# Association between Polymorphism in *p21*$^{Waf1/Cip1}$ Cyclin-dependent Kinase Inhibitor Gene and Human Oral Cancer[1]

**Ranju Ralhan,[2, 3] Sandhya Agarwal,[2] Meera Mathur, Bohdan Wasylyk, and Anurag Srivastava**

Departments of Biochemistry [S. A., R. R.], Pathology [M. M.], and Surgical Disciplines [A. S.], All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India, and Institut de Genetique et de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, ULP, 67404 Illkirch, France [B. W.]

## ABSTRACT

**The cyclin-dependent kinase inhibitor gene *p21*$^{Waf1/Cip1}$ plays a central role in inducing cellular growth arrest, terminal differentiation, and apoptosis. Alterations in this gene may adversely affect regulation of these processes and increase susceptibility for cancer. We have recently reported a novel polymorphism in the *p21*$^{Waf1/Cip1}$ gene in the Indian population and its association with esophageal cancer. An *A→G* transition at codon 149 resulted in amino acid substitution from aspartate to glycine in the proliferating cell nuclear antigen binding COOH-terminal domain of *p21*$^{Waf1/Cip1}$ that may affect PCNA-p21$^{Waf1/Cip1}$ interactions, thereby affecting regulation of cellular proliferation, and may increase susceptibility for development of cancer. In a parallel study in our laboratory, we searched for putative *p21*$^{Waf1/Cip1}$ mutations in oral premalignant and malignant lesions. No somatic mutation was detected in exon 2 of *p21*$^{Waf1/Cip1}$. Interestingly, a codon 149 polymorphism variant (A→G) was identified in 11 of 30 (37%) premalignant lesions (7 of 19 hyperplastic lesions and 4 of 11 dysplastic lesions) and 11 of 30 (37%) squamous cell carcinomas (SCCs). This codon 149 variant was also identified in paired lymphocytes of all of the patients with premalignant lesions and SCCs harboring the variant allele, suggesting the occurrence of a polymorphism. Lymphocyte DNA isolated from 50 unrelated age- and gender-matched healthy subjects was screened for this polymorphism. Seven of 50 (14%) normal controls harbored the A→G codon 149 variant allele. Immunohistochemical analysis of p21$^{Waf1/Cip1}$ protein expression showed immunoreactivity in 19 of these 30 (63%) oral premalignant lesions and 16 of 30 (53%) SCCs. The most intriguing features of the study were: (*a*) the significant increase in frequency of this polymorphism not only in patients with oral SCCs ($P = 0.038$), but also in patients with premalignant lesions ($P = 0.038$), compared with normal controls; and (*b*) the significantly higher frequency of p21$^{waf1/cip1}$ variants (codon 149) in oral premalignant lesions (10 of 11 cases) and SCCs (11 of 11 cases) with wild-type *p53* ($P = 0.045$) than in lesions with *p53* mutations, suggesting that this polymorphism affects the p53 pathway and may play a vital role in oral tumorigenesis. Furthermore, overexpression of p21 protein in oral lesions harboring missense mutations in the *p53* gene suggest a p53-independent role for p21 in the pathogenesis of oral cancer.**

## INTRODUCTION

Oral cancer is one of the 10 most common cancers in the world, and its rising incidence poses a formidable challenge to oncologists. SCC[4] of the oral cavity is a major cause of morbidity and mortality in India, causally associated with the commonly prevailing habit of chewing tobacco. Clinical, epidemiological, and laboratory studies confirm the etiological relationship between prolonged tobacco chewing and the high incidence of oral cancer in India (1). Early premalignant oral lesions, such as leukoplakia, appear as a white patch in the oral cavity of betel and tobacco consumers; five percent to 10% progress toward malignancy (1). Therefore, identification of a biomarker for screening the high-risk population for increased predisposition to cancer is of utmost importance for primary prevention and early intervention.

p21$^{Waf1/Cip1}$ is a universal inhibitor of cdks, which suggests a widespread role for p21 in regulating the cell cycle. In normal cells p21 exists predominantly in quaternary complexes with cyclins, cdks, and PCNA to inhibit the activity of cdks and control the $G_1$ to S phase transition (2). The *p21* gene has a p53 transcriptional regulatory motif, and the cells lacking functional p53 express very low levels of p21, suggesting that p53 regulates p21 expression directly (3). p21 expression induces differentiation of normal and transformed cells (4). The involvement of p21 in terminal differentiation has been observed in several cell systems (4, 5). Differential regulation of p21 by p53 and retinoblastoma has been reported in cellular response to oxidative stress (6). In addition, several recent studies suggest a role for p21 in apoptosis. Caspase-3-mediated cleavage of p21$^{Waf1Cip1}$ protein switches cancer cells from growth arrest to

Received 12/7/99; revised 3/8/00; accepted 3/8/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Indo French Centre for the Promotion of Advanced Research. S. A. was supported by Senior Research Fellowship from University Grant Commission, India.

[2] These authors contributed equally to this study.

[3] To whom requests for reprints should be addressed, at Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India. Phone: 91-11-6593478; Fax: 91-11-6862663; E-mail: rralhan@medinst.ernet.in or ralhanr@hotmail.com.

[4] The abbreviations used are: SCC, squamous cell carcinoma; ESCC, esophageal SCC; cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; Mab, monoclonal antibody; Wt, wild-type.

undergoing apoptosis (7). Quercetin-induced apoptosis in hepatocytes was also associated with the regulation of p21 protein expression in a p53-independent pathway (8).

The human *p21* gene consists of three exons of 68, 450, and 1600 bp. The first ATG codon appears at nucleotide 76 in exon 2, and a termination codon appears at nucleotide 570 in exon 3 (9). The cdk and PCNA inhibitory activities of p21 have been mapped to different domains of the protein. The amino terminus of p21 binds cdk and cyclins and plays an important role in suppression of tumor cell growth, whereas the COOH terminus of p21 contains the PCNA binding and inhibitor activity (10). In view of the central role of p21Waf1/Cip1 in inducing growth arrest, terminal differentiation, or apoptosis, alterations in the *p21\Waf1/Cip1* gene and its expression may play a vital role in the pathogenesis of cancer. Alterations in *p21\Waf1/Cip1* expression have been observed in a wide variety of human cancers, including ovarian, uterine, cervix, colorectal, hepatocellular, and head and neck carcinomas (11–13).

Currently, knowledge of the biological and clinical relevance of p21 alterations in oral lesions is meager (14–15). We have previously reported differential expression of $p21^{Waf1/Cip1}$ protein in tobacco-related oral tumorigenesis in the Indian population (16). Recently, we reported a novel polymorphism in the $p21^{Waf1/Cip1}$ gene in the Indian population and its association with human esophageal cancer (17). In a parallel study, we sought to examine whether mutational inactivation of the *p21* gene and/or altered expression of p21 mRNA or protein may occur in oral lesions and whether such alterations may occur in cells containing Wt p53, thus abrogating normal p53 function. The goal of the study was to understand the mechanism of deregulation of $p21^{Waf1/Cip1}$ expression in oral tumorigenesis.

## MATERIALS AND METHODS

**Patients and Controls.** Thirty oral SCC cases, 30 leukoplakia patients with histological evidence of hyperplasia (19 cases) or dysplasia (11cases), enrolled in the Out Patients clinic (Department of Surgical Disciplines, All India Institute of Medical Sciences, New Delhi, India) and 50 age- and gender-matched healthy controls were inducted into the study with prior consent. The patients and controls were interviewed. A prestructured questionnaire was used to collect information on various demographic, socioeconomic, occupational, and lifestyle variables, including the consumption of alcohol, betel quid, and tobacco (chewing and/or smoking). These parameters were taken into account while selecting the patients and healthy controls with overlapping habitual patterns to minimize the contribution of these exogenous risk factors as far as feasible. The gender, age, habitual patterns, and vital status distribution of the control group (healthy individuals) overlapped adequately with the patient group.

**Tissue Specimens.** Surgical tissue specimens from patients with oral SCCs (30 cases) and premalignant lesions (leukoplakia, 30 cases) were used in this study. Histologically, the premalignant lesions were characterized by epithelial hyperplasia (19 cases) or dysplasia (11 cases). The distribution of patients according to the site of leukoplakia (with hyperplasia) included buccal mucosa (nine cases), tongue (nine cases), and lip (one case), and those with dysplasia included buccal mucosa (nine cases) and tongue (two cases) The site distribution of oral tumors included buccal mucosa (18 cases), tongue (7 cases), palate (2 cases), alveolus (2 cases), and lip (1 case). The patients were grouped based on tumor stage following the tumor, node, metastasis classification of Union International Contre Cancer (16). The oral SCC cases included $T_1$ (1 case), $T_2$ (5 cases), $T_3$ (7 cases), and $T_4$ (17 cases); nodal metastasis [pNo (19 cases) and $pN_1$ (19 cases)]; and distal organ metastasis [$M_0$ (25 cases) and $pM_1$ (5 cases)]. For immunohistochemical analysis, one piece of tissue was snap-frozen and stored at −80°C until use, whereas another was put in formalin for histopathological examination. Blood samples (10 ml) were collected from normal control subjects and patients with oral premalignant lesions and malignant lesions. Peripheral blood mononuclear cells isolated using Lymphoprep were used for DNA extraction as described (16).

**Mabs.** p21 is a mouse monoclonal IgG1 antibody produced by immunization with full-length p21 of human origin obtained from Santa Cruz Biotechnology (Santa Cruz, CA). p53 Mab DO1 is a mouse monoclonal $IgG_{2a}$ antibody, specific for the epitope corresponding to amino acids 11–25 of p53 of human origin. It recognizes both Wt and mutant p53 proteins. DO-1 was obtained from Santa Cruz Biotechnology.

**Immunohistochemistry.** Cryosections of oral tissue specimens (5-μm thickness) were fixed in acetone. Representative sections were stained with H&E for histological grading, and immunostaining was done in serial sections as described by us previously (16). For immunohistochemical analysis, endogenous peroxidase activity was blocked by immersing the sections in methanol containing 0.3% (v/v) hydrogen peroxide for 20 min. Nonspecific binding was blocked by incubation with 0.1% (w/v) BSA in PBS for 1 h. The sections were then incubated with the primary antibody overnight at 4°C. Mouse Mabs (1 μg/ml) p21 and DO-1 (Santa Cruz Biotechnology) were used for detecting the p21 and p53 proteins, respectively. The primary antibody was detected using biotinylated secondary antimouse IgG antibody, avidin-biotin complex, and 3,3′-diamino-benzidine tetrachloride as the chromogen. All incubations were performed at room temperature in a moist chamber. Slides were washed several times with PBS after each step. In negative controls, the primary antibody was replaced by PBS or nonimmune mouse IgG of the same isotype to ensure specificity. Human ESCC tissue sections known to overexpress $p21^{Waf1/Cip1}$ protein were used as the positive control in each batch of sections analyzed (data not shown). The intensity of immunohistochemical staining was evaluated in five areas of the slide sections for correlation and confirmation of the tissue analysis. For $p21^{Waf1/Cip1}$ and p53 protein expression, specific staining in the nucleus was defined as positive staining (16). The p21- and p53-positive cases were evaluated semiquantitatively on a 4-point scale based on the percentage of cells showing p21 or p53 staining: −, <10%; +1, 10–30%; +2, 30–50%; and +3, >50% positively stained cells. The grading of H&E-stained sections as well as the immunostained sections of hyperplastic lesions, dysplastic lesions, and SCCs was carried out by three of us independently.

*Table 1* *p21* polymorphism in normal subjects and patients with oral premalignant and malignant lesions

| *p21* polymorphism codon 149 | Positive | Negative | *P* | Odds ratio | 95% confidence intervals |
|---|---|---|---|---|---|
| Normal ($n = 50$) | 7 | 43 | | | |
| Premalignant lesions ($n = 30$) | 11 | 19 | 0.038 | 3.56 | 1.06–12.23 |
| Hyperplasia ($n = 19$) | 7 | 12 | 0.0002[a] | 10.5 | 2.67–44.25 |
| Dysplasia ($n = 11$) | 4 | 7 | 0.09[b] | | |
| SCC ($n = 30$) | 11 | 19 | 0.038 | 3.56 | 1.06–12.23 |
| Premalignant lesions and SCC ($n = 60$) | 22 | 38 | 0.013 | 3.56 | 1.26–10.39 |

[a] $\chi^2$ test.
[b] Fisher's exact test.

**Genomic DNA Extraction.** Genomic DNA was extracted from peripheral blood mononuclear cells and tissues from premalignant and malignant lesions by suspending and incubating in lysis buffer [10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, and 0.5% Tween] overnight at 37°C in the presence of 10 mg/ml proteinase K. Genomic DNA was purified using standard phenol/chloroform extraction method. Purified genomic DNA samples were used as template in the PCR for amplification of DNA sequences (16).

**PCR and Sequencing.** The *p21* exon 2 containing 90% of the coding sequences was amplified and sequenced. Primers (oligodeoxynucleotide amplimers), complimentary to adjacent target sequences were synthesized (Rama Biotechnology, Inc., New Delhi, India) as described (17).

Primer 1 (exon 2-AFwd): 5′-GCGCCATGTCAGAACCGGC-3′

Primer 2 (exon 2-A.Rev): 5′-GAGAATCCTGGTCCCTTAC-3′

The PCR reaction mixture consisted of 10 μl of 10× PCR buffer, 20 pmol of each primer, 1.875 mM deoxynucleotide triphosphates, 1.5 units of Taq DNA polymerase (Perkin-Elmer Corp.), and 200 ng of genomic DNA in a final volume of 100 μl. Samples were denatured by amplified preheating at 94°C for 4 min and amplified for 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and primer extension at 72°C for 30 s, followed by autoextension at 72°C for 5 min.

The *p53* gene exons 5–9, shown to have high incidence of mutations, were the target sequences amplified. Samples were amplified for 35 cycles of 94°C for 1 min, 60°C for 1 min, and 78°C for 30 s, with initial preheating at 94°C for 4 min and a final extension of the cycle at 78°C for 5 min. Primers (oligonucleotides amplimers) complimentary to adjacent target sequences were used (Rama Biotechnology Inc.):

Exon 5 Fwd: 5′′-GTTTCTTTGCTGCCGTGTTC-3′′
Rev: 5′′-AGGCCTGGGGACCCTGGGCA-3′′
Exon 6 Fwd: 5′′-TGGTTGCCCAGGGTCCCCAG-3′′
Rev: 5′′-GGAGGGCCACTGACAACCA-3′′
Exon 7 Fwd: 5′′-ACCATCCTGGCTAACGGTGA-3′′
Rev: 5′′-AGGGGTCAGCGGCAAGCAGA-3′′
Exon 8 and 9 Fwd: 5′′-TTGGGAGTAGATGGAGCCT-3′′
Rev: 5′′-AGTGTTAGACTGGAAACTTT-3′′

Samples were amplified using 200 ng of genomic DNA, 40 pmol of each primer, 1.875 mM deoxynucleotide triphosphates, 10 μl of 10× PCR buffer, and 2.5 units of Taq DNA polymerase in a total volume of 100 μl. The PCR products were purified on low-melting agarose gels. The purified PCR products were sequenced directly using automated DNA sequencer (Applied Biosystems 373 Sequencer and ABI Prism terminators). All of the sequencing data were obtained by sequencing with both the forward and reverse primers. The sequencing data obtained was confirmed twice by performing a complete repeat of the experimental procedure: amplification of stock genomic DNA, fragment purification, and sequencing of both the DNA strands. Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information (NCBI) web site.[5]

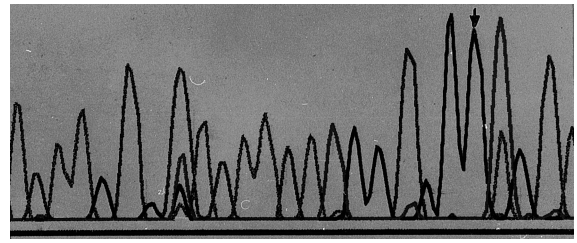

*Fig. 1* PCR amplification and DNA sequence analysis of exon 2 of the *p21* gene in oral cancer. Automated DNA sequence scan of the *p21* gene showing G***A***T→G***G***T polymorphism in codon 149, exon 2. The position of base change is indicated by an *arrow*.

**Statistical Analysis.** The correlation between *p21$^{Waf/Cip1}$* polymorphism, p21 or p53 protein expression, and clinicopathological parameters of oral cancer patients was statistically evaluated using $\chi^2$ test or Fisher's exact test.

## RESULTS

**Genetic Analysis of p21 in Oral Normal, Premalignant, and Malignant Lesions.** Genetic analysis of *p21* was carried out in DNA isolated from lymphocytes and oral tissue specimens of 30 patients with premalignant lesions and 30 patients with malignant oral lesions, as well as lymphocytes of 50 normal individuals, by PCR, followed by direct sequencing (Table 1 and Fig. 1). Because 90% of the coding region is present in exon 2 of the *p21* gene, exon 2 was analyzed in all of the cases. Of the 30 patients with oral premalignant lesions examined, 11 cases (37%; 7 of 19 hyperplastic lesions and 4 of 11 dysplastic lesions) showed A→G base substitution in codon 149, leading to replacement of aspartic acid with glycine in oral

[5] http:/www.ncbi.nlm.nih.gov.

*Table 2* Analysis of *p21* and *p53* gene and protein status in human oral premalignant lesions

| Case no. | Gender/age (yr) | *p21* gene | p21 expression[a] | *p53* gene | *p53* expression[a] |
|---|---|---|---|---|---|
| Hyperplastic lesions | | | | | |
| 1 | F/45 | Polymorphic | +3 | Wt[b] | +2 |
| 2 | M/70 | Wt | +1 | Wt | +1 |
| 3 | M/36 | Wt | — | Wt | +1 |
| 4 | M/27 | Wt | +1 | Wt | +1 |
| 5 | F/32 | Wt | — | Wt | +1 |
| 6 | F/57 | Wt | — | Wt | — |
| 7 | M/26 | Wt | +3 | MS | +3 |
| 8 | M/30 | Polymorphic | +2 | Wt | — |
| 9 | M/34 | Wt | — | MS | +1 |
| 10 | M/65 | Wt | — | Wt | +1 |
| 11 | F/57 | Polymorphic | — | Wt | — |
| 12 | M/55 | Wt | — | Wt | +1 |
| 13 | F/36 | Wt | — | Wt | +2 |
| 14 | M/35 | Polymorphic | +1 | Wt | +2 |
| 15 | F/68 | Polymorphic | +3 | Wt | +1 |
| 16 | M/18 | Wt | +2 | Wt | — |
| 17 | M/40 | Polymorphic | +1 | Wt | +3 |
| 18 | F/68 | Wt | +3 | NS | +1 |
| 19 | M/42 | Polymorphic | +3 | Wt | — |
| Dysplastic lesions | | | | | |
| 1 | M/49 | Wt | +1 | Wt | — |
| 2 | M/32 | Polymorphic | — | NS | — |
| 3 | F/65 | Wt | +1 | Wt | +1 |
| 4 | M/45 | Wt | +2 | Wt | +2 |
| 5 | M/55 | Wt | +3 | Wt | — |
| 6 | M/29 | Wt | — | Wt | — |
| 7 | M/60 | Wt | +2 | Wt | +2 |
| 8 | F/55 | Polymorphic | +1 | Wt | +2 |
| 9 | M/20 | Polymorphic | +2 | Wt | +3 |
| 10 | M/50 | Polymorphic | +3 | Wt | — |
| 11 | M/42 | Wt | — | MS | +2 |

[a] p21 and p53 immunostaining: —, >10%; +1, 10–30%; +2, 30–50%; +3, <50% of the cells positive.
[b] Wt, wild type; NS, nonsense; MS, missense.

lesions (Table 2). The codon149 A→G base substitution was also detected in the lymphoctye DNA isolated from all of the patients with oral premalignant lesions (11 cases) harboring this base substitution. Of the 30 oral SCC cases examined, 11 (37%) cases showed A→G base substitution in codon 149 in DNA isolated from the tumors as well as matched lymphocytes of the patients, suggesting the occurrence of a polymorphism in codon149 (Table 3). To determine the occurrence of this polymorphism in normal healthy controls, lymphocyte DNA was analyzed from 50 age- and gender-matched individuals. The A→G base substitution in codon 149 was also observed in lymphocytes from 7 of 50 (14%) normal individuals (Table 1).

The frequency of this polymorphism was significantly higher in patients with premalignant lesions compared with normal individuals ($P < 0.038$, with an odds ratio of 3.56 and a 95% confidence interval of 1.06–12.23). The oral cancer patients also showed significantly higher occurrence of the p21 codon 149 variant compared with normal control population ($P = 0.038$, with an odds ratio of 3.56 and a 95% confidence interval of 1.26–10.39). Furthermore, when pooled, the total number of patients with premalignant and malignant lesions harboring p21 polymorphism was 22 of 60 (37%) and was significantly higher compared with the normal control population ($P = 0.013$, with an odds ratio of 3.56 and a 95% confidence interval of 1.26–10.39; Table 1). There was no apparent correlation between the presence of codon 149 polymorphism and the subjects' age, gender, site of lesion, tumor grade and tumor, node, metastasis stage in this cohort.

To determine the biological significance of codon 149 polymorphism in oral tumorigenesis, we investigated its correlation with *p53* gene status. The *p53* mutational spectrum had been determined by PCR-single-strand conformational polymorphism and direct DNA sequencing of exons 5–9 of the *p53* gene[6] Interestingly, 10 of 11 oral premalignant lesions and 11 of 11 SCCs showing the codon 149 polymorphic variant harbored Wt *p53* (Tables 2 and 3), reflecting a significant association between p21 codon 149 variant in oral SCCs and Wt p53 in SCCs ($P = 0.045$). However, the inverse correlation between *p21* polymorphic frequency and *p53* mutation just failed to reach statistical significance due to the small sample size of *p53* mutations in premalignant lesions in this cohort. It is worthwhile to note that accumulation of p53 protein was observed in 6 of these 11 premalignant lesions and 7 of these 11 SCCs harboring wild type p53.

[6] Ranju Ralhan and Sandhya Agarwal, unpublished observations.

*Table 3* Analysis of *p21* and *p53* gene and protein status in oral SCCs

| Case no. | Gender/age (yr) | *p21* gene | p21 expression[a] | *p53* gene | p53 expression[a] |
|---|---|---|---|---|---|
| 1 | M[b]/60 | Polymorphic | — | Wt | — |
| 2 | M/24 | Wt | — | Wt | +2 |
| 3 | F/52 | Polymorphic | +3 | Wt | +2 |
| 4 | M/45 | Wt | +1 | Wt | — |
| 5 | M/50 | Wt | +2 | Wt | +2 |
| 6 | M/50 | Wt | — | Wt | +2 |
| 7 | M/48 | Polymorphic | +1 | Wt | — |
| 8 | F/56 | Wt | +1 | MS | +2 |
| 9 | M/40 | Wt | — | Wt | — |
| 10 | M/66 | Wt | +1 | Wt | +2 |
| 11 | M/42 | Wt | +1 | MS | +2 |
| 12 | M/52 | Wt | +1 | Wt | +2 |
| 13 | F/49 | Polymorphic | — | Wt | — |
| 14 | M/65 | Wt | — | NS | — |
| 15 | F/52 | Wt | — | MS | +2 |
| 16 | F/59 | Wt | — | Wt | +3 |
| 17 | M/58 | Polymorphic | — | Wt | +3 |
| 18 | M/40 | Wt | — | Wt | +1 |
| 19 | M/60 | Wt | +1 | Wt | +2 |
| 20 | M/52 | Wt | +1 | Wt | — |
| 21 | M/54 | Polymorphic | — | Wt | +2 |
| 22 | M/52 | Wt | +3 | MS | +2 |
| 23 | M/56 | Polymorphic | +1 | Wt | +3 |
| 24 | M/60 | Polymorphic | +3 | Wt | — |
| 25 | M/82 | Wt | +3 | MS | — |
| 26 | F/53 | Polymorphic | +1 | Wt | +2 |
| 27 | M/45 | Polymorphic | — | Wt | +1 |
| 28 | M/36 | Polymorphic | — | Wt | +1 |
| 29 | M/50 | Wt | +2 | MS | +2 |
| 30 | M/70 | Wt | — | Wt | +3 |

[a] p21 and p53 immunostaining: —, >10%; +1, 10–30%; +2, 30–50%; +3, <50% of the cells positive.
[b] M, male; F, female; Wt, wild type; MS, missense.

**Analysis of p21 Protein Expression in Oral Premalignant and Malignant Lesions.** To investigate the clinical relevance of codon 149 variant allele, we determined its relationship with p21$^{Waf1/Cip1}$ protein expression in the same set of oral lesions. The results of the immunohistochemical analysis of p21 protein in different stages of oral tumorigenesis [*i.e.*, premalignant (30 cases) and malignant (30 cases) lesions] are summarized in Tables 2 and 3. Normal oral tissue sections did not show detectable p21 immunoreactivity (Fig. 2*a*). Histopathological examination of clinically identified premalignant (leukoplakic) lesions showed either hyperplastic (19 cases) or dysplastic epithelium (11 cases). Nineteen of 30 (63%) premalignant lesions (11 of 19 hyperplastic lesions and 8 of 11 dysplastic lesions) showed detectable expression of p21$^{Waf1/Cip1}$ protein (Fig. 2*b*), and 16 of 30 (53%) oral SCCs showed positive staining. The well-differentiated tumors showed intense nuclear staining (Fig. 2*c*). However, no significant association was observed between p21 codon 149 variant and p21 protein expression in oral premalignant lesions as well as SCCs in this cohort ($P > 0.05$). The frequent accumulation of p21 protein in SCCs may be due to increased transcription or translation. Hence, *p21* mRNA transcripts were analyzed. *In situ* hybridization analysis of p21 mRNA did not show an increased level of transcripts in 9 of 11 oral premalignant lesions and 10 of 11 SCCs harboring the codon 149 variant (data not shown). No significant association was observed between p53 and p21 protein expression in oral premalignant lesions as well as SCCs ($P > 0.05$). However, the $p53^{+}/p21^{-}$ phenotype showed significant association with p21 codon 149 variant in oral premalignant lesions ($P = 0.025$). The data are in support of our previous findings that induction of p21 may occur by both p53-dependent and p53-independent pathways during oral tumorigenesis (16).

## DISCUSSION

p21 has a central role in arresting the cell cycle; hence, it is possible that alterations in *p21* itself may be responsible for progression in tumors harboring Wt p53. Herein, whereas no somatic mutations were detected in the *p21* gene, one polymorphism was identified at codon 149. Eleven of 30 oral premalignant lesions (7 of 19 hyperplasias and 4 of 11 dysplasias) and 11 of 30 SCCs harbored an alteration at codon 149, involving a single nucleotide substitution, G*A*T→G*G*T, changing the predicted amino acid from aspartate to glycine. The consistent nature of the alterations observed in only a single codon in paired lymphocytes and oral lesions was suggestive of a polymorphism. It is of interest to note that in a parallel study carried out in our laboratory, this polymorphism in codon 149 was also observed in 42 of 50 (84%) esophageal cancer patients (17). In addition, the codon 149 variant was also observed in lymphocytes of 7 of 50 (14%) normal individuals in the Indian population. The significantly higher frequency of the codon 149 polymorphic variant in oral premalignant lesions (37%) and SCCs (37%) compared with normal individuals (14%; $P =$

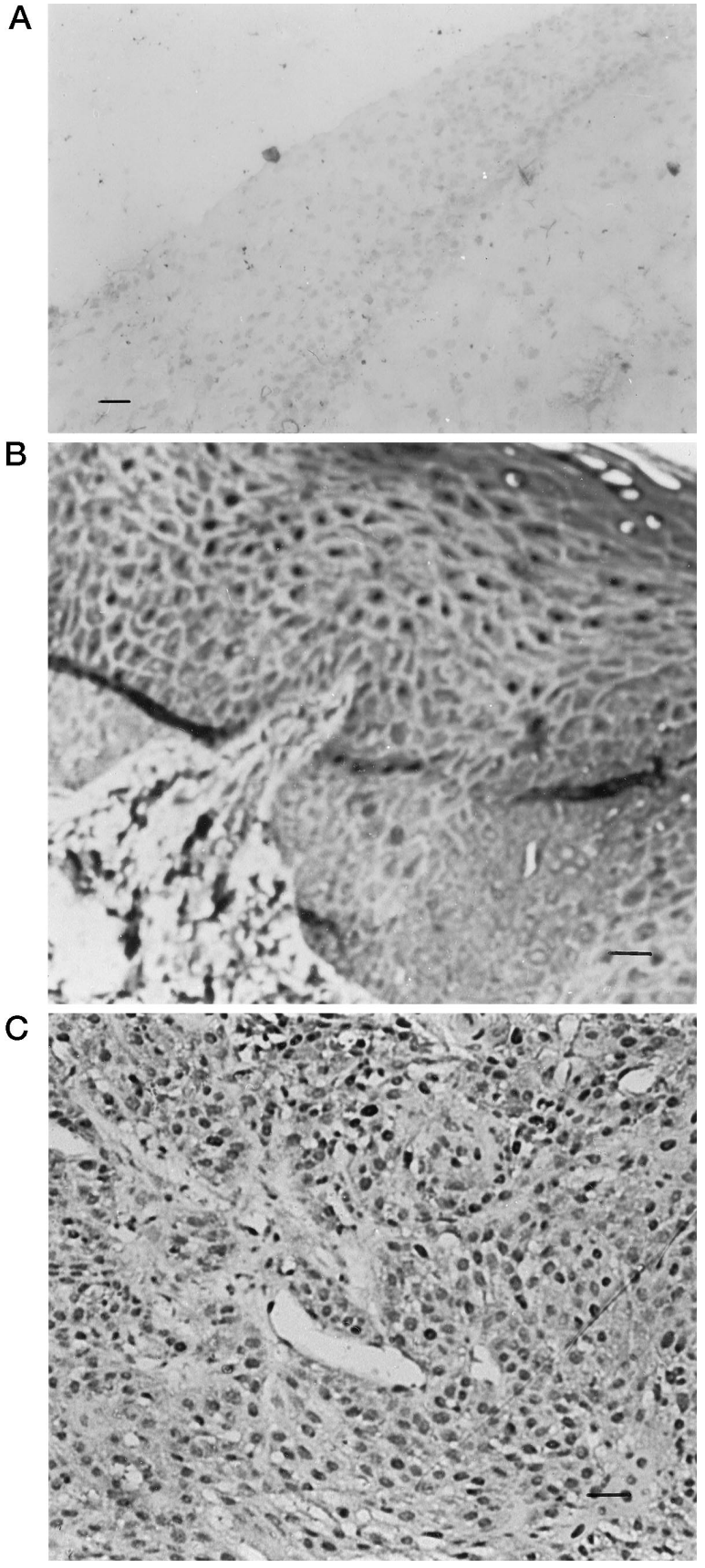


*Fig. 2* Immunohistochemical detection of p21 protein in oral lesions using Mab p21. *a*, normal epithelium showing no staining for p21 protein. *b*, dysplasia showing nuclear staining for p21 mostly in the superficial epithelium with more differentiated cells. *c*, well-differentiated SCC showing intense nuclear staining for p21 protein. *Bar*, ×200 m.

0.038) suggests a plausible association with increased susceptibility for cancer. Furthermore, when the premalignant and malignant lesions were pooled, the polymorphic allelic frequency were significantly increased compared with the normal control population ($P = 0.013$, with an odds ratio of 3.56 and a 95% confidence interval of 1.26–10.39). Interestingly, an inverse correlation was observed between p21 polymorphic frequency and p53 mutations in oral cancer patients ($P = 0.045$). The occurrence of this polymorphism in patients harboring Wt p53 in oral premalignant, as well as malignant, lesions suggests that the function of the variant $p21^{Waf1/Cip1}$ is altered in such a way as to obviate the requirement for *p53* mutations to deregulate the cell cycle. These data support our earlier observations in ESCCs harboring the codon 149 variant (17).

PCNA has been shown to be the prime target of $p21^{Waf1/Cip1}$-mediated growth inhibition induced by $G_1$ and $G_2$-M cell cycle blocks in p53-deficient DLD1 human colon cancer cells, and alterations in the $p21^{Waf1/Cip1}$ COOH-terminal domain, especially at codons 147–151, have been proposed to affect the cell cycle regulation by $p21^{Waf1/Cip1}$ (18). We proposed that the Asp→Gly substitution in codon 149 variant may change the net charge of this domain and inhibit $p21^{Waf1/Cip1}$/PCNA interactions (17). The putative implications of codon 149 polymorphic variant on PCNA/p21 interactions and cell cycle regulation have been discussed (17).

A number of tumor cell lines and tumors of different origin revealed polymorphism in the *p21* gene (19–21). In an ELISA-based pepscan assay, peptides spanning amino acids 1–20, 141–160, and 144–164 of human p21 were shown to interact with Gadd45 (22). The Gadd45 interacts with PCNA and can compete with p21 for binding to PCNA (23). The codon 149 resides in the COOH-terminal domain (residues 144–151) of the *p21* gene, which is involved in binding to PCNA (10). Chedid *et al.* (19) demonstrated polymorphism at codon 31 wherein serine is replaced by arginine, both Ser 31 allele and the Arg 31 allele showed almost equal abilities to inhibit colony formation, suggesting that both forms of p21 had comparable tumor-suppressing ability, indicating lack of association between this *p21* polymorphism and tumor formation. Interestingly, the codon 31 polymorphism was not observed in any of the cases analyzed in our study and in only 2 of 50 (4%) ESCCs (17), suggesting that codon 31 variant occurs at a relatively lower frequency in the Indian population than that reported in the West. Moreover, our study corroborates the previous reports, which suggest that the coding region of the *p21* gene is not frequently mutated in cancer (14, 20, 24).

We found premalignant lesions (Table 2, case 7) and SCCs (Table 3, cases 8, 11, 22, and 29) with mutant p53 protein overexpressing p21. p21 immunopositive well-differentiated tumors with *p53* missense mutations probably harbor a p21-dependent differentiation pathway activated through a p53-independent mechanism. Sato *et al.* (25) showed induction of $p21^{waf1/cip1}$ expression and its association with inhibition of cell growth and induction of differentiation in human salivary adenosquamous carcinoma-forming cell line harboring a mutant *p53* gene at codon 280 Asp→His. Taken together, these effects reflect the complexity of the p53/p21 pathways of cell cycle regulation and differentiation in the pathogenesis of oral cancer.

In conclusion, we report: (*a*) a significantly higher frequency of polymorphism at codon 149 in the *p21* gene in oral premalignant and malignant lesions compared with normal controls; and (*b*) an inverse correlation between p21 polymorphic variant and p53 mutation in oral tumorigenesis.

## ACKNOWLEDGMENTS

We thank the Institut de Genetique et de Biologe Moleculaire et Cellulaire core and oligonucleotide synthesis and DNA sequencing facilities for tremendous help and support; the members of Boh Wasylyk's laboratory for help and constructive discussion; Pascal Dolle and his group, Brigitte and Valerie, for helping with *in situ* hybridization studies; and the members of Centre Franco-Indien pour la Promotion de la Recherche Avancee for scientific support.

## REFERENCES

1. Daftary, D. K., Murti, P. R., Bhonsle, R. R., Gupta, P. C., Mehra, F. S., and Pindborg, J. J. Risk factors and risk markers for oral cancer in high risk areas of the world. *In:* N. W. Johnson (ed.), Oral Cancer: The Detection of Patients and Lesions at Risk, pp. 29–63. Cambridge, United Kingdom: Cambridge University Press, 1991.

2. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinases. Nature (Lond.), *366:* 701–704, 1993.

3. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Veleuliesous, V. E., Canman, C. E., Jackman, J., and Pietenpol J. A. WAF1/CIP1 is induced in p53-mediated $G_1$ arrest and apoptosis. Cancer Res., *54:* 1169–1174, 1994.

4. Jiang, H., Lin, J., Su, Z., Collart, F. R., Huberman, E., and Fisher, P. B. Induction of differentiation in human promyelocytic leukemia HL-60 cell activates $p21^{WAF1/CIP1}$ expression in the absence of *p53*. Oncogene, *9:* 3397–3406, 1994.

5. Michieli, P., Chedid, M., Lin, D., Pierce, J. H., Mercer, W. E., and Givol, D. Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res., *54:* 3391–3395, 1994.

6. Yin, Y., Solomon, G., Deng, C., and Barrett, J. C. Differential regulation of p21 by p53 and Rb in cellular response to oxidative stress. Mol. Carcinog., *24:* 15–24, 1999.

7. Zhang, Y., Fujita, N., and Tsuruo, T. Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. Oncogene, *18:* 1131–1138, 1999.

8. Iwao, K., and Tsukamoto, I. Quercetin inhibited DNA synthesis and induced apoptosis associated with increase in c-fos mRNA level and the up-regulation of p21WAF1CIP1 mRNA and protein expression during liver regeneration after partial hepatectomy. Biochim. Biophys. Acta, *1427:* 112–120, 1999.

9. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 cdk-interacting protein CIP1 is a potent inhibitor of $G_1$ cyclin-dependent kinases. Cell, *75:* 805–816, 1993.

10. Chen, J., Jackson, P. K., Kirchner, M. W., and Dutta, A. Separate domains of p21 involved in the inhibition of cdk kinase and PCNA. Nature (Lond.), *374:* 386–388, 1995.

11. El-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescu, V. E., Burrell, M., Hill, D. E., Healy, E., Rees, J. L., Hamilton, S. R., Kinzler, K. W., and Vogelstein, B. Topological control of p21 $^{WAF1/CIP1}$ expression in normal and neoplastic tissues. Cancer Res., *55:* 2910–2919, 1995.

12. Elbendary, A. A., Cirisano, F. D., Evans, A. C., Davis, P. L., Iglehart, J. D., Marks, J. R., and Berchuck, A. Relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. Clin. Cancer Res., *2:* 1571–1575, 1996.

13. Erber, R., Klein, W., Andl, T., Enders, C., Born, A. I., Conradt, C., and Bartek, J. Aberrant $p21^{waf1/cip1}$ protein accumulation in head and neck cancer. Int. J. Cancer Pred. Oncol., *74:* 383–389, 1997.

14. Heinzel, P. A., Balaram, P., and Bernard, H. O. Mutations and polymorphisms in the *p53*, *p21* and *p16* genes in oral carcinomas of India betel quid chewers. Int. J. Cancer, *68:* 420–423, 1996.

15. Facher, E. A., Becich, M. J., Deka, A., and Law, J. C. Association between human cancer and two polymorphisms occurring together in *p21*$^{waf1/cip1}$ cyclin-dependent kinase inhibitor gene. Cancer (Phila.), *79:* 2424–2429, 1997.

16. Agarwal, S., Mathur, M., Shukla, N. K., and Ralhan, R. Expression of cyclin dependent kinase inhibitor p21$^{waf1/cip1}$ in premalignant and malignant oral lesions: relationship with p53 status. Eur. J. Cancer B Oral Oncol., *34:* 353–360, 1998.

17. Bahl, R., Arora, S., Nath, N., Mathur, M., Shukla, N. K., and Ralhan, R. Novel polymorphism in *p21*$^{Waf1/Cip1}$ cyclin dependent kinase inhibitor gene: association with human esophageal cancer. Oncogene, *19:* 323–328, 2000.

18. Cayrol, C., Knibiehler, M., and Ducommun, B. p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. Oncogene, *16:* 311–320, 1998.

19. Chedid, M., Michieli, P., Lengel, C., Huppi, K., and Givol, D. A single nucleotide substitution at codon 31 (Ser/Arg) defines a polymorphism in a highly conserved region of the p53-induicble gene *waf1/cip1*. Oncogene, *9:* 3021–3024, 1994.

20. Shiohara, M., El-Deiry, W. S., Wada, M., Nakamaki, T., Takeuchi, S., Yang, R., Chen, D. L., Vogelstein, B., and Koeffler, H. P. Absence of waf1 mutations in a variety of human malignancies. Blood, *84:* 3781–3784, 1994.

21. Law, J. C., and Deka, A. Identification of a Pst1 polymorphism in the *p21*$^{waf1\diagdown cip1}$ cyclin-dependent kinase inhibitor gene. Hum. Genet., *95:* 459–460, 1995.

22. Kearsey, J. M., Coates, P. J., Prescott, A. R., Warbrick, E., and Hall, P. A. Gadd45 is a nuclear cell cycle regulated protein which interacts with p21$^{cip1}$. Oncogene, *11:* 1675–1681, 1995.

23. Canman, C. E., Gilmer, T. M., Coutts, S. B., and Kastan, M. B. Growth factor modulation of p53-mediated growth arrest *versus* apoptosis. Genes Dev., *9:* 600–611, 1995.

24. Shimizu, T., Miwa, W., Nakamori, S., Ishikawa, O., and Konishi, Y. Absence of a mutation of the *p21*$^{waf1}$ gene in human lung and pancreatic cancers. Jpn. J. Cancer Res., *87:* 275–278, 1996.

25. Sato, M., Kawamata, H., Harada, K., Nakashiro, K., Ikeda, Y., Gohda, H., Yoshida, H., Nishida, T., Ono, K., Kinoshita, M., and Adachi, M. Induction of cyclin-dependent kinase inhibitor, p21 WAF1, by treatment with 3,4-dihydro-6[4-(3,4)-dimethoxybenzoyl)-1-piperazinyl]-2(1H)-quinoline (vesnarinone) in a human salivary cancer cell line with mutant p53 gene. Cancer Lett., *112:* 181–189, 1997.