauber et al [36]. One to three 8 μm sections were dissected from each SCC sample depending on the size of the tumour and normal reticular dermal tissue.

**PCR amplifications**

The microsatellite markers chosen for analysis of the integrity of chromosome 9p21 were D9S916, D9S974, D9S942, D9S1604, D9S1814. The order of the genes of interest on 9p21 is telomere- MTAP- CDKN2A- p15INK4B- centromere. The D9S916 marker is within intron 4 of the MTAP gene, D9S974 and D9S942 are within the CDKN2A locus. D9S1604 is located between the CDKN2A and p15INK4B
genes, and D9S1814 is centromeric to p15 INK4B. The D9S171 microsatellite marker at 9p13 was also examined. The DNA sequences of the primers chosen for amplification of these microsatellite markers were obtained from The Genome Database (http://www.gdb.org). In each primer set, the forward primer had a CY-5 label at the 5’ end.

PCR amplifications were performed in a final volume of 20µl and consisted of 1 x PCR reaction buffer, 200 µM of dNTPs, 1.25–1.5mM of MgCl₂ (Promega, WI, USA), 10–40 pmol of each primer (MWG Biotech, UK), and 2 µl of sample DNA digest. The concentration of MgCl₂, and of primers used in the PCR reactions varied according to the primers used (Table 1). All PCR protocols were carried out using a hot start in which after an initial denaturation step of 95°C for 5 min. the PCR reactions were heated at 80°C for 5 min during which time 2 units of Taq DNA polymerase (Promega, WI, USA) was added to each PCR reaction.

A touchdown PCR was then used.[37] This consisted of two cycles of 95°C for 1 min, 1 min at an initial annealing temperature (Table 1), and extension at 72°C for 1 min. Subsequent steps consisted of two cycles each with annealing temperature reduced by 2°C until 32 cycles were done at the final annealing temperature. A final extension step at 72°C for 10 min completed the PCR reactions.

PCRs and subsequent fragment analysis of the microsatellite markers were carried out in duplicate for each SCC sample DNA and its corresponding normal tissue DNA.

Following PCR, 4 µl of PCR products mixed with agarose loading dye were electrophoresed through 12% polyacrylamide mini gels. Gels were stained with ethidium bromide (20 mg/ml) and viewed under UV light to assess the efficiency of amplification. The intensity of the bands seen was used to determine the dilutions necessary for further analysis.

Table 1 PCR conditions for amplification of the microsatellite markers

| Primer pair | Initial annealing temperature (°C) | Final annealing temperature (°C) | Product Size | [MgCl₂] | [Primer] |
|-------------|------------------------------------|----------------------------------|--------------|---------|---------|
| D9S916      | 57                                 | 55                               | 258–285 base pairs | 1.25mM  | 30pmol  |
| D9S974      | 57                                 | 55                               | 90–135 base pairs  | 1.38mM  | 40pmol  |
| D9S942      | 57                                 | 55                               | 274–279 base pairs  | 1.25mM  | 10pmol  |
| D9S1604     | 57                                 | 55                               | 188–192 base pairs  | 1.25mM  | 20pmol  |
| D9S1814     | 59                                 | 55                               | 274–279 base pairs  | 1.25mM  | 10pmol  |
| D9S171      | 59                                 | 55                               | 157–175 base pairs  | 1.50mM  | 40pmol  |

Analysis of allelic imbalance/ loss of heterozygosity (AI/LOH)

The CY5 labelled PCR products (diluted in distilled water if necessary) were mixed with a CY5.5 labelled DNA size ladder and loading dye, then denatured by heating at 95°C for 10 min and placed on ice.

The samples were then analysed for LOH using the OpenGene Long-Read Tower™ system DNA Sequencer (Visible Genetics, France). This involved electrophoresis of the samples in a denaturing 6% polyacrylamide gel heated to 55°C. Normal and corresponding tumour samples were loaded side by side in the gel. The Sequencer used a laser to excite the fluorescent CY5 label on the PCR products and the CY5.5 label on the internal size markers as they moved through the gel. The Sequencer Fragment analysis software was then able to accurately size the microsatellite alleles in each sample in relation to the internal size markers, and was also able to plot the measured fluorescence in terms of peak area for each microsatellite allele.

A ratio of allele peak areas was calculated by dividing the area of the first allele by the area of the second allele in each of the normal and corresponding tumour DNA samples i.e. T1:T2 and N1:N2 for the tumour samples and their corresponding normals, respectively.

A ratio of ratios between the tumour and normal PCR products was then calculated to give an overall allele ratio, i.e. T1:T2 / N1:N2. In cases where the overall allele ratio calculated by this equation was over 1.00 the inverse was used to give a result between 0.00 and 1.00 [38].

An experimentally established AI/LOH allele ratio cut-off point of 0.88 was previously calculated in our laboratory using the microsatellite marker D13S267 and corresponding heterozygote normal samples to
sixteen of the SCC used in the present study. Two aliquots of DNA from each of the sixteen normal DNA samples were amplified by PCR. Then the pairs of PCR products from each of the sixteen normal DNA samples were loaded side by side in the 6% polyacrylamide gel attached to the Visible Genetics Sequencer in order to mimic the normal/tumour relationship. This was done in duplicate, and the mean allele ratios of the duplicated sixteen paired normal DNA samples was calculated. From these means, the overall mean and the overall standard deviation from the mean was calculated. Assuming a normal distribution it was found that 99.5% of normal DNA samples (i.e. DNA with retention of heterozygosity) would have allele ratios greater than or equal to 0.89 (i.e. less than or equal to 11% loss of allele signal in the tumour DNA as compared to the corresponding normal DNA).

**Immunohistochemical detection of p16 expression**

The twenty-two cutaneous SCC samples, which were analysed for AI/LOH or instability at the D9S916, D9S974, D9S942, D9S1604, D9S1814 and D9S171 microsatellite markers were analysed immunohistochemically for the expression of the tumour suppressor protein, p16.

For immunohistochemical detection of the p16 protein, the antibody used was the anti-p16INK4a (Ab-7) antibody from Neomarkers (Lab Vision UK Ltd., Suffolk, England). A case of cervical intraepithelial neoplasia grade III (CIN III) known to strongly express P16 protein was used as a positive control. Another section of the CIN III to which no p16 antibody was added served as a negative control. Extra sections were taken from two of the cutaneous SCC samples, and these served as additional negative controls by omitting the p16 antibody.

Four μm sections of the formalin fixed paraffin embedded samples were deparaffinized and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked by incubating the sections in two changes of 0.9% hydrogen peroxide for 15 min each change at room temperature. The antigenic sites were unmasked by pressure-cooking the sections in 0.01 M Sodium Citrate solution (pH 6) for 2 min. The Vectastain® Universal ABC kit (Vector Laboratories, USA) was used as described by the manufacturer. Incubation with the p16 antibody was at a dilution of 1/100, for 30 min at room temperature. Immunostaining was

![Fig. 1 AI/LOH results at the D9S916, D9S974, D9S942, D9S1604, D9S1814 and D9S171 microsatellite markers and p16 expression for the 22 cutaneous SCC samples.](image-url)
visualized using 3, 3’-diaminobenzidine (DAB) (Zymed, CA, USA) as the substrate chromagen, and sections were counterstained with Harris’ Haematoxylin.

The samples were assessed for nuclear staining indicative of p16 expression.

**Results**

**AI/LOH analysis**

A summary of the AI/LOH analysis results obtained for the twenty-two cutaneous SCC samples at the six microsatellite markers is given in Fig. 1.

Of the twenty-two SCC samples analysed for AI/LOH, the average informative rate per marker was 73% (range from 55% to 95%). A high rate of AI/LOH was seen at all markers. AI/LOH was most frequent at the D9S1814 marker (100% of informative samples). At the D9S916 locus, 92% of the informative samples showed AI/LOH (Fig. 2). At the D9S974 and the D9S942 markers, both within the CDKN2A gene, 89% and 76% of informative samples showed AI/LOH, respectively. One sample, sample 22, displayed MSI at both the D9S974 and the D9S942 markers (Fig. 3). At the D9S1604 marker, 76% of the informative samples displayed AI/LOH and at the D9S171 marker, 87% of the informative samples displayed AI/LOH (Fig. 2).

**Immunohistochemical analysis of p16 protein expression in the cutaneous SCC samples**

All of the twenty-two samples of cutaneous SCC were analysed by immunohistochemistry for p16 expression. Only two out of the twenty-two of the cutaneous SCC samples, stained positive for p16 expression. All other cutaneous SCC samples examined were negative for p16 expression.
Examples of p16 protein expression patterns are illustrated in Fig. 4 (A–D).

The adjacent normal epithelium of twenty-one of the twenty-two SCC samples was negative for p16 expression (sample 1 had no adjacent normal epithelium to evaluate). The normal adjacent reticular dermis of all SCC samples was also negative for p16 expression.

**Correlation between p16 protein expression and AI/LOH results**

The relationship between AI/LOH at the six microsatellite markers flanking p16 and expression of the p16 protein in the cutaneous SCC samples is shown in Fig. 1.

In Fig. 1 it can be seen that AI/LOH of the five markers within the 9p21 region and of the D9S171 marker within 9p13 is frequent. Of particular interest are the two markers closest to the CDKN2A locus. These are the D9S974 marker, which has been mapped within the CDKN2A locus, and the D9S942 marker (centromeric to D9S974), which has been mapped within intron 1 of the CDKN2A locus (NCBI genemap ’99). Twenty out of the twenty-two SCC samples were negative for p16 expression. Two of the SCC samples negative for p16 expression showed retention of heterozygosity (ROH) at the D9S942 marker with AI/LOH at the D9S974 marker. Another sample, sample 15, also showed ROH at the D9S942 marker, and also at the D9S974 marker. All other samples that were negative for p16 expression showed AI/LOH at the D9S974 and D9S942 markers (where informative), except for sample 22 which showed MSI at both D9S974 and D9S942.

Only two of the SCC samples were positive for p16 expression (Fig. 4 A, B). These were samples 1 and 9. Sample 1 was non-informative at D9S974, but displayed AI/LOH at D9S942, where as sample 9 showed AI/LOH at D9S974 and ROH at D9S942.

Overall both AI/LOH within the CDKN2A locus and absence of p16 protein expression were common among the cutaneous SCC samples analysed.

**Discussion**

Previous studies on cutaneous SCC have shown that AI/LOH is frequent on chromosome 9p [20, 39–40]. The present study focused in particular on the 9p21 region of chromosome 9. This region contains the CDKN2A, p15 and MTAP genes.[4, 41] In the present study, the integrity of these genes in cutaneous SCC was examined by analysing the presence of AI/LOH at microsatellite markers located within or flanking the genes of interest.
The results of the present study show that AI/LOH is frequent in the 9p21 region in invasive cutaneous SCC. This high rate of AI/LOH was also seen in the D9S974 and D9S942 microsatellite markers, both of which have been mapped within the CDKN2A gene (NCBI genemap '99), suggesting that inactivation of the CDKN2A gene may play a role in cutaneous SCC. Immunohistochemical analysis of p16 expression in these cutaneous SCC samples found that p16 was not expressed in 91% of the samples. Fifteen samples that lacked p16 protein expression showed AI/LOH at the D9S942 marker and also showed AI/LOH at the D9S974 marker (where informative). As an absence of protein expression requires biallelic inactivation, it appears likely that in these SCC samples AI/LOH has inactivated one p16 allele, with the other allele having been inactivated by mutation and/or methylation.

Another three samples that lacked p16 protein expression, showed retention of heterozygosity (ROH) at the D9S942 marker, two of which displayed AI/LOH at the D9S974 marker and the other displayed ROH at the D9S974 marker. One SCC that lacked p16 expression showed retention of het-
erozygosity at both the D9S942 and D9S974 markers. In such fluorescent analysis of AI/LOH as employed in this study, apparent ROH may sometimes, though not always, mask homozygous deletions. Therefore further analysis of these samples would be required to rule out if the p16 gene was inactivated as a result of homozygous deletion or mutations and/or methylation. The one remaining SCC sample that did not express p16 protein was the only sample to display MSI, and showed MSI at both the D9S974 and the D9S942 markers. In this sample it appears likely that MSI has caused inactivating frameshift mutations of the p16 gene, however analysis of the methylation status of the p16 gene in this sample would be required to confirm if MSI was contributing to biallelic inactivation or not.

In normal epithelial tissues P16 expression is not detectable by immunohistochemistry and this is believed to reflect the basal physiological level of p16, however in tumours in which p16 was active, p16 expression detectable by immunohistochemistry would be expected [42–43]. Only two of the examined SCCs were positive for p16 expression, with sample 1 showing particularly intense expression. Such strong expression of p16 in cervical SCC has been associated with inactivation of Rb as a result of infection with high risk or intermediate risk types of human papilloma viruses (HPV) [44–46]. Sample 1, which showed very strong p16 expression, displayed AI/LOH at the D9S942 marker and was non-informative at the D9S974 marker. Therefore the presence of p16 expression in this sample suggests that although one allele was deleted within intron 1 of the CDKN2A gene, the other allele was wild type and able to compensate for the mutated allele, or that perhaps the deletion was a breakpoint deletion that did not extend far into the CDKN2A locus and both copies of the p16 gene were intact. The overexpression of p16 protein in this sample also suggests that Rb was inactivated in this SCC sample, though this has yet to be confirmed and whether such Rb inactivation was due to HPV infection or not also needs to be established.

The only other SCC to express p16 protein reported in the present study was SCC sample number 9. P16 protein was not overexpressed in sample 9 (as in sample 1), suggesting that Rb as well as p16 is intact in this sample. Sample 9 showed AI/LOH at the D9S974 marker, but retention of heterozygosity at the other marker. As with sample 1, the presence of p16 expression in this sample rules out biallelic inactivation of the p16 gene, however AI/LOH at the D9S974 marker may have been enough to inactivate one of the p16 alleles.

Previous studies have examined p16 expression immunohistochemically in cutaneous SCC with conflicting results [40, 47–48] Hodges and Smoller have found that 100% of their cutaneous SCC samples displayed strong p16 expression, whereas Mortier et al. and Brown et al. found that only 10% and 38%, respectively, of their samples were positive for p16 expression. [40, 47–48] The results of the present study are in agreement with the results found by Mortier et al. and Brown et al., that the majority of cutaneous SCC lack p16 expression. The reason for the conflicting results by Hodges and Smoller is unclear. It is possible that some of the samples that stain positive may be expressing a mutant inactive p16. However, further analysis of their samples at the DNA level would be required to ascertain if that was the case [40, 47–48].

The results of the present study show that a high proportion of the SCC analysed have AI/LOH within the CDKN2A locus. As the CDKN2A locus also codes for the p19ARF protein, this AI/LOH may be disrupting expression of the p19ARF protein. Although analysis of p19ARF protein expression was not carried out in the present study, Brown et al. have reported an absence of p19ARF expression in 58% of cutaneous SCC samples analysed, suggesting a role for inactivation of the p19ARF tumour suppressor gene in cutaneous SCC [47].

In the present study, AI/LOH at the D9S916 microsatellite marker located within the MTAP gene was also examined in the cutaneous SCC samples. The number of SCC with AI/LOH at this marker was very high, with 12/13 (92%) of informative samples showing AI/LOH. This suggests that inactivation of the MTAP gene may be playing a role in cutaneous SCC development.

Two microsatellite markers, the D9S1604 marker (which flanks the p15 gene telomerically) and the D9S1814 marker (which flanks the p15 gene centromerically) were also examined for AI/LOH in the cutaneous SCC samples. At the D9S1604 marker 11/12 (92%) informative samples displayed AI/LOH and at the D9S1814 marker 14/14 (100%) of informative samples displayed AI/LOH. This very high rate of AI/LOH at the markers flanking the p15 gene suggest that inactivation of the p15 may also have a role to play in cutaneous SCC development.
AI/LOH at the D9S171 microsatellite marker, located within 9p13 was also examined in the cutaneous SCC. Using the technique of silver staining, Saridaki et al. have previously found that 20% of their analysed cutaneous SCC displayed AI/LOH at the D9S171 marker. [20] Mortier et al. and Brown et al., using the more sensitive fluorescent technique reported that 24% and 29%, respectively, of their cutaneous SCC samples showed AI/LOH at D9S171. [40, 47]. In the present study, the incidence of AI/LOH at D9S171 was found to be higher, with 59% of the cutaneous SCC samples displaying AI/LOH.

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