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# TUMOR SUPPRESSION IN ONCOGENESIS OF ORAL SQUAMOUS CELL CARCINOMA

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## Abstract

Oral cancer is relatively uncommon in the industrialized world but accounts for about 5% of all cancer deaths worldwide, and up to 40% of all malignancies in South and South-East Asia. The present paper aims to review the role and significance of tumor suppressor genes in the genetic and molecular pathways to oral squamous cell carcinoma (SCC), which is the most common form of oral cancer and frequently associated with poor prognosis. The high incidence of SCC in betel quid users is due to the severe chemical insult resulting in multiple alterations of oncogenes and tumor suppressing genes. Of the latter, SCC in betel quid users is more often associated with alterations in *p16* and *pRb* (typically at least 60% of the cases) than in *p53* (typically less than 20%). However, in most parts of the world SCC is mostly attributed to smoked tobacco and alcohol, which inflict a synergistic effect when used in combination. The characteristic prevalent alterations are common (50 to 100%) in the tumor suppressor gene *p53*. Potential applications of the genes, corresponding expressed proteins and related other markers are discussed in brief.

## Abstrak

Kanker mulut relatif jarang terjadi dinegara industri, namun mencapai sekitar 5% dari seluruh kematian akibat kanker diseluruh dunia, dan mencapai 40% dari seluruh kasus keganasan di Asia selatan dan Asia tenggara. Makalah ini bertujuan untuk membahas peran dan kemaknaan gen supresor tumor pada jalur genetik dan molekuler karsinoma sel skuamosa mulut (SCC), yang merupakan jenis kanker mulut yang paling umum terjadi, dan seringkali dihubungkan dengan prognosis yang buruk. Tingginya insiden SCC pada pemakai pinang, disebabkan oleh iritasi kimia berat yang berakibat terjadinya perubahan-perubahan multipel onkogen dan gen supresor tumor. Dari yang disebut terakhir, SCC pada pengguna pinang lebih sering dihubungkan

dengan perubahan pada p16 dan pRb ( secara spesifik paling sedikit 60% dari seluruh kasus) dibandingkan dengan p53 (yang kurang dari 20%). Namun demikian, di sebagian besar belahan dunia, SCC dianggap disebabkan oleh asap tembakau dan alkohol, yang menimbulkan suatu efek sinergistik bila digunakan secara kombinasi. Perubahan-perubahan spesifik yang umum terjadi (50-100%) adalah pada gen supresor p53. Aplikasi penting dari gen-gen, ekspresi protein serta penanda lain yang berhubungan akan dibahas secara singkat.

## Introduction

Oral squamous cell carcinoma (SCC) is the most common form of oral cancer, which is the sixth most common malignancy worldwide representing 5.6% of new cancer cases and 5.1% of all cancer deaths.[1] SCC is particularly resistant to therapeutic treatment: about 40-50% of the cases are fatal within 5 years of first diagnosis.[2] Until recently, there has been little change in the relative survival rates since 1950.[3] For further progress, it is clearly important to better understand the mechanisms that lead to oral tumors and control their progression.

Oral cancer is thought to be predominantly initiated and promoted by the chemical insult from oral exposure to certain etiological agents, such as tobacco, ethanol and betel quid.[4,5] Other contributing though less influential factors appear to include viral infections, poor oral hygiene, consumption of saturated fats and deficiencies in vitamins A and C, and iron.[6,7,8,9] A rare inherited genetic disorder, Fanconi's anaemia, can also induce oral cancer but probably more important inherited factors are the inter-individual variations in sensitivity to carcinogens.[10] As the patterns of predisposing factors can differ in various parts of the world, so do the incidence and other characteristics of oral cancer. The genetic and molecular pathways in oral cancer can be expected to show corresponding regional differences.

The early premalignant phases of SCC initiation result in the development of lesions in the form of leukoplakia (white patches) or erythroplakia (red patches), which can exhibit varying degrees of hyperplasia or dysplasia. Leukoplakia is much more common but erythroplakia is a more potent precursor of oral squamous cell carcinoma; in fact histopathological examination of the latter type of lesions suggests that about half of these lesions are actually invasive squamous cell carcinomas.[11] Of advanced but premalignant dysplastic lesions, 36% on avarage have been found to transform to a malignant state within 8 years in USA.[12]

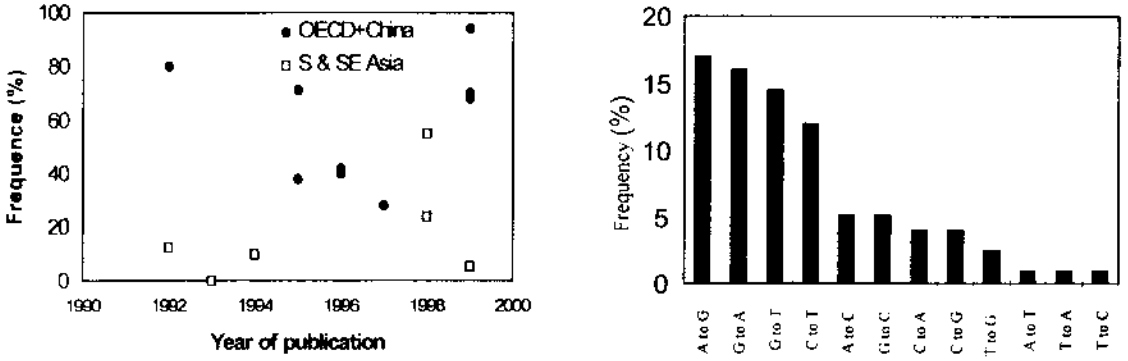
There are many oncogenes, or genes which when inappropriately activated, amplified or inactivated can promote some form of cancer: more than 100 oncogenes have been identified.[13,14] However, cancer is a multistep process where singular gene alterations are necessary but not sufficient for carcinogenesis, and different genetic events are involved in the initiation, promotion, progression and metastasis stages of cancer. Based on epidemiological, molecular and statistical studies it has been suggested that 6 to 10 genetic events are required for oral cancer.[15] In most cases, mechanisms of DNA repair and apoptosis will deal with the deviations from normal cellular function. Cancer is more likely with additional dysfunction, i.e. inappropriate activation or silencing of the tumor suppressing genes (anti-oncogenes) which control some critical events of the cell cycle, including the processes of DNA repair and apoptosis. It is the purpose of the present paper to elucidate the role of tumor suppressor genes in oncogenesis of oral squamous cell carcinomas. Below, a brief review is given on the role of the tumor suppressor genes in the oncogenesis of oral SCC.

## Observed Patterns of SCC Oncogenesis and Alteration of Tumor Suppressor Genes

In Europe, USA, Japan, China and Australia the main established etiological agents of oral cancer are smoked tobacco and alcohol (ethanol), which are known to exert a disproportionately stronger effect when used in combination. The synergy is thought to arise sequentially so that carcinogenic and cocarcinogenic chemicals from tobacco induce DNA damage and ethanol promotes its progression by immunosuppressive action and by preventing damage from being repaired. The carcinoma is typically found either endophytically in the trough formed by the lateral border of the tongue, mouth floor and the lingual aspect of the lower alveolus (possibly due to local pooling of carcinogens), or in case of tobacco chewing, exophytically at the site of application, frequently in the buccal sulcus and buccal mucosa. Oral cancer is more common in males than in females, and the incidence rate increases sharply after about 40-50 years of age. In Europe, USA, Japan and Australia about 1-2% of all cancers are oral in origin.[14]

In Europe, USA, Japan and China oral carcinomas show a high prevalence of mutations and allelic loss in the important tumor suppressor gene *p53* (Fig 1a), the reported incidence varying generally from about 30% to 95%.[16-26] Mutations have been mostly reported from a range of codons from exons 4 to 9, with highest frequencies in hot spots between codons 100 and 300. Particularly common are mutations A→G, G→A, G→T and C→T (Fig 1b). Of these, at least G→T mutations are typical for tobacco derived carcinogens such as benzpyrene.[27] The studies reporting highest incidence range (90% or more) of mutations tend to be more recent and to employ direct sequencing of mRNA followed by additional DNA sequencing. The same method also shows higher concordance between primary tumors and lymph node metastases, and 100% concordance has been reported when using additionally mutation specific oligo ligation assay. This suggests that the *p53* mutations are stable in the progress of cancer.[26] Also DNA viruses such as human papilloma virus (HPV) can bind to and potentially inactivate *p53*. As *p53* has multiple important roles (see Table 1) in regulating cell proliferation, DNA repair and apoptosis, its inactivation can lead to cancer in a wide range of tissues including those of the oral cavity.[13,28] Alterations of p53 can be already present in pre-malignant lesions, in about 15% of leukoplakias[21,29] and 30-50% of erythroplakias.[30]

Frequent inactivation of the tumor suppressing *p16* gene has been reported for primary tumors and metastases of oral SCC and also from about 40% of premalignant lesions.[31,32] The p16 protein regulates cell proliferation by binding to cyclin-dependent kinases (CDK) 4 and 6, inhibiting the catalytic activity of the CDK-cyclin D-1 complexes at the G1/S interface by phosphorylation of pRb in the retinoblastoma (Rb) pathway. Allelic loss and mutations of *p16* have been observed in 70-80% of SCC tumors.[31,33,34] However, in at least 20-30% of the reported cases of SCC, *p16* is not expressed in spite of a normal and intact gene. This is apparently due to DNA methylation at a specific 5' CpG island of the gene. In cultivated SCC cell lines the frequency of altered p16 can reach 100% but is often observed to be lower in primary tumors. The difference may be partly due to mutations in culture, partly due to techniques used in assessing the altered frequency.[31] Of the two alternative transcripts (α and β) coded by *p16*, the β transcript gives rise to the $p14^{ARF}$ protein, which by binding to MDM2 leads to stabilization and accumulation of tumor suppressing p53. However, the α transcript which carries the main function of *p16* is much more commonly altered in oral SCC tumors.[31,35,36]




a) b)

Fig 1. a) Reported frequency of p53 mutations in SCC tumors in Europe, USA, Japan and China (OECD+China) and in South and South-East Asia (S&SE Asia)[16-26,39,41-45] ; b) frequency of transitions/transversions in human oral SCC tumors (redrawn from the review data of [4]).

A different path of carcinogenesis appears to occur in the well differentiated oral verrucous carcinoma (VC), which shows no metastasis although it is originally described as a subclass of SCC. VC is though to be initiated by HPV virus[25], and the resulting tumor expresses the p16 protein much more strongly (and p53 less) than usual SCC (Fig 2). After emergence of metastasis, the prognosis of SCC is worse but even at this stage there is at least one characteristic tumor suppressing gene, *nm-23*, which is thought to code the NDP kinase involved in transfer of terminal phosphate from a nucleoside triphosphate to NDP.[37]

The *p16* gene is functionally complementary to *pRb*, which can also be seen as a tumor suppressor gene. In normal cells active pRb prevents cellular transition from G1 to S phase by sequestering the necessary transcription factors. The pRb protein can also bind with the HPV oncoproteins E6 and E7, and high pRb expression together with high levels of p16 is typical in HPV-induced VC.[25] In contrast, functional inhibition of p16 but elevated expression of pRb can be observed in usual forms of SCC, particularly in terms of the intensity of the protein expression (Fig 2b).[25]

Another suggested tumor suppressor gene is *p130*, which is structurally and functionally a relative to *pRb*, and therefore also involved in cell cycle regulation in the G1 phase, with affinity to CDK2. Immunohistochemical activity of p130 is seen in the nuclei and mitochondria.[38] Allelic loss in *p130* has been reported in many forms of cancer, and *p130* is apparently particularly affected in poorly differentiated forms of SCC (Fig 3).[38]

In India, Sri Lanka, Papua New Guinea and parts of Southeast Asia including Indonesia and Taiwan, a particular agent carrying a high risk to oral cancer is chewed tobacco in the form of betel quid. This consists of areca nut (*pinang*) and lime, with or without and tobacco and spices, wrapped into a leaf of the betel vine (*Piper betle, sirih*). Exophytical origin of oral cancer is then common, often in the buccal regions. Habitual use of the betel quid is thought to be the main reason for oral cancer in South Asia accounting for 18-40% of all malignancies.[5,39,40]

Generally less than 20% of the malignant oral tumors have been reported to show mutations in the *p53* gene in South and South East Asia where betel quid is

habitually used (Fig 1a).[39,41,42,43,44,45] Instead, frequent (35%) mutations of the *H-ras* gene, loss of heterozygosity at the *H-ras* locus (30%), some mutation of the *K-ras* gene (7%), as well as amplification of *N-ras* (28%), *N-myc* (29%), *bcl-2* (23%), c-*myc* (20%) and *K-ras* (17%) oncogenes have been observed.[39,40,42,46] This multiplicity of genetic alterations appears characteristic for betel quid users, probably reflecting the severity of chemical attack. The *ras* family of oncogenes, often involved early in the formation of chemically induced cancers, result in abnormal G proteins which in normal cells regulate the information exchange across the cell membrane.[13] The *bcl-2* oncogene expression inhibits both p53-dependent and p53-independent apoptosis pathways.[2] The *c-myc* oncogene is involved both in driving the cell cycle and in apoptosis, depending on the level of the growth factor IGF1.[47] Overexpression of p53 has also been reported but is not well correlated with incidence of mutations.[45,48] Apparently p53 is inversely expressed with respect to bcl-2 so that taken together, about 40% of Indian SCC cases may involve dysfunctional apoptosis pathway either via p53 or bcl-2.[40] Perhaps more importantly, frequent loss of both *p16* and *pRb* expression have been reported in SCC tumors and pre-malignant leukoplakias of betel quid users (Fig 2b).[5]

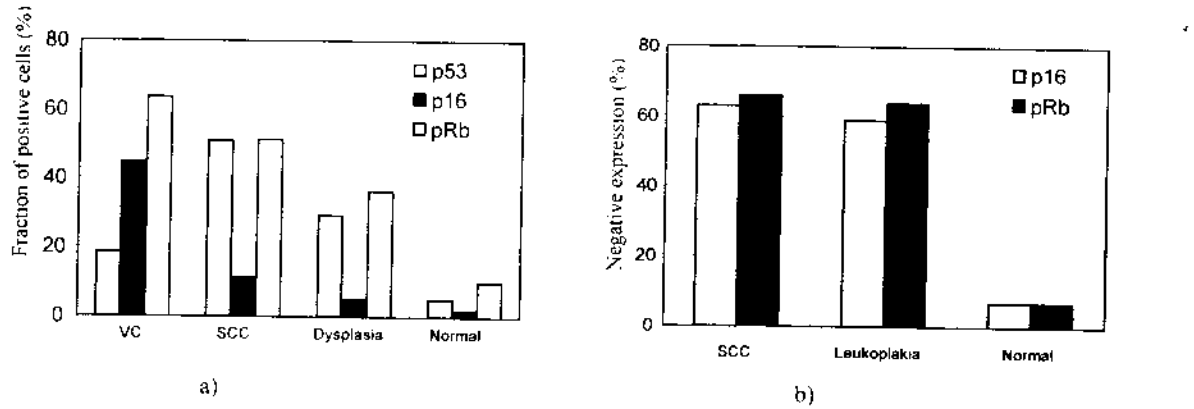



Fig 2. a) Fraction of cells expressing p53, p16 and pRb in common oral SCC tumors, oral verrucous carcinomas (VC), pre-malignant (severe) dysplasias and normal oral tissue, redrawn from data of [25] on Japanese subjects. b) Loss of p16 and pRb expression in SCC tumors, oral premalignant leukoplakias and normal oral tissue from habitual Indian betel/tobacco users (redrawn from data of [5])

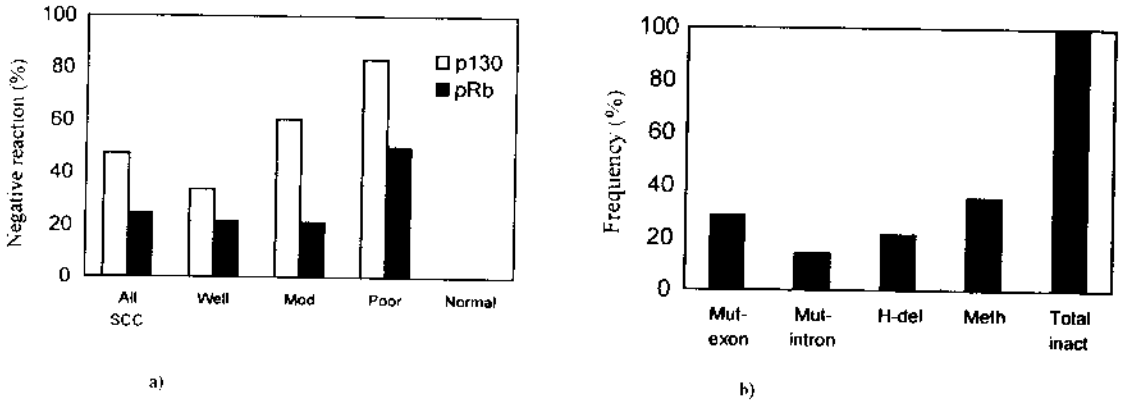



Fig 3. a) Loss of expression of p130 and pRb in oral SCC tumors in all SCC cases and well, moderately and poorly differentiated tumors, and in normal oral tissue (redrawn from data of [38] on Japanese subjects). Note that loss of expression for pRb is much less than that reported for Indian subjects in Fig 2b, with the possible exception of poorly differentiated tumors. b) Frequency of different types of p16 inactivation (mutations in exon or intron, homozygous deletion and methylation) in 14 SCC cell lines (redrawn from data of [31]).

Some of the characteristics of observed oncogenes and tumor suppressor genes associated with oral SCC are summarised in Tables 1 and 2.

Table 1 Chromosomal location and carcinogenetic mechanisms of some recognized oncogenes and anti-oncogenes (tumor suppressor genes) related to oral cancer.[13,40]

| Oncogene | Anti-oncogene | Mechanism [1] | Chromosome |
|---|---|---|---|
| *N-ras* | | IA, PM | 1 |
| *N-myc* | | IA | 2 |
| *K-ras-1* | | IA, PM | 6 |
| *c-erb-B-1* | | IA | 7 |
| *c-myc* | | IA, TL | 8 |
| | *p16* | IA, PM, AL, M | 9p21-22 (short arm) |
| *bl-ras-1, bcl-1, int-2* | | PM, TL | 11 |
| *K-ras-2* | | IA, PM | 12 |
| | *pRb* | I, IA, AL, PM | 13 |
| | *p130* | I, PM, AL | 16q12,2-13 |
| | *p53* | IA, AL, PM | 17p13 |
| *bcl-2* | | IA | 18q21 |

[1] carcinogenesis: IA = inappropriate activation/amplification, I = inactivation, PM = point mutation, AL = allelic loss, TL = translocation, M = methylation

Table 2. Features of known tumor suppressor genes associated with oral SCC.[5,13,25,37]

| Gene | Normal function | Notes |
|---|---|---|
| p53 | Induction of p21 for DNA repair or apoptosis; block of replication of cells with damaged DNA | Altered in many types of tumors including SCC |
| p16 | α-transcript: inactivates cyclin D-dependent kinases 4 and 6 that are needed for phosphorylation of pRb to mediate passage through G1/S | Inactivated (α) in many types of tumors including SCC |
| p130 | Cell cycle regulation, specific for G1 phase, with affinity to CDK2, structural/functional similarity to *pRb* | Allelic loss in many forms of cancer |
| pRb | When activated, suppresses cell proliferation by inhibiting G1/S transition via sequestering transcription factors like E2F | Inactivated in many types of tumors including SCC |
| nm23 | Encodes NDP kinase involved in transfer of terminal phosphate from a nucleoside triphosphate to NDP | Reduces (in wt-α form) metastatic potential in some cancer types |

## Other Related Gene/ Protein Markers with Potential Clinical Significance

Apart from those listed above, there is a wide range of cell cycle related proteins that can show important reactions to the status of oral SCC. For example, the antigen protein Ki-67 is a proliferation marker that is active in all phases of the cell cycle except $G_0$. Increased Ki-67 expression has been reported as an indicator of proliferation activity (Fig 4a) in pre-malignant and malignant lesions.[25,49,50] However, the marker efficien-

cy shows considerable scatter and the marker can also fail to reflect the prolifiration rate of the tumor, because different antibodies of heterogeneous cell populations recognize different epitopes of the antigen.[50]

The CDK inhibitor p27 has a role in preventing the transition from G1 to S phase.[25,51] Expression of p 27 generally decreases with increased cell proliferation and is inversely related to Ki-67 expression.[25] Significantly elevated apoptotic index has been reported in p27 positive SCC tumors,[2] and hence p27 may be able to slow down cancer progression. However, generally p27 is less intensively expressed in SCC cells than in normal oral tissue (Fig 4a), and lack of p27 expression may be associated with poor prognosis.[2] The corresponding gene *p27* is not sensitive to mutations, but adenovirus E1A and human papillomavirus (HPV) E7 can block the beneficial action of p27.[52]

The apoptosis regulating protein Bax is a negative regulator of Bcl-2 and is therefore usually inversely distributed in normal tissues with respect to Bcl-2.[53] Over-expression of Baxα enhances apoptosis by modulating the p27 protein levels.[2] Based on experimental evidence on mouse SCC (Fig 4b) it has been suggested that exogenous administering of the *bax* gene in association with chemotherapy may have clinical significance.[54]

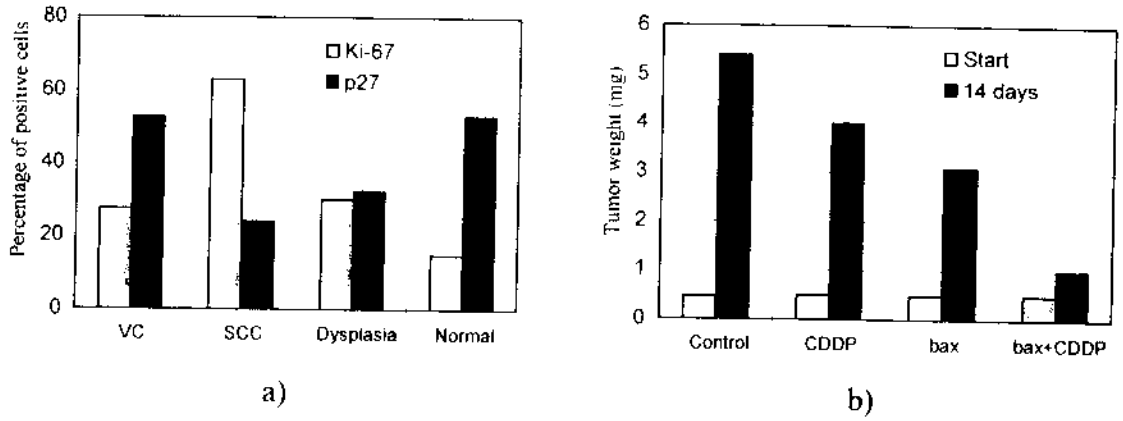



Fig 4. a) Fraction of cells expressing the proteins Ki-67 and p27 in oral VC and SCC tumors and in pre-malignant moderate dysplasia (from data of [25]). b) Effect on tumor size of gene gun injection of the bax gene with and without chemotherapy (CDDP or cisplatin) initially and after 14 days (4 treatments) in mice inoculated with SCC cells 7 days before therapy (from data of [54]).

## Conclusive Remarks

The mechanisms of carcinogenesis of oral SCC are similarly complex as in all cancers, with central role attributed to the accumulating damage inflicted by the etiological agents on the genetic and molecular regulation of the cell cycle. The observed large difference in the incidence of oral SCC between Southern/ Southeastern Asia and the rest of the world reflects the respective DNA damaging action of the carcinogenic chemicals in chewed betel quid and smoked tobacco. Important part of the damage affects the tumor suppressor genes, of which *p53* and *p16* are particularly sensitive to alteration by mutations, allelic loss and methylation. With the development

efficient analysis techniques, full quantification of the DNA alterations in oral SCC tumors has only recently become possible.

Although many details of the genetic and molecular mechanisms of carcinogenesis and its suppression remain poorly known, the observations made so far hold promise for clinical applications both in diagnosis and potential therapy. Further research is needed before new applications can make a significant contribution to the currently dominating therapeutic methods of irradiation, surgery and chemotherapy, which in aggressive combination have recently improved the 5 year survival rates of oral SCC to about 80%.[55] Positive contribution would be much needed. however, for cases which do not respond well to conventional therapy. In particular, improved survival probability can be expected, if the incidence of complete remission in the therapeutic treatment were to increase.[55] Current survival rates are lowest and recurrence rates highest in advanced carcinomas with metastasis, which also show the lowest rates of complete remission in the course of therapy. At least to evaluate the potential for carcinogenesis and to discern between primary carcinoma and metastasis, the DNA alterations of the tumor suppressor genes *p53* and *p16* appear promising.[56,57,58]

The risk of developing primary oral SCC tumors is mainly associated with the habitual use of tobacco. betel quid and alcohol, and the obvious preventive measure is to avoid these carcinogenic agents. The risk of developing second malignancy after treatment for oral SCC tumors has been estimated to be 10-30 times higher than for general population, but recent follow-up studies show that second cancers are likely to appear outside the oral region.[55,59] This suggests that to prevent secondary tumors, it is important to dissuade the patients from continuing to smoking, betel chewing or excessive alcohol consumption.

The new developments have also opened new ways towards cancer chemoprevention, where specific drugs are administered to suppress carcinogenesis and to prevent the development of invasive cancer. Although larger scale confirmation is needed, promising results have been obtained by using 13-*cis*-retinoic acid, which in a limited study resulted in a six-fold decrease in the rate of development of second primary tumors.[60] Equally important are developments to improve the therapeutic treatments of diagnosed oral SCC. Such promising but as yet experimental methods to enhance the efficiency of chemotherapy include e.g. gene therapy by gene gun injection of the *bax* gene[54] to the SCC tumors to promote tumor cell apoptosis.

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