Loss of Tumour Suppressor p16 Expression in Initial Stages of Oral Oncogenesis

ELEFTHERIOS VAIRAKTARIS[1], CHRISTOS YAPIJAKIS[1,2], AMANDA PSYRRI[1], SOFIA SPYRIDONIDOU[1], ATHANASIOS YANNOPOULOS[3], ANDREAS LAZARIS[4], STAVROS VASSILIOU[1], ELEFTHERIOS FEREKIDIS[5], ANTONIS VYLLIOTIS[1], EMEKA NKENKE[6] and EFSTRATIOS PATSOURIS[4]

[1]Department of Oral and Maxillofacial Surgery, University of Athens Medical School, Vas. Sofias 93 and Dim. Soutsou 1, Athens 11521;
[2]Department of Neurology, University of Athens Medical School, Eginition Hospital, Vas. Sofias 74, Athens 11528, Greece;
Departments of [3]First Surgery, [4]Pathology and [5]First Otolaryngology, University of Athens Medical School, Mikras Asias 75, Athens 11527, Greece;
[6]Department of Maxillofacial Surgery, Universität, Erlangen, Klinik und Poliklinik fur Mund-, Kiefer-, Gesischtschirurgie, Glueckstrasse 11, Erlangen D-91054, Nurnberg, Germany

Abstract. *Background: The p16 tumour suppressor gene is known to be involved in regulation of the cell cycle. p16 expression in sequential histological stages of oral squamous cell carcinoma (OSCC) formation was investigated using an experimental model of induced oral carcinogenesis in Syrian golden hamsters. Materials and Methods: Thirty-seven animals were divided into one control group (N=7) and three experimental groups (N=10 each) which were treated with a carcinogen and sacrificed at 10, 14 and 19 weeks after treatment. Tumour sections were studied immunohistochemically using monoclonal antibodies against p16 protein. Results: p16 was found significantly increased in hyperplasia, sharply decreased in dysplasia and in the subsequent stages of oral carcinogenesis. Conclusion: Inactivation of p16 occurs at the early stage of oral mucosal dysplasia in the multistep process of oral tumourigenesis. Therefore, p16 may be considered as a useful prognostic marker for the progression of oral cancer.*

Oral squamous cell carcinoma (OSCC) consistently ranks as one of the top seven cancers worldwide (1-3). Its incidence has recently increased to an extent that cannot be fully explained by increased exposure to known risk factors, such as tobacco and alcohol (1-3). Regardless of the accelerating factors, neoplasms arise clonally from transformed cells that have undergone specific genetic and epigenetic alterations in oncogenes or tumour suppressor genes (4).

During the process of carcinogenesis, the dysregulation of the cell cycle is a critical event. Tumour suppressor protein p16, a negative regulator of cell proliferation, is the founder member of a family of proteins with the ability to bind to CDK4 and CDK6 and inhibit the kinase activity of the CDK4-6/cycD complexes (5, 6). CDK 4-6/cycD kinases are critical in the phosphorylation and subsequent suppression of the retinoblastoma susceptibility protein, Rb, which inhibits several transcription factors required for proliferation (7). These functional relations are known as the p16INK4a/CDK4/cycD1/Rb pathway (5).

Expression of Ras in primary cells is able to strongly induce expression of cell cycle inhibitory molecules such as p53 and p16 (8). This activated Ras-dependent response causes an arrest in the cell cycle progression of rodent and human primary fibroblasts and shows characteristics indistinguishable from cellular senescence. Cells develop a flat morphology and express specific markers of senescence (8).

The abrogation of p16 occurs frequently in human oral cancers (9). The loss of expression of p16 has been observed in oral premalignant lesions (10, 11) and primary tumours of the oral cavity (12-14). Mechanisms of inactivation include homozygous gene deletion, gene mutation and hypermethylation of upstream CpG island regions (15). Accumulation of p16 mRNA and protein has been reported in response to a short list of stimuli and conditions: cellular senescence, oncogenic hyperactivity of Ras and inactivation of Rb (5). We have previously investigated the expression

Correspondence to: Prof. Dr. Eleftherios Vairaktaris, MD, DDS, Ph.D., Department of Oral and Maxillofacial Surgery, University of Athens Medical School, Vas. Sofias 93 and Dim. Soutsou 1, Athens 11521, Greece. Tel: +30 210 6443035, Fax: +30 210 6443803, e-mail: lvairakt@med.uoa.gr

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of Ras and cell cycle marker molecules such as p53 and Ki-67 in various stages of oral oncogenesis (16-18).

The present study was designed to determine p16 expression by employing immunohistochemical methods in sequential histological stages of OSCC formation in an experimental system of induced oral carcinogenesis in Syrian golden hamsters. In these animals, the chemically induced oral premalignant lesions and OSCC resemble both pathologically and ultrastructurally the equivalent ones that develop in humans exposed to tobacco and alcohol (19, 20). Although p16 expression has been previously studied in biopsies and cell lines of oral cancer and precancerous lesions in humans and rodents (13, 15, 21), the present study is the first to our knowledge that investigates p16 in sequential stages of oral oncogenesis.

Materials and Methods

Experimental carcinogenesis. Thirty seven male Syrian golden hamsters (*Mesocricetus auratus*) purchased from the Hellenic Pasteur Institute (Athens, Greece) at the age of five weeks, weighing approximately 100 g each, were used in this study. The hamsters were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animals were randomly divided into four groups: one control group (n=7) and three experimental groups for carcinogen treatment (A, B and C; n=10 animals each). The left buccal pouches of animals in experimental groups were treated with 0.5% 9,10-dimethyl-1,2-benzanthracene (DMBA) (Sigma, St. Louis, MO, USA) dissolved in paraffin oil, for 10 weeks (group A) and for 14 weeks (groups B and C), as described elsewhere (16). The pouches of all animals were examined weekly in order to observe the growth of tumours on the mucosa. The treated buccal pouches were removed after the animals were sacrificed at 10 weeks from the application of the carcinogen (group A), at 14 weeks (group B) and at 19 weeks (group C). The control animals were sacrificed at 10 weeks and a left buccal pouch sample was taken. The biopsies were given a number and examined blindly.

Pathological evaluation. Each section was examined under light microscopy and all possible different lesion types were evaluated. The tissue profiles were classified into totally normal oral mucosa, non-cancerous (hyperkeratosis, hyperplasia), precancerous (high grade dysplasia) and cancerous conditions (early invasion, well- and moderately differentiated carcinoma).

Immunohistochemical analysis. The biopsies from the 37 animals were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Three sections of 4 μm were prepared from each specimen and were mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany). One section was stained with hematoxylin and eosin for routine histological evaluation, while the other two were used for immuno-histochemical detection of p16 protein. The sections were incubated with monoclonal primary antibody against p16 (p16 F-12: sc-1661, dilution 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described elsewhere (16).

Tonsil tissue, which strongly expresses p16, was used as positive control. Negative controls of tonsil tissue were processed in the same manner, using phosphate-buffered saline (PBS) instead of the primary antibody. A positive reaction for p16 was quantitatively determined when the nucleus in the cancer cells was stained more strongly than in the cytoplasm and when squamous cells were stained more strongly than stroma cells (Figure 1).

All slides were independently reviewed by two investigators blindly. The consecutive hematoxylin-eosin-stained slides were evaluated by a pathologist experienced in oral pathology, without knowing the p16 staining pattern.

Statistical analysis. The mean value of percentages of positively stained cells was calculated for all the different lesions present in each sample. These values were tabulated for each group of animals (control group, experimental groups A, B, C) and every group's percentages were compared with those corresponding to the previous group.

In order to evaluate the pattern of antibody expression in relation to the histological status, the various lesions were divided according to tumour progression into a) normal tissue, b) non-cancerous and precancerous conditions (hyperkeratosis, hyperplasia, dysplasia), c) tumour (early invasion, well-differentiated carcinoma, moderately differentiated carcinoma). In every lesion, the percentage of positively-stained cells from each non-cancerous and precancerous category were compared with those of the normal tissue, while the percentage of positively stained cells from each tumour category was compared with the average percentage of the two non-cancerous and one precancerous lesions.

A two-tailed Student's *t*-test was applied for statistical analysis using the SPSS 10.0 program for Windows™ (SPSS Inc. Headquarters, Chicago, IL, USA). In addition, in every group and every histological category a normal distribution check was performed using the Kolmogorov-Smirnov Z test (SPSS). If a group or a histological category was not normally distributed, additional statistical analysis was performed with the Wilcoxon test using SPSS. A *p*-value less than 0.05 was considered statistically significant.

Results

The histological status of biopsies in the control group and in the three experimental groups is shown in Table I. A progression towards OSCC formation with increased time of carcinogen application is evident. Therefore, as expected, this experimental model seems valid and further analysis of data was implemented.

The percentages of p16 in the various categories of histological status are shown in Table II. No statistical difference in the expression of p16 was observed between oral mucosa with hyperkeratosis and normal mucosa. Nevertheless, p16 was significantly greater in hyperplasia ($p=0.006$) and much lower in dysplastic tissues ($p=0.008$). In early invasion and in the last stages of oral oncogenesis p16 expression continued to be minimal and remained at significantly lower levels than p16 expression in normal mucosa (Figure 1).

The percentage of positive cells for p16 in the control group and in the three experimental groups of animals are

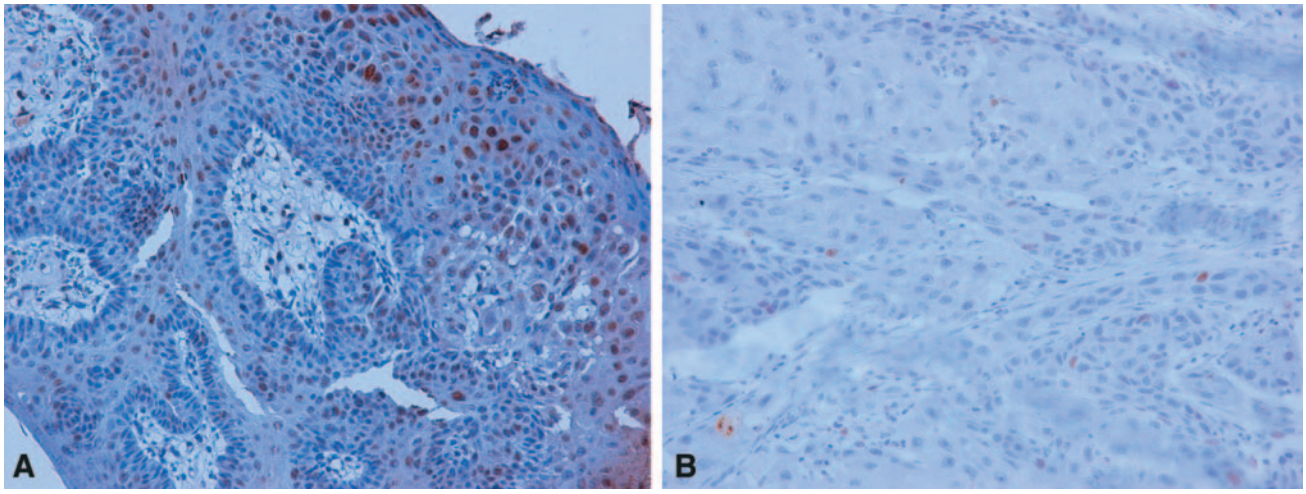

Figure 1. *(a) Increased p16 nuclear immunoreaction in a hyperplastic/dysplastic squamous epithelium (x200); (b) Minimal p16 nuclear immunoreactivity in invasive squamous cells (x200).*

shown in Table III. The statistical analysis revealed that in the control group and group A, p16 expression remained stable showing no statistical difference while a significant decrease in p16 expression was observed between group A and group B ($p=0.011$). In group C, p16 expression was lower than in the control group but this decrease was not statistically significantly lower than group B.

Discussion

In this study, an experimental model of chemically induced oral carcinogenesis was used to investigate the expression levels of tumour suppressor p16 in various stages of OSCC formation. Our findings revealed that p16 expression increased in the stage of oral mucosal hyperplasia, decreased sharply during oral mucosal dysplasia and remained at low levels in the subsequent stages of oral oncogenesis.

A possible explanation for the increased amount of p16 protein during hyperplasia observed in our study may be the unscheduled transactivation of the *p16* gene. Increased accumulation of p16 mRNA and protein has been reported in response to cellular senescence, oncogenic hyperactivity of Ras and inactivation of Rb (5). Since hyperplasia does not correspond to senescence, and we have previously shown that H-Ras and N-Ras expression does not significantly change during the same stage of oral oncogenesis (18), the only remaining possibility is the inactivation of Rb protein (22). Indeed, it has been reported that about 60% of human premalignant oral lesions do not exhibit detectable levels of Rb protein (13, 23). In accordance with our findings, previous studies in oral premalignant lesions have detected a strong reciprocal relationship between Rb and p16 expression (13, 23). Since the *p16* gene is transcriptionally repressed by Rb protein, p16 mRNA and its encoded protein accumulate to high levels in cells lacking Rb (22).

Table I. *Histological status of biopsies in the control group and in the three experimental groups.*

	Number of hamsters			
	Control group	Group A	Group B	Group C
Normal tissue	4			
Hyperkeratosis	2			
Hyperplasia	1			
Dysplasia		6		
Early invasion		3	1	
Well-differentiated carcinoma		1	6	1
Moderately-differentiated carcinoma			3	9

Furthermore, the findings of the present study indicated a comparative decrease of p16 protein expression in dysplastic lesions, in accordance with certain studies of human oral oncogenesis. Studies have investigated the expression of cell cycle proteins in human OSCC, including p16, and their data suggested alterations of the p16 pathway which occur at an early stage in the multistep process of oral oncogenesis, most commonly in the precancerous stage of dysplasia (24-26). Accordingly, in advanced stages of oral oncogenesis,

Table II. *Percentage of p16-positive cells in the various categories of histological status.*

	Non-cancerous and precancerous				Tumour		
	Normal oral mucosa	Oral mucosa with hyperkeratosis	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	OSCC	
						Well-differentiated	Moderately-differentiated
	5	5	10	2	2	2	2
	5	5	10	2	2	5	2
	10	5	10	5	2	5	2
	5	5	10	5	0	0	2
	5	10	5	2	0	2	2
	5		5	5	0	2	2
	5		10	2	2	2	5
	10		10	0	0	2	5
	10		10	5	0	2	2
	5		15	2	0	5	0
	5		20	2	2	2	2
	10		10	5	0	2	2
	10		20	2	2	0	2
	5		10	2	2	2	2
	5		10	2	2	0	0
			20	2	2	0	0
				2	0	0	0
				0	2	0	5
				5	0	5	5
				2		2	2
				0		0	2
				0		0	2
						2	2
						2	2
							2
Mean value of percentages	6.67	6	11.56	2.45	1.05	1.83	2.16
Mean value of non-cancerous and precancerous conditions			6.25				
Probability of *t*-test		N.S.	0.006	0.008	<0.0001	<0.0001	<0.0001

N.S.: not significant.

decreased levels of p16 were found in this study. Possible mechanisms of *p16* gene inactivation may include accumulation of events such as homozygous gene deletion (40-50%), mutations (10-15%) and hypermethylation of the promoter region of the *p16* gene (40-55%) (14, 15, 27-29).

The present study provides comprehensive evidence regarding the loss of p16 gene function in oral carcinogenesis, complementing the findings of other animal model studies. A study of a hamster oral cancer cell line has indicated that homozygous deletion of the *p16* gene plays a prominent role in oral oncogenesis (15). Another study in a mouse model of oral and esophageal carcinogenesis found that p16 expression decreased in neoplastic epithelia of tongue and esophagous compared to normal controls (21). Finally, p16 inactivation by promoter hypermethylation was observed at high frequency in severe dysplasia and OSCCs in rat carcinogenesis (30).

Conclusion

The findings of our experimental model, as well as those of other researchers, seem to suggest that inactivation of p16 occurs at the early stage of oral mucosal dysplasia in the multistep process of oral cancer progression, before the acquisition of an invasive phenotype. Therefore, loss of p16 function in precancerous oral lesions may be considered as a prognostic marker for the progression of malignancy.

Table III. *Percentage of p16-positive cells in the control group and in the three experimental groups.*

	Control	Group A	Group B	Group C
	6.67	8	3	1.33
	6.67	4	4	4
	5	8.33	1.2	0
	6.67	5.67	1.5	1.67
	6.67	7.33	1	2
	5	8.33	5.8	1.33
	8.33	4	2	0
		8	2.25	3.5
		5.67	8.33	2
		1.33	1.33	2
Mean value of percentages	6.43	6.06	3.04	1.78
Probability of *t*-test		N.S.	0.011	N.S.

N.S.: not significant.

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References

1 Rodrigues VC, Moss SM and Tuomainen H: Oral cancer in the UK: to screen or not screen. Oral Oncol *34*: 454-465, 1998.
2 Parkin DM, Pisani P and Ferlay J: Global cancer statistics. CA Cancer J Clin *49*: 33-64, 1999.
3 Annertz K, Anderson H, Biorklund A, Moller T, Kantola S, Mork J, Olsen JH and Wennerberg J: Incidence and survival of squamous cell carcinoma of the tongue in Scandinavia, with special reference to young adults. Int J Cancer *101*: 95-99, 2002.
4 Ponder BA: Cancer genetics. Nature *411*: 336-341, 2001.
5 Serrano M: The Tumour Suppressor Protein p16INK4a. Exp Cell Res *237*: 7-13, 1997.
6 Serrano M, Hannon GJ and Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*: 704-707, 1993.
7 Weinberg RA: The retinoblastoma protein and cell cycle control. Cell *81*: 323-330, 1995.
8 Serrano M, Lin AW, McCurrach ME, Beach D and Lowe SW: Oncogenic ras Provokes Premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*: 593-602, 1997.
9 Todd R, Hinds PW, Munger K, Rustgi AK, Opitz OG, Suliman Y and Wong DT: Cell cycle dysregulation in oral cancer. Crit Rev Oral Biol Med *13*: 51-61, 2002.
10 Papadimitrakopoulou V, Izzo J, Lippman SM, Lee JS, Fan YH, Clayman G, Ro JY, Hittelaman WN, Lotan R, Hong WK and Mao L: Frequent inactivation of p16INK4a in oral premalignant lesions. Oncogene *14*: 1799-1803, 1997.
11 Kresty LA, Mallery SR, Knobloch TJ, Song H, Lloyd M, Casto BC and Weghorst CM: Alterations of p16INK4a and p14ARF in patients with severe oral epithelial dysplasia. Cancer Res *62*: 5295-5300, 2002.
12 Riese U, Dahse R, Fiedler W, Theuer C, Koscielny S, Ernst G, Beleites E, Claussen U and von Eggeling F: Tumour suppressor gene *p16 (CDKN2A)* mutation status and promoter inactivation in head and neck cancer. Int J Mol Med *4*: 61-65, 1999.
13 Nakahara Y, Shintani S, Mihara M, Kiyota A, Ueyama Y and Matsumura T: Alterations of Rb, p16INK4A and cyclin D1 in the tumourigenesis of oral squamous cell carcinomas. Cancer Lett *160*: 3-8, 2000.
14 Namazie A, Alavi S, Olopade OI, Pauletti G, Aghamohammadi N, Aghamohammadi M, Gornbein JA, Calcaterra TC, Slamon DJ, Wang MB and Srivatsan ES: Cyclin D1 amplification and *p16(MTS1/CDK4I)* deletion correlate with poor prognosis in head and neck tumours. Laryngoscope *112*: 472-481, 2002.
15 Muscarella P, Knobloch TJ, Ulrich AB, Casto BC, Moniaux N, Wittel UA, Melvin WS, Pour PM, Song H, Gold B, Batra SK and Weghorst CM: Identification and sequencing of the Syrian Golden hamster (*Mesocricetus auratus*) *p16 (INK4a)* and *p15 (INK4b)* cDNAs and their homozygous gene deletion in cheek pouch and pancreatic tumour cells. Gene *278(1-2)*: 235-243, 2001.
16 Derka S, Vairaktaris E, Papakosta V, Vassiliou S, Acil Y, Vylliotis A, Spyridonidou S, Lazaris AC, Mourouzis C, Kokkori A, Moulavassili P, Perrea D, Donta I, Yapijakis C and Patsouris E: Cell proliferation and apoptosis culminate in early stages of oral oncogenesis. Oral Oncol *42(5)*: 540-550, 2006.
17 Papakosta V, Vairaktaris E, Vylliotis A, Derka S, Nkenke E, Vassiliou S, Lazaris A, Mourouzis C, Rallis G, Spyridonidou S, Anagnostopoulou S, Perrea D, Donta I, Yapijakis C and Patsouris E: The co-expression of c-myc and p53 increases and reaches a plateau early in oral oncogenesis. Anticancer Res *26(4B)*: 2957-2962, 2006.
18 Vairaktaris E, Papageorgiou G, Derka S, Moulavassili P, Nkenke E, Kessler P, Vassiliou S, Papakosta V, Spyridonidou S, Vylliotis A, Lazaris AC, Anagnostopoulou S, Mourouzis C, Yapijakis C and Patsouris E: Expression of ets-1 is not affected by N-ras or H-ras during oral oncogenesis. J Cancer Res Clin Oncol *133(4)*: 227-233, 2007.
19 Chang KW, Saraj S, Lin SC, Tsai PI and Solt D: p53 expression, *p53* and *Ha-ras* mutation and telomerase activation during nitrosamine-mediated hamster pouch carcinogenesis. Carcinogenesis *21(7)*: 1441-1451, 2000.
20 Balasenthil S, Saroja M, Ramachandran CR and Nagini S: Of humans and hamsters: comparative analysis of lipid peroxodation, glutathione and glutathione-dependent enzymes during oral carcinogenesis. Br J Oral Maxillofac Surg *38*: 267-270, 2000.
21 Tang XH, Knudsen B, Bemis D, Tickoo S and Gudas LJ: Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. Clin Cancer Res *10*: 301-313, 2004.
22 Li Y, Nichols MA, Shay JW and Xiong Y: Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product Rb. Cancer Res *54(23)*: 6078-6082, 1994.
23 Pande P, Mathur M, Shukla NK and Ralhan R: Rb and p16 protein alterations in human oral tumourigenesis. Oral Oncol *34*: 396-403, 1998.

24 Bradley KT, Budnick SD and Logani S: Immunohistochemical detection of $p16^{INK4a}$ in dysplastic lesions of the oral cavity. Mod Pathol *19(10)*: 1310-1316, 2006.

25 Gonzales-Moles MA, Rodriguez-Archilla A, Ruiz-Avila I, Martinez AB, Morales-Garcia P and Gonzalez-Moles S: p16 Expression in squamous carcinomas of the tongue. Onkologie *25(5)*: 433-436, 2002.

26 Shintani S, Mihara M, Nakahara Y, Kiyota A, Ueyama Y, Matsumura T and Wong DT: Expression of cell cycle control proteins in normal epithelium, premalignant and malignant lesions of oral cavity. Oral Oncol *38*: 235-243, 2002.

27 Huang MJ, Yeh KT, Shih HC, Wang YF, Lin TH, Chang JY, Shih MC and Chang JG: The correlation between *CpG* methylation and protein expression of P16 in oral squamous cell carcinomas. Int J Mol Med *10(5)*: 551-554, 2002.

28 Kresty LA, Mallery SR, Knobloch TJ, Song H, Lloyd M, Casto BC and Weghorst CM: Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. Cancer Res *62(18)*: 5295-5300, 2002.

29 Yakushiji T, Noma H, Shibahara T, Arai K, Yamamoto N, Tanaka C, Uzawa K and Tanzawa H: Analysis of a role for p16/CDKN2 expression and methylation patterns in human oral squamous cell carcinoma. Bull Tokyo Dent Coll *42(3)*: 159-168, 2001.

30 Nakahara Y, Shintani S, Mihara M, Matsumura T and Hamakawa H: High frequency methylation of *p16INK4A* gene during 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis. Oncol Rep *12(1)*: 101-106, 2004.

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