

Role of p16/MTS1, cyclin D1 and RB in primary oral cancer and oral cancer cell lines

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Summary One of the most important components of G1 checkpoint is the retinoblastoma protein (pRB¹¹⁰). The activity of pRB is regulated by its phosphorylation, which is mediated by genes such as *cyclin D1* and *p16/MTS1*. All three genes have been shown to be commonly altered in human malignancies. We have screened a panel of 26 oral squamous cell carcinomas (OSCC), nine premalignant and three normal oral tissue samples as well as eight established OSCC cell lines for mutations in the *p16/MTS1* gene. The expression of *p16/MTS1*, *cyclin D1* and pRB¹¹⁰ was also studied in the same panel. We have found *p16/MTS1* gene alterations in 5/26 (19%) primary tumours and 6/8 (75%) cell lines. Two primary tumours and five OSCC cell lines had p16/MTS1 point mutations and another three primary and one OSCC cell line contained partial gene deletions. Six of seven p16/MTS1 point mutations resulted in termination codons and the remaining mutation caused a frameshift. Western blot analysis showed absence of p16/MTS1 expression in 18/26 (69%) OSCC, 7/9 (78%) premalignant lesions and 8/8 cell lines. One cell line, H314, contained a frameshift mutation possibly resulting in a truncated p16/MTS1 protein. pRB was detected in 14/25 (56%) of OSCC but only 11/14 (78%) of these contained all or some hypophosphorylated (active) pRB. In premalignant samples, 6/8 (75%) displayed pRB, and all three normal samples and eight cell lines analysed contained RB protein. p16/MTS1 protein was undetectable in 10/11 (91%) OSCCs with positive pRB. Overexpression of cyclin D1 was observed in 9/22 (41%) OSCC, 3/9 (33%) premalignant and 8/8 (100%) of OSCC cell lines. Our data suggest p16/MTS1 mutations and loss of expression to be very common in oral cancer cell lines and less frequent in primary OSCC tumours. A different pattern of p16/MTS1 mutations was observed in OSCC compared to other cancers with all the detected p16/MTS1 mutations resulting in premature termination codons or a frameshift. The RB protein was expressed in about half (44%) of OSCCs and its expression inversely correlated with p16/MTS1 expression. In conclusion, we show that abnormalities of the RB pathway are a common mechanism of oral carcinogenesis.

Keywords: oral cancer; p16/MTS1; cyclin D1; retinoblastoma; G1 checkpoint

Oral squamous cell carcinoma (OSCC) is the most common malignancy in South Asia. Carcinogens such as alcohol, tobacco and nitrosamines contained in areca nut are known to cause the development of most oral cancers but the molecular mechanisms involved in the malignant transformation of oral epithelial cells are still unclear (Johnson, 1991). Aberrations in the *p53* gene have been shown to be the most common genetic alterations in oral cancers (Wong et al, 1996). However, about 50% of oral cancers seem to have wild-type (wt) *p53*, suggesting that other genes may contribute to the development of oral malignancies. Recently, much attention has been focused on the role of the G1 checkpoint in human cancer. Apart from *p53*, the retinoblastoma (RB) gene product, pRB¹¹⁰, is another key component of this checkpoint (Weinberg, 1995). pRB¹¹⁰ was the first of the 'pocket' proteins to be characterized (Goodrich and Lee, 1993). The other known members of this family of proteins are p130 and p107 (Ewen et al, 1993; Mayol et al, 1993). pRB binds a number of viral proteins

such as E7 from certain types of papillomaviruses (Li et al, 1993), large T antigen of SV40, E1A of certain adenoviruses (Wang et al, 1991) and IE2 of cytomegaloviruses (CMV) (Hagemeier et al, 1994). It also binds several cellular proteins including cyclin-dependent kinases and the E2F transcription factor (Bagchi et al, 1991; Chellappan et al, 1991), suggesting a very important role for RB in controlling cellular growth. pRB¹¹⁰ activity is regulated by phosphorylation, which occurs in a cell cycle-dependent manner (Chen et al, 1989). Dephosphorylation of pRB¹¹⁰ renders it active, leading to G1 arrest. The phosphorylation and inactivation of pRB is thought to be induced by successive waves of cyclins D1, D2 and D3 together with CDK4 or CDK6 and cyclin E together with CDK2. The function of these complexes is counteracted by the activity of cyclin-dependent kinase inhibitors (CDKIs) (reviewed by Hunter and Pines, 1994; Sherr, 1994). Two CDKI families are known, the prototype genes of these families being p16/CDKN2/MTS1 (Serrano et al, 1993) and p21/WAF1/CIP1 respectively. p21/WAF1 is thought to be a 'universal' CDKI, inhibiting the activity of both Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes (Xiong et al, 1993) while p16/MTS1 only inhibits cyclin D1-CDK4/6 complexes (Serrano et al, 1993). The expression of p16/MTS1 is constantly low during most of the cell cycle, peaking with a slight increase at G1 phase (Tam et al, 1994;

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Stone et al, 1995). Ectopic overexpression of p16/MTS1 has been shown to result in G1 arrest (Koh et al, 1995; Lukas et al, 1995; Serrano et al, 1996). This arrest is dependent on the presence of (wt) pRB (Medema et al, 1995).

p16/MTS1 is located on 9p21 in humans, a region commonly deleted in many tumour types (Fountain et al, 1992; Kamb et al 1994). As well as gene deletion (van der Riet et al, 1994; Reed et al., 1996), point mutations (Zhou et al, 1994; Liu et al, 1995; Arap et al, 1997), methylation (Gonzalez-Zulueta et al, 1995; Otterson et al, 1995; Shapiro et al, 1995) and the TAX protein of HTLV1 virus (Suzuki et al, 1996) have been found to inactivate p16/MTS1 in several tumour types including head and neck (Reed et al, 1996; Olshan et al, 1997; Papadimitrakopoulou et al, 1997).

Cyclin D1/PRAD1/BCL1 is located on 11q13 in humans, a region commonly amplified in several types of cancer (Berenson et al, 1989; Bartkova et al, 1995a, 1995b). As well as amplification, mutations which result in the stabilization of cyclin D1 protein have also been suggested to be a mechanism for the abnormal accumulation of cyclin D1 (Welcker et al, 1996). Ectopic expression of cyclin D1 results in the acceleration of the G1/S phase transition, showing that cyclin D1 is rate-limiting in this step (Jiang et al, 1993; Ohtsubo and Roberts 1993). The acceleration through the G1 checkpoint due to cyclin D1 overexpression has been shown to result in an increase in genomic instability (Zhou et al, 1996).

Despite the importance of pRB¹¹⁰ in regulating the G1 checkpoint, mutations in pRB¹¹⁰ are uncommon in head and neck squamous cell carcinomas (Yoo et al, 1994). This suggests that not only pRB¹¹⁰, but also the proteins regulating RB function, may be involved in carcinogenesis of head and neck epithelium.

In this study we have assessed aberrations in genes upstream of RB including p16/MTS1, cyclin D1 as well as pRB¹¹⁰ itself in a panel of OSCC and premalignant oral lesions as well as OSCC cell lines. Our data suggest that loss of expression of p16/MTS1 is very common in OSCC. Also, cyclin D1 was frequently found to be overexpressed in malignancies of the oral cavity. While the RB gene itself seems to be less prone to alterations in this type of cancer its expression and/or activity is altered by other proteins such as p16/MTS1, cyclin D1 and possibly E7 of HPV-16.

MATERIALS AND METHODS

Sample selection

Fresh oral biopsies were collected from 38 patients with lesions clinically and histologically diagnosed as OSCCs or premalignant mucosal lesions from six hospitals in South East England. Three pathologically normal specimens were also included. The total panel consisted of 22 male and 16 female and their mean age was 65.6 ± 2.1 years. Histology confirmed that 26 of these were malignant OSCC; nine were keratoses with varying grades of dysplasia (Tables 1 and 2). OSCC were histologically graded as well, moderate and poorly differentiated and the severity of dysplasia as mild, moderate or severe by the Smith and Pindborg criteria (1969). The samples were stored in the gas liquid phase of liquid nitrogen until further use. The oral cancer cell lines were gifts from Profs Stephen Prime, Bristol (H103, H157, H314, H357, H376, H400) and Barry Gusterson, Sutton (HN5 and HN6). The cells were grown under the conditions which have been previously described (Bartkova et al, 1995a; Yeudall et al, 1995).

DNA preparation and mutational analysis

The p16/MTS1 polymerase chain reaction (PCR) was performed using primers; exon 1 (sense: 5'-CGG CTG CGG AGA GGG GGA GA-3', antisense: 5'-CCG CTG CAG ACC CTC TAC CCA CCT-3'), exon 2 part 1 (sense: 5'-ACA AGC TTC CTT TCC GTC ATG CCG- 3', antisense: 5'-CCA GGC ATC GCG CAC GTC CA-3') exon 2 part 2 (sense: 5'-TTC CTG GAC ACG CTG GTG GT-3', antisense: 5'-TCT GAG CTT TGG AAG CTC TCA G-3') exon 3 (sense: 5'-CGC CTG TTT TCT TTC TGC CCT CTG-3', antisense: 5'-GAA AGC GGG GTG GGT TGT GG-3'). Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal PCR control. Oligonucleotide primers for GAPDH were (sense: 5'-AGT ACG CTG CAG GGC CTC ACT CC TT-3', antisense 5'-AAG AGC CAG TCT CTG GCC CCA GCC A-3'). The PCR reaction consisted of 1 µl of DNA extract, 25 pmol of each primer, 1X PCR Promega Formula buffer containing 1.5 mM magnesium, 200 µM dNTPs, 5% dimethyl sulphoxide (DMSO) and 1.5 U *Taq* polymerase (Advanced Biotechnology) in a total volume of 50 µl. The PCR mix was denatured at 94°C for 5 min, followed by 30 cycles (94°C, 30 s; 62°C, 1 min and 72°C, 1 min).

DNA was isolated from 2 × 6 µm cryostat sections from frozen specimens for which adjacent sections were examined by microscopy for assessment of the presence of adequate tumour tissue and the proportion of stromal tissue. All OSCC samples used for DNA extraction showed > 60% tumour tissue in each case. Cellular DNA was extracted as we have previously described (Steingrimsdottir et al, 1997). Briefly, the samples were lysed in sodium dodecyl sulphate (SDS) buffer [100 mM sodium chloride (NaCl), 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0] and were incubated with 0.1 mg ml⁻¹ proteinase K for approximately 5 h. The lysate was then treated with an equal volume of phenol-chloroform-isoamyl alcohol pH 8.0 (25:24:1). DNA was recovered from the aqueous phase by the addition of 2 volumes of ethanol and was stored in 100 µl TE containing 2 µg RNase A. For each PCR reaction, 1 µl DNA extract equal to approximately 100 ng was used.

PCR amplification and SSCP analysis of the p16/MTS1 gene

For SSCP analysis, the PCR products were labelled after 15 cycles by the addition of 1 µCi [α -³²P]dCTP. A total of 3 µl of the labelled PCR products were diluted with 4 µl of 95% deionized formamide containing bromophenol blue-xylene cyanol, 3.7 µl EDTA (0.5 M) and 26.25 µl water. The PCR mixtures were denatured for 5 min at 95°C, and were snap frozen by placing in liquid nitrogen. The denatured products (5 µl) were loaded on an 8% non-denaturing polyacrylamide gel electrophoresis (PAGE) gel and separated using 1 × TBE in the upper chamber (cathode) and 2 × TBE in the lower chamber (anode). Electrophoresis was carried out at 350 volts at room temperature overnight. The gels were dried and exposed to X-ray film developing after 16 h.

For sequencing, 40 µl of the PCR products were electrophoresed in a 1% low melting point agarose gel and the target bands were excised from the gel. The selected agarose fragments were sliced and digested with Agarase (B-Agarase I, Calbiochem) according to the manufacturer's recommendation. The purified DNA products were then sequenced directly using both 5' and 3' primers together with dye terminators in an ABI 373A automated sequencer.

Expression analysis

For Western blotting, samples were lysed in Laemmli sample buffer (Laemmli, 1970), which were then boiled and resolved by SDS-PAGE, 6–12.5%. The gel was transferred to 0.45 µm nitrocellulose membrane (Schleicher and Schuell) as previously described (Towbin et al, 1992). Probing of the blot and detection of the antibodies were performed according to the manufacturer's instructions for the enhanced chemiluminescent reaction (ECL, from Amersham). The membrane was blocked for 1 h in 4% milk powder containing 1% bovine serum albumin (BSA) in TBST buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20). For p16, the antibody used was DCS-50 and for cyclin D1, DCS-6 (both gifts of Dr Jiri Bartek and Dr Gordon Peters). Both p16 and cyclin D1 antibodies were diluted 1:1000 in TBST. For pRB, the antibody used was 14001A (Pharmingen), diluted 1:500 in TBST. Secondary antibodies tagged to horseradish peroxidase were detected using the enhanced chemiluminescence method according to the manufacturer's instructions (ECL, from Amersham). p16-positive cell line MDAMB 468 and cyclin D1-positive cell line MCF7 were used as positive controls.

RESULTS

p16/MTS1 gene deletion/mutation analysis

DNA from 26 OSCC tumours, nine premalignant and three normal samples as well as eight OSCC cell lines was analysed by PCR. Exons 1α, 2 and 3 of p16/MTS1 were independently amplified using GAPDH primers in the same reactions as an internal control. Because of the large size of p16/MTS1 exon 2 (305 bp), this was amplified as two separate fragments (parts I and II).

Samples 13, 14 and 24 failed to amplify the 218 bp fragment expected for exon 1α. These samples amplified a 474 bp fragment corresponding to the GAPDH fragment, albeit a weaker band was observed for 24 (Figure 1). These results suggested that at least part of exon 1α is deleted in these three tumours (Figure 1).

To examine the p16/MTS1 gene mutations the PCR products were then analysed by single-stranded conformation polymorphism (SSCP) and samples that showed abnormal shifts on the SSCP gel were subsequently sequenced directly. Point mutations were detected in 2/26 (8%) of the primary oral tumours, 6 and 15. Both of these were non-sense mutations at codon 58 (CGA>TGA, arg>term). Sample 6 also showed a transition at codon 148 (GCG>ACG, ala>thr), which is a known p16/MTS1 polymorphism (Tables 1 and 2).

Analysis of the p16/MTS1 gene in the cell lines by PCR amplification showed deletion of exon 3 in one cell line, HN6. Point mutations in the p16/MTS1 gene were found in 5/8 (63%) of OSCC cell lines (Tables 1 and 2). We found non-sense mutations at codons 58 (CGA>TGA, arg>term) in H103 and H357, and codon 80 (CGA>TGA, arg>term) in H157. In HN5, codon 88 was mutated substituting GAG>TAG, glu>term. In H314, the deletion of a single G at codon 69 caused a frameshift. This frameshift results in several termination codons in the reading frame, the first one being in codon 119. None of the premalignant or normal samples showed either deletions or point mutations in the p16/MTS1 gene.

p16/MTS1 expression

Expression of p16/MTS1 was analysed in primary samples and OSCC cell lines by Western blotting, using the DCS-50 antibody.

The expression results are summarized in Tables 1 and 2. Absence of p16 was observed in 18/26 (69%) OSCC, whilst 8/26 (31%) OSCCs and 2/9 (22%) premalignant lesions expressed p16/MTS1 (Table 1 and Figure 2). All 8/8 (100%) cell lines lacked the expression of p16/MTS1. Sample 6, which had a p16/MTS1 mutation resulting in a termination codon (Tables 1 and 2), did not express normal size p16/MTS1. However, sample 15, which had the same termination mutation as 6, expressed p16/MTS1 protein. Also, sample 24 with exon 1 deletion lacked p16/MTS1 expression, while both samples 13 and 14 with a similar deletion expressed moderate to high levels of p16/MTS1. Western blot analysis detected a protein of approximately 25 kDa in the H314 cell line using C-20 p16/MTS1 antibody (Santa Cruz). However, such protein was not detected using another p16/MTS1 antibody, DCS-50.

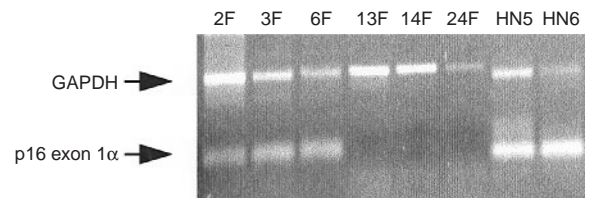


Figure 1 Analysis of p16/MTS1 exon 1α by PCR. Exon 1α of p16/MTS1 gene was co-amplified with the internal control GAPDH and 10 µl of PCR products were separated on a 2% agarose gel. The bands corresponding to the predicted molecular weights of the GAPDH (474 bp) and p16/MTS1 exon 1α (218 bp) amplicons are indicated on the gel by arrows. Lane numbers correspond to the sample references in Table 1

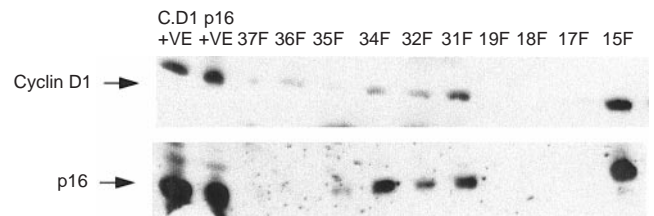


Figure 2 Western blot analysis of cyclin D1 and p16 in primary oral lesions and oral cancer cell lines. Total cell or tissue lysates were separated in a 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and the membrane was hybridized to antibodies DCS-50 for p16 and DCS-6 for cyclin D1. The lanes indicated +C. D1 for MCF-7 cell lysate as cyclin D1-positive control; +p16 for MDAMB 453 cell lysate as p16-positive control. The rest of the lanes correspond to the sample references in Table 1

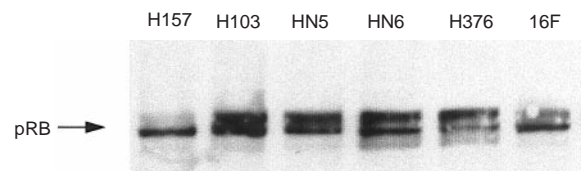


Figure 3 Western blot analysis of pRB in oral samples. Total cell or tissue lysates were separated on a 6% SDS-PAGE and the membrane was hybridized to antibody 14001A. Samples are marked according to the sample references in Table 1

Table 1 Comparison of p16/MTS1 mutation analysis and expression of p16, cyclin D1 and pRB in primary oral lesions

Sample number	Sample histology	p16 Mutation analysis	p16 expression	Cyclin D1 expression	pRB expression	pRB phosphorylation status
2	OSCC	WT	-	NA	+	RB ¹⁰⁵
3	OSCC	WT	-	-	+	RB ¹⁰⁵
4	OSCC	WT	-	NA	+	RB ¹⁰⁵
6	OSCC	$\alpha\alpha 58;CGA>TGA$ $\alpha\alpha 148;GCG>ACG$	-	NA	-	no RB
8	OSCC	WT	+	+	+	RB ¹¹⁰
9	OSCC	WT	-	+	-	no RB
10	OSCC	WT	++	+	+	RB ¹¹⁰
11	OSCC	WT	+	-	+	RB ^{105/110}
13	OSCC	deletion exon 1	+	-	-	no pRB
14	OSCC	deletion exon 1	++	+	-	no RB
15	OSCC	$\alpha\alpha 58;CGA>TGA$	++	+	NA	NA
17	OSCC	WT	-	-	+	RB ¹⁰⁵
18	OSCC	WT	-	-	+	RB ¹⁰⁵
19	OSCC	WT	-	-	-	no RB
20	OSCC	WT	-	-	+	RB ¹⁰⁵
22	OSCC	WT	-	-	+	RB ^{105/110}
23	OSCC	WT	-	-	-	no pRB
24	OSCC	deletion exon 1	-	-	+	RB ¹¹⁰
26	OSCC	WT	-	+	+	RB ¹⁰⁵
27	OSCC	WT	-	+	+	RB ¹⁰⁵
30	OSCC	WT	-	-	-	no RB
31	OSCC	WT	+	+	-	no RB
32	OSCC	WT	+	-	-	no RB
36	OSCC	WT	-	-	-	no RB
37	OSCC	WT	-	-	-	no RB
42	OSCC	WT	-	+	+	RB ^{105/110}
1	pre-cancer	WT	-	-	+	RB ^{105/110}
12	pre-cancer	WT	+	+	+	RB ¹⁰⁵
16	pre-cancer	WT	-	-	+	RB ^{105/110}
25	pre-cancer	WT	-	+	NA	NA
34	pre-cancer	WT	+	+	-	no RB
35	pre-cancer	WT	-	-	+	RB ¹⁰⁵
38	pre-cancer	WT	-	-	+	RB ¹⁰⁵
40	pre-cancer	WT	-	-	+	RB ¹⁰⁵
43	pre-cancer	WT	-	-	-	no RB
33	normal	WT	+	-	+	RB ¹⁰⁵
39	normal	WT	-	-	+	RB ¹⁰⁵
41	normal	WT	-	-	+	RB ¹⁰⁵

The pRB expression column refers to '+' for samples which expressed either, one or both hypo- and hyperphosphorylated forms of pRB. The pRB phosphorylation column describes the phosphorylation status of pRB. Samples expressing only dephosphorylated pRB are denoted as 'RB¹⁰⁵', only phosphorylated pRB as 'RB¹¹⁰', no expression as 'no pRB' and expression of both forms as 'RB^{105/110}'. WT, wild-type sequence, NA, not analysed.

Cyclin D1 and RB expression

Western blot analysis using DCS-6 antibody detected high levels of cyclin D1 in 9/22 (41%) of the tumours, 3/9 (33%) potential malignancies and in 100% (8/8) of the OSCC cell lines. Both normal samples analysed expressed low levels of cyclin D1.

pRB was expressed in 14/25 (56%) of the cancers, 6/8 (75%) premalignant lesions and all three normals. The level of pRB phosphorylation varied in different samples. pRB was hyperphosphorylated in 3/14 malignant samples (8, 10 and 24), indicating the presence of an inactive pRB. Surprisingly, all (8/8) cell lines expressed pRB but in one cell line, H157, mainly the unphosphorylated form of pRB was detected (Tables 1 and 2 and Figures 2 and 3).

Comparison between pRB, cyclin D1 and p16/MTS1 expression

The pRB expression results obtained by Western blotting were compared to those for p16/MTS1 and cyclin D1. We found an inverse association between the expression of p16/MTS1 and the presence of pRB. Lack of p16/MTS1 expression was observed in 10/11 (91%) OSCCs which contained active pRB. Also 5/6 (83%) of the premalignant lesions which were pRB-positive lacked p16/MTS1 protein (Table 3) and (8/8) 100% cell lines expressed pRB but no p16/MTS1. Comparison between cyclin D1 and p16/MTS1 showed 10/13 (77%) OSCCs and 6/6 premalignant samples did not express either Cyclin D1 or p16/MTS1 (Table 4). Cyclin D1 and pRB expression also showed some correlation: 5/6

Table 2 Comparison of p16/MTS1 mutation/deletion and expression of p16, cyclin D1 and pRB in OSCC cell lines

Cell line	p16 mutation status	p16 expression	Cyclin D1 expression	pRB expression	pRB phosphorylation status
HN5	αα88; GAG>TAG	–	++	+	RB ^{105/110}
HN6	deletion exon 3	–	++	+	RB ^{105/110}
H103	αα58; CGA>TGA	–	++	+	RB ^{105/110}
H157	αα80;	–	++	+	RB ¹⁰⁵
H314	αα69; deletion of G	–	++	+	RB ^{105/110}
H357	αα58; CGA>TGA	–	++	+	RB ^{105/110}
H376	WT	–	++	+	RB ^{105/110}
H400	WT	–	++	+	RB ^{105/110}

The pRB expression column refers to '+' for samples which expressed either, one or both hypo- and hyperphosphorylated forms of pRB. The pRB phosphorylation column describes the phosphorylation status of pRB. Samples expressing only dephosphorylated pRB are denoted as 'RB¹⁰⁵', only phosphorylated pRB as 'RB¹¹⁰', no expression as 'no pRB' and expression of both forms as 'RB^{105/110}'. WT, wild-type sequence, NA, not analysed.

Table 3 Comparison between expression of p16/MTS1 and active pRB

Samples	Positive pRB		Negative pRB	
	Positive p16	Negative p16	Positive p16	Negative p16
OSCC	1/11 (9%)	10/11 (91%)	6/14 (43%)	8/14 (57%)
Pre-cancer	1/6 (17%)	5/6 (83%)	1/2 (50%)	1/2 (50%)

pRB was scored as active if either only the hypophosphorylated or both hypo- and hyperphosphorylated forms were detected.

Table 4 Comparison between expression of cyclin D1 p16/MTS1 by Western blotting

Samples	Positive cyclin D1		Negative cyclin D1	
	Positive p16	Negative p16	Positive p16	Negative p16
OSCC	4/9 (56%)	4/9 (44%)	3/13 (23%)	10/13 (77%)
Pre-cancer	2/3 (67%)	1/3 (33%)	0/6 (0%)	6/6 (100%)

(83%) of the premalignant lesions expressed pRB but no cyclin D1 while only 6/9 (67%) of the OSCCs showed that pattern of expression (Table 5). The number of normal samples in this study was too small to make any correlation.

Table 5 Comparison between expression of cyclin D1 and active pRB

Samples	Positive pRB		Negative pRB	
	Positive cyclin D1	Negative cyclin D1	Positive cyclin D1	Negative cyclin D1
OSCC	3/9 (33%)	6/9 (67%)	5/12 (42%)	7/12 (58%)
Pre-cancer	1/6 (17%)	5/6 (83%)	1/2 (50%)	1/2 (50%)

pRB was scored as active if either only the hypophosphorylated or both hypo- and hyperphosphorylated forms were observed.

DISCUSSION

In order to understand the role of the RB pathway in oral cancer we studied the expression of pRB as well as its upstream regulators p16/MTS1 and cyclin D1 in primary oral lesions and oral cancer cell lines. We also analysed p16/MTS1 mutations in the same panel. To our knowledge, few studies have analysed the expression of these three key proteins in primary OSCCs (Andl et al, 1998).

In primary OSCCs, p16/MTS1 point mutations/deletions occurred relatively frequently (19%) but in the cell lines the p16/MTS1 gene was altered with a much higher frequency (75%). No p16/MTS1 gene alterations were found in premalignant or normal samples. This large difference in the frequencies, also reported by others (Zhang et al, 1994), could be due to instability of the p16/MTS1 gene in the cell lines maintained for long duration in culture. Mutations in the p16/MTS1 gene could also confer a growth advantage to cells and therefore cells with such mutations are selected for establishing lines. Another reason for the lower incidence of p16/MTS1 mutations in primary tumours could be due to the heterogeneity of the tissues analysed. The presence of normal cells in the specimens can mask the detection of p16/MTS1 point mutations/deletions.

Mechanisms other than gene mutation have also been shown to be responsible for down-regulation of the p16/MTS1 gene (Hara et al, 1996). DNA methylation has been shown to play an important role in silencing p16/MTS1 gene transcription (Otterson et al, 1995). In this study, 16/26 (62%) OSCCs, 5/9 (56%) premalignant

lesions and 2/8 cell lines which had no detectable p16/MTS1 mutation, lacked p16/MTS1 protein. This indicates that epigenetic mechanisms such as DNA methylation could be responsible for inducing gene silencing in these samples.

In three primary tumours p16/MTS1 exon 1 α was not amplified despite amplification of exons 2 and 3 as well as the GAPDH gene. We interpreted this to be a partial deletion of *p16/MTS1* gene. Surprisingly, two of these three samples expressed p16/MTS1 protein (Tables 1 and 2). This could be due to the presence of small deletions or point mutations preventing primer annealing and hence gene amplification, but not affecting the reading frame and expression of *p16/MTS1* gene. Alternatively, this discrepancy could be due to the use of different sections taken from different parts of the tumour for use in the PCR and Western blot analyses.

In sample 24, which failed to amplify exon 1 α , the level of GAPDH was also lower, which was probably due to the presence of less DNA in this particular sample available for the PCR reaction. We checked and confirmed the partial deletions in the *p16/MTS1* gene in these three tumour samples in three independent PCR reactions. However, we are aware of the limitations of the semiquantitative PCR technique for deletion detection, therefore we are cautious interpreting our PCR results. To minimize artefacts, only sections with more than 60% malignant cells were used for DNA and protein analysis. The option of tissue microdissection was also considered, but preliminary experiments showed that this process greatly increases the risk of tissue contamination caused by the extensive manipulations performed on the tissues. The frequency of deletions within or encompassing the *p16/MTS1* gene has been found to vary greatly. For example, one study (Reed et al, 1996) showed homozygous deletions of 9p in 67% of head and neck tumour samples, whilst Zhang et al (1994) could not detect any p16/MTS1 deletions in 68 head and neck tumours that they analysed. Also, it has been shown that deletions on 9p do not necessarily correlate with loss of p16/MTS1 expression. Indeed, it has been shown that loss of p16/MTS1 expression can occur at a much higher frequency than deletion of the *p16/MTS1* gene (Gonzalez-Zulueta et al, 1995). The converse is also true: deletions in 9p do not necessarily affect p16/MTS1 expression (Cheng et al, 1994; Farrell et al, 1997).

Deletions encompassing exon 1 α could affect other genes upstream of *p16/MTS1*, such as the CDKI p15/MTS2 (Hannon and Beach, 1994) or p19^{ARF} (Quelle et al, 1995, 1997). Two recent papers discuss the importance of p19^{ARF} in the regulation of the p53 pathway (Pomerantz et al, 1998; Zhang et al, 1998). p19^{ARF} can suppress cellular proliferation in cells bearing wt p53, but not otherwise (Kamijo et al, 1997). This is thought to occur by virtue of p19^{ARF} ability to destabilize the MDM2 protein (Pomerantz et al, 1998; Zhang et al, 1998). The MDM2 protein has also been shown to play a role in the pRB regulated cell cycle control (Martin et al, 1995; Xiao et al, 1995). Alterations in p19^{ARF} protein as a result of p16/MTS1 mutations could affect its function, leading to pRB deregulation and development of cancer.

Interestingly, we found that all but one of the *p16/MTS1* gene point mutations resulted in a termination codon. The remaining mutation identified in the cell line H314 created a frameshift. This frameshift results in several termination codons after codon 119, thus presumably a truncated p16/MTS1 protein is produced. This observation shows that the spectrum of p16/MTS1 mutation in oral cancer is different to other tumour types and may be related to

carcinogens contained in the aetiological factors for oral cancer such as tobacco and alcohol. For example, most p16/MTS1 mutations in glioblastomas (Kyritsis et al, 1996), oesophageal (Mori et al, 1994), lung, leiomyosarcoma, chondrosarcoma, prostate and non-small cell lung cancer cell lines analysed are mis-sense mutations (Liu et al, 1995; Ruas and Peters, 1998). However, melanomas have also been shown to frequently contain non-sense mutations in the *p16/MTS1* gene (Liu et al, 1995). Of the OSCCs analysed by Zhang et al (1994) only a small percentage contained non-sense mutations in the *p16/MTS1* gene, though in other studies no non-sense mutations were found in the OSCCs analysed (Yoshida et al, 1995).

Western blot analysis detected pRB in 14/25 (56%) OSCCs, 6/8 (75%) premalignant samples as well as all 8 cell lines. Down-regulation of pRB expression occurred in about half of the OSCC primary tumours.

The function of pRB has shown to be normally regulated by phosphorylation (Chen et al, 1989). Hyperphosphorylation of pRB, which is controlled by cyclin D1-CDK4/6 renders pRB inactive. Cyclin-dependent kinase inhibitors such as p16/MTS1 are responsible for inhibiting pRB phosphorylation and thus induce pRB activity. To understand the role of pRB in the control of cell growth, it is important to examine not only the level but also the phosphorylation status of RB protein. We examined the status of RB phosphorylation in the panel of OSCCs studied and observed that in most samples both hyper- and hypophosphorylated forms of pRB were present. The samples which expressed both hypo- and hyper- or only hypophosphorylated pRB were classed as samples containing active pRB; the samples containing only hyperphosphorylated pRB were classed as having inactive pRB.

To understand the association between different components of the pRB pathway, we compared the status of p16, pRB and cyclin D1. In normal cells both p16 and cyclin D1 are expressed at low levels throughout the cell cycle. p16 is known to slightly peak at entry into S phase. Thus in non-malignant cells low or undetectable levels of cyclin D1 and p16 is expected. The absence or high steady state levels observed in some tumours therefore indicates the presence of abnormal regulatory mechanisms.

The results shown above suggested an inverse relationship (91%) between the presence of pRB and p16/MTS1 proteins (Table 2). Such correlation has previously been reported by Parry et al (1995) who have shown absence of pRB function to result in the accumulation of p16. However, our data suggest that the lack of p16/MTS1 expression is most likely due to its gene aberrations rather than due to inadequate regulation by pRB.

Cyclin D1 overexpression was found in 9/21 (43%) OSCC and in 3/9 (33%) premalignant samples. HN5 and HN6 have been previously reported not to bear amplification of the *cyclin D1* gene (Bartkova et al, 1995b). However, when compared to the positive control cell line MCF7, comparable expression of cyclin D1 was detected in HN5 and HN6. Thus, the expression of cyclin D1 in these cell lines was recorded as high. Surprisingly, 100% of the OSCC cell lines showed high levels of cyclin D1 expression. These results suggest that cyclin D1 overexpression is very common and possibly an early event in oral carcinogenesis. Other studies have shown moderate overexpression of cyclin D1 in up to 40% of OSCC cell lines (Timmermann et al, 1997). Also, amplification of the *cyclin D1* gene has been observed in 25% of oral dysplasias (Kyomoto et al, 1997). Overexpression of cyclin D1 in

our samples could be due to gene amplification. Although we have not examined cyclin D1 amplification, high frequency of *cyclin D1* gene amplification (between 30 and 50%) in head and neck cancers has been previously reported (Bartkova et al, 1995b).

When cyclin D1 expression was compared to pRB we found that 6/9 (67%) malignant tumours and 5/6 (83%) premalignant lesions had pRB expression but lacked cyclin D1. Whether such correlation bears any significance is not known.

The cyclin-dependent kinases 4 and 6 are dependent on cyclin D1 for their activity and regulate the activity of pRB by phosphorylation. Two recent reports have shown increased activity of CDK4 and/or 6 in OSCC cell lines compared to normal keratinocytes (Patel et al, 1997; Timmermann et al, 1997). It would have been desirable to include such an analysis in this study; however, these functional studies were not feasible with the small amounts of tissue available from our primary tumours.

In this study absence of cyclin D1 expression in 6/6 premalignant and 10/13 (77%) OSCC samples was generally accompanied by no p16/MTS1 expression. This suggests that both p16/MTS1 and cyclin D1 are tightly regulated in wild-type cells and that deregulation of one of the components of the G1 checkpoint leads to rapid accumulation of other abnormalities.

Infection with human papillomavirus can result in the down-regulation of pRB by virtue of its interaction with the E7 viral gene product (Li et al, 1993). Comparison of the presence of HPV 16 (Elamin et al, 1998) and pRB protein expression revealed that of the 15 samples which were HPV 16-positive, eight did not express pRB, two expressed only hyperphosphorylated pRB, four expressed only hypophosphorylated pRB and one OSCC sample expressed both hypo- and hyperphosphorylated pRB. The lack of functional pRB in 10/16 (63%) samples suggests that inactivation of pRB by E7 could be a major event in OSCC development.

In conclusion, we found deregulated expression of some of the components of the 'pRB pathway' to be consistent with the notion that this pathway is important for cell cycle control. Deregulation of pRB by aberrations in several cellular proteins such as p16/MTS1 and cyclin D1 and/or viral proteins such as E7 of HPV 16 can lead to the development of oral cancer.

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