Epigenetic Inactivation of IκB Kinase-α in Oral Carcinomas and Tumor Progression

Genta Maeda,[1,2] Tadashige Chiba,[1] Shuichi Kawashiri,[3] Tazuko Satoh,[2] and Kazushi Imai[1]

Abstract

Purpose: The loss of epithelial phenotypes in the process of carcinoma progression correlates with clinical outcome, and genetic/epigenetic changes accumulate aggressive clones toward uncurable disease. IκB kinase-α (IKKα) has a decisive role in the development of the skin and establishes keratinocyte phenotypes. We assessed clinical implications of IKKα expression in oral carcinomas and epigenetic aberrations for the loss of expression.

Experimental Design: We examined IKKα expression in oral carcinomas by immunostaining ($n = 64$) and genetic instability by microsatellite PCR ($n = 46$). Promoter methylation status was analyzed by bisulfite-modified sequence ($n = 11$).

Results: IKKα was expressed in the nucleus of basal cells of normal oral epithelium, but not or marginally detected in 32.8% of carcinomas. The immunoreactivity was significantly decreased in less differentiated carcinomas ($P < 0.05$) and correlated to long-term survival of patients ($P < 0.01$) with an independent prognostic value ($P < 0.05$). Although allelic/biallelic loss of the gene was limited to four cases, we detected microsatellite instability in 63.0% cases in which the immunoreactivities were decreased and the promoter was hypermethylated.

Conclusion: These results showed that oral carcinomas exhibiting genetic instability and promoter hypermethylation down-regulate expression of IKK and suggest that the epigenetic loss of the expression closely associates with disease progression toward unfavorable prognosis.

Squamous cell carcinoma is the most common neoplasm of the oral cavity, and worldwide, the annual incidence of new cases exceeds 300,000. The disease causes great morbidity, and the 5-year survival rate has not sufficiently improved in more than two decades (1, 2). With few exceptions, carcinomas are derived from single somatic cells and their progeny. Carcinoma cells in the emerging neoplastic clone accumulate within them genetic and/or epigenetic changes that are subjected to selection for tumor progression (3). Multiple epigenetic alterations have been characterized during the progression and may trigger alterations in regulatory sequences of correct gene expression. Inappropriate gene activation and inactivation are typical for carcinoma cells and predispose tumors to a more advanced state of progression (4–6). Identifying the genes that contribute to phenotypic alterations of tumor cells provides insights into understanding the mechanism of disease progression.

Authors' Affiliations: Departments of [1]Biochemistry and [2]Oral Surgery, School of Life Dentistry at Tokyo, The Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo, Japan; and [3]Department of Oral Surgery, Kanazawa University, 13-1 Takaramachi, Kanazawa, Japan

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Requests for reprints: Kazushi Imai, Department of Biochemistry, School of Life Dentistry, The Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan. Phone: 81-3-3261-8870; Fax: 81-3-3261-8875; E-mail: KIMAI@Tokyo.ndu.ac.jp.

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Structural aberration at chromosome 10q24 is frequently found in many types of carcinomas, and this region is suggested to carry a tumor suppressor gene (7, 8). In particular, chromosomal deletion at this position has been described in squamous cell carcinomas (nasal and oral cavities, larynx, lung, and vagina) by the National Cancer Institute Cancer Genome Anatomy Project.[4] Numbers of investigations represent that *PTEN*, which locates at chromosome 10q23, suppresses initiation and progression of tumors (9). However, chromosomal aberration at the *PTEN* locus is a rare event and does not correlate with progression of oral carcinomas (10, 11). Therefore, it is attractive to speculate that there is an unidentified gene(s) closely linked to tumor suppression in and around 10q24.

IκB kinases (IKK) consist of three members, including IKKα (IKK1, CHUK), IKKβ (IKK2), and IKKγ (NEMO). Oligomerized IKK complex phosphorylates IκB-α, leading to dissociation from IκB-α and translocation of nuclear factor κB (NFκB) to the nucleus. NFκB binds to the κB element in the target gene promoter region and regulates the transcription. Although accumulating evidence emphasizes that NFκB activation promotes progression and therapeutic resistance of head and neck squamous cell carcinomas (12–14), activation of NFκB canonically depends on the kinase activity of IKKβ (15, 16). IKKα, which is located at chromosome 10q24.31, has been shown to have an uncharacterized role in the epithelial cell fates independent of NFκB. Gene-targeting mouse models show that the loss of *IKKα* perturbs the differentiation program of the epidermis (17–19). Thus, it is widely predicted that IKKα takes

[4] http://cgap.nci.nih.gov/Chromosomes/Mitelman

a decisive role in the epithelial cell phenotype(s), and it seems likely that the expression status of IKKα has an impact on the phenotypic definition of carcinoma cells of the epithelial origin. However, little is known about the expression and role of IKKα in solid tumors. In the present study, we examined the expression and clinical implications of IKKα in oral squamous cell carcinomas and the mechanism of inactivation of *IKKα* expression.

Materials and Methods

Cell lines and tissue samples. Immortalized oral carcinoma cell lines, human skin keratinocytes (HaCaT), and primary cultured normal gingival keratinocytes were used (4). Incisional or excisional biopsy specimens from 64 patients with primary oral squamous cell carcinomas were collected from the files of Kanazawa University Hospital and were subjected to experiments that were reviewed and approved by the Institutional Review Boards of the Nippon Dental University and Kanazawa University. Patients underwent surgery (n = 55) or radiation and surgery (n = 9), and their clinical and pathologic data were summarized in Table 1. The median age of the study patients was 63.2 years (range, 41-93 years) at the time of diagnosis. Histologic grading and staging were assessed according to the 1987 International Union against Cancer tumor-node-metastasis classification (20). Control normal oral tissues (n = 5) were also used (4).

Reverse transcription-PCR. Total RNA were reverse transcribed and subjected to semiquantitative PCR (21) using gene-specific primer sets for *IKKα* or *GAPDH*: for *IKKα*, forward 5′-TTGGAGAGATACAG-CCAGCAGA-3′ and reverse 5′-CCAATGACACCAACCTCAGCAT-3′; and for *GAPDH*, forward 5′-GTCAGTGGTGGACCTGACCT-3′ and reverse 5′-AGGGGAGATTCAGTGTGGTG-3′.

Immunoblotting. Nuclear and cytoplasmic fractions or total cell lysates (5 μg protein) of HaCaT cells were used for immunoblotting with a standard protocol. The membrane was probed with antibodies specific to IKKα (Cell Signaling Technology), involucrin (Sigma), cytokeratin 10 (Progen Biotechnik GmbH), histone H3 (Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ambion), or β-actin (Sigma).

Immunostaining. Carcinoma tissue sections were reacted with anti-IKKα antibody, and the percentage of positive staining was evaluated by counting at least 3,000 carcinoma cells in randomly selected areas of each specimen. They were blinded as to the clinicopathologic parameters and verified by two independent observers (G.M. and K.I.).

DNA extraction. Microdissection and DNA extraction from 8-μm-thick paraffin-embedded tissue sections (n = 46) were carried out using a laser capture microdissection apparatus (Leica Microsystems). Tumor cells and adjacent normal cells were separately microdissected to neglect DNA contamination, and genomic DNA was extracted by a standard protocol (22) and quantified by a spectrophotometer.

Microsatellite markers and PCR. Genetic instability or allelic imbalance was determined by genomic PCR using microsatellite markers AFM205tg7, D10S603, and AFMA086wg9 (at *PTEN* intron 2) around the *IKKα* locus. Because there are no established microsatellite markers within the *IKKα* locus, we made designed intragenic three makers (IKKαe1 at intron 1, IKKαe2 at intron 2, and IKKαe3 at intron 5) spanning (TG)n, (GA)n, and (TT)n, respectively: for IKKαe1, forward 5′-GGGGACTGCCTTTGGGAATG-3′ and reverse 5′-CCATGCT-TCCAAAGGCTTAACTCTG-3′; for IKKαe2, forward 5′-GAAGCTAGCTT-TCCCCAGAA-3′ and reverse; 5′-TGGAAAAGGATGGAGTCTCAC-3′; and for IKKαe3, forward 5′-CAGTGGTTCCATCAGAGAAC-3′and reverse 5′-CAGCCTGTATCTTTGCTAGA-3′. Genomic DNA (20 ng) was amplified in a volume of 20 μL by 35 cycles of touchdown PCR (denaturation for 45 s at 94°C, annealing for 1 min, and elongation for 1 min at 72°C). Annealing temperature was started at 66°C and lowered 1°C per cycle. About 15 μL of the amplified products were electrophoresed through acrylamide gels that were stained with Vistra Green (Amersham Bioscience) and scanned by Typhoon 9410 Image Analyzer (Amersham Biosciences).

Analysis of the loss of heterozygosity and microsatellite instability. The allelic profile was initially scored for microsatellite instability (MSI) according to the criteria for alterations in allelic length. If novel allelic bands that were not observed in normal DNA were found in the corresponding tumor DNA, they were interpreted as MSI. Sometimes, it was not possible to distinguish between MSI and the loss of heterozygosity (LOH), especially in cases with intimately close heterozygous allelic bands. A signal reduction in one of two alleles might result not only from allelic loss but also from allelic shift attributable to comigration with an adjacent allele. Thus, MSI was considered to be present when the amplified tumor DNA contained multiple bands or bands that differed from those seen in DNA from normal cells.

Because of the nature of microsatellite markers on electrophoresis, minor shadow bands were sometimes observed in addition to the main bands. The shadow bands are thought to result from slipped strand mispairing at dinucleotide repeats (23). If the allelic size difference is small, this artifact confounds the definition of heterozygosity status. To avoid this problem, we considered the heterozygosity by the presence of a ≥2 bp difference between the alleles according to a study (24). The LOH of tumor DNA was scored as relative allelic ration, which was calculated by dividing the tumor allelic ratio by the normal allelic ration. We used a criteria described by Choi et al. (25) to determine borderline LOH in some cases, which settled a 1.55-fold value as a cutoff point because this provided the best discrimination between wild-type heterozygosity and LOH.

Bisulfite-modified sequencing of IKK promoter region. The 5′ proximal promoter region of *IKKα* was analyzed on the computer

Table 1. Clinicopathologic parameters and IKKα expression in 64 primary oral carcinomas

Parameters	*n*	IKKα staining, mean ± SD	*P*
Sex			
Female	24	28.88 ± 34.61	
Male	40	36.60 ± 34.88	0.3930*
T stage †			
T1	18	42.56 ± 36.85	
T2	24	28.92 ± 33.39	
T3	8	24.63 ± 37.50	
T4	14	35.71 ± 33.56	0.5375 ‡
N stage †			
N0	42	33.60 ± 34.61	
N1	13	45.54 ± 36.62	
N2	8	18.88 ± 29.67	
N3	1	3.00 ± 0.00	0.2937 ‡
Clinical stage †			
Stage 1	17	40.94 ± 37.33	
Stage 2	18	30.50 ± 33.74	
Stage 3	12	26.92 ± 35.77	
Stage 4	17	34.65 ± 34.04	0.7234 ‡
Histologic differentiation			
Well	26	45.85 ± 34.68	
Moderate	26	33.08 ± 34.81	
Poor	12	9.00 ± 20.50	0.0107 ‡

*Probability of statistical difference (P) was analyzed by Mann-Whitney *U* test.
†Patients were categorized by tumor size (T stage) and clinical stages according to the International Union against Cancer (UICC) WHO grading system and by its presence.
‡Probability of statistical difference (P) was analyzed by analysis of covariance.

program (CpG Island Finder)[5] to find CpG islands. The program was developed by the modification of criteria by Takai and Jones (26), which defined the sequence as CpG islands according to the criterion as follows: (*a*) >500 bp long; (*b*) ≥55% of G + C nucleotides; (*c*) >0.65 of observed CpG/expected CpG ratio; and (*d*) exclude Alu-repetitive elements. Because the CpG islands were identified in the region spanning core promoter and exon 1, we treated genomic DNA with bisulfite (27) and did direct sequence analyses using a primer pair from -253 to +66; forward 5′-TAGGAGAGATTGGGTTGTTTTGAAAAGTGG-3′ and reverse 5′-CTCAAATTCCACAATTATTCCAA-3′. The nucleotides in bold correspond to the uracils generated from cytosines by the effect of bisulfite.

Statistical analysis. Distribution of the clinicopathologic factors, IKKα immunoreactivities, and MSI were analyzed using the analysis of covariance or the Mann-Whitney *U* test. For survival analysis, we divided patients into two groups as a negative/weakly positive group (≤10% of immunoreactivity) and a positive group (>11% of immunoreactivity) and used the Kaplan-Meier method, and the statistical difference was analyzed by the log-rank test. To determine whether the prognostic levels of immunostaining are independent of clinicopathologic parameters, the influence of these factors on patient survival was analyzed by the multivariate Cox proportional hazards method. In survival analysis and the Cox hazards method, we excluded six cases that died of other diseases during the follow-up period.

Results

Expression and localization of IKKα. A single 113-bp band of *IKKα* was augmented by semiquantitative reverse transcription-PCR (RT-PCR) using an exon 13 and 14 primer set. In comparison with normal gingival keratinocytes, 7 of 13 oral carcinoma cell lines did not or marginally expressed the gene below 35% (Fig. 1A).

Because the data above suggest that *IKKα* is expressed in normal oral keratinocytes but the expression is frequently decreased in carcinoma cells, we wished to determine whether IKKα expression correlates with clinicopathologic parameters of carcinoma patients. IKKα was immunolocalized to the nucleus at basal and suprabasal cells of the normal epithelium and carcinoma cells (Fig. 1B-E). However, IKKα was not detected in 13 of 64 carcinomas (20.3%) and weakly reacted in 8 carcinomas (12.5%). Then, we calculated the percentage of nuclear staining in carcinoma cells and compared this to the clinicopathologic parameters of the samples. Immunoreactive carcinoma cells were observed in 33.7 ± 34.7% (mean ± SD) of total carcinoma cells. Although the immunoreactivity was retained in well-differentiated carcinomas (45.8 ± 34.7%; Fig. 1C), it was decreased in less differentiated carcinomas (Fig. 1D) and moderately (33.1 ± 34.8%) and poorly differentiated carcinomas (9.0 ± 20.5%; $P = 0.0107$; Table 1). Other clinicopathologic parameters, including age of patients at diagnosis, sex, T stage, N stage, and clinical stage, did not correlate with IKKα staining.

Prognostic significance of IKKα expression in primary oral carcinomas. Univariate analysis of age (≤50 years versus >50 years), T stage (T1/2 versus T3/4), lymph node status (N-, absence of lymph node metastasis versus N+, presence of lymph node metastasis), clinical stage (stage 1/2 versus stage 3/4), histologic tumor differentiation (well versus moderately/poorly differentiated), and IKKα expression status (positive versus negative/weakly positive) for disease-specific survival in oral carcinomas is summarized in Table 2. Log-rank testing on 58 carcinomas except patients that died of other diseases ($n = 6$) revealed that advanced T and clinical stages and histologic tumor differentiation were significantly correlated with poor patient survival (T stage, $P = 0.0003$; clinical stage, $P = 0.0034$; histologic differentiation, $P = 0.0024$). When the oral carcinoma patients were categorized along a Kaplan-Meier survival curve according to positive (>11%) versus negative (≤10%) expression of IKKα, a statistically significant association between IKKα expression levels and patient survival was observed ($P = 0.0011$, Fig. 2).

Multivariate risk factor analyses on age (≤50 years versus >50 years), T stage (T1/2 versus T3/4), lymph node metastasis (N- versus N+), clinical stage (stage 1/2 versus stage 3/4), histologic differentiation (well-differentiated versus moderately/poorly differentiated), and IKKα expression status (positive versus negative/weakly positive) were done. The Cox proportional hazards method showed that IKKα staining was a significant independent predictor of death from carcinomas ($P = 0.0293$; Table 3). For disease-specific survival, reduced IKKα staining was an independent predictor of survival with a hazard ratio of 1.696 and a 95% confidence interval ranging from 1.055 to 2.727. No other variables including age, sex, T stage, N status, clinical stage, and histologic differentiation were associated with survival according to the multivariate analysis.

IKKα localization in the nucleus and up-regulation upon differentiation. Because immunostaining data suggest that the majority of IKKα protein localized within the nucleus, we fractionated HaCaT cells and did immunoblotting (Fig. 3A). IKKα was exclusively detected in the histone H3-positive nuclear fraction, but not in the GAPDH- and β-actin–positive cytoplasmic fraction. Collectively, the data above show the nuclear localization of IKKα in the keratinocyte lineage.

Statistical analysis suggests an association between IKKα expression and tumor differentiation. To address a question about an alteration of IKKα expression status in response to cellular differentiation, we used the HaCaT differentiation model (28). HaCaT cells are immortalized human normal skin keratinocytes and can represent a differentiation process in dishes. In the absence of Ca^{2+} in culture medium, cells do not express differentiation markers (involcurin and high-molecular-weight cytokeratins) and dedifferentiate (29). In fact, HaCaT cells expressed involucrin and cytokeratin 10, but negligibly in the Ca^{2+}-stripped medium (Fig. 3B). Differentiated HaCaT cell lysates expressed IKKα as a single 85-kDa band, but the expression was remarkably decreased upon dedifferentiation.

Genetic instability of the IKKα gene. Genetic instability has been documented in many types of genes and malignant tumors and considered to be involved in the loss of gene expression (30–32). Therefore, we analyzed the instabilities at the *IKKα* locus by microsatellite PCR. Among 46 cases analyzed, 63.0% of carcinomas ($n = 29$) shifted the electrophoretic mobility of PCR amplicons relative to normal counterparts, reflecting the changes of dinucleotide repeat number in carcinoma cells (Fig. 4A and B). A total of 16 cases are associated with two or three MSI (34.8%), and 13 cases are associated with one MSI (28.3%). The LOH was found in two cases, and two cases showed a homozygous deletion. The other 17 cases exhibited microsatellite stability (MSS). IKKα staining was increased in MSS carcinomas (56.7 ± 33.0%), but decreased in MSI carcinomas (23.9 ± 31.9%;

[5] http://cpgislands.usc.edu/

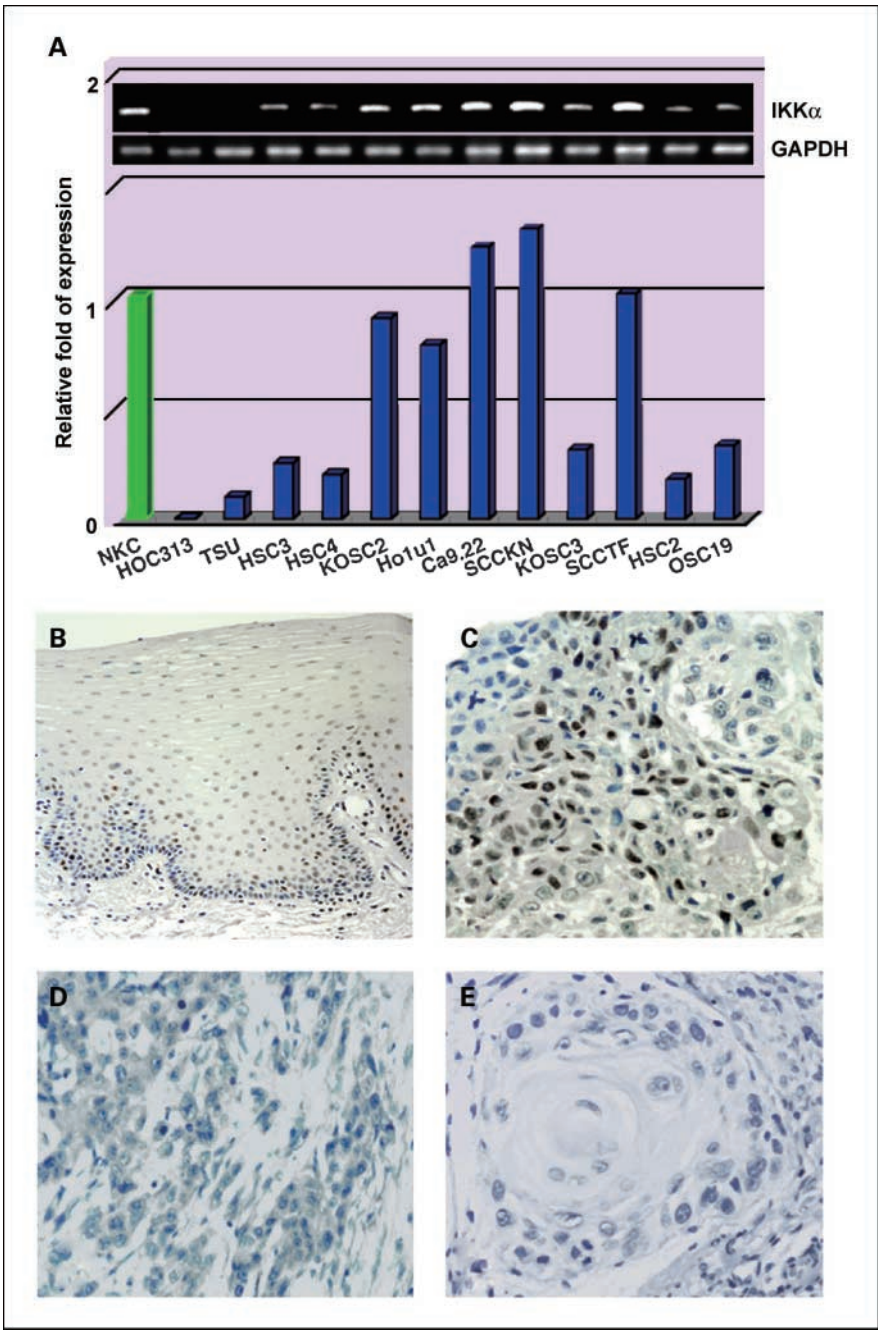

Fig. 1. Expression of IKKα in oral carcinoma cell lines and tissues. GAPDH-normalized semiquantitative RT-PCR for IKKα was done on oral carcinoma cell lines (n = 12) and compared the expression level in normal gingival keratinocytes (*A*). Immunolocalization of IKKα in normal gingiva (*B*) and oral carcinoma tissues (*C-E*). IKKα was detected in well-differentiated carcinoma (*C*) but not in poorly differentiated carcinomas (*D*). Negative control staining using nonimmune immunoglobulin G showed no nuclear localization of IKKα in well-differentiated carcinoma (*E*). Bars, 300 μm (*B*) and 75 μm (*C-E*).

P = 0.0017; Fig. 5A). Additional microsatellite markers in the vicinity (AFM205tg7 and D10S603) and at the *PTEN* locus (AFMA086wg9) limited MSI in 10, 17, and 10 cases, respectively, but did not statistically associate with *IKKα* MSI data (data not shown), indicating that genetic instability at the *IKKα* locus is selectively generated.

Hypermethylation of CpG islands. Because the genetic instability has been documented to frequently associate with promoter hypermethylation resulting in epigenetic inactivation of gene transcription (33, 34), we determined the presence of 5′-methyl cytosine (5mC) from -253 to +66 in CpG islands. Among two MSS carcinomas, one case with high IKKα immunoreactivity restricted 5mC at two positions, whereas another case was extensively methylated (Fig. 5B). This hypermethylated MSS carcinoma was negligibly stained IKKα. In contrast to MSS carcinomas, MSI carcinomas increased the number of 5mC as an equal frequency between carcinomas with one third and two thirds of the intragenic microsatellites. Most of the MSI carcinomas were methylated from the Ets-binding element at -55, which is critical for *IKKα* expression (35), to exon 1. However, some carcinomas did not correlate methylation status with the immunoreactivities. There may be some other mechanisms regulating the gene expression.

Discussion

Stratified squamous epithelium requires strict proliferation and differentiation programs to develop and maintain tissue

Table 2. Univariate analysis of clinicopathologic parameters and IKKα expression for patient survival

Factors	P*
Age (≤50 vs. >50 y)	0.1855
T stage (T1/2 vs. T3/4)	0.0003
Lymph node status (N- vs. N+) †	0.0814
Clinical stage (stage 1/2 vs. 3/4)	0.0034
Histologic differentiation (well vs. moderately/poorly differentiated)	0.0024
IKKα expression (≤10% vs. >10%)	0.0011

*Log-rank test.
† N+, cases with lymph node metastasis.

integrity (36). Although the detailed mechanism of the programs remains to be elucidated, NFκB is considered to be a prognostic risk factor and therapeutic target of head and neck carcinomas (12). However, IKKα localizes in the nucleus of basal keratinocytes (18, 37) and did not play a central role in the activation of canonical NFκB pathway in squamous carcinoma cells (14). *IKKα* gene targeting mice show hyperproliferation of the epithelium and prevent the expression of differentiation markers (involucrin, loricrin, and filaggrin) independent of IκB-phosphorylating activity (17–19). In this study, we also detected the nuclear localization of IKKα in basal cells of oral epithelium and reduction of IKKα protein expression in dedifferentiated keratinocytes. Although the exact role in the nuclear milieu is not clearly understood, it can be expected that IKKα directly binds to estrogen receptor-α, which activates the expression of involucrin (38, 39). A crucial role of IKKα on keratinocyte differentiation is also designated by the fact that an unknown differentiation-inducing protein is secreted from keratinocytes in an IKKα-dependent manner (37, 40). More recently, IKKα was shown to accelerate the degradation of cyclin D1 and NFκB and phosphorylate histone H3 (41, 42). Therefore, it seems likely that IKKα governs differentiation and/or other aspects of phenotypic definition of keratinocytes.

We did immunostaining to clarify the expression pattern of IKKα in carcinoma tissues and analyzed its prognostic significance in comparison with clinicopathologic parameters and patient survival. IKKα staining was decreased in 44.9% of carcinomas, and the multivariate Cox hazards method indicated that IKKα expression was closely associated with the disease-specific survival rate of patients independent of other risk factors. Tumor development is triggered by the loss of gene function, which takes an important role in the process of normal cell differentiation (43). Emerging evidence highlighting an involvement of IKKα in cell differentiation, proliferation, and survival (17–19, 37, 40) suggests that the loss of IKKα impacts on multiaspects of carcinoma cells and facilitates disease progression toward poor prognosis. While this article was being prepared, Liu et al. (44) have shown that IKKα expression is reduced in high-grade and less differentiated squamous cell carcinomas of the skin, and that the reintroduction of the *IKKα* gene in the null mouse represses chemically induced tumor development and progression. Although our data are highly supported by these findings, future avenues of research will be required to clearly find out the role of *IKKα* transcriptional inactivation in the pathology of oral carcinomas.

Immunostaining of IKKα in the normal oral epithelium and oral carcinomas suggests that the loss of expression does not occur at an early stage of carcinogenesis. We studied genetic instabilities and promoter methylation status and showed a close association between MSI and promoter hypermethylation. MSI arises through defective DNA mismatch repair genes, which exhibit allelic imbalance in 80% of squamous cell carcinomas of the head and neck (45), and closely associates with gene silencing through promoter hypermethylation (31, 33, 46). Recent findings indicate that MSI and aberrant promoter hypermethylation selectively but not randomly occurred to specific susceptible sites, resulting in a functional impact on the initiation and progression of tumors (47). Because MSI at the *PTEN* locus was observed in a low frequency (10 cases, 17.2%) and did not correlate with *IKKα* MSI status, genetic instability at the *IKKα* locus may be a selective phenomenon.

Expression of the IKKα protein was significantly decreased in MSI carcinomas compared with MSS carcinomas and negligible

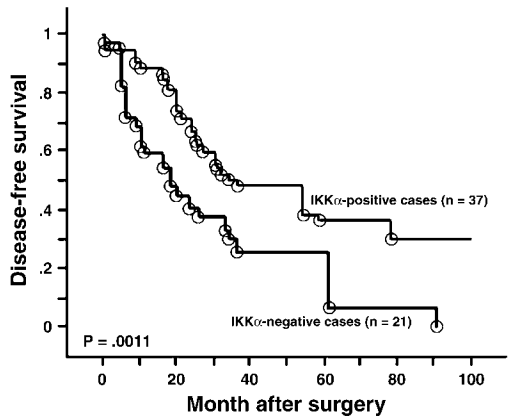

Fig. 2. Disease-specific survival in 58 oral carcinoma patients based on the expression of IKKα. The graph summarizes Kaplan-Meier survival analysis for patients with positive (>10% immunoreactivity) and negative staining (≤10% immunoreactivity). Statistical differences were examined between positive and negative IKKα staining ($P = 0.0011$).

Table 3. Contribution of various potential prognostic factors to disease-free survival

Factors	Risk ratio (95% CI)*	P †
Age (≤50 vs. >50 y)	1.170 (0.639-2.142)	0.6105
T stage (T1/2 vs. T3/4)	1.500 (0.823-2.735)	0.1853
Lymph node status (N- vs. N+) ‡	0.881 (0.467-1.661)	0.6948
Clinical stage (stage 1/2 vs. 3/4)	1.409 (0.662-2.998)	0.3731
Histologic differentiation (well vs. moderately/poorly differentiated)	1.227 (0.785-1.918)	0.3694
IKKα expression (≤10% vs. >10%)	1.696 (1.055-2.727)	0.0293

*Confidence interval.
† Cox proportional hazards method.
‡ N-; cases without lymph node metastasis, N+; cases with lymph node metastasis.

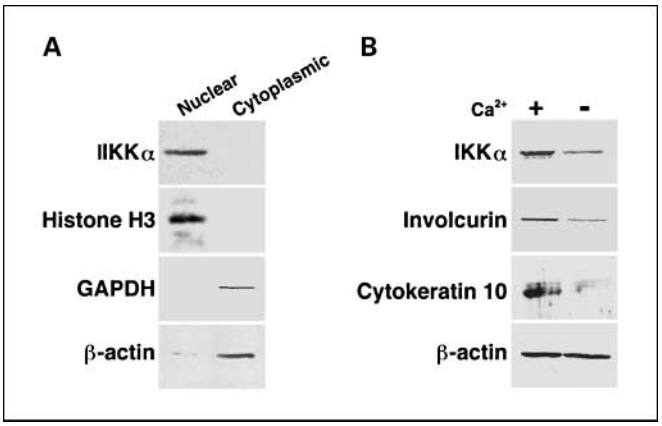

Fig. 3. IKKα expression in HaCaT keratinocytes. *A,* nuclear and cytoplasmic fractions of HaCaT cells were subjected to the immunoblotting for IKKα, histone H3, GAPDH, or β-actin. *B,* differentiated HaCaT cells maintained in the presence of Ca^{2+} (+) and dedifferentiated cells in the absence of Ca^{2+} (-) were proven by antibodies to IKKα, involcurin, cytokeratin 10, and β-actin.

in hypermethylated carcinomas, indicating that the aberrant promoter hypermethylation reflects the expression status. The promoter hypermethylation determines the transcriptional status of a gene by blocking the access of certain transcription factors that are sensitive to the cytosine methylation in their binding motifs (48–50). We observed a high frequency of 5mC

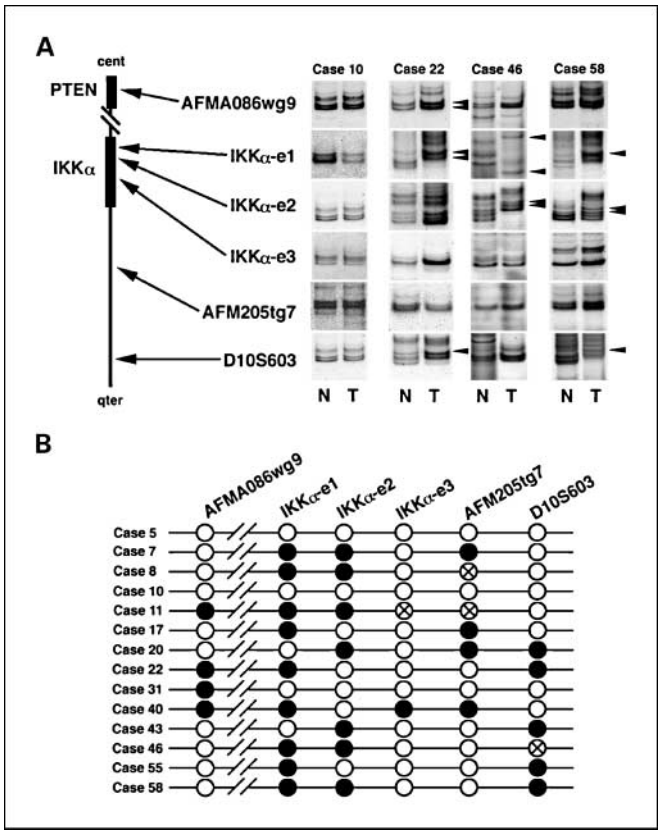

Fig. 4. Microsatellite instabilities at the *IKKα* locus in oral carcinomas. *A,* representative cases (case 10, 22, 46, and 58) were shown with a positional map of microsatellite markers used in this study. Arrowheads, MSI with the changes of electromobilities of bands. *B,* a summary of MSI status of representative 14 oral carcinomas. ●, MSI; ⊗, not informative.

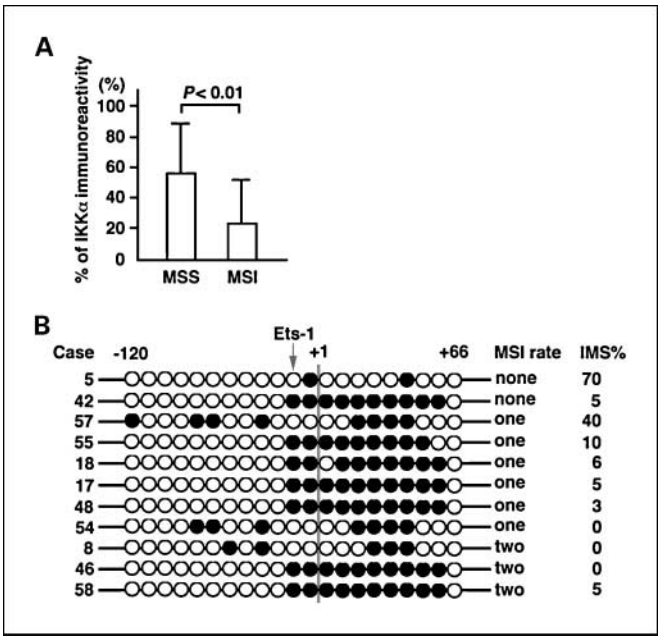

Fig. 5. Microsatellite instabilities and IKKα protein expression and promoter methylation status. *A,* IKKα immunoreactivity of carcinoma cells was compared between groups with microsatellite stability (MSS, $n = 17$) and microsatellite instability (MSI, $n = 29$). Columns, mean; bars, SD. Significant difference was analyzed by Mann-Whitney *U* test. *B,* schematic illustration for position of 5′-methyl cytosines. ○, relative position of potential methylation-sensitive cytosines in the *IKKα* CpG island. ●, methylated cytosines were analyzed by the bisulfite-modified sequence. MSI rate, number of MSI-positive sites within three *IKKα* intragenic markers. IMS%, percentage of IKKα immunoreactive carcinoma cells in each case.

in the CpG island including an Ets-binding element, which is critical for *IKKα* gene transcription (35). Promoter hypermethylation observed at this motif may disturb the accessibility of a key transcription factor and repress *IKKα* transcription. Two carcinomas (cases 8 and 54) did not exhibit a close relationship between hypermethylation and protein expression, suggesting that other factors may also be involved in the loss of IKKα protein expression. Although our preliminary examination surveying point mutations in the all 21 exons in five different oral carcinoma cell lines showed no nonsense mutation responsible for the gene expression loss (data not shown), missense mutations in the exon 15 were reported in the skin carcinomas (44). However, the data above strongly suggest that the promoter hypermethylation and genetic instability negatively regulate the IKKα expression in oral carcinoma cells.

Treatment failure of oral carcinomas can be attributed to multiple factors but remains difficult to predict because no reliable molecular marker is currently available as indicators of prognosis. Identifying the mechanisms that provide insights into understanding the pathway of tumor progression contributes to long-term survival of patients. Collectively, our findings indicated that the *IKKα* gene is epigenetically inactivated in an aggressive and less differentiated subset of oral carcinomas, and the loss of IKKα expression is an independent prognostic factor in patients with oral carcinoma. Although emerging evidence highlights a role of NFκB in the progression of carcinomas and poor prognosis of patients, the present study suggests that the IKKα present in the nucleus suppresses malignancy through the effects on carcinoma cell differentiation independent of canonical NFκB activation.

References

1. Lippman SM, Hong WK. Molecular markers of the risk of oral cavity. N Engl J Med 2001;344:1323–6.
2. Jemal A, Murray T, Ward E, et al. Cancer statistics. CA Cancer J Clin 2005;55:10–30.
3. Ponder BAJ. Cancer genetics. Nature 2001;411:336–41.
4. Miyazawa J, Mitoro A, Kawashiri S, Chada KK, Imai K. Expression of mesenchyme-specific gene HMGA2 in squamous cell carcinomas of the oral cavity. Cancer Res 2004;64:2024–9.
5. Maeda G, Chiba T, Aoba T, Imai K. Epigenetic inactivation of E-cadherin by promoter hypermethylation in oral carcinoma cells. Odontology 2007;1:24–9.
6. Maeda G, Chiba T, Okazaki M, et al. Expression of SIP1 in oral squamous cell carcinomas: implications for E-cadherin expression and tumor progression. Int J Oncol 2005;27:1535–41.
7. Richter J, Wagner U, Schraml P, et al. Chromosomal imbalances are associated with a high risk of progression in early invasive (pT1) urinary bladder cancer. Cancer Res 1999;59:5687–91.
8. Alers JC, Rochat J, Krijtenburg PJ, et al. Identification of genetic markers for prostatic cancer progression. Lab Invest 2000;80:931–42.
9. Sansai I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004;22:2954–63.
10. Mavros A, Hahn M, Wieland I, et al. Infrequent genetic alterations of the tumor suppressor gene PTEN/MMAC1 in squamous cell carcinoma of the oral cavity. J Oral Pathol Med 2002;31:270–6.
11. Chen Q, Samaranayake LP, Zhou H, Xiao L. Homozygous deletion of the PTEN tumor-suppressor gene is not a feature in oral squamous cell carcinoma. Oral Oncol 2000;36:95–9.
12. van Waes C. Nuclear factor-κB in development, prevention, and therapy of cancer. Clin Cancer Res 2007;13:1076–82.
13. Mishra A, Bharti AC, Varghese P, Saluja D, Das BC. Differential expression and activation of NF-κB family proteins during oral carcinogenesis. Role of high risk human papillomavirus infection. Int J Cancer 2006;119:2840–50.
14. Yu M, Yeh J, van Waes C. Protein kinase casein kinase 2 mediates inhibitor-κB kinase and aberrant nuclear factor-κB activation by serum factor(s) in head and neck squamous carcinoma cells. Cancer Res 2006;66:6722–31.
15. Li Q, Antwerp DV, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the IκB kinase 2 gene. Science 1999;284:321–5.
16. Makris C, Godfrey VL, Krahn-Senftleben G, et al. Female mice heterozygous for IKK γ/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. Mol Cell 2000;5:969–79.
17. Takeda K, Takeuchi O, Tsujimura T, et al. Limb and skin abnormalities in mice lacking IKKα. Science 1999;284:313–6.
18. Hu Y, Baud V, Delhase M, et al. Abnormal morphogenesis but intact IKK activation by mice lacking the IKKα subunit of IκB kinase. Science 1999;284:316–20.
19. Li Q, Lu Q, Hwang JY, et al. IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev 1999;13:1322–8.
20. Hermanek P, Sobin LH, editors. International Union against Cancer. TNM classification of malignant tumors. 4th ed. Berlin: Springer-Verlag; 1987.
21. Okuse T, Chiba T, Katsuumi I, Imai K. Differential expression and localization of WNTs in an animal model of skin wound healing. Wound Repair Regen 2005;13:491–7.
22. Emmert-Buck MR, Bonner RF, Smith PD, et al. Laser capture microdissection. Science 1996;274:998–1001.
23. Strachan T, Read AP, editors. Human molecular genetics. 2nd ed. New York: John Wiley & Sons; 1999.
24. Shen CY, Yu JC, Lo YL, et al. Genome-wide search for loss of heterozygosity using laser capture microdissected tissue of breast carcinoma: an implication for mutator phenotype and breast cancer pathogenesis. Cancer Res 2000;60:3884–92.
25. Choi SW, Lee KJ, Bae YA, et al. Genetic classification of colorectal cancer based on chromosomal loss and microsatellite instability predicts survival. Clin Cancer Res 2002;8:2311–22.
26. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002;99:3740–5.
27. Sighal RP. Modification of *Escherichia coli* glutamate transfer ribonucleic acid with bisulfite. J Biol Chem 1971;246:5848–51.
28. Breitkreutz D, Stark HJ, Plein P, Baur M, Fusenig NE. Differential modulation of epidermal keratinization in immortalized (HaCaT) and tumorigenic human skin ketatinocytes (HaCaT-ras) by retinoic acid and extracellular Ca^{2+}. Differentiation 1993;54:201–17.
29. Kawabata H, Kawahara K, Kanekura T, et al. Possible role of transcriptional coactivator P/CAF and nuclear acetylation in calcium-induced keratinocyte differentiation. J Biol Chem 2002;277:8099–105.
30. An C, Choi IS, Yao JC, et al. Prognostic significance of CpG island methylator phenotype and microsatellite instability in gastric carcinoma. Clin Cancer Res 2005;11:656–63.
31. Dhillon VS, Aslam M, Husain A. The contribution of genetic and epigenetic changes in granulosa cell tumors of ovarian origin. Clin Cancer Res 2004;10:5537–45.
32. Brock MV, Gou M, Akiyama Y, et al. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res 2003;9:2912–9.
33. Ahuja N, Mohan AL, Li Q, et al. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 1997;57:3370–4.
34. Koinuma K, Yamashita Y, Liu W, et al. Epigenetic silencing of AXIN2 in colorectal carcinoma with microsatellite instability. Oncogene 2006;25:139–46.
35. Gu L, Zhu N, Findley HW, Woods WG, Zhou M. Identification and characterization of the IKKα promoter. J Biol Chem 2004;279:52141–9.
36. Fuch E, Raghavan S. Getting under the skin of epidermal morphogenesis. Nat Rev Genet 2002;3:199–209.
37. Sil AK, Maeda S, Sano Y, Roop DR, Karin M. IκB kinase-α acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature 2004;428:660–4.
38. Park KJ, Krishnan V, O'Malley BW, Yamamoto Y, Gaynor RB. Formation of an IKKα-dependent transcription complex is required for estrogen receptor-mediated gene activation. Mol Cell 2005;18:71–82.
39. Kurita T, Cooke PS, Cunha GR. Epithelial-stromal tissue interaction in paramesonephric (Mullerian) epithelial differentiation. Dev Biol 2001;240:194–211.
40. Hu Y, Baud V, Oga T, Kim K, Yoshidda K, Karin M. IKKα controls formation of the epidermis independently of NF-κB. Nature 2001;410:710–4.
41. Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB. Histone H3 phosphorylation by IKK-α is critical for cytokine-induced gene expression. Nature 2003;423:655–9.
42. Anest V, Hanson JL, Cogswell PC, Steinbrecher KA, Strahl BD, Baldwin AS. A nucleosomal function for IκB kinase-α in NF-κB – dependent gene expression. Nature 2003;423:659–63.
43. Harris H. Putting on the brakes. Nature 2004;427:201.
44. Liu B, Park E, Zhu F, et al. A critical role for IκB kinase α in the development of human and mouse squamous cell carcinomas. Proc Natl Acad Sci U S A 2006;103:17202–7.
45. Nunn J, Nagini S, Risk JM, et al. Allelic imbalance at the DNA mismatch repair loci, hMSH2, hMLH1, hPMS1, hPMS2 and hMSH3, in squamous cell carcinoma of the head and neck. Oral Oncol 2003;39:115–29.
46. Nakagawa H, Nuova GJ, Zervos EE, et al. Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Res 2001;61:6991–5.
47. Haydon AMM, Jass JR. Emerging pathways in colorectal-cancer development. Lancet Oncol 2002;3:83–8.
48. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
49. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 2000;405:482–5.
50. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 2000;405:486–9.

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