

# Genomic Structure of the Human *ING1* Gene and Tumor-specific Mutations Detected in Head and Neck Squamous Cell Carcinomas<sup>1</sup>

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## Abstract

We characterized the genomic structure of the human *ING1* gene, a candidate tumor suppressor gene, and found that the gene has three exons. We also demonstrated that four mRNA variants were transcribed from three different promoter regions. Of 34 informative cases of head and neck squamous cell carcinoma, 68% of tumors showed loss of heterozygosity at chromosome 13q33–34, where the *ING1* gene is located. Here we present the first report that three missense mutations and three silent changes were detected in the *ING1* gene in 6 of 23 tumors with allelic loss at the 13q33–34 region. These missense mutations were found within the PHD finger domain and nuclear localization motif in *ING1* protein, probably abrogating the normal function.

## Introduction

Tumor suppressor genes are defined as genetic elements whose loss or mutational inactivation allows cells to display one or more phenotypes of neoplastic growth (1). Tumor suppressor proteins are known to be involved in negative regulation of the cell cycle by different mechanisms (2). A candidate tumor suppressor gene, *ING1*, has recently been cloned (3). Forced overexpression of the *ING1* gene led the cells to arrest in the G<sub>1</sub> phase of the cell cycle and induced apoptosis in several cell types (3, 4). Conversely, inhibition of *ING1* expression by antisense constructs promoted the transformation of mouse breast epithelial cells and increased the frequency of focus formation with NIH3T3 cells and protected the cells from apoptosis (3, 4). The *ING1* gene was mapped on human chromosome 13q34 by radiation hybrid analysis (5). Deletion of the chromosomal 13q33–34 region has been reported in HNSCCs<sup>3</sup> (6–8). Limited analysis of the gene demonstrated rearrangement of the *ING1* gene in one neuroblastoma cell line and reduced expression in primary breast cancers and cell lines (3, 9). Only one germ-line mutation in this gene has been found (9). However, no tumor-specific somatic mutation in the gene has been reported thus far. Here we report the genomic structure of human *ING1* gene and some significant tumor-specific mutations of the *ING1* gene in HNSCCs.

## Materials and Methods

**Tissue Samples.** Paired normal and tumor samples were obtained from 55 patients with primary HNSCCs at the Department of Otolaryngology, Okayama University Hospital after acquisition of informed consent from each

patient. All tissues were frozen in liquid nitrogen immediately after surgery and stored at –80°C until the extraction of DNA and RNA. Histological studies were also performed at the Department of Pathology, and all tumors were confirmed as squamous cell carcinoma.

**DNA and RNA Extraction.** Genomic DNAs were isolated from frozen tissues by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Total RNAs were prepared by using a modified acid guanidinium phenol chloroform method (ISOGEN; Nippon Gene Co., Tokyo, Japan).

**Microsatellite Analysis.** Primers for amplification of microsatellite markers D13S285, D13S796, D13S278, D13S158, and D13S779 are available through the internet genome database.<sup>4</sup> PCR was carried out in 20 μl of reaction mixture with 10 pmol of each primer, 100 ng of genomic DNA, 1× PCR buffer, 200 μM of each deoxynucleotide triphosphate, and 0.5 unit of Taq DNA polymerase (Takara, Kyoto, Japan). Initial denaturation at 94°C for 3 min was followed by 25 cycles of a denaturation step at 94°C for 30 s, an annealing step at 52°C (D13S278 and D13S158) or 56°C (D13S285, D13S796, and D13S779) for 30 s, and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added. After amplification, 2 μl of the reaction mixture were mixed with 8 μl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heat denatured, chilled on ice, and then electrophoresed through an 8% polyacrylamide gel containing 8 M urea. The DNA bands were visualized by silver staining (10). LOH was scored if one of the heterozygous alleles showed at least 50% reduced intensity in tumor DNA as compared with the corresponding normal DNA.

**Analysis of the Genomic Structure of the Human *ING1* Gene.** PCR analysis with *ING1*-specific primers was used to screen a BAC library, and a positive BAC clone (118N21) was purchased from Genome Systems, Inc. (St. Louis, MO). The BAC DNA was digested with appropriate restriction enzymes, and regions containing *ING1* exons were mapped by Southern blotting with *ING1* cDNA probes. Some restriction fragments containing *ING1* exons were subcloned into pBluescript KS(–) (Stratagene, La Jolla, CA) and sequenced.

**Luciferase Assay.** Each putative promoter region around exon 1a and exon 1b was subcloned, and sequential 5′ truncation was introduced by restriction digestion. These DNA fragments were inserted into the promoterless luciferase plasmid pOA-luciferase (11) to produce reporter plasmids for the luciferase assay (see Fig. 2). Each reporter plasmid (0.5 μg) was cotransfected with 0.25 μg of an internal control Renilla luciferase plasmid, pRLTK (Promega, Madison, WI), into 293 human embryonic kidney cells or Saos2 osteosarcoma cells using the calcium phosphate coprecipitation method. The cells were harvested 48 h after transfection. The activities of firefly and Renilla luciferase were measured simultaneously using the Dual-Luciferase Reporter Assay kit (Promega) and normalized for the variation in transfection efficiency. These assays were repeated at least twice in duplicate.

**SSCP Analysis.** The coding region of exon 1a was amplified by PCR with primers on the flanking regions, primers GS9 (5′-TGCAGTGCTATTTTT-GAGGGG) and GAS9 (5′-CGCCCCGCCATCCATCA). Exon 2 was amplified as four overlapping fragments with four primer sets: (a) GS1 (5′-ACGCCTGTCTTCTTGCCCC) and AS8 (5′-CTTGCCGCTGTGGCC-GCTG); (b) S8 (5′-TTCGAGGCGCAGCAGGAGT) and AS5 (5′-CTTG-GCCTTCTTCTCTTGGG); (c) S6 (5′-CAGCAACCACGACCACGACG) and AS3 (5′-TGAGCCCCACGACGAGAAG); and (d) S3 (5′-CCTC-CCCATCGACCCCAACG) and AS1 (5′-ACATTTTACACTCCTTGAC-

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<sup>3</sup> The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity; BAC, bacterial artificial chromosome; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism; aa, amino acid(s).

<sup>4</sup> <http://gdbwww.gdb.org>.

CTCA). The PCR mixture contained 100 ng of DNA, 1.2 mM MgCl<sub>2</sub>, 1× PCR buffer, 200 μM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 unit of rTth DNA polymerase XL (Perkin-Elmer Applied Biosystems, Foster City, CA) in a 50-μl volume. Initial denaturation at 94°C for 3 min was followed by 30 cycles of a denaturation step at 94°C for 30 s, an annealing step at 64°C for 1 min, and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added. One μl of PCR product was mixed with 8 μl of loading dye as described above, heat denatured, chilled on ice, applied onto an 8% polyacrylamide gel with or without 5% glycerol, and run at variable temperatures. Bands were detected by silver staining (10). Aberrantly migrating bands on the gels were excised, reamplified with the same sets of primers, cloned into *EcoRV*-digested pBluescript KS(-) vector, and sequenced.

## Results and Discussion

**LOH Analysis.** We examined DNA from 55 pairs of matched HNSCCs and normal tissues for losses at five microsatellite markers (D13S285, D13S796, D13S278, D13S158, and D13S779) on the chromosome 13q33–34 region. Fig. 1 shows the summary and representative examples. Twenty-three of 34 informative tumors (68%) showed LOH of at least one microsatellite marker (Fig. 1A). This prevalence may be underestimated because most of the specimens were not microdissected, and some apparently normal tissues might have loss by field cancerization. Five tumors (samples 27, 28, 36, 37, and 44) displayed a large deletion that included most polymorphic markers tested. In the other 18 cases, a partial deletion was found, providing information about the areas of preferential loss. The most frequent loss (49%) was seen at marker D13S278. In particular, two cases (samples 14 and 15) exhibited LOH with marker D13S278 but retained heterozygosity with flanking markers D13S796 and

D13S158. This deleted area was estimated to be, at most, 6 cM. It has been reported that a candidate tumor suppressor gene, *ING1*, is located in this region (5). By using the Stanford G3 radiation hybrid panel, *ING1* was linked to marker SHGC-5819, with a LOD score of 10.35 (5). The markers SHGC-5819 and D13S278 are colocalized within less than 3 cM.<sup>4</sup> We therefore determined the genomic structure of the *ING1* gene and searched for its mutation in HNSCCs.

**Genomic Structure of the Human *ING1* Gene.** We screened a BAC clone library by PCR using specific primers for *ING1* cDNA and obtained BAC clone 118N21, which contains the entire region of the *ING1* gene, from Genome Systems, Inc. We identified and cloned two contiguous *Xba*I fragments (about 8 and 3.5 kb) that together contained the whole coding region of the *ING1* gene.<sup>5</sup> Three exons and two introns were identified by sequence analysis, as shown in Fig. 2. Recently, three alternatively spliced forms of *ING1* mRNA have been described (12). Our results showed that the *ING1b* form (GenBank accession number AF181850), which encodes a *M<sub>r</sub>* 33,000 protein, was composed of exons 1a and 2 and that the *ING1a* form (GenBank accession number AF181849), which is predicted to encode a *M<sub>r</sub>* 47,000 protein, was composed of exons 1b and 2 in our map (Fig. 2). The third spliced form, *ING1c* (GenBank accession number AB031269), which is predicted to encode a *M<sub>r</sub>* 24,000 protein, was composed of a truncated *ING1a* message including the first ATG codon in exon 2 on our map. The size of the coding region in exons 1a, 1b, and 2 is 136, 565, and 701 bp, respectively.

Our sequence results showed some differences from previously published cDNAs (3): (a) GTG to GCG at codon 123; (b) GTT to GCT at codon 129; (c) AAT to AAA at codon 135; (d) GAT to GAG at codon 137; (e) GTA to GCA at codon 139; and (f) TCT to GCT at codon 142, according to numbering from exon 1a. These new sequences were reconfirmed by our genomic DNA and cDNA analyses and by the database corrections made recently by Ma *et al.* (12).

When our study was almost complete, the genomic organization of the mouse *ING1* gene and the functional diversity of alternatively spliced variants of the gene product were reported (13). In the mouse *ING1* gene, there are three alternative exons corresponding to human exons 1a and 1b, but only two final protein products were predicted [p37 (279 aa) and p31 (185 aa), corresponding to human p33ING1b (279 aa) and p24ING1c (210 aa), respectively]. The largest form, p47ING1a, appears to be unique to the human. Three putative promoters in the mouse gene were also inferred by sequencing (13).

**Promoter Analysis of the Human *ING1* Gene.** To analyze the promoter for the p33ING1b form, the 5'-flanking 3.5-kb region containing exon 1a was digested at six restriction sites described in Fig. 2, (1), and six luciferase reporter plasmids were constructed and assayed. Four luciferase constructs with the promoter region between the *Xba*I and *Not*I sites showed about a 39–59-fold increase in luciferase activity as compared with the promoterless luciferase plasmid. A promoter region of about 1.7 kb from the *Apa*LI site to the translational initiation site was sequenced and searched for binding sites of transcription factors. There were some motifs such as the GC box, the GATA box, and the TATA box-like and CCAAT box-like motifs (6). The CCAAT box-like and TATA box-like motifs were detected at almost the same position as shown in the putative promoter for exons 1a and 1b of mouse *ING1* (13). The promoter analysis for the p47ING1a form was performed by using the 1.1-kb fragment between the *Not*I site in intron 1 and the *Pfl*MI site located 12 nucleotides upstream from the initiation codon for p47ING1a as shown in Fig. 2, (2). The *Not*I-*Pfl*MI luciferase construct and the four derivative constructs showed a 2–12-fold increase in luciferase activity as compared with the promoterless luciferase plasmid. To analyze the pro-

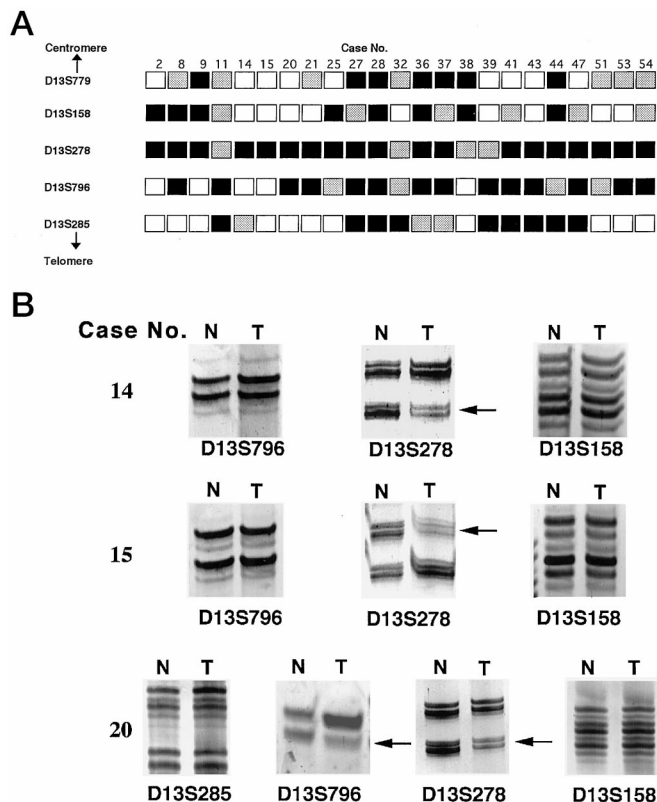


Fig. 1. LOH analysis on chromosome 13q33–34 in HNSCCs. A, schematic representation of LOH distribution. Case numbers are shown at the top. Microsatellite markers used to detect LOH are shown to the left. ■, LOH; □, retention of heterozygosity; □, not informative (homozygous). B, primary LOH data from three representative HNSCCs. N, normal DNA; T, tumor DNA. Cases 14 and 15 show LOH of marker D13S278 but retention of the flanking markers. Case 20 also shows LOH of markers D13S796 and D13S278 but retention of the flanking markers.

<sup>5</sup> The nucleotide sequences reported in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB037386 and AB037387.

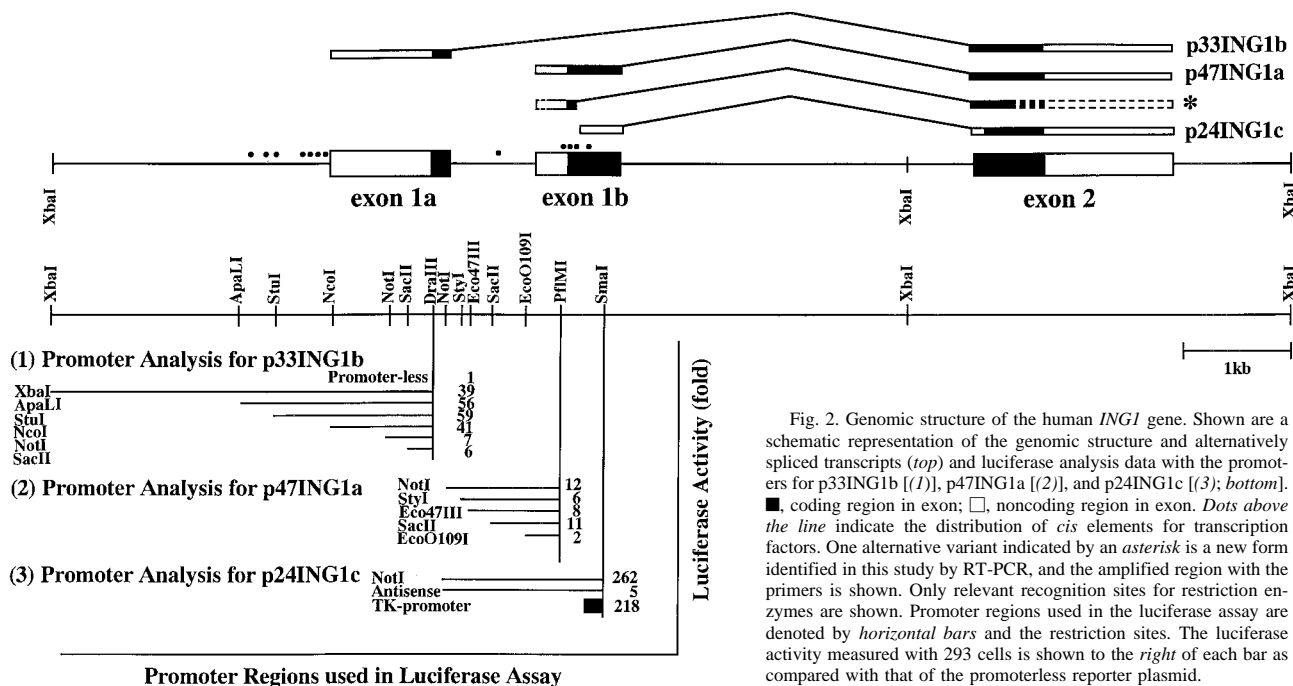


Fig. 2. Genomic structure of the human *ING1* gene. Shown are a schematic representation of the genomic structure and alternatively spliced transcripts (*top*) and luciferase analysis data with the promoters for p33ING1b [(1)], p47ING1a [(2)], and p24ING1c [(3); *bottom*]. ■, coding region in exon; □, noncoding region in exon. Dots above the line indicate the distribution of *cis* elements for transcription factors. One alternative variant indicated by an asterisk is a new form identified in this study by RT-PCR, and the amplified region with the primers is shown. Only relevant recognition sites for restriction enzymes are shown. Promoter regions used in the luciferase assay are denoted by horizontal bars and the restriction sites. The luciferase activity measured with 293 cells is shown to the right of each bar as compared with that of the promoterless reporter plasmid.

motor for the p24ING1c form, the 1.6-kb fragment between the *NorI* site in intron 1 and the *SmaI* site in exon 1b was used in the luciferase assay as shown in Fig. 2, (3). This region showed a 262-fold increase in luciferase activity, despite the lower luciferase activity on the *NorI-PflMI* region described above, suggesting that this strong promoter existed in the coding exon between the *PflMI* and *SmaI* sites. To confirm these results, we analyzed each spliced form by PCR with mRNA from head and neck tissues or cDNA libraries from human fetal lung cells and HeLa cells. The *ING1c* form was readily detected with a sense primer locating at a 150-nucleotide downstream region from the initiation codon for p47ING1a and an antisense primer on exon 2, whereas no *ING1a* form was detected with another sense primer set on the initiation codon for p47ING1a and an antisense primer on exon 2. Instead, we detected a weakly expressed form composed of the first 29 codons of exon 1b and exon 2 in frame that contained a limited region amplified with the primer pair used<sup>6</sup> (summarized in Fig. 2), but the importance of this form is not yet known. The promoter for p47ING1a was relatively weak, suggesting that it may be activated in a tissue-specific manner. We could not identify the transcriptional start site for the *ING1a* form as reported (12) by 5' extension of cDNA ends as long as it was reported (12). In the promoter regions for the human *ING1a* and *ING1c* forms, we found three motifs for transcriptional binding factors: (a) the GC box; (b) the CCAAT box-like sequence; and (c) the TATA box-like sequence<sup>6</sup> (summarized in Fig. 2). RT-PCR analysis also showed that the *ING1b* form was expressed as a major transcript in human normal head and neck tissues and in cDNA libraries from human fetal lung and HeLa cells.<sup>6</sup>

Mouse exon 1c is included within human exon 1b with a limited homology, whereas the putative promoter sequence for mouse exon 1c was conserved with a higher homology (about 70%) within the intraexonic promoter in human exon 1b. In contrast, coding and noncoding regions in human exon 1a as well as the upstream promoter region conserved a high homology (about 80%) with mouse *ING1* exons 1b and 1a and promoter sequences (13). It is noteworthy that human sequences similar to mouse exon 1a or 1c did not show any proper structure as a functional exon.

**Mutation Analysis.** To investigate whether the *ING1* gene is the target of functional loss in tumors, we searched for mutations in the coding regions of the gene in 23 samples of HNSCC with LOH at chromosome 13q34. Six possible mutations were identified by SSCP analysis. Three samples showed missense mutations with aa change (13%) (Fig. 3). The other three samples displayed a common alteration from TCG to TCA at codon 173 of p33ING1b without an aa change. All of these point mutations were confirmed by repeated, independent PCR amplification and SSCP analyses. The *ING1* gene encodes nuclear proteins that contain a zinc finger motif in their COOH-terminal half (3). Zinc finger motifs are classified into several subfamilies, and most of them have a role in the recognition of macromolecules such as DNA, RNA, and protein (14). The PHD finger, a C4HC3-type zinc finger spanning 50–80 aa residues, has been found in a number of chromatin-mediated transcriptional regulators from a variety of sources (15). One of the mutations in this study showed an aa substitution from cysteine (TGC) to serine (TCC) at codon 215, which is one of the seven cysteine residues composing the C4HC3 motif in *ING1*. This change may affect the PHD finger and break the three-dimensional structure of *ING1* protein, leading to loss of the function. The second mutation at codon 216 is just next to the above-mentioned cysteine residue. This missense mutation showed an aa change from asparagine (AAC) to serine (AGC) and may also affect the conformation of the zinc finger domain and the *ING1* protein. The third mutation we detected caused an aa change from alanine (GCC) to aspartic acid (GAC) at codon 192. This aa was next to a lysine within the possible nuclear localization signal of *ING1*. This substitution from a nonpolar to an acidic aa may affect the nuclear localization signal that is abundant in positively charged aa and may ultimately interfere in the accumulation of *ING1* protein in the nucleus. All three mutations were located within the area of exon 2 encoding the common COOH-terminal portion of all *ING1* protein species.

Thus, it would be likely that a considerable subset of HNSCCs harbors inactivating mutations in the *ING1* gene accompanied by selective loss of another allele, in accordance with the definition of a tumor suppressor gene (16). This is, to our knowledge, the first report describing the inactivating point mutations in the *ING1* gene from human primary cancer. None of a total of 452 breast cancers, ovarian

<sup>6</sup> M. Gunduz and M. Ouchida, unpublished results.



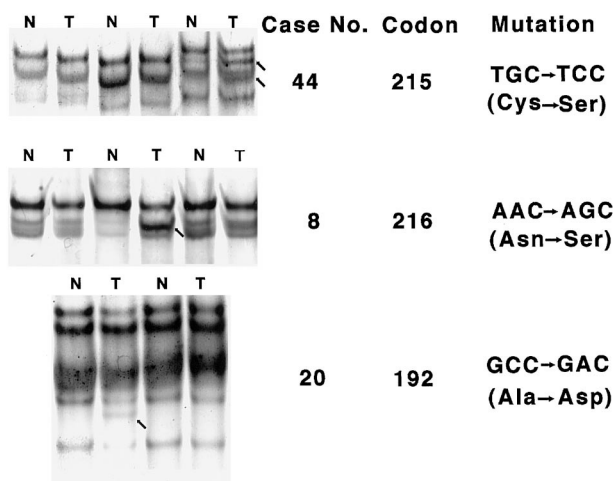


Fig. 3. SSCP analysis of the *ING1* gene in genomic DNA from HNSCCs. SSCP analysis demonstrated the altered migration pattern of PCR products containing a mutated nucleotide. N, normal DNA; T, tumor DNA. Arrows point to the bands altered in tumor DNA as compared with the corresponding normal DNA. The aa substitutions of p33ING1b are shown to the right.

cancers, and cell lines examined exhibited tumor-specific somatic mutation (9). Because LOH at chromosome 13q33–34 has been observed only in HNSCCs (6–8), *ING1* gene mutation may be restricted to only this type of cancer.

As compared with the LOH value (49%), the frequency of missense mutation of the *ING1* gene (13%) is still low in HNSCC. Several possible explanations may explain this result.

First, the *ING1* gene and flanking regions are highly GC rich, and methylation of the promoter region may cause a loss of gene products. A well known tumor suppressor gene, *p16*, has rarely demonstrated homozygous loss or point mutations because the gene is usually inactivated by aberrant methylation in head and neck cancers (17). Research aimed at examining methylation in the promoter region of the *ING1* gene may uncover another mechanism for inactivation of this gene. Although we have just started to look for methylation of the *ING1* gene, one sample with LOH showed a tumor-specific complete loss of the *ING1* transcripts by RT-PCR.<sup>6</sup> This suggests that methylation of the *ING1* promoter region does occur. Our data on the structure of the promoters of *ING1* may be useful for these analyses. Second, it has been shown that ING1 protein interacts physically with p53 and that the growth-inhibitory effect of ING1 requires the activity of wild-type p53 (18, 19). Because mutations of p53 have been reported frequently in various human tumors including HNSCCs (20), ING1 may not exhibit its growth-inhibitory activity in cells in which wild-type p53 is lost, and alterations in ING1 may not be necessary in these cases. With regard to this issue, it was recently reported that the p33 equivalent of the mouse *ING1* gene acts as a p53 suppressor and not as an activator, as p24ING1c does (13). Thus, the situation may be complicated. Because the control mechanism(s) of the *ING1* gene in the human and mouse forms appears to have both common and different pathways, the exact nature of each protein variant must be analyzed separately, including the p53-dependent and -independent pathways as suggested previously (13). No matter what ING1 protein variant is responsible for tumor suppression, we suppose that the observed mutations in the COOH-terminal portion of the protein influence the tumor-suppressing ability of ING1 proteins. The high prevalence of LOH at 13q33–34 in HNSCCs suggests the importance of tumor suppressor ability rather than oncogenic ability for the gene(s) located here.

Thirdly, Knudson's definition (16) of a tumor suppressor gene requires the demonstration of inactivation of both alleles of a candidate gene in

tumors. However, a new class of tumor suppressor gene with haploid insufficiency, in which one allele is lost and the remaining allele is haploinsufficient, has been described recently, and these hemizygous tumor suppressor genes show a tumor-prone phenotype when challenged with carcinogens (21, 22). Most of the head and neck cancer patients continued smoking for a long time, and inactivation of only one allele of the *ING1* gene or a moderately decreased expression may be sufficient to predispose cells to tumorigenesis. Finally, it can also be thought that there may be another tumor suppressor gene in chromosomal region 13q33–34. More detailed studies will clarify this possibility in the near future.

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