Epigenic Inactivation of 14-3-3 σ in Oral Cancer: Association with p16INK4a Silencing and Human Papillomavirus Negativity

Milena Gasco, Alexandra K. Bell, Victoria Heath, Alex Sullivan, Paul Smith, Louise Hiler, Isik Yulug, Giannamo Numico, Marco Merlano, Paul J. Farrell, Mahvash Tavassoli, Barry Gusterson, and Tim Crook

UO Oncologia Medica, Azienda Ospedaliera S Croce e Carle, 12100 Cuneo, Italy [M. G., G. N., M. M.]; Ludwig Institute for Cancer Research, Imperial College of Science and Medicine, St. Mary’s Campus, Norfolk Place, London W2 1PG, United Kingdom [A. S., P. J. F., T. C.]; Institute of Cancer Genetics, Brunel University, Uxbridge UB8 4SP, United Kingdom [P. J. F.]; CRC Trials Unit, Institute for Cancer Studies, Edgbaston, Birmingham B15 2TT, United Kingdom [L. H.]; Department of Molecular Biology and Genetics, Bilkent University, 06533 Ankara, Turkey [I. Y.]; Cancer Gene Therapy Group, King’s College London, The Rayne Institute, London SE5 9NU [M. T.]; and University Department of Pathology, Western Infirmary, Glasgow G11 6NT, Scotland [A. K. B., V. H., B. G.]

ABSTRACT

In vitro studies have identified 14-3-3-σ as a regulator of senescence in human keratinocytes. To assess its contribution to squamous neoplasia, we have analyzed genetic and epigenetic changes in this gene in squamous cell carcinomas (SCCs) and dysplastic lesions of the oral cavity. No mutations were detected in the coding sequence of 14-3-3-σ in 20 oral carcinomas, and there was loss of heterozygosity in only 7 of 40 informative cases. In contrast, to the absence of genetic change, aberrant methylation within 14-3-3-σ was detected in 32 of 92 squamous cell carcinomas and in 3 of 6 oral dysplasias and was associated with reduced or absent expression at both mRNA and protein levels. Methylation was not detected in matched, normal epithelial tissue controls. Carcinomas in which 14-3-3-σ was methylated were significantly more likely to lack DNA sequences from human papillomavirus and to have coincident methylation of p16INK4a than cases that expressed 14-3-3-σ. Methylation was detected in SCC, both wild-type and mutant for p53, but was more commonly detected in cancers with wild-type p53. These results implicate coincident epigenetic abrogation of function in both σ and p16INK4a in a subset of SCCs of the oral cavity.

INTRODUCTION

SCC1 of the head and neck, including carcinoma of the oral cavity, is the sixth most common cancer worldwide. Epidemiological data strongly link smoking and alcohol consumption to the development of this malignancy (1). There is also evidence that a subset of oral SCCs is associated with infection by oncogenic HPV; a recent study identified HPV 16 in ~25% of cases (2).

σ (also known as stratatin) was first identified as a gene expressed specifically in stratified squamous epithelium (3). In that study, it was shown that expression of σ was absent from a few breast carcinoma cell lines but was not down-regulated in other cancer cell lines. Down-regulation of σ was also reported in a head and neck SCC cell line (4). σ is a regulator of senescence in epithelial cells. Down-regulation of σ allows keratinocytes to escape from replicative senescence (5). Steady-state levels of p16INK4a increase as keratinocytes approach replicative senescence, and conversely, p16INK4a is almost always inactivated in immortalized keratinocytes (6, 7). Consistent with these observations, there is loss of p16INK4a expression in human keratinocytes immortalized after transduction by retroviruses expressing antisense σ (5). Thus, in vitro evidence suggests that σ may have an important function in malignant development in epithelial tissues.

σ is induced by p53 in response to DNA damage (8) and mediates a G1 checkpoint. Such a mechanistic model might imply that down-regulation of σ would not occur in cancers with p53 mutation or in HPV-associated carcinomas, because p53 is targeted by HPV-encoded E6 (9).

The involvement of σ in human cancer has been established in studies of breast cancer in which methylation-dependent silencing of the gene was observed in a majority of cases of ductal carcinoma (10). Furthermore, loss of σ occurs early in neoplastic development in breast epithelium (11). Silencing of σ expression has also been reported in 43% of primary gastric adenocarcinomas (12) and 89% of hepatocellular carcinomas (13).

To determine the contribution of changes in σ to carcinogenesis in squamous epithelium, we have analyzed genetic and epigenetic changes in the gene in a series of malignant and premalignant neoplastic oral lesions.

MATERIALS AND METHODS

Tissues and Nucleic Acid Isolation. Cancers, with patient-matched normal epithelium where available, were obtained at operation. Tissues were snap frozen and stored in liquid nitrogen before analysis. The presence of a majority of neoplastic tissue was verified in each carcinoma by histopathological analysis of tissue sections. Genomic DNA was isolated by proteinase K digestion, and total RNA was isolated by phenol/guanidinium. cDNA was synthesized from 5 μg of total RNA using the Prostar system (Stratagene). Fifty-six SCCs were available for analysis from this series. A second series of cancers, comprising 36 paraffin-embedded tissue sections, was also analyzed. For isolation of genomic DNA, sections were treated with xylene to remove paraffin, dehydrated in ethanol, and then subjected to extended digestion in proteinase K.

Analysis of Structure and Expression of σ. Mutation analysis and analysis of LOH were performed using primers and conditions essentially as described by Ferguson et al. (10). Analysis of SCC for σ mutations was performed by amplification of the open reading frame with Pfu DNA polymerase and sequencing of clones in the vector pCRblunt (Invitrogen). For analysis of methylation, genomic DNA (1 μg) was modified with sodium bisulfite using the CpG modification system (Intergen) as directed by the manufacturers. Bisulfite sequencing was done with primers described previously (10). After PCR, reactions were cleaned with the Qiagen PCR purification kit and then directly sequenced on an ABI sequencer. MSP was done according to Ferguson et al. (10) with the exception that for DNA isolated from paraffin sections, PCR was performed for 40 rather than 35 cycles. PCR products were resolved on 10% polyacrylamide gels and visualized by staining with ethidium bromide. For analysis of σ expression, cDNA was synthesized as above from RNA isolated from matched pairs of normal and tumor. This was used as substrate for PCR with primers as described (10). PCR was performed for 28 cycles, under which conditions the reaction is in the exponential phase of amplification. PCR products were resolved on 2% agarose gels, transferred to nylon, and then hybridized with oligonucleotides complementary to the amplified sequences, end-labeled with [γ-32P]ATP by polynucleotide kinase. For immunocytochemical analysis, 3 μm unstained sections were taken from 38 samples also analyzed for HPV, p53 mutations, and sequence and methylation in σ. Tissue sections were dewaxed in xylene, rinsed well in ethanol, and then washed in tap water. Antigen retrieval was performed

Received 10/23/01; accepted 1/30/02.

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 Received in the laboratory of B. A. G. is supported by Breakthrough Breast Cancer. 2 To whom requests for reprints should be addressed, at Ludwig Institute for Cancer Research, Imperial College Faculty of Medicine, St. Mary’s Campus, Norfolk Place, London W2 1PG, United Kingdom. Phone: 0207-563-7721; Fax: 0207-724-8586; E-mail: t.crook@ic.ac.uk.

* The abbreviations used are: SCC, squamous cell carcinoma; HPV, human papillomavirus; σ, 14-3-3-σ; MSP, methylation-specific PCR; LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR.
by pressure cooking in 0.01 M citric acid buffer (pH 6.0). Sections were blocked in 1% hydrogen peroxide for 10 min, rinsed in tap water, and then placed in a humid staining chamber and covered with TBS buffer (pH 7.6) for 5 min. Sections were covered in primary antibody (Neomarkers; 14-3-3σ Ab-1) at 2 μg/ml incubated for 1 h at room temperature.

**Analysis of p16<sup>NK4α</sup>.** PCR primers for analysis of the p16<sup>NK4α</sup> locus were as described by Zhang et al. (14). The methylation status of p16<sup>NK4α</sup> was studied using MSP. Bisulfite-modified DNA was subjected to 35 cycles of PCR (40 cycles for paraffin-extracted DNA) using primers and conditions described by Herman et al. (15). Reaction products were resolved on 10% polyacrylamide gels and visualized under UV light after staining with ethidium bromide. RT-PCR analysis of expression was done as described by Gonzalez-Zulueta et al. (16).

**p53 Analysis.** Mutations in p53 were sought using single-strand conformation analysis. Suspected mutations were identified by reamplification with Pfs polymerase, ligation into pCRBlunt (Invitrogen), and sequencing of multiple plasmid clones.

**HPV Typing.** HPV sequences were sought in DNA from frozen tissue using the MY09/MY11 (HPV) and PC04/GP20 (globin) primers (17). For detection of HPV in DNA from paraffin sections, each genomic DNA was initially checked by amplification with the PC03/PC04 primers (18) and then analyzed with the CPI/CPIIG consensus primer pair (19). HPV type was determined by direct sequencing.

**Statistical Analysis.** All P values were obtained from χ² tests with continuity corrections.

**RESULTS**

**Inactivation of σ in Oral Cancer Occurs Predominantly by Epigenetic Silencing.** LOH in σ was sought using a microsatellite described previously (10). LOH was detected in 7 of 40 informative cases. To determine the presence of mutations, the σ open reading frame was amplified as a single fragment from genomic DNA of 20 SCCs, and the sequence of individual plasmid clones was determined. No mutations were detected in these cases. Next, evidence for epigenetic change in σ was sought in DNA from all 92 primary oral SCCs, 56 from fresh-frozen SCCs, and 36 from paraffin-embedded archival SCCs. Using MSP and bisulfite sequencing, methylation within the σ gene was detected in 32 of 92 (35%) cancers but was not detected in matched normal tissue (Fig. 1). To assess the effect of methylation on expression of σ, we performed RT-PCR of mRNA isolated from matched pairs of normal and tumor tissue (Fig. 2). These studies revealed either a marked reduction, or absence of expression, in all cases available for study in which methylation had been detected by either bisulfite sequencing or MSP. To verify results of RT-PCR, we performed immunocytochemical analysis of σ expression on the 36 paraffin-embedded cases. Expression of σ was reduced or absent in each case with methylation (Fig. 2).

**Methylation of σ Is Detected in Premalignant Oral Lesions.** We were interested to determine whether σ methylation was present in oral premalignant lesions. Using MSP, methylated σ DNA was detected in 3 of 6 oral dysplasias (Fig. 1).

**Silencing of σ Predominantly Targets Oral Cancers Lacking HPV DNA.** The etiological association between oral cancer and HPV is well established (2). We therefore analyzed each case for HPV DNA. HPV DNA sequences were detected in 17 of 92 (18%) cases (Fig. 3). HPV 16 was detected in 13 cases, HPV 6 in 3 cases, and 1 case contained types 6 and 16. Of the 17 HPV-positive cancers, there was methylation of σ in only 1 case (containing HPV 16; 6%), whereas methylation was detected in 31 of 75 (41%) HPV-negative cases (P = 0.013; Table 1). The 3 oral dysplasias with σ methylation were all negative for HPV DNA. These results indicate that methylation of σ is significantly more common in HPV-negative than in HPV-positive oral cancers.

**Methylation of σ Occurs More Commonly in Cancers with Wild-Type p53.** σ expression is induced by p53 (8), and we investigated the hypothesis that inactivation of σ would be less likely to occur in cancers with mutant p53. Mutations in p53 were identified in a total of 37 of 92 (40%) cancers. Methylation of σ was detected in 24 of 55 (44%) cases with wild-type p53 and 8 of 37 (22%) cases with
p53 mutations (Table 1; $P = 0.074$). Although this $P$ just fails to reach statistical significance, these results nevertheless indicate that silencing of $\sigma$ occurs more commonly in cases lacking p53 mutations.

**Concomitant Methylation of $\sigma$ and $p16^{INK4a}$ in Oral Cancer.** Down-regulation of $\sigma$ is accompanied by loss of $p16^{INK4a}$ expression during keratinocyte immortalization (5). We determined whether there was methylation in the $p16^{INK4a}$ gene in the series of oral SCCs characterized for $\sigma$. Methylated $p16^{INK4a}$ DNA was detected in 38 of 92 (41%) SCCs (Fig. 1); these comprised 5 of 17 HPV-positive and 33 of 75 HPV-negative cases. Methylation of $p16^{INK4a}$ was associated with reduced expression (Fig. 2). Of the 32 SCCs with methylated $\sigma$ DNA, there was concomitant methylation of $p16^{INK4a}$ in 25 cases (78%; Table 1), whereas of the 60 SCCs with unmethylated $\sigma$, there was methylation of $p16^{INK4a}$ in 13 (22%; $P = 0.001$). Of the 3 dysplasias with $\sigma$ methylation, there was concomitant methylation of $p16^{INK4a}$ in 1 case, whereas a further dysplasia had methylated $p16^{INK4a}$ but not $\sigma$. The previously described polymorphism (Ala→Thr) at codon 148 occurred in 148 cases, mutations in $p16^{INK4a}$ were detected in 3 of 92 SCCs, all of which were negative for HPV DNA and unmethylated in $\sigma$ (Table 2).

Taken together, these results indicate a significant association between epigenetic silencing of $\sigma$ and $p16^{INK4a}$ in HPV-negative oral SCCs.

**DISCUSSION**

The importance of $\sigma$ as a regulator of senescence in human keratinocytes has been demonstrated clearly (5). In the present study, we demonstrate that $\sigma$ is subject to methylation-dependent transcriptional silencing in primary oral SCC and in premalignant oral dysplastic lesions.

The first conclusion to be drawn from our studies is that genetic changes in $\sigma$ are uncommon in oral cancer, with a complete absence of mutations and a relatively low (18%) frequency of LOH in the gene. Inactivation of $\sigma$ in oral neoplasia, therefore, appears to occur almost exclusively by epigenetic, transcriptional silencing. Absence of mutations and low frequency of LOH are consistent with studies of breast cancer (10, 11) and other adenocarcinomas (12, 13). The common silencing of $\sigma$ seen strongly implies that loss of expression is an important event in malignant transformation in a proportion of oral SCC and suggests that $\sigma$ functions as a tumor suppressor gene in squamous as well as glandular epithelium. Furthermore, detection of methylated $\sigma$ DNA in dysplastic oral lesions suggests that epigenetic silencing of the gene occurs as an early event in a subset of oral SCCs.

**Characteristics of oral SCC with and without methylation of $\sigma$**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>p53 WT ($n = 55$)</th>
<th>p53 MT ($n = 37$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma$ methylated ($n = 32$)</td>
<td>24/55 (0.44)</td>
<td>8/37 (0.56)</td>
<td>0.074</td>
</tr>
<tr>
<td>$\sigma$ unmethylated ($n = 60$)</td>
<td>31/55 (0.56)</td>
<td>24/55 (0.44)</td>
<td></td>
</tr>
<tr>
<td>HPV +ve ($n = 17$)</td>
<td>17/17 (1.00)</td>
<td>17/17 (1.00)</td>
<td></td>
</tr>
<tr>
<td>HPV -ve ($n = 75$)</td>
<td>31/75 (0.41)</td>
<td>44/75 (0.59)</td>
<td>0.013</td>
</tr>
<tr>
<td>$\sigma$ methylated ($n = 32$)</td>
<td>16/17 (0.94)</td>
<td>17/17 (1.00)</td>
<td></td>
</tr>
<tr>
<td>$\sigma$ unmethylated ($n = 60$)</td>
<td>16/17 (0.94)</td>
<td>17/17 (1.00)</td>
<td></td>
</tr>
<tr>
<td>p16 meth. ($n = 38$)</td>
<td>25/32 (0.78)</td>
<td>7/32 (0.22)</td>
<td>0.001</td>
</tr>
<tr>
<td>p16 unmeth. ($n = 54$)</td>
<td>25/32 (0.78)</td>
<td>7/32 (0.22)</td>
<td></td>
</tr>
</tbody>
</table>

* p53 WT, wild-type p53; p53 MT, mutant p53; +ve, HPV positive; −ve, HPV negative; meth., methylated; unmeth., unmethylated.
As such, the loss of σ expression in premalignant lesions resembles the situation in breast neoplasia wherein methylated σ is detectable in a significant proportion of ductal carcinomas in situ (11). There is compelling evidence that inactivation of p16\(^{INK4a}\) is an important event in immortalization of keratinocytes (6, 7). Consistent with this, immortalization of primary keratinocytes by antisense σ is accompanied by down-regulation of p16\(^{INK4a}\) expression (5). In our series, the majority of oral SCCs with σ methylation also had methylation of p16\(^{INK4a}\). HPV sequences were detected in ~20% of the SCCs in our series, comparable with a previous large study (2), and cancers with concomitant methylation of σ and p16\(^{INK4a}\) were almost invariably HPV negative. In contrast, cases in which only p16\(^{INK4a}\) was methylated were both HPV positive and HPV negative. Taken together, these observations are consistent with the hypothesis that down-regulation of σ has effects equivalent to expression of E6 and E7 proteins of HPV (5), because these proteins cooperate to immortalize primary keratinocytes (20).

The observation that σ expression is induced by p53 (8) suggested that silencing of σ might represent a response to the presence of the wild-type protein in cancers lacking a mechanism for inactivating p53. One recognized mechanism of p53 inactivation is expression of HPV 16 E6 protein, which mediates ubiquitin-dependent proteolysis via E6-AP. It is of interest that cancers with methylated σ sequences were predominantly those lacking HPV DNA. This observation supports the hypothesis that abrogation of p53-dependent induction is, at least in part, the mechanistic basis for silencing of σ to predominantly target HPV-negative oral cancers. Analysis of the p53 sequence of the cases also supported this; SCC with σ methylation was more commonly wild-type for p53.

This raises the question of why some cancers containing p53 mutations also have σ methylation. One likely explanation is that mutation of p53 is required to abrogate transcriptional induction of other genes in the p53 pathway that cannot be or are not epigenetically silenced. p53 regulates expression of a large number of effector proteins including mediators of cell cycle arrest, differentiation, and apoptosis (21). Inactivation of p53, by mutation or other means, will abrogate induction of the entire p53-dependent program of gene expression and thereby have more profound effects than mutation or inactivation of individual downstream genes (21). An alternative but not mutually exclusive explanation is that silencing of σ is an early event in a subset of oral SCCs. Selection for p53 mutation would then be proposed to operate later during neoplastic progression to favor outgrowth of clones unable to undergo p53-dependent apoptosis or perhaps those expressing “gain of function” p53 mutants. Supporting this possibility, loss of σ is detectable in a high proportion of early breast lesions (11) and was detected in oral premalignant lesions in our series. What then is the role of σ down-regulation in squamous carcinomas? One obvious possibility is that σ loss is sufficient to immortalize squamous epithelium. As such, the role of silencing in squamous neoplasia would be analogous to that observed in vitro in primary keratinocytes (5). Detection of σ methylation in preneoplastic oral epithelium and preneoplastic vulval intraepithelial neoplasia is consistent with such a hypothesis (22). A further possibility is that silencing of σ is not required for immortalization but occurs at a stage after immortalization. Loss at this stage may result in impaired differentiation, altered response to apoptotic stimuli, or higher proliferation, because σ is known to have functions that regulate all of these characteristics (5, 23, 24). Resolution of these issues will require detailed immunocytochemical and molecular genetic characterization of large series of neoplastic and preneoplastic lesions.

Loss of σ expression sensitizes cells to γ irradiation and DNA-damaging chemotherapeutic agents (25). Optimal treatment of head and neck cancer remains controversial. In view of the common loss of σ in oral cancer, it will be of interest to determine whether cases with σ silencing show differences in response to treatment regimens based on radiotherapy or chemotherapy. Moreover, the increased radio- and chemosensitivity of σ−/− cells may facilitate identification of patients likely to derive greater benefit from specific strategies.

**REFERENCES**


Table 2 Mutations in p16\(^{INK4a}\) in oral carcinomas

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Change</th>
<th>σ status</th>
</tr>
</thead>
<tbody>
<tr>
<td>34, 35</td>
<td>2-bp deletion (GAgCG→TAGC)</td>
<td>Frameshift</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>26</td>
<td>1-bp deletion (GAg→Ag)</td>
<td>Frameshift</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>27</td>
<td>GAg→TAG</td>
<td>Glu→Ter</td>
<td>Unmethylated</td>
</tr>
</tbody>
</table>
Epigenetic Inactivation of 14-3-3 σ in Oral Carcinoma: Association with p16INK4a Silencing and Human Papillomavirus Negativity

Milena Gasco, Alexandra K. Bell, Victoria Heath, et al.


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/7/2072

This article cites 24 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/7/2072.full#ref-list-1

This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/7/2072.full#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/62/7/2072.
Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.