

Genetic Alterations of Candidate Tumor Suppressor *ING1* in Human Esophageal Squamous Cell Cancer¹

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Abstract

Overexpression of *ING1*, a candidate tumor suppressor gene, efficiently blocks cell growth or induces apoptosis in different experimental systems. *ING1* maps to chromosome 13q33–34, and because loss of the terminal region of chromosome 13q has been implicated in esophageal squamous cell cancer (ESCC), we examined ESCC for genetic alterations of *ING1*. Among 31 informative cases of ESCC, 58.9% of the tumors showed allelic loss at chromosome 13q33–34, and we detected four tumor-specific missense nucleotide changes. These alterations were found within the PHD finger domain and nuclear localization motif of the *ING1* and may be functionally involved in the development of ESCC. Because immunohistochemical study revealed that all of the ESCC samples showed loss of *ING1* protein expression, genetic or epigenetic alterations that abrogate the normal function of *ING1* may contribute to esophageal squamous cell carcinogenesis.

Introduction

ING1 is a recently cloned novel growth inhibitor and candidate tumor suppressor gene that was detected using a method of subtractive hybridization of cDNAs from normal and cancer cells followed by an *in vitro* selection assay (1–3). It appears to have a role in programmed cell death, because its overexpression confers sensitivity to apoptosis, whereas antisense *ING1* protects cells from apoptosis in different experimental systems (4). Expression of *ING1* is also regulated through the cell cycle, and its growth-inhibitory effects are abrogated by SV40 large T antigen (5). Recently, *ING1* protein has been reported to cooperate directly with p53 in growth regulation by modulating the ability of p53 to act as a transcriptional activator (6), although the effects upon gene expression control may be attributable to effects on gene expression through histone acetyl transferase activity (7). The biological effects of *ING1* and its ability to regulate the expression of growth inhibitors indicate that *ING1* may be a tumor suppressor and that functional loss of *ING1* might contribute to tumorigenesis.

The *ING1* gene localizes to chromosome 13q33–34 (8), a site that has been implicated in the development of various human tumors (9), especially oral/esophageal/squamous cell cancers. Esophageal cancer ranks among the 10 most common cancers in the world and is almost uniformly fatal. The genetic events leading to the development of esophageal carcinoma are not well established. ESCC³ shows frequent

LOH on terminal regions of chromosome 13q where *ING1* is located (10, 11). This correlation prompted us to investigate its potential role in esophageal squamous cell carcinogenesis.

Accordingly, we examined whether genetic alterations, such as allelic imbalance or mutations of the *ING1* gene, as well as altered protein expression of *ING1*, might be responsible for the emergence and progression of human ESCC.

Materials and Methods

Tumor Tissue. Tumor tissue and matched normal mucosal tissue or matched lymphocytes were obtained from ESCC patients undergoing surgical resections as primary therapy for their disease after informed consent. All patients were from the Okayama University Medical School. Tumors had to be large enough to provide sufficient amounts of tissue for genetic analysis without compromising pathological diagnosis. Tissues of nonnecrotic areas of the tumor, as well as normal mucosa, were placed on ice immediately upon removal from the patient and subsequently frozen in liquid N₂. Tissues were stored at –80°C until RNA and DNA isolation were completed.

RT-PCR. *ING1* mRNA expression in tumors and in normal tissues was monitored by RT-PCR. Each 20- μ l cDNA synthesis reaction contained 1 μ l of total RNA from tumor and normal cells, buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 1.5 mM MgCl₂], 1 mM each of deoxynucleoside triphosphates, 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.), and 100 ng of pd(N)₆ random hexamer (Pharmacia). One μ l of each RT-PCR reaction was amplified using 1 unit of rTth DNA polymerase, XL (Perkin-Elmer, Foster City, CA). PCR for 35 cycles was performed for the amplification of the *ING1* conserved shared COOH domain encoded by the common 3' exon for both *ING1a* and *ING1b* (*ING1ex-S*: 5'-CTGAAGGAGCTAGACGAGTGC-3' and *ING1exAS*: 5'-ACGCACGAGAAGTGAACCA-3'), specific for *ING1a* (*ING1int-S*: 5'-CCGCATCTTTGCTGACCCGA-3' and *ING1int-AS*: 5'-GCCTTCTTCTTGGGTGT-3') and specific for *ING1b* (*ING1B-S*: 5'-CTCCATCGAGTCCCTGCCTT-3' and *ING1B-AS*: 5'-GCCTTCTTCTTGGGTGT-3'). PCR for GAPDH were also done for 25 cycles using primer pair GAPDH-S: 5'-CGGAGTCAACGGATTTGGTGCAT-3' and GAPDH-AS: 5'-AGCCTTCTCATGGTGGYGAAGAC-3'.

LOH Analysis Using a Microsatellite Marker on 13q. To study allelic deletion in esophageal cancer, we examined DNA for LOH at *D13S285*, *D13S796*, *D13S278*, *D13S158*, and *D13S779*, which are located close to the *ING1* locus. Primer sets were available through the Internet genome database. PCR was performed in 25- μ l reaction mixtures comprising 100 ng of template genomic DNA, 5 pmol of each oligonucleotide primer pair (one end-labeled with Texas Red), 0.5 unit of Taq DNA polymerase (Takara, Kyoto, Japan), 2.5 μ l of 10 \times buffer, and 1 μ l of 1.25 mM deoxynucleotide triphosphate. After denaturation for 2 min at 94°C, each PCR was carried out for 30 cycles consisting of denaturation for 1 min at 94°C, annealing for 2 min at 54°C, and extension for 10 min at 72°C. PCR products were denatured in 98% formamide for 2 min at 80°C and then electrophoresed on denaturing 8% polyacrylamide sequencing gels on HITACH Autosequencer SQ-5500 (Hitachi Electronics Engineering Co., Ltd., Tokyo, Japan).

Direct Sequencing of PCR Products. Cycle sequence was done using a Thermo Sequenase core sequencing kit PRN2440 (Molecular Dynamics, Inc., Sunnyvale, CA). Briefly, 400 ng of template DNA, 2 pmol of primer, and 3 μ l of Sequenase with 4 dye termination mix were mixed with distilled H₂O to make a final reaction volume of 22 μ l, and the cycle sequence reaction was

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³ The abbreviations used are: ESCC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry.

performed as the following profile: denaturation at 95°C for 30 s and annealing at 60°C for 30 s. The number of cycles used was 25. Sequencing of the products was conducted with site-directed primers to cover the entire length of cDNA from both the 5' and 3' ends under the manufacturer's guidelines (Hitachi Autosequencer SQ-5500). Site-directed primer pairs were as follows: ING1int-S: 5'-CCGCATCTTTGCTGACCCGA-3' and ING1int-AS: 5'-GCCTTCTTCTTGGGTGT-3'; and ING-S1: 5'-AAGCGGCGGATGCTGCACTGT-3' and ING-AS4: 5'-CTACCTGTTCTAAGCCCTCTC-3'. Reproduction of the result was confirmed by sequencing all samples at least twice from both sides.

IHC. The expression of ING1 protein in paraffin-embedded histological sections was determined using the avidin-biotin-peroxidase complex method (12). Histological sections (4 μm) on 0.02% poly-L-lysine-coated slides (Sigma Chemical Co., St. Louis, MO) were deparaffinized and rehydrated, and the endogenous peroxidase activity was blocked by incubation with 2% H₂O₂ in phosphate buffer, followed by pretreatment with proteinase K. Nonspecific binding was blocked with serum, and sections were incubated with ING1 antibody (supplied by the Southern Alberta Cancer Research Center hybridoma facility). After washing with phosphate buffer, the sections were incubated with biotinylated secondary antibody and washed with phosphate buffer, followed by incubation with an avidin-biotin-peroxidase complex. The reac-

tion was developed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), washed, and counterstained with methyl green. Sections from normal stomach were used as positive controls for the expression of ING1, and sections incubated in goat serum instead of the corresponding primary antibody were used for comparison as negative controls.

Results and Discussion

LOH Analysis. Loss of terminal regions of chromosome 13q has been implicated in ESCC. Because the gene encoding the ING1 candidate tumor suppressor is located in 13q33–34, we first tested for LOH in that region using microsatellite markers. DNA samples from tumors and normal adjacent noncancerous epithelium of patients with ESCC were subjected to a study using microsatellite markers *D13S285*, *D13S796*, *D13S278*, *D13S158*, and *D13S779* on different locations on chromosome 13q33–34. Overall, allelic loss on 13q33–34 was observed in 17 of 31 patients. The pattern of allelic loss of chromosome 13q33–34 for each sample is presented schematically in Fig. 1A. Considering informative tumors only, LOH was observed in 17 of 29 informative cases (58.9%). Among 17 samples with allelic

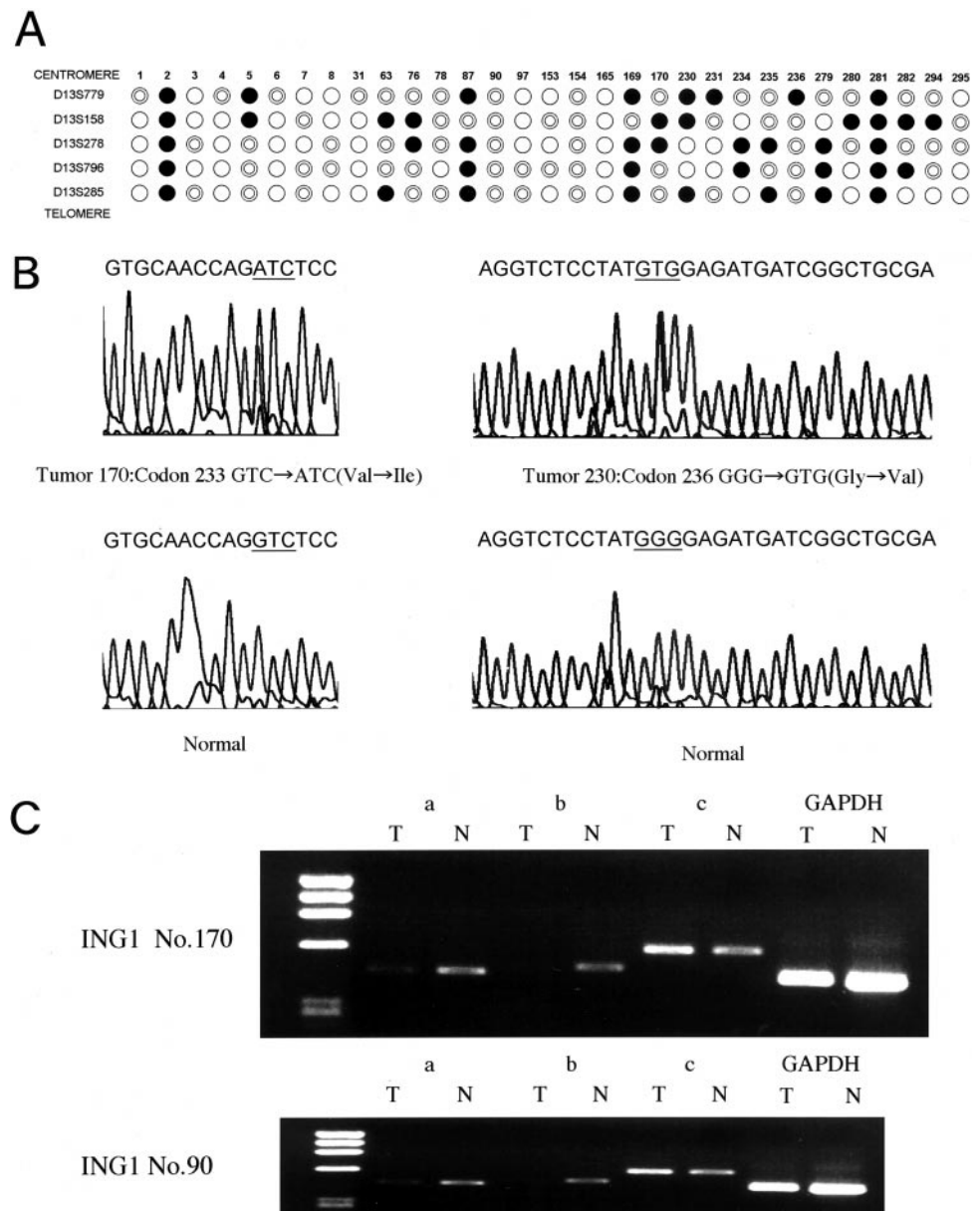


Fig. 1. Analysis of chromosome 13q33–34 in ESCC. **A**, LOH analysis on chromosomal 13q33–34 in ESCC. ●, informative and with LOH; ○, informative and retention of heterozygosity; ◎, not informative (homozygous). **B**, two mutations of *ING1* among the 31 cases of ESCC. Tumor 170 represents a missense mutation at codon 233. Guanine in the corresponding mucosa was replaced by alanine in the primary lesion. This mutation resulted in a change from valine to isoleucine in the gene product (*left*). Tumor 230 represents a missense mutation of guanine to thymine transversion at codon 236, resulting in a change from glycine to valine (*right*). **C**, *ING1* mRNA expression analysis in selected samples. T, tumor; N, normal.

Table 1 *ING1* alterations in esophageal cancer patients

Patient no.	Codon	Nucleotide change	Amino acid change	LOH	Effect on coding sequence
90	270	AAG-AAT	Lys-Asn	NI ^a	Missense mutation
170	233	GTC-ATC	Val-Ile	+	Missense mutation
230	236	GGG-GTG	Gly-Val	+	Missense mutation
281	214	GCG-GAG	Ala-Glu	+	Missense mutation
234	219	CCC-CCT	Pro-Pro	+	Silent mutation
279	223	AAC-AAT	Asn-Asn	+	Silent mutation

^a NI, not informative.

loss, 10 showed LOH in all informative loci examined. The frequency of LOH for each of the loci was >39%. In two samples (90 and 154), all loci examined were not informative (homozygous).

Mutation Analysis. To investigate whether the *ING1* gene is the target of functional inactivation in tumors, we searched for mutations in the coding regions of the *ING1* in 31 samples of ESCC by direct sequencing of RT-PCR products. We also sequenced syngeneic normal counterparts of tumors to determine whether the variant was tumor specific. Four somatic missense mutations and two somatic silent mutations were detected in 31 ESCC patients (Table 1). The tumor DNA from patient 170 showed a G to A transition at the first nucleotide of codon 233 and resulted in a valine to isoleucine substitution (Fig. 1*b*). The DNA from tumor sample 90 showed a G to T transversion at the third nucleotide of codon 270, resulting in a lysine to asparagine substitution. The ESCC DNA from patients 230 and 281 showed missense substitution on codons 236 and 214, respectively (Fig. 1*b*). These two alterations were G to T and C to A nucleotide changes of the middle nucleotide of the codons, resulting in glycine to valine and alanine to glutamic acid substitutions, respectively. All of the point mutations were confirmed by repeated, independent PCR amplification and sequencing from both ends.

All of the missense mutations detected were located between codons 214 and 270. These lesions are within a conserved region of *ING1* isoforms having a high degree of homology to PHD fingers, which are implicated in transcriptional regulation. Because our studies showed that tumors 170, 230, and 281 showed LOH, we conclude that there is biallelic inactivation of *ING1* in them. Because we could not

confirm LOH on tumor sample 90 and because all of the microsatellite markers we examined were not informative, it is also possible that tumor 90 had both alleles inactivated.

Expression of Subtypes of *ING1* mRNA. Recently, it was reported that the human *ING1* gene has two splicing variants encoding p47^{ING1a} and p33^{ING1b}, which have one large conserved common 3' exon (13). Accordingly, we also examined the RNA expression of both variants in our series of ESCC with primer sets specific for *ING1a* or *ING1b*, as well as primer sets amplifying a region common to both genes. Almost all of the cancers and their normal counterparts express both *ING1a* and *ING1b*. Some tumors express less *ING1a*, and some express less *ING1b*, but there were no obvious correlations between the subtype, expression levels, and genetic alterations (Fig. 1*C*).

Protein Expression by IHC. *ING1* protein expression was examined in paraffin-embedded material from 31 ESCC patients (Fig. 2). Normal stomach sections of paraffin-embedded materials from the series of ESCC patients were examined for *ING1* expression as a control. All of the normal gastric fundic glands were positive for *ING1* antibody. *ING1* expression in normal esophageal squamous epithelia was positive weekly, and *ING1* protein expression was absent in all ESCC samples (Fig. 2). Albeit all ESCC epithelia did not express *ING1* protein, the four tumors with amino acid changes in *ING1* showed no obvious correlation to the expression levels of this protein.

Clinicopathological factors of all of the patients are shown in Table 2. One ESCC was stage IV, and the others except three cases were

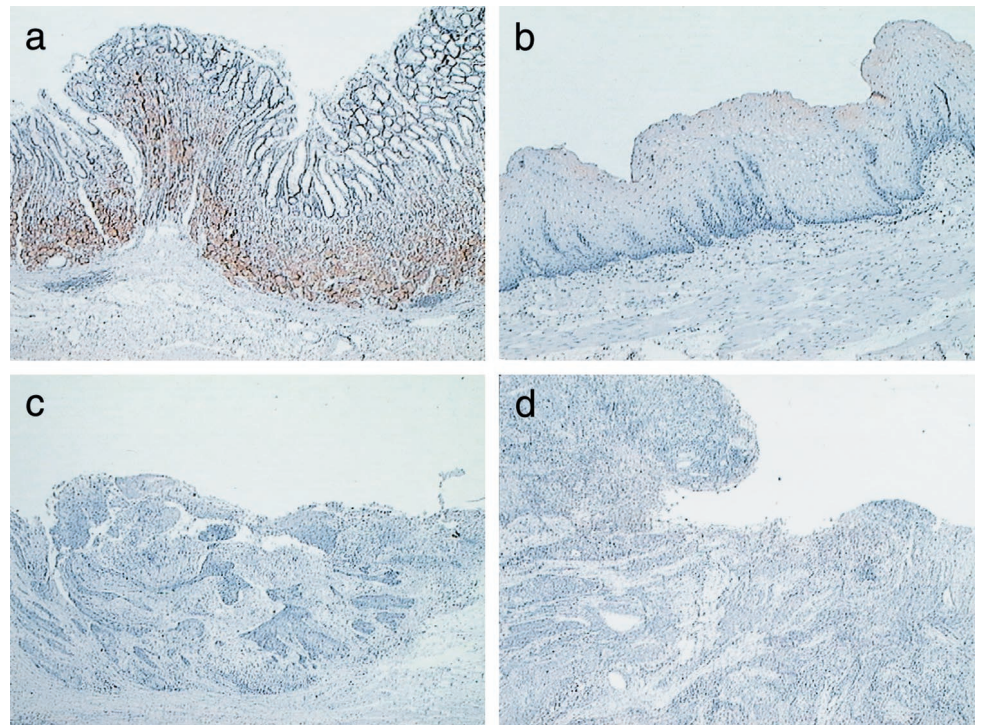


Fig. 2. Representative examples of IHC staining for *ING1* ($\times 200$) on normal stomach epithelium (*a*), normal esophagus epithelium (*b*), and ESCC (*c* and *d*). In *a*, gastric fundic glands were positive for *ING1*. In *b*, esophageal squamous epithelia were positive weekly for *ING1*. In *c* and *d*, *ING1* was not detected in ESCC samples (*c*, tumor 230 with *ING1* missense mutation; *d*, tumor 97 without *ING1* mutation).

Table 2 Patient characteristics and tumor samples

Patient no.	Age at diagnosis (yr)	Sex	Histology	TNM ^a	Stage	Survival after diagnosis (m)	Clinical outcome
1	74	M	mod. SCC ^b	T ₂ N ₀ M ₀	II A	9	NED
2	64	M	mod. SCC	T ₃ N ₁ M ₀	III	9	NED
3	64	M	mod. SCC	T ₃ N ₁ M ₀	III	10	NED
4	61	M	mod. SCC	T _x N ₀ M ₀		9	NED
5		F	mod. SCC	T ₁ N ₁ M ₀	II B	2	DOD
6	69	M	well. SCC	T ₃ N ₁ M ₀	III	10	NED
7	73	M	mod. SCC	T ₁ N ₀ M ₀	I	12	NED
8	66	M	mod. SCC	T ₂ N ₁ M ₀	IV	11	NED
31	69	M	well. SCC	T ₄ N ₁ M ₀	III	8	DOD
63	69	M	por. SCC	T ₁ N ₁ M ₀	I	26	DOD
76	58	M	well. SCC	T ₂ N ₁ M ₀	II B	57	NED
78	53	M	por. SCC	T ₃ N ₁ M ₀	III	57	NED
87	66	M	dif. SCC	T ₂ N ₁ M ₀	II B	11	DOD
90	70	F	well. SCC	T ₄ N ₁ M ₀	III	7	DOD
97	64	M	mod. SCC	T ₃ N ₁ M ₀	III	54	NED
153	63	M	mod. SCC	T ₃ N ₁ M ₀	III	9	DOD
154	70	M	mod. SCC	T ₂ N ₁ M ₀	II B	37	NED
165	70	M	mod. SCC	T ₂ N ₁ M ₀	II B	8	DOD
169	57	M	por. SCC	T ₃ N ₁ M ₀	III	43	AWD
170	60	M	well. SCC	T ₄ N ₁ M ₀	III	34	NED
230	54	M	well. SCC	T ₂ N ₁ M ₀	II B	16	DOD
231	71	M	mod. SCC	T ₃ N ₁ M ₀	III	28	NED
234	46	M	mod. SCC	T ₃ N ₁ M ₀	III	10	DOD
235	66	M	mod. SCC	T ₂ N ₁ M ₀	II B	2	DOD
236	73	F	undif. SCC	T ₂ N ₀ M ₀	II A	5	DOD
279	80	M	por. SCC	T ₂ N ₁ M ₀	II B	3	DOD
280	60	M	mod. SCC	T ₁ N ₁ M ₀	II B	33	NED
281	62	M	por. SCC	T ₁ N ₁ M ₀	II B	34	NED
282	55	M	well. SCC	T ₂ N ₀ M ₀	II A	21	NED
295	66	M	mod. SCC	T ₂ N ₁ M ₀	II B	12	DOD

^a TNM, Tumor-Node-Metastasis.

^b mod. SCC, moderately differentiated; por., poorly differentiated; well., well differentiated, undif., undifferentiated; dif., differentiated; SCC, squamous cell carcinoma; NED, non—evidence of disease; DOD, died of disease; AWD, alive with disease.

stages II to III cancers. ESCC in general shows poor prognosis, as shown in Table 2. Among the four ESCC with missense mutations, two were dead with disease, and the other two were alive without evidence of disease, but there was no statistically significant correlation between mutation status and prognosis. The tumors harboring mutations also had no relationship to other clinicopathological features.

Although the sequence of *ING1* was not altered frequently, our data represent the first report of detecting candidate mutations in ESCC; however, a previous study has also noted the presence of mutations in head and neck squamous cell cancers (14). The reported mutations detected in head and neck squamous cancers were also located in PHD finger domains with missense mutations, which are similar to our ESCC results. The reported mutations in head and neck cancers were infrequent as well. There are several possible explanations for our detection of a low frequency of *ING1* mutation in ESCC: (a) our sequencing analysis was done within the common exon. Because the common exon of both genes accounts for approximately 75 and 80% of the coding regions of the major *ING1* isoforms, the actual *ING1* mutation frequency may be underestimated; and (b) this frequency may also be underestimated because the examined specimens were not microdissected, or field carcinogenesis might have obliterated the LOH. Because there is evidence that *ING1* may serve as a “class 2 tumor suppressor” being inactivated at the level of RNA rather than DNA, in our study we have accumulated data that suggest such epigenetic modification. Our IHC study shows that the down-regulation of *ING1* protein in ESCC is very substantial. Because in some tumors *BRCA1* or *p16* gene expression was reported to be reduced by hypermethylation of their promoter sequence, such epigenetic modifications operate to functionally inactivate the *ING1* gene (15–17). Examination of the hypermethylation status of the *ING1* promoter region, which is in progress, should address this possibility.

Although functional analyses of the detected *ING1* variants to determine whether they were mutants or polymorphisms were not

done, all of the missense mutations found in ESCC were located within the highly conserved PHD finger-like domain that is implicated in transcriptional regulation. The PHD finger, a C₄HC₃-type zinc finger spanning 50–80 amino acid residues, has been found in a number of chromatin-mediated transcriptional regulators from a variety of sources (18).

In addition to *ING1*, it is likely that additional, presently unidentified growth-regulatory tumor suppressor genes that are important for ESCC carcinogenesis are located in 13q33–34. *ING1* may, therefore, represent only one critical component leading to human squamous esophageal carcinogenesis, which is supported by the low level of expression of *ING1* protein and the detection of some missense mutations in this study within a functionally important region of the gene. More detailed studies will elucidate this possibility in the near future.

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References

- Lisitsyn, N., Lisitsyn, N., and Wigler, M. Cloning the differences between two complex genomes. *Science* (Wash. DC), 259: 946–951, 1993.
- Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA*, 93: 6025–6030, 1996.
- Garkavtsev, I., Kazarov, A., Gudkov, A., and Riabowol, K. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. *Nat. Genet.*, 14: 415–420, 1996.
- Helbing, C. C., Veillette, C., Riabowol, K., Johnston, R. N., and Garkavtsev, I. A novel candidate tumor suppressor, *ING1*, is involved in the regulation of apoptosis. *Cancer Res.*, 57: 1255–1258, 1997.
- Garkavtsev, I., and Riabowol, K. Extension of the replicative life span of human diploid fibroblasts by inhibition of the p33ING1 candidate tumor suppressor. *Mol. Cell. Biol.*, 17: 2014–2019, 1997.

6. Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. *Nature (Lond.)*, *391*: 295–298, 1998.
7. Loewith, R., Meijer, M., Lees-Miller, S. P., Riabowol, K., and Young, D. Three yeast proteins related to the human candidate tumor suppressor p33(ING1) are associated with histone acetyltransferase activities. *Mol. Cell. Biol.*, *20*: 3807–3816, 2000.
8. Garkavtsev, I., Demetrick, D., and Riabowol, K. Cellular localization and chromosome mapping of a novel candidate tumor suppressor gene (*ING1*). *Cytogenet. Cell Genet.*, *76*: 176–178, 1997.
9. Toyama, T., Iwase, H., Watson, P., Muzik, H., Saettler, E., Magliocco, A., DiFrancesco, L., Forsyth, P., Garkavtsev, I., Kobayashi, S., and Riabowol, K. Suppression of *ING1* expression in sporadic breast cancer. *Oncogene*, *18*: 5187–5193, 1999.
10. Shibagaki, I., Shimada, Y., Wagata, T., Ikenaga, M., Imamura, M., and Ishizaki, K. Allelotype analysis of esophageal squamous cell carcinoma. *Cancer Res.*, *54*: 2996–3000, 1994.
11. Pack, S. D., Karkera, J. D., Zhuang, Z., Pak, E. D., Balan, K. V., Hwu, P., Park, W. S., Pham, T., Ault, D. O., Glaser, M., Liotta, L., Detera-Wadleigh, S. D., and Wadleigh, R. G. Molecular cytogenetic fingerprinting of esophageal squamous cell carcinoma by comparative genomic hybridization reveals a consistent pattern of chromosomal alterations. *Genes Chromosomes Cancer*, *25*: 160–168, 1999.
12. Yoshino, T., Miyake, K., Ichimura, K., Mannami, T., Ohara, N., Hamazaki, S., and Akagi, T. Increased incidence of follicular lymphoma in the duodenum. *Am. J. Surg. Pathol.*, *24*: 688–693, 2000.
13. Garkavtsev, I. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. *Nat. Genet.*, *23*: 373, 1999.
14. Gunduz, M., Ouchida, M., Fukushima, K., Hanafusa, H., Etani, T., Nishioka, S., Nishizaki, K., and Shimizu, K. Genomic structure of the human *ING1* gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. *Cancer Res.*, *60*: 3143–3146, 2000.
15. Dobrovic, A., and Simpfendorfer, D. Methylation of the *BRCA1* gene in sporadic breast cancer. *Cancer Res.*, *57*: 3347–3350, 1997.
16. Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I. C., Repasky, E. A., Gabrielson, E., Schutte, M., Baylin, S. B., and Herman, J. G. Promoter hypermethylation and *BRCA1* inactivation in sporadic breast and ovarian tumors. *J. Natl. Cancer Inst. (Bethesda)*, *92*: 564–569, 2000.
17. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*, *93*: 9821–9826, 1996.
18. Aasland, R., Gibson, T. J., and Stewart, A. F. The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.*, *20*: 56–59, 1995.