

## Epigenetic Inactivation of I $\kappa$ B Kinase- $\alpha$ in Oral Carcinomas and Tumor Progression

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**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 incurable disease. I $\kappa$ B kinase- $\alpha$  (IKK $\alpha$ ) has a decisive role in the development of the skin and establishes keratinocyte phenotypes. We assessed clinical implications of IKK $\alpha$  expression in oral carcinomas and epigenetic aberrations for the loss of expression. **Experimental Design:** We examined IKK $\alpha$  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:** IKK $\alpha$  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.

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.<sup>4</sup> 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.

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

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<sup>4</sup> <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 IKK $\alpha$  has an impact on the phenotypic definition of carcinoma cells of the epithelial origin. However, little is known about the expression and role of IKK $\alpha$  in solid tumors. In the present study, we examined the expression and clinical implications of IKK $\alpha$  in oral squamous cell carcinomas and the mechanism of inactivation of IKK $\alpha$  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 IKK $\alpha$  or GAPDH: for IKK $\alpha$ , forward 5'-TTGGAGAGATACAGCCAGCAGA-3' and reverse 5'-CCAATGACCAACCTCAGCAT-3'; and

for GAPDH, forward 5'-GTCAGTGGTGGACCTGACCT-3' and reverse 5'-AGGGGAGATTCAGTGTGGTG-3'.

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

**Immunostaining.** Carcinoma tissue sections were reacted with anti-IKK $\alpha$  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- $\mu$ m-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 IKK $\alpha$  locus. Because there are no established microsatellite markers within the IKK $\alpha$  locus, we made designed intragenic three makers (IKK $\alpha$ e1 at intron 1, IKK $\alpha$ e2 at intron 2, and IKK $\alpha$ e3 at intron 5) spanning (TG) $n$ , (GA) $n$ , and (TT) $n$ , respectively: for IKK $\alpha$ e1, forward 5'-GGGGACTGCCTTTGGGAATG-3' and reverse 5'-CCATGCTTCCAAAGGCTTAACCTCTG-3'; for IKK $\alpha$ e2, forward 5'-GAAGCTAGCTTCCCCAGAA-3' and reverse; 5'-TGGAAAAGGATGGAGTCTCAC-3'; and for IKK $\alpha$ e3, forward 5'-CAGTGGTTCCATCAGAGAAC-3' and reverse 5'-CAGCCTGTATCTTTGCTAGA-3'. Genomic DNA (20 ng) was amplified in a volume of 20  $\mu$ L 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  $\mu$ L of the amplified products were electrophoresed through acrylamide gels that were stained with Vistra Green (Amersham Biosciences) 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  $\geq 2$  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 IKK $\alpha$  was analyzed on the computer

**Table 1.** Clinicopathologic parameters and IKK $\alpha$  expression in 64 primary oral carcinomas

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

\*Probability of statistical difference (P) was analyzed by Mann-Whitney U test.

<sup>†</sup>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.

<sup>‡</sup>Probability of statistical difference (P) was analyzed by analysis of covariance.

program (CpG Island Finder)<sup>5</sup> 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)  $\geq 55\%$  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'-CTCAAATTCACAATTATTCCAA-3'. The nucleotides in bold correspond to the uracils generated from cytosines by the effect of bisulfite.

**Statistical analysis.** Distribution of the clinicopathologic factors, IKK $\alpha$  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 ( $\leq 10\%$  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 IKK $\alpha$ .** A single 113-bp band of *IKK $\alpha$*  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 *IKK $\alpha$*  is expressed in normal oral keratinocytes but the expression is frequently decreased in carcinoma cells, we wished to determine whether IKK $\alpha$  expression correlates with clinicopathologic parameters of carcinoma patients. IKK $\alpha$  was immunolocalized to the nucleus at basal and suprabasal cells of the normal epithelium and carcinoma cells (Fig. 1B-E). However, IKK $\alpha$  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  $33.7 \pm 34.7\%$  (mean  $\pm$  SD) of total carcinoma cells. Although the immunoreactivity was retained in well-differentiated carcinomas ( $45.8 \pm 34.7\%$ ; Fig. 1C), it was decreased in less differentiated carcinomas (Fig. 1D) and moderately (33.1  $\pm$  34.8%) and poorly differentiated carcinomas ( $9.0 \pm 20.5\%$ ;  $P = 0.0107$ ; Table 1). Other clinicopathologic parameters, including age of patients at diagnosis, sex, T stage, N stage, and clinical stage, did not correlate with IKK $\alpha$  staining.

**Prognostic significance of IKK $\alpha$  expression in primary oral carcinomas.** Univariate analysis of age ( $\leq 50$  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 IKK $\alpha$  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 ( $\leq 10\%$ ) expression of IKK $\alpha$ , a statistically significant association between IKK $\alpha$  expression levels and patient survival was observed ( $P = 0.0011$ , Fig. 2).

Multivariate risk factor analyses on age ( $\leq 50$  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 IKK $\alpha$  expression status (positive versus negative/weakly positive) were done. The Cox proportional hazards method showed that IKK $\alpha$  staining was a significant independent predictor of death from carcinomas ( $P = 0.0293$ ; Table 3). For disease-specific survival, reduced IKK $\alpha$  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.

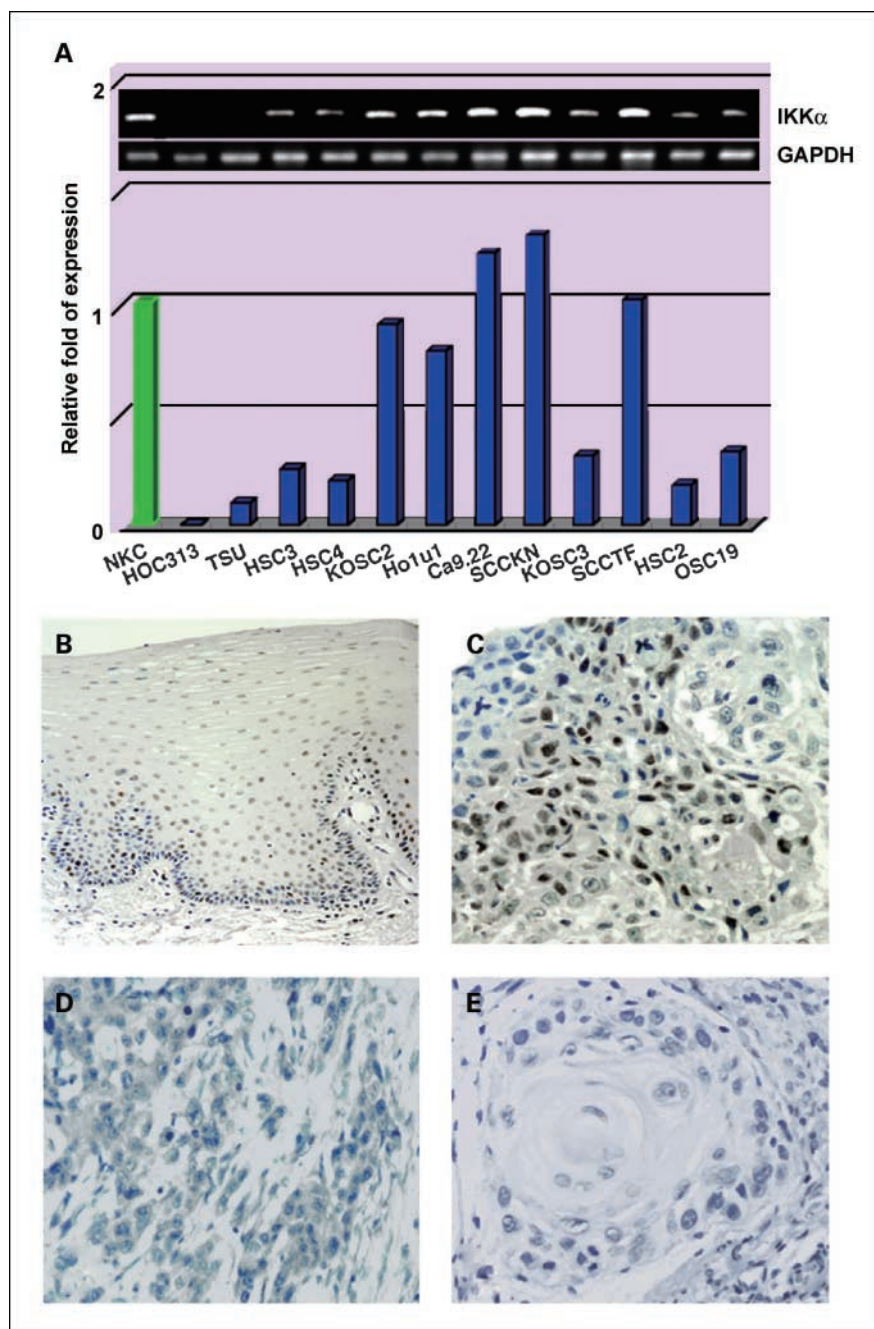
**IKK $\alpha$  localization in the nucleus and up-regulation upon differentiation.** Because immunostaining data suggest that the majority of IKK $\alpha$  protein localized within the nucleus, we fractionated HaCaT cells and did immunoblotting (Fig. 3A). IKK $\alpha$  was exclusively detected in the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

Statistical analysis suggests an association between IKK $\alpha$  expression and tumor differentiation. To address a question about an alteration of IKK $\alpha$  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 (involucrin 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 IKK $\alpha$  as a single 85-kDa band, but the expression was remarkably decreased upon dedifferentiation.

**Genetic instability of the IKK $\alpha$  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 *IKK $\alpha$*  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). IKK $\alpha$  staining was increased in MSS carcinomas ( $56.7 \pm 33.0\%$ ), but decreased in MSI carcinomas ( $23.9 \pm 31.9\%$ ;

<sup>5</sup> <http://cpgislands.usc.edu/>





**Fig. 1.** Expression of IKK $\alpha$  in oral carcinoma cell lines and tissues. GAPDH-normalized semiquantitative RT-PCR for IKK $\alpha$  was done on oral carcinoma cell lines ( $n = 12$ ) and compared the expression level in normal gingival keratinocytes (A). Immunolocalization of IKK $\alpha$  in normal gingiva (B) and oral carcinoma tissues (C-E). IKK $\alpha$  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 IKK $\alpha$  in well-differentiated carcinoma (E). Bars, 300  $\mu$ m (B) and 75  $\mu$ m (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 IKK $\alpha$  MSI data (data not shown), indicating that genetic instability at the IKK $\alpha$  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 IKK $\alpha$  immunoreactivity restricted 5mC at two positions, whereas another case was extensively methylated (Fig. 5B). This hyper-

methylated MSS carcinoma was negligibly stained IKK $\alpha$ . 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 IKK $\alpha$  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 IKK $\alpha$  expression for patient survival

| Factors   | P*     |
|---|--------|
| Age ( $\leq 50$ vs. $> 50$ y)   | 0.1855 |
| T stage (T1/2 vs. T3/4)   | 0.0003 |
| Lymph node status (N- vs. N+) <sup>†</sup>                                | 0.0814 |
| Clinical stage (stage 1/2 vs. 3/4)  | 0.0034 |
| Histologic differentiation<br>(well vs. moderately/poorly differentiated) | 0.0024 |
| IKK $\alpha$ expression ( $\leq 10\%$ vs. $> 10\%$ )                      | 0.0011 |

\*Log-rank test.

<sup>†</sup> N+, cases with lymph node metastasis.

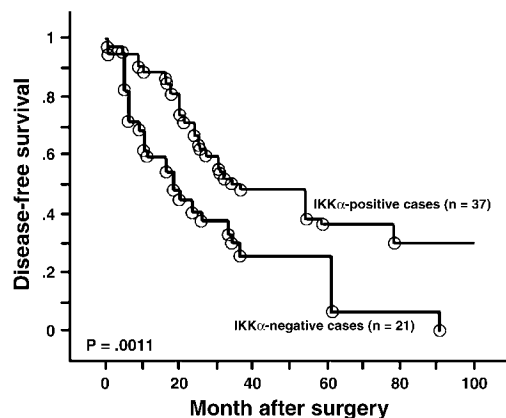
integrity (36). Although the detailed mechanism of the programs remains to be elucidated, NF $\kappa$ B is considered to be a prognostic risk factor and therapeutic target of head and neck carcinomas (12). However, IKK $\alpha$  localizes in the nucleus of basal keratinocytes (18, 37) and did not play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

differentiation and/or other aspects of phenotypic definition of keratinocytes.

We did immunostaining to clarify the expression pattern of IKK $\alpha$  in carcinoma tissues and analyzed its prognostic significance in comparison with clinicopathologic parameters and patient survival. IKK $\alpha$  staining was decreased in 44.9% of carcinomas, and the multivariate Cox hazards method indicated that IKK $\alpha$  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 IKK $\alpha$  in cell differentiation, proliferation, and survival (17–19, 37, 40) suggests that the loss of IKK $\alpha$  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 IKK $\alpha$  expression is reduced in high-grade and less differentiated squamous cell carcinomas of the skin, and that the reintroduction of the IKK $\alpha$  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 IKK $\alpha$  transcriptional inactivation in the pathology of oral carcinomas.

Immunostaining of IKK $\alpha$  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 IKK $\alpha$  MSI status, genetic instability at the IKK $\alpha$  locus may be a selective phenomenon.

Expression of the IKK $\alpha$  protein was significantly decreased in MSI carcinomas compared with MSS carcinomas and negligible



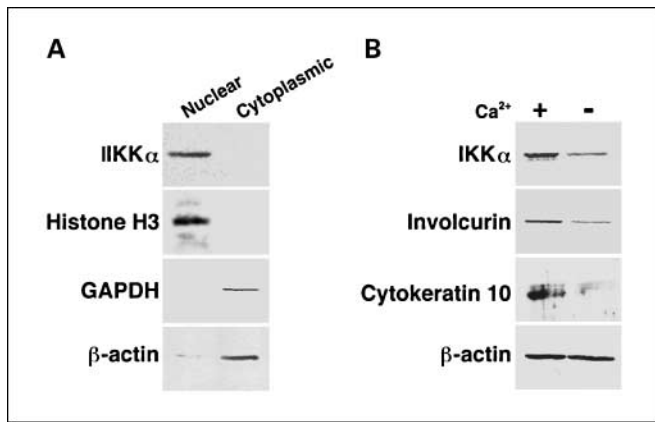
**Fig. 2.** Disease-specific survival in 58 oral carcinoma patients based on the expression of IKK $\alpha$ . The graph summarizes Kaplan-Meier survival analysis for patients with positive ( $> 10\%$  immunoreactivity) and negative staining ( $\leq 10\%$  immunoreactivity). Statistical differences were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

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

| Factors   | Risk ratio (95% CI)* | P <sup>†</sup> |
|---|----------------------|----------------|
| Age ( $\leq 50$ 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+) <sup>‡</sup>                                    | 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<br>(well vs. moderately/<br>poorly differentiated) | 1.227 (0.785-1.918)  | 0.3694         |
| IKK $\alpha$ expression<br>( $\leq 10\%$ vs. $> 10\%$ )                       | 1.696 (1.055-2.727)  | 0.0293         |

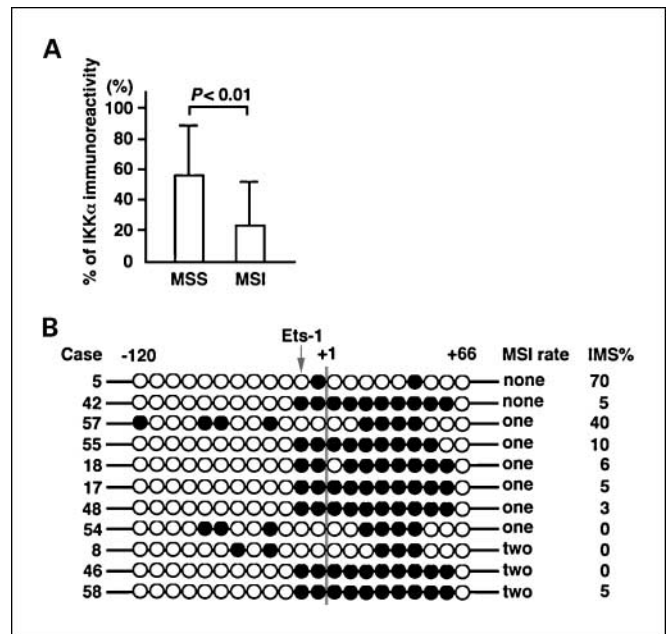
\*Confidence interval.

<sup>†</sup> Cox proportional hazards method.<sup>‡</sup> N-; cases without lymph node metastasis, N+; cases with lymph node metastasis.

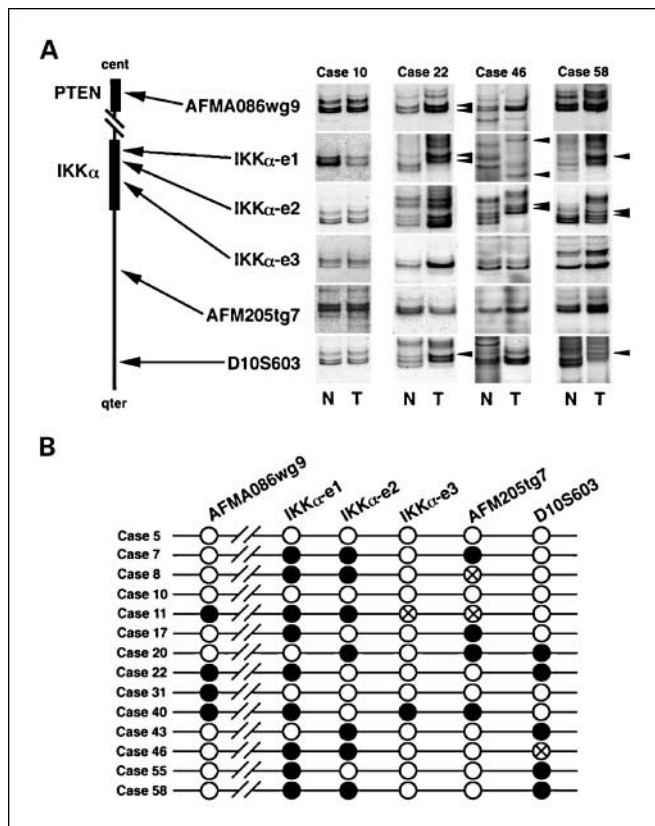


**Fig. 3.** IKK $\alpha$  expression in HaCaT keratinocytes. *A*, nuclear and cytoplasmic fractions of HaCaT cells were subjected to the immunoblotting for IKK $\alpha$ , histone H3, GAPDH, or  $\beta$ -actin. *B*, differentiated HaCaT cells maintained in the presence of Ca<sup>2+</sup> (+) and dedifferentiated cells in the absence of Ca<sup>2+</sup> (-) were proven by antibodies to IKK $\alpha$ , involucrin, cytokeratin 10, and  $\beta$ -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. 5.** Microsatellite instabilities and IKK $\alpha$  protein expression and promoter methylation status. *A*, IKK $\alpha$  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 IKK $\alpha$  CpG island. ●, methylated cytosines were analyzed by the bisulfite-modified sequence. MSI rate, number of MSI-positive sites within three IKK $\alpha$  intragenic markers. IMS%, percentage of IKK $\alpha$  immunoreactive carcinoma cells in each case.



**Fig. 4.** Microsatellite instabilities at the IKK $\alpha$  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.

in the CpG island including an Ets-binding element, which is critical for IKK $\alpha$  gene transcription (35). Promoter hypermethylation observed at this motif may disturb the accessibility of a key transcription factor and repress IKK $\alpha$  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 IKK $\alpha$  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 IKK $\alpha$  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 IKK $\alpha$  gene is epigenetically inactivated in an aggressive and less differentiated subset of oral carcinomas, and the loss of IKK $\alpha$  expression is an independent prognostic factor in patients with oral carcinoma. Although emerging evidence highlights a role of NF $\kappa$ B in the progression of carcinomas and poor prognosis of patients, the present study suggests that the IKK $\alpha$  present in the nucleus suppresses malignancy through the effects on carcinoma cell differentiation independent of canonical NF $\kappa$ B activation.



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