Overexpression of the DNA sensor proteins, absent in melanoma 2 and interferon-inducible 16, contributes to tumorigenesis of oral squamous cell carcinoma with p53 inactivation

Yuudai Kondo,1,2 Kentaro Nagai,1,2 Shingo Nakahata,2 Yusuke Saito,2 Tomonaga Ichikawa,2 Akira Suekane,2 Tomohiko Taki,3 Reika Iwakawa,4 Masato Enari,5 Masafumi Taniwaki,3 Jun Yokota,4 Sumio Sakoda1 and Kazuhiro Morishita2,6

1Division of Oral and Maxillofacial Surgery, Medicine of Sensory and Motor Organs, and 2Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, Miyazaki; 3Department of Hematology and Oncology, Kyoto Prefectural University of Medicine, Kyoto; 4Division of Multistep Carcinogenesis, and 5Division of Refractory Cancer, National Cancer Center Research Institute, Tokyo, Japan

(Received September 1, 2011 / Revised December 27, 2011 / Accepted December 29, 2011 / Accepted manuscript online February 9, 2012 / Article first published online February 23, 2012)

The development of oral squamous cell carcinoma (OSCC) is a multistep process that requires the accumulation of genetic alterations. To identify genes responsible for OSCC development, we performed high-density single nucleotide polymorphism array analysis and genome-wide gene expression profiling on OSCC tumors. These analyses indicated that the absent in melanoma 2 (AIM2) gene and the interferon-inducible gene 16 (IFI16) mapped to the hematopoietic interferon-inducible nuclear proteins. The 200-amino-acid repeat gene cluster in the amplified region of chromosome 1q23 is overexpressed in OSCC. Both AIM2 and IFI16 are cytoplasmic double-stranded DNA sensors for innate immunity and act as tumor suppressors in several human cancers. Knockdown of AIM2 or IFI16 in OSCC cells results in the suppression of cell growth and apoptosis, accompanied by the downregulation of nuclear factor kappa-light-chain-enhancer of activated B cells activation. Because all OSCC cell lines have reduced p53 activity, wild-type p53 was introduced in p53-deficient OSCC cells. The expression of wild-type p53 suppressed cell growth and induced apoptosis via suppression of nuclear factor kappa-light-chain-enhancer of activated B cells activity. Finally, the co-expression of AIM2 and IFI16 significantly enhanced cell growth in p53-deficient cells; in contrast, the expression of AIM2 and/or IFI16 in cells bearing wild-type p53 suppressed cell growth. Moreover, AIM2 and IFI16 synergistically enhanced nuclear factor kappa-light-chain-enhancer of activated B cells signaling in p53-deficient cells. Thus, expression of AIM2 and IFI16 may have oncogenic activities in the OSCC cells that have inactivated the p53 system. (Cancer Sci 2012; 103: 782–790)

Oral squamous cell carcinoma is commonly found in low-income communities. This cancer mainly affects older men; 90% of cases are in men over 45 years old who have been exposed to risk factors including tobacco and/or alcohol (International Agency for Research on Cancer [IARC] 2004). OSCC is the sixth most common cancer worldwide and affects approximately 270 000 people each year.1 The incidence and rate of mortality from OSCC are rising in several regions of Europe, Australia and Asia, including Japan. Despite recent progress in OSCC diagnosis and therapy, the 5-year survival rate has not improved in more than two decades.2

Oral carcinogenesis is a multifactorial cascade involving numerous genetic changes that affect the activity of oncogenes, tumor suppressor genes and other classes of disease-related genes. Chronic exposure to carcinogens, such as tobacco, causes genetic changes in the epithelial cells of the oral mucosa. The activation of the COX-2 (3) epidermal growth factor receptor,(13) and cyclin D1 oncogenes and the inactivation of the p16 and p53 tumor suppressor genes have also been reported in OSCC.5,7 In addition to tobacco smoke exposure, chronic alcohol use and chronic inflammation can both induce genetic alterations.7 The causative agent of cervical cancer, HPV is also reportedly associated with head and neck cancers, including OSCC.8 Compared to HPV-negative cases, HPV-positive OSCC have an intact p16 gene and wild-type p53, and harbor frequent genetic alterations of the p16 and p53 genes.9,10 The HPV oncoproteins E6 and E7 exploit the ubiquitin-proteasome system to degrade and functionally inactivate negative cell-regulatory proteins, including members of the p110 (Rb) family and p53; this process may primarily contribute to HPV-induced carcinogenesis.11

The innate immune system provides nonspecific protection and enhances the adaptive immune response against a variety of pathogens, including HPV. The IFI16 and AIM2 proteins were recently found to be innate immune sensors for cytosolic dsDNA. Upon sensing dsDNA, the IFI16 protein induces the expression of IFN-β, whereas the AIM2 protein forms an inflammasome that promotes the secretion of interleukin-1β.12,13 Both IFI16 and AIM2 belong to the HIN-200 gene family found on human and mouse chromosome 1; they are positively regulated by type I and II INF and have been described as regulators of cell proliferation, differentiation, apoptotic and inflammatory processes.14 The overexpression of IFI16 in cells inhibits cell proliferation by potentiating the p53/p21- and Rb/E2F-mediated inhibition of cell-cycle progression, and IFI16 downregulation contributes to oncogenicity.15,16 Also, AIM2 expression suppresses cell proliferation and tumorigenicity of human breast cancer cells.17 Therefore, it has been proposed that AIM2 and IFI16 function as tumor suppressor genes.

To identify genes involved in OSCC tumorigenesis, OSCC tumors were submitted to genomic analysis by high-density SNP array analysis. A number of amplified or deleted genomic regions in OSCC cells were identified, and a series of genes in the genetically altered regions were selected by expression
profile analysis. Of these genes, the NIH-200 gene family locus on chromosome 1q23 was frequently amplified, and AIM2 and IFI16 in the NIH-200 gene family were highly expressed in most OSCC tumors. Although AIM2 and IFI16 were reported to be tumor suppressors, the expression of AIM2 and IFI16 enhanced the cell growth of OSCC cell lines. Therefore, we describe a mechanism by which AIM2 and IFI16 may be functioning as oncogenes in OSCC.

**Materials and Methods**

Materials and Methods are given in Data S1 and Data S2 in the supporting information.

**Results**

**Higher expression of AIM2 and IFI16 with frequent amplification at 1q23 in OSCC.** To identify novel genes responsible for tumorigenesis in OSCC, we performed high-density SNP array analysis on 28 OSCC tumor samples using an Affymetrix Human Mapping 250K Sty Array (Affymetrix, Santa Clara, CA, USA). The most frequent gains involved segments of chromosomes 1, 3q, 5p, 6p, 7p, 8q, 9q, 14, 15, 16, 17, 19, 20 and 22, whereas the most frequent losses involved segments of chromosomes 3p, 4, 5q, 8p, 10p, 18q and 21q (Fig. S1). Of these, 77 were amplified and ranged in size from 0.3 to 49.3 Mb, and four were found to be deleted and ranged in size from 0.2 to 0.6 Mb, in more than 14 of 28 OSCC tumor samples (Table S1).

To select candidate genes within the regions with altered copy numbers, we analyzed a data set from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), which contained the gene expression data of four oral tissue samples from healthy volunteers and 16 tumor samples from OSCC patients. Of the genes in the amplified regions, 27 were expressed at more than two-fold higher levels (P < 0.01) (Table S1-1). However, no genes that were downregulated more than two-fold were identified in the deleted region (Table S1-2). Interestingly, 15 of the 27 candidates were previously reported to be cancer-related genes. The genes linked to OSCC included keratin 19, lectin, galactoside-binding, soluble, 1 and S1 2). Interestingly, 15 of the 27 candidates were previously (Table S1-1). However, no genes that were downregulated and HIN domain family, member 1 (MNDA), were observed (Fig. S2a). Furthermore, five of the 27 upregulated genes were also found to be IFI1 genes, including IFI16, IFI35, AIM2, IFI16 and bone marrow stromal cell antigen-2. Among them, the AIM2 and IFI16 genes are located within the HIN-200 gene cluster on 1q23 and have been identified as a new family of innate immune DNA sensors for intracellular DNA called IFI16-like receptors. Because chronic inflammation and infection contribute to the development of several types of cancer, we analyzed the HIN-200 gene cluster locus on 1q23.

Based on the SNP array analysis from 28 OSCC cases, four members of the HIN-200 family, along with other 19 genes, were located in the 1.3-Mb common region of amplification at 1q23 (Fig. 1a). The expression profiles showed that IFI16 and AIM2 are highly expressed in OSCC, but no significant differences in the expression levels of the other 21 genes, including myeloid cell nuclear differentiation antigen (MNDA) and pyrin and HIN domain family, member 1 (PYHIN), were observed between the OSCC and control oral tissues (Fig. S2a-c). Using semi-quantitative and quantitative real-time PCR, we confirmed statistically significant higher expression of AIM2 and IFI16 in tumor samples from OSCC patients and OSCC cell lines (P < 0.05) (Figs 1b-e, S2d). Moreover, the expression of AIM2 was significantly higher in the group with metastasis (N1) than in that without metastasis (N0) (P < 0.05). To confirm the relationship between genomic amplification and mRNA expression levels of AIM2 and IFI16, a scatter plot was used to evaluate the correlation between the two variables in 20 OSCC tumor samples and eight cell lines. As shown in Figure 1(f), we found weak but positive correlations between the DNA copy numbers and mRNA expression levels for the two genes. Although a few cases did not show gene amplification or overexpression of the AIM2 and IFI16 genes, the majority of cases presented gene amplification and high expression of these two genes. Thus, in the HIN-200 family of genes, AIM2 and IFI16 are overexpressed in OSCC, and this overexpression is frequently accompanied by gene amplification.

**High expression of AIM2 and IFI16 enhanced cell growth by preventing apoptosis in OSCC cells.** An important inflammatory component, AIM2 senses potentially dangerous cytoplasmic DNA and regulates caspase-1 activation. Cytoplasmic overexpression of AIM2 also reportedly reduces cell proliferation and increases susceptibility to cell death in transfected murine fibroblasts. To determine whether the high expression levels of AIM2 or IFI16 have an effect on OSCC cell growth, we introduced a shRNA expression vector against AIM2 (shAIM2), IFI16 (shIFI16) or luciferase (shLuc) as a control into the human OSCC cell line SAS. SAS cells expressing the shRNA for either AIM2 (SAS/shAIM2) or IFI16 (SAS/shIFI16) had decreased growth rates relative to control-transfected cells (SAS/shLuc) (Fig. 2a-b). Notably, downregulation of both AIM2 and IFI16 expression had the most significant effect on growth inhibition. Similar effects were observed in the HSC4-OSCC cell line (Fig. S3). Next, apoptosis and cell cycle were investigated by flow cytometry with propidium iodide (PI) and Annexin V, respectively. The cell cycle profiles of SAS/shAIM2 and SAS/shIFI16 cells were not significantly different from those of the control SAS/shLuc cells, but the SAS/shAIM2 and SAS/shIFI16 cells exhibited a higher percentage of cell death (sub-G1 population) than the SAS/shLuc cells (Figs 2c, S4). In the SAS/shAIM2 and SAS/shIFI16 cells, the percentage of cells that bound Annexin V increased approximately 10- and 3-fold, respectively, compared with the SAS/shLuc cells (Fig. 2d). These data suggest that high expression of AIM2 and IFI16 enhances cell survival by preventing OSCC cells from entering apoptosis.

**Activation of NF-κB signaling by AIM2 and IFI16 in OSCC.** Once bound to the DNA in the cytoplasm, AIM2 activates both NF-κB and caspase-1 (15), and cytosolic DNA also triggers NF-κB activation by IFI16. To clarify the mechanisms by which constitutive expression of AIM2 and/or IFI16 prevent apoptosis in OSCC cells, we studied caspase-1 and NF-κB for constitutive activation in OSCC. Initially, the SAS-OSCC cell lines transfected with the shLuc, shIFI16 or shAIM2 vectors were examined for the expression of cleaved caspase-1 by immunoblot analysis using a cleaved caspase-1 specific antibody. Cleaved caspase-1 was not detected in any of these cell lines but was detected in the human acute monocytic leukemia cell line (THP-1) transfected with poly deoxyadenylyl-deoxythymidylic acid (poly[dA:dT]) as a positive control (Fig. S5a). The cleaved form of caspase-1 was also not detected in seven of the eight OSCC cell lines except for HOSQ89 (data not shown). In addition, the treatment of SAS-OSCC cells with poly[dA:dT] had no effect on caspase-1 cleavage (Fig. S5b), suggesting that dsDNA could not trigger the formation of the AIM2 inflammasome in OSCC cells.

To assess NF-κB activation in OSCC, we measured 1xB protein in eight OSCC cell lines and 10 primary OSCC tumors by immunoblot analysis. We observed significantly higher levels of phosphorylated 1xB and lower levels of total 1xB in most OSCC cell lines and primary tumor samples compared to control gingival tissues (Fig. 3a). This result suggests that NF-κB signaling is often activated in OSCC. To confirm this hypothesis, four OSCC cell lines (HOSQ89, HSC3, HSC4 and SAS) were treated with various concentrations of the NF-κB inhibitor.
inhibitor Bay 11-7082 for 48 h and examined for cell viability. In three of the four cell lines (not HSQ89), cell viability was inhibited by Bay 11-7082 treatment, although the effect varied among the cell lines (Fig. 3b). To determine whether the observed cell death was due to apoptosis, two cell lines, SAS and HSC3, treated with or without Bay 11-7082 were stained with Annexin V/PI and analyzed by flow cytometry. Over 90% of the OSCC cell lines underwent apoptosis 48 h after treatment with 10⁻¹ M Bay11-7082 (Figs 3c, S6). Moreover, there was a dose-dependent increase in the total IκBα and decrease in the phosphorylated IκBα levels after exposure to 1–10-µM Bay11-7082 (Fig. 3d). To determine whether high expression of AIM2 and/or IFI16 contributes to the constitutive NF-κB activation in OSCC, SAS cells were transfected with either shIF116 and/or shAIM2 vectors and analyzed for IκBα expression. As expected, the SAS cells treated with shIF116, shAIM2 or both shRNA significantly increased protein levels of phosphorylated-IκBα and reduced the total IκBα levels compared with control cells transfected with shLuc and parental cells (Fig. 3e). The reduction of NF-κB activation by shIF116 or

**Fig. 1.** Overexpression of IFI16 and AIM2 mRNA in OSCC. (a) Recurrent genetic changes are depicted based on the copy number analyzer for GeneChip (CNAG) output of the single nucleotide polymorphism array analysis of 28 oral squamous cell carcinoma (OSCC) samples, which include gains at the IFI16 and AIM2 loci on 1q23. Regions showing copy number gains are indicated by horizontal lines. AIM2, absent in melanoma 2; APCS, amyloid P component; CADM3, cell adhesion molecule 3; CCDC19, coiled-coil domain containing 19; CRP, C-reactive protein, pentraxin-related; DARC, Duffy blood group, chemokine receptor; DUSP23, dual specificity phosphatase 23; FCER1A, Fc fragment of IgE, high affinity I, receptor for: alpha polypeptide; FCRL6, Fc receptor-like 6; IFI16, interferon, gamma-inducible protein 16; IGSF9, immunoglobulin superfamily, member 9; KCNJ9, potassium inwardly-rectifying channel, subfamily J, member 9; KCN10, potassium inwardly-rectifying channel, subfamily J, member 10; MND2, myeloid cell nuclear differentiation antigen; OR10J1, olfactory receptor, family 10, subfamily J, member 1; OR10J3, olfactory receptor, family 10, subfamily J, member 3; OR10J5, olfactory receptor, family 10, subfamily J, member 5; PIGM, phosphatidylinositol glycan anchor biosynthesis, class M; PYHIN1, pyrin and HIN domain family, member 1; SLAMF8, SLAM family member 8; SLAMF9, SLAM family member 9; TAGLN2, transgelin 2; VSIG8, V-set and immunoglobulin domain containing 8, (b,d) Expression of the AIM2 and (c,e) IFI16 mRNA were examined in 11 primary OSCC and eight OSCC cell lines (Ca9-22, Hoc1u1, HSC2, HSC3, HSC4, HSQ89, SAS and Sa3) by quantitative real-time PCR. Normal gingival tissues from normal volunteers were used as controls. (f) Scatter plot of DNA copy number versus mRNA expression for AIM2 (left) and IFI16 (right). Correlations were quantified using the Spearman’s rank correlation coefficient. AIM2, absent in melanoma; HIN-200, Hematopoietic interferon-inducible nuclear proteins with a 200-amino-acid repeat; IFI16, interferon-inducible 16; N0, without metastasis; N1, with metastasis.

© 2012 Japanese Cancer Association
ShAIM2 was also confirmed by NF-κB-dependent luciferase reporter activity assay (Fig. 3f). The overexpression of IFI16 and AIM2 may enhance IκBα kinase activity and promote the degradation of IκBα and NF-κB activation, leading to the acceleration of cell growth in OSCC cells.

Restoration of p53 function inhibits constitutive NF-κB activation in OSCC cells. Studies have reported that the overexpression of HIN-200 proteins can decrease cell proliferation and block cell cycle progression at the G1-S phase transition. It has been shown that IFI16-mediated growth arrest is partly dependent on

Fig. 2. Effects of knockdown of AIM2 and IFI16 expression on the growth of OSCC cells. (a) Retroviral constructs containing shRNA against AIM2 and/or IFI16 or luciferase (Luc) as a control were transfected into SAS cells. Forty-eight hours after transfection, ZsGreen-positive cells were sorted, and total RNA was extracted to analyze expression levels of AIM2 and IFI16 by RT-PCR. (b) The growth of sorted cells was analyzed with MTT. The data are shown as the mean ± SD of triplicate samples. The statistical analysis was performed using the Student’s t-test (*P < 0.01). (c) Cell cycle phase distribution was analyzed by FACS with PI staining. Each cycle phase distribution was analyzed by the cell recruited into the cell cycle. (d) The percentage of sorted cells undergoing apoptosis was quantitated by Annexin V staining and FACS. The lower bar graph shows the mean ± SD from three independent experiments. An asterisk indicates a statistically significant difference (P < 0.01). AIM2, absent in melanoma; IFI16, interferon-inducible 16.
the function of p53. Because a high frequency of mutations in p53 was noted in OSCC, we determined whether p53 dysfunction results in abrogation of the growth suppressive effects of AIM2 and IFI16 in OSCC cells. Initially, we determined the expression and genomic alterations of p53 in eight OSCC cell lines. All cell lines showed normally high expression levels and/or point mutations of p53 (Table S2-1, Doc. S2) and five (Ca9-22, HSC4, HSQ89, SAS and Sa3) expressed a detectable level of p53 protein, including a truncated form of p53 (Fig. 4a)(20,21) In primary OSCC samples, five out of 11 tumors had abnormally high expression levels and/or point mutations of p53 (data not shown) (Table S2-2). We introduced the wild-type p53 expression vector into the SAS cell line and confirmed (24) with various combinations of the AIM2, IFI16 and p53 expression vectors. The transfection of AIM2, IFI16, or p53 alone had no significant effect on the growth rate of H1299 cells (Fig. 5a,b). However, co-transfection of AIM2 and IFI16 strongly promoted cell proliferation with increased IκB phosphorylation. Strikingly, the growth-promoting effect of AIM2 and IFI16 was abrogated in the presence of wild-type p53. To further confirm these results, we performed cell proliferation assays using the human mammary tumor cell line MCF-7 expressing wild-type p53, which has been used to study the role of IFI16 in p53-dependent apoptosis. Transfection with either or both of the AIM2 and IFI16 expression vectors retarded the growth rate of MCF-7 cells. The shRNA inhibition of p53 expression caused a slight increase in cell growth rate (Fig. 5c,d). Importantly, co-transfection of AIM2 and IFI16 with a shRNA expression vector for p53 led to an approximately two-fold higher proliferation rate with significant upregulation of phosphorylated IκBα. Thus, the simultaneous high expression of AIM2 and IFI16 confers a proliferative advantage in cells with functionally inactive p53, in part, through the activation of NF-κB signaling.

Finally, we examined whether co-expression of both AIM2 and IFI16 can activate NF-κB in an OSCC cell line. Very low expression levels of AIM2 and IFI16 and a low level of NF-κB signaling was observed in the HSC4 cell line treated with the indicated concentration of Bay 11-7082 for 48 h (Fig. 3a). The percentage of apoptotic cells was measured by FACS analysis in two normal gingival tissue samples, eight OSCC cell lines (Ca9-22, Ho1u1, HSC2, HSC3, HSC4, HSQ89, SAS and Sa3) and 10 OSCC primary tumors by Western blot analysis. (b) Four OSCC cell lines were treated with various concentrations of Bay 11-7082 for 48 h, and viable cell numbers were counted by MTT assay. The results are shown as percentages of the values obtained from the control Bay 11-7082-free culture. The data are shown as the means ± SD of triplicate samples. (c) The percentage of apoptotic cells was measured by FACS after Annexin V/PI staining in untreated and 10-μM Bay 11-7082-treated SAS and HSC3 cell lines at 48 h. (d) Levels of phosphorylated IκBα and total IκBα in the HSC4 cell line treated with the indicated concentration of Bay 11-7082 for 48 h were examined by Western blot analysis. (e) The SAS cell transfectants described in Figure 2 were examined for the levels of phosphorylated IκBα and total IκBα by Western blot analysis. (f) The SAS cell transfectants were co-transfected with the NF-κB-Luc reporter and an internal control Renilla luciferase (pRL-TK) plasmid. After 36 h, luciferase activities were measured with a dual-luciferase reporter assay system. The data are shown as mean ± SD of triplicate transfections, and statistical analysis used the Student’s t-test. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

The overexpression AIM2 and IFI16 synergistically promotes cell growth only in the absence of functional p53. To determine whether a lack of functional p53 is associated with the growth-promoting effect of AIM2 and IFI16 in OSCC cells, we transfected human lung cancer H1299 cells, which lack endogenous p53, with various combinations of the AIM2, IFI16 and p53 expression vectors. The transfection of AIM2, IFI16, or p53 alone had no significant effect on the growth rate of H1299 cells (Fig. 5a,b). However, co-transfection of AIM2 and IFI16 strongly promoted cell proliferation with increased IκBα phosphorylation. Strikingly, the growth-promoting effect of AIM2 and IFI16 was abrogated in the presence of wild-type p53. To further confirm these results, we performed cell proliferation assays using the human mammary tumor cell line MCF-7 expressing wild-type p53, which has been used to study the role of IFI16 in p53-dependent apoptosis. Transfection with either or both of the AIM2 and IFI16 expression vectors retarded the growth rate of MCF-7 cells. The shRNA inhibition of p53 expression caused a slight increase in cell growth rate (Fig. 5c,d). Importantly, co-transfection of AIM2 and IFI16 with a shRNA expression vector for p53 led to an approximately two-fold higher proliferation rate with significant upregulation of phosphorylated IκBα. Thus, the simultaneous high expression of AIM2 and IFI16 confers a proliferative advantage in cells with functionally inactive p53, in part, through the activation of NF-κB signaling.

Finally, we examined whether co-expression of both AIM2 and IFI16 can activate NF-κB in an OSCC cell line. Very low expression levels of AIM2 and IFI16 and a low level of NF-κB signaling was observed in the HSC4 cell line treated with the indicated concentration of Bay 11-7082 for 48 h (Fig. 3a). The percentage of apoptotic cells was measured by FACS analysis in two normal gingival tissue samples, eight OSCC cell lines (Ca9-22, Ho1u1, HSC2, HSC3, HSC4, HSQ89, SAS and Sa3) and 10 OSCC primary tumors by Western blot analysis. (b) Four OSCC cell lines were treated with various concentrations of Bay 11-7082 for 48 h, and viable cell numbers were counted by MTT assay. The results are shown as percentages of the values obtained from the control Bay 11-7082-free culture. The data are shown as the means ± SD of triplicate samples. (c) The percentage of apoptotic cells was measured by FACS after Annexin V/PI staining in untreated and 10-μM Bay 11-7082-treated SAS and HSC3 cell lines at 48 h. (d) Levels of phosphorylated IκBα and total IκBα in the HSC4 cell line treated with the indicated concentration of Bay 11-7082 for 48 h were examined by Western blot analysis. (e) The SAS cell transfectants described in Figure 2 were examined for the levels of phosphorylated IκBα and total IκBα by Western blot analysis. (f) The SAS cell transfectants were co-transfected with the NF-κB-Luc reporter and an internal control Renilla luciferase (pRL-TK) plasmid. After 36 h, luciferase activities were measured with a dual-luciferase reporter assay system. The data are shown as mean ± SD of triplicate transfections, and statistical analysis used the Student’s t-test. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

Fig. 3. Activation of NF-κB signaling in oral squamous cell carcinoma (OSCC). (a) The levels of total and phosphorylated (Ser32/36) inhibitor of kappa B alpha (IκBα) were examined in two normal gingival tissue samples, eight OSCC cell lines (Ca9-22, Ho1u1, HSC2, HSC3, HSC4, HSQ89, SAS and Sa3) and 10 OSCC primary tumors by Western blot analysis. (b) Four OSCC cell lines were treated with various concentrations of Bay 11-7082 for 48 h, and viable cell numbers were counted by MTT assay. The results are shown as percentages of the values obtained from the control Bay 11-7082-free culture. The data are shown as the means ± SD of triplicate samples. (c) The percentage of apoptotic cells was measured by FACS after Annexin V/PI staining in untreated and 10-μM Bay 11-7082-treated SAS and HSC3 cell lines at 48 h. (d) Levels of phosphorylated IκBα and total IκBα in the HSC4 cell line treated with the indicated concentration of Bay 11-7082 for 48 h were examined by Western blot analysis. (e) The SAS cell transfectants described in Figure 2 were examined for the levels of phosphorylated IκBα and total IκBα by Western blot analysis. (f) The SAS cell transfectants were co-transfected with the NF-κB-Luc reporter and an internal control Renilla luciferase (pRL-TK) plasmid. After 36 h, luciferase activities were measured with a dual-luciferase reporter assay system. The data are shown as mean ± SD of triplicate transfections, and statistical analysis used the Student’s t-test. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.
activation were found in HSQ89 with p53 mutation. Although transfection of either AIM2 or IFI16 alone did not have a significant effect on NF-κB activation and cell growth, co-transfection of AIM2 and IFI16 resulted in accelerated cell proliferation, a decrease in total IκBa, and an increase in phosphorylated IκBa (Fig. 5e,f). This result suggests that co-expression of AIM2 and IFI16 activates NF-κB signaling in OSCC cells. Taken together, these results suggest that co-expression of AIM2 and IFI16 can promote cell proliferation through activation of NF-κB signaling pathway in the absence of p53.

Discussion

The oral cavity contains some of the most varied and extensive flora in the entire human body. A member of a group of DNA-based viruses, HPV infects the skin and mucous membranes within the human body. Infection with HPV 16 and 18 increases the risk of oral cavity cancer and oropharyngeal cancer. Because the interferon-inducible AIM2 and IFI16 genes act as innate immune sensors for cytosolic double-stranded DNA, the expression of AIM2 and/or IFI16 might be activated by recurrent infections in the oral cavity. In this study, we showed that constitutive high expression levels of AIM2 and IFI16, along with other interferon-inducible genes, were associated with genomic alterations in OSCC. Upregulation of a series of interferon-inducible genes and enhancement of interferon-signaling pathways has previously been reported in OSCC by protein expression analysis using tandem mass spectrometry or by mRNA expression profile analysis using a DNA microarray. The expression of the interferon-inducible gene is an important characteristic of OSCC. Moreover, OSCC cells were shown to become resistant to IFN-β-mediated inhibition of cell growth. The constitutive expression of the interferon-inducible genes may affect the development of OSCC, and the expres-
Fig. 5. Expression of AIM2 and IFI16 confers growth-stimulating effects on transformed cells in the absence of wild-type p53. (a) The growth of human lung cancer H1299 cells after transfection with various combinations of AIM2, IFI16 and wild-type p53 (SN3-p53wt) expression vectors was examined by MTT assay. The data are shown as the mean ± SD of triplicate samples. The statistical analysis was performed using the Student’s t-test (*P < 0.05; **P < 0.01). (b) Western blot analysis of extracts from untransfected or transfected H1299 cells described in panel (a) at 48 h. (c) The growth of human mammary tumor MCF7 cells after transfection with various combinations of the AIM2, IFI16 and shp53 expression vectors was examined by MTT assay. The data are shown as the mean ± SD of triplicate samples. The statistical analysis was performed using the Student’s t-test (*P < 0.05; **P < 0.01). (d) Western blot analysis of extracts from untransfected or transfected MCF7 cells described in panel (c) at 48 h. (e) The growth of HSQ89 cells after transfection with AIM2 and/or IFI16. (f) Western blot analysis of extracts from untransfected or transfected HSQ89 cells described in panel (e) at 48 h. AIM2, absent in melanoma; IκBα, inhibitor of kappa B alpha; IFI16, interferon-inducible 16.
Because the loss of p53 and/or IFI16 function in cells a component of the positive feedback loop between p53 and OSCC. In normal aged human cells, increased levels of the IFI16 and IFI16 enhanced the cell growth and prevented apoptosis in suppressors in a series of cancers, high expression levels of AIM2 NF-κBα nuclear factor kappa-light-chain-enhancer of activated B cells OSCC oral squamous cell carcinoma SNPs single-nucleotide polymorphism.

References
1 Shah JP, Singh B. Keynote comment: why the lack of progress for oral cancer? Lancet Oncol 2006; 7: 356–7.
2 Gibson MK, Forastiere AA. Reassessment of the role of induction chemotherapy for head and neck cancer. Lancet Oncol 2006; 7: 565–74.
3 Perez-Sayans M, Somoza-Martín JM, Barros-Angueira F, Reboiras-Lopez MD, Gandara Rey JM, Garcia-Garcia A. Genetic and molecular alterations associated with oral squamous cell cancer (Review). Oral Rep 2009; 22: 1277–82.
4 Rogers SJ, Harrington KJ, Phsys-Evans P, P OC, Eccles SA. Biological significance of c-erbB family oncogenes in head and neck cancer. Cancer Metastasis Rev 2005; 24: 47–69.
5 Rousseau A, Lim MS, Lin Z, Jordan RC. Frequent cyclin D1 gene amplification and protein overexpression in oral epithelial dysplasia. Oral Oncol 2001; 37: 268–75.
6 Reed AL, Califano J, Cairns P et al. High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res 1996; 56: 3630–3.
7 Choi S, Myers JN. Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. J Dent Res 2008; 87: 14–32.
8 Sisk EA, Solity SG, Zha S, Fisher SG, Carey TE, Bradford CR. Human papillomavirus and p53 mutational status as prognostic factors in head and neck carcinoma. Head Neck 2002; 24: 841–9.
9 Min BM, Baek JH, Shin KH, Gujuluva CN, Cherrick HM, Park NH. Inactivation of the p53 gene by either mutation or HPV infection is extremely frequent in human oral squamous cell carcinoma cell lines. Eur J Cancer B Oral Oncol 1994; 30B: 338–45.
10 Boyer SN, Wazer DE, Band V. E7 protein of human papillomavirus virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res 1996; 56: 4620–4.
11 Feller L, Wood NH, Khammisa RA, Lemberm J. Human papillomavirus-mediated carcinogenesis and HPV-associated oral and oropharyngeal squamous cell carcinoma. Part 1: human papillomavirus-mediated carcinogenesis. Head Face Med 2010; 6: 14.
12 Unterholzer L, Keating SE, Baran M et al. IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol 2010; 11: 997–1004.
13 Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 2009; 458: 509–13.
14 Asea B, Klarman KD, Copeland NG, Gilbert DJ, Jenkins NA, Keller JR. The interferon-inducible p200 family of proteins: a perspective on their roles in cell cycle regulation and differentiation. Blood Cells Mol Dis 2004; 32: 155–67.
15 Ludlow LE, Johnstone RW, Clarke CJ. The HIN-200 family: more than interferon-inducible genes? Exp Cell Res 2005; 308: 1–17.

Disclosure Statement
The authors have no conflict of interest to declare.

Abbreviations

AIM2 absent in melanoma 2

HIN-200 hematopoietic interferon-inducible nuclear proteins with a 200-amino-acid repeat

HPV human papillomavirus

IFN interferon

IF0 interferon

AIM2 absent in melanoma 2

NK-κBα nuclear factor kappa-light-chain-enhancer of activated B cells

OSCC oral squamous cell carcinoma

SNPs single-nucleotide polymorphism

Kondo et al.
Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Genome-wide measurement of DNA copy number alterations in oral squamous cell carcinoma (OSCC).

**Fig. S2.** IFI16 and AIM2 are upregulated in oral squamous cell carcinoma (OSCC).

**Fig. S3.** Downregulation of either AIM2 or IFI16 expression decreases the growth rate of HSC4 cells.

**Fig. S4.** Downregulation of either AIM2 or IFI16 expression increases apoptosis in SAS cells.

**Fig. S5.** Caspase-1 is not activated in oral squamous cell carcinoma cells.

**Fig. S6.** Treatment with Bay 11-7082 induced apoptosis in oral squamous cell carcinoma (OSCC) cells.

**Table S1.** Summary of DNA copy number aberrations in 28 oral squamous cell carcinoma (OSCC) tumors.

**Table S2.** p53 status in primary oral squamous cell carcinoma tumors (OSCC) and cell lines.

**Data S1.** Materials and methods.

**Data S2.** Additional references in Table S2 and Data S1.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.