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Cytoplasmic expression of p33^{ING1b} is correlated with tumorigenesis and progression of head and neck squamous cell carcinoma

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Summary. To clarify the role of p33^{ING1b} in tumorigenesis and progression of head and neck squamous cell carcinoma (HNSCC), we examined the expression and subcellular localization of p33^{ING1b} in 214 HNSCC cases in parallel with 60 dysplasia samples and 48 normal epithelium samples by immunohistochemistry, and analyzed correlations of expression of p33^{ING1b} in HNSCC cases with clinicopathological variables, apototic index and expression of 14-3-3η, p300, p21 and PCNA. Although 12% of HNSCC cases lost expression of p33^{ING1b}, most cases of HNSCC retained expression of p33^{ING1b} with levels similar to those in non-cancerous epithelia. Nuclear expression of p33^{ING1b} was significantly decreased in HNSCC compared to normal epithelia. In contrast, cytoplasmic expression of p33^{ING1b} was found to be significantly higher in HNSCC. An abundance of p33^{ING1b} in cytoplasm positively correlated with poor differentiation and tumor progression. Corresponding to those clinicopathogical features, high expression of p33^{ING1b} in the cytoplasm correlated with PCNA labelling index but in contrast, that in the nuclei correlated with apoptosis. In nuclei, p33^{ING1b} is coexpressed with p300 and p21, implying its roles in tumor suppression. Elevated expression of 14-3-3η was associated with cytoplasmic expression of p33^{ING1b} and immunofluorescence study suggested association of p33^{ING1b} and 14-3-3η. Among three cell lines derived from oral SCC, poorly-differentiated SAS cells showed a relatively high expression of p33^{ING1b} in cytoplasm with increased level of 14-3-3η. The results obtained here suggest that relocation of p33^{ING1b} from the nucleus to the cytoplasm, where the protein is tethered by 14-3-3η, participates in tumorigenesis and progression in HNSCC.

Key words: p33^{ING1b}, 14-3-3η, HNSCC, Tumorigenesis

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Introduction

ING1 (inhibitor of growth 1), the founding member of the *ING* gene family, was discovered through a subtractive hybridization assay between normal mammary epithelium and breast cancer cell lines and was shown to play a role in neoplastic transformation (Garkavtsev et al., 1996). *ING1* is considered to be a candidate type II tumor suppressor gene (TSG). TSGs, which include *p53* and *p16*, are often referred to as "gatekeepers" because they prevent cancer development through direct control of cell growth. The *ING1* gene is located on chromosome 13q33-34 and alternatively spliced mRNAs encoding four isoforms, p24I^{NG1c}, p27^{ING1d}, p33^{ING1b} and p47^{ING1a}, which vary in mass between 24 and 47 kDa (Coles and Jones, 2009). Collectively, we will refer to these isoforms as ING1. The ING1 proteins contain a conserved plant homeodomain (PHD)-finger motif in the carboxyl-terminal region and a nuclear localization signal (NLS) (Coles and Jones, 2009). The PHD domain, a zinc finger domain that binds histone H3 in a methylation-sensitive manner, is the key structural feature required for ING protein function (Peña et al., 2006). ING1 proteins can both physically and functionally associate with several

histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes and, thereby, exhibit tumor suppressor function through chromatin remodeling (Doyon et al., 2006). *ING* genes are conserved across many species, including human, mouse, rat, and yeast, suggesting that these genes play important roles in biological processes central to life (He et al., 2005).

Alternatively spliced ING1 mRNAs are expressed in various human tissues. Northern blot analysis of specific ING1 multiplex PCR products demonstrated that $p33^{ING1b}$ is the most widely expressed isoform in normal tissues, although the levels of expression are different among tissues (Saito et al., 2000). Current evidence suggests that $p33^{ING1b}$ plays a role in regulation of cell growth, apoptosis, DNA repair, and cell senescence. $p33^{ING1b}$ has been shown to be involved in regulation of the G_1/S and G_2/M cell cycle checkpoints (Garkavtsev et al., 1998; Takahashi et al., 2002) and in induction of apoptosis (Scott et al., 2001; Tallen et al., 2007). $p33^{ING1b}$ activates transcription of the p21/WAF1 promoter, a key mechanism required for p53-mediated cell growth control (Garkavtsev et al., 1998). In addition, $p33^{ING1b}$ can inhibit cell growth or death in a p53-independent manner through regulation of cyclin B1 expression or Hsp70 transcription (Takahashi et al., 2002; Feng et al., 2006). It has been reported that $p33^{ING1b}$ cooperates with p53 in nucleotide excision repair of UV-damaged DNA (Cheung et al, 2001). Also, $p33^{ING1b}$ is suggested to mediate interaction between Proliferating Cell Nuclear Antigen (PCNA) and p300, an association of which is proposed to link DNA repair to chromatin remodeling (Scott et al., 2001; Vieyra et al., 2002).

ING1 expression has been reported to be downregulated or lost in many cancers, including breast (Toyama et al., 1999), esophagus (Chen et al., 2001) and lung (Kameyama et al., 2003). Previous studies have shown that a decrease of ING1 mRNA results in loss of protein in tumor cells. Analysis of the *ING1* gene in a wide variety of human tumors and cancer cell lines revealed that mutation of *ING1* is rather infrequent in tumor cells. Mutations so far identified were primarily detected either in the NLS or in the PHD domain finger (Ythier et al., 2008). Instead, increased cytoplasmic expression of $p33^{ING1b}$ has been found to associate with concomitant loss of nuclear expression in melanoma, papillary thyroid carcinoma and breast carcinoma compared with corresponding normal tissue (Nouman et al., 2002b).

At present, it is yet to be determined to what extent HNSCC accompanies genetic alterations of the *ING1* gene and/or loss of nuclear expression of the protein. Previously, loss of heterozygosity (LOH) at chromosome 13q33-34 region containing the *ING1* was shown in 23/34 HNSCC cases (68%) and 3 of the 23 LOH cases carried missense mutations in the *ING1* (Gunduz et al., 2000), implying a role of the *ING1* in tumor suppression. In contrast, another study identified no mutations in *ING1* in 5 HNSCC cell lines and 20 HNSCC cases (Sanchez-Cespedes et al., 2000), suggesting that if *ING1* is a TSG in HNSCC, mutation is possibly not the predominant reason for the loss of ING1 function, as in the case of the *p53*.

In order to determine the roles of $p33^{ING1b}$ in trumorigenesis and progression of HNSCC, the expression and subcellular localization of $p33^{ING1b}$ were investigated by immunohistochemistry (IHC) in a large number of cases of HNSCC, in comparison with adjacent dysplastic epithelium and normal epithelium. We analyzed the correlation of expression of $p33^{ING1b}$ with clinicopathological variables. In addition, we examined correlations of the status of $p33^{ING1b}$ with those of 14-3-3η, PCNA, p300 and p21 which may be involved in ING1 tumor suppressor-function. Also, we examined the levels of expression of the *ING1* gene by RT-PCR in 3 oral SCC-derived cell lines, and the subcellular localization of $p33^{ING1b}$, as well as those of 14-3-3η, PCNA, p300 or p21. The results obtained in this study suggest that decreases in nuclear expression of $p33^{ING1b}$, either through downregulation or relocation of the protein from the nucleus to the cytoplasm, may participate in tumorigenesis and progression of HNSCC.

Materials and methods

Cases

One hundred and thirty-one punch biopsy specimens and 83 surgical specimens of head and neck squamous cell carcinoma (HNSCC), consisting of 128 laryngeal SCCs, 45 pharyngeal SCCs, and 41 tongue SCCs, were selected from the patient files of the Kouseiren Takaoka Hospital and the affiliated hospital of Toyama University from 1993 to 2008. Additionally, 30 specimens of moderate dysplasia, 30 specimens of severe dysplasia and 48 specimens of non-cancerous squamous epithelium were obtained from corresponding surgically resected specimens. HNSCC patient samples were from 177 men and 37 women (mean age, 66.2 years old; range, 34-90 years old). Eighty-three surgical patients were followed up by consulting case documents. Twenty-nine cases had lymph node metastases, but no case had distant metastases. None of the patients underwent chemotherapy or radiotherapy before surgery. All patients consented to the use of their tumor tissues for clinical research, and the Ethical Committee approved the research protocol.

Tissue microarray (TMA)

All specimens were fixed in 10% formalin and embedded in paraffin. Typical lesions corresponding to HNSCC and dysplasia, as well as non-cancerous epithelium, were selected according to hematoxylin and eosin (H&E) staining. A 2 mm diameter tissue core was punched out of each sample and transferred to a recipient block using a Tissue Microarray Instrument (AZUMAYA KIN-1, Japan) with a maximum of 48

cores. The tissue microarrays (TMAs) consisted of SCC, dysplasia, and normal epithelium.

Cell culture

Three human oral SCC cell lines were used in the present study. HSC-2 was established from a highly differentiated SCC of the mouth floor and tongue, B88 from a moderately differentiated SCC of the tongue, and SAS from a poorly differentiated SCC of the tongue. All cell lines were obtained from the Human Science Research Resource Bank (Osaka, Japan). Cells were cultured in their respective media supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37°C. Cultured cells were collected by centrifugation and rinsed with PBS, then either fixed in 10% formalin or embedded in paraffin.

Immunohistochemistry

Consecutive 4 μm sections were cut from the TMAs and cell line blocks, as well as some whole tissue specimens containing both cancerous lesion and normal epithelium. After routine deparaffinization, antigen retrieval was carried out by microwave treatment in target retrieval solution (DAKO, CA, USA) for 15 minutes. IHC was performed as previously described (Li et al., 2010a). Primary antibodies, sources, and working dilutions are listed in Table 1. HRP-conjugated anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG (DAKO, CA, USA) antibodies were used as secondary antibodies to detect the appropriate primary antibodies. Bound antibodies were visualized with 3,3´-diaminobenzidine (DAB). The sections were counterstained with Mayer's hematoxylin for TMAs and with methyl green for cell line sections. Additionally, sections incubated with normal goat or rabbit or mouse IgG served as a negative reaction control, respectively.

IHC evaluation

The sections stained with antibodies were examined by three independent observers (XH Li, Y Takano and A Noguchi). The percentage of positive cells was assessed semi-quantitatively using a four-tiered scoring system: negative (-), 0-5% positive cells; weakly positive (+), 6-25% positive cells; moderately positive (++), 26-50% positive cells; and strongly positive (+++), 51-100% positive cells. Because the majority of stained tissues were positive for expression of $p33^{ING1b}$, immunohistochemical results for $p33^{ING1b}$ staining were grouped into two categories: low expression (- or +) and high expression (++ or +++). Immunohistochemical data for 14-3-3η, p300, and p21 were also divided into two groups: negative expression (-) and positive expression (+, ++, or +++). PCNA-positive nuclei were counted in three fields at x 400 magnification. The PCNA labeling index was defined as the percentage of PCNA-positive cells in the given field.

TUNEL assay

To evaluate levels of apoptosis in HNSCC samples, we utilized the ApopTag® Plus Peroxidase In situ Apoptosis Detection Kit (CHEMICON International, Inc, CA, USA) and performed the assay as previously described (Li et al., 2010a). Apoptotic cell nuclei were stained brown, and non-apoptotic nuclei were stained green. Apoptotic and total nuclei were counted in three fields at 400x magnification. The apoptotic index (AI) was defined as the percentage of apoptotic cells in a given field.

Double immunofluorescence staining

Double immunofluorescence staining was used to analyze colocalization of $p33^{ING1b}$ with 14-3-3η, p300, and p21 in HNSCC samples. Staining was performed as previously described (Li et al., 2010b). Briefly, tissue sections were incubated with primary antibodies against $p33^{ING1b}$ and 14-3-3η, p300, or p21 for 16h at 4°C. The antibodies were then detected by Alexa Fluor 594-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500, Invitrogen Corporation, CA, USA) for 1 h. The sections were mounted using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, CA, USA). Samples were imaged using an Olympus Ax80 fluorescence microscope (Japan).

RNA isolation, reverse-transcription PCR (RT-PCR), and sequencing

Total RNA was isolated from the cells using the PARIS™ (Protein And RNA Isolation System) kit

Table 1. List of primary antibodies used in immunohistochemical studies.

Antibody	Clone	Source	Company	Dilution
$p33^{ING1b}$ (C-19)	polyclonal	Goat	Santa Cruz, Biotechnology, CA, USA	1:50
14-3-3η	polyclonal	Rabbit	Enzo Life Sciences, Inc, PA, USA	1:600
p300 (N-15)	polyclonal	Rabbit	Santa Cruz, Biotechnology, CA, USA	1:50
p21	polyclonal	Rabbit	Santa Cruz, Biotechnology, CA, USA	1:50
PCNA (PC10)	monoclonal	mouse	DAKO, Denmark	1:200

(Ambion, Inc, TX, USA) and treated with RNase-free DNase I (Takara, Japan) to eliminate residual DNA. Total RNA was reverse transcribed into cDNA using the PrimeScript® II 1st strand cDNA Synthesis Kit (Takara, Japan). For PCR analysis, cDNA was amplified using Taq DNA polymerase (TAKARA, Japan). ß-actin was used as the endogenous expression standard. Gene-specific primers for RT-PCR included the following: 5'-tgggcgacgagaagatccag-3' (forward) and 5'-gctttgtc catggtcttctcgttc-3' (reverse) for p33ING1b (NM_198219) and 5'-cttcaacaccccagccatgt-3' (forward) and 5'-gacgtagcacagcttctcgt-3' (reverse) for ß-actin. Reaction mixtures (25 μL) contained 0.125 μl Takara Ex Taq HS (TaKaRa), 2.5 μl 10x PCR buffer, 2.5 μl dNTP mixture, 1 μM of each gene-specific primer set, and 100 ng of template cDNA. PCR cycling conditions were as follows: (1) initial denaturation at 94°C for 8 min, (2) 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s, and (3) a final extension in the last cycle of 10 min. Negative controls for RT-PCR were treated identically, but lacked template cDNA. Amplified products were resolved by electrophoresis on ethidium bromide (EB)-stained 2% agarose gels.

Amplified products were purified using MicroSpin S-300 HR Columns (GE Healthcare, UK). The products were then sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) with each pair of forward and reverse primers. Sequencing data was compared with the p33ING1b sequence (Gene bank NM_198219) using GENETYX analysis.

Western blot analysis

Nuclear and cytoplasmic proteins were isolated from the three cell lines using the PARIS™ kit (Ambion, Inc, TX, USA). Equal amounts (30 μg) of protein were separated on SDS-polyacrylamide gel and transferred to PVDF membranes (GE Healthcare, UK). The membranes were blocked in 5% skim milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 2h and incubated with primary antibodies directed against p33ING1b (1:200), 14-3-3η (1:1000), p300 (1:500), p21 (1:500), H2AX (1:500, Signalway Antibody, TX), or ß-actin (1:500, AnaSpec, Inc, CA) at 4°C overnight. They were detected using appropriate secondary antibodies and the Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, UK).

Statistical analysis

Statistical evaluation was performed using non-parametric test. The correlation of p33ING1b expression in normal epithelium, dysplasia and HNSCC was analyzed by chi-square or Exact Fisher test. Spearman correlation test was used to analyze the association between p33ING1b expression with clinicopathological features and tumor-related molecules. P-values (p) less than 0.05 were considered to be statistically significant. The SPSS 10.0 software program was employed for all data analysis.

Results

p33ING1b expression in normal epithelium, dysplasia, and HNSCC

We examined the levels of expression and subcellular localization of p33ING1b in normal epithelium, dysplasia and HNSCC. Total loss of p33ING1b expression in HNSCC was observed in 25 cases out of 214 cases (11.7%) (Fig. 1), which is significantly fewer than that of the previous report (Zhang et al., 2008). Thus most cases of HNSCC that we examined here retained expression of p33ING1b. Subcellular localizations of p33ING1b were, however, significantly different in non-cancerous epithelia and HNSCC (Fig. 1). In normal epithelia and dysplasia, expression of p33ING1b was predominantly observed in the nucleus (Fig. 2a,b, respectively). By contrast, a significant portion of the HNSCC samples showed both nuclear- and cytoplasmic- localization of p33ING1b (Figs. 1, 2c). Notably, the cases with nuclear-low and cytoplasmic-high expression of p33ING1b were evidently increased in HNSCC (Fig. 1), including some cases where only high cytoplasmic expression of p33ING1b was detectable (Fig. 2d). In total, high nuclear p33ING1b expression significantly decreased in HNSCC (p=0.045, Table 2). In contrast, high expression of cytoplasmic p33ING1b was observed more frequently in HNSCC than in normal epithelium (p=0.002) or in dysplasia (p=0.014, Table 2).

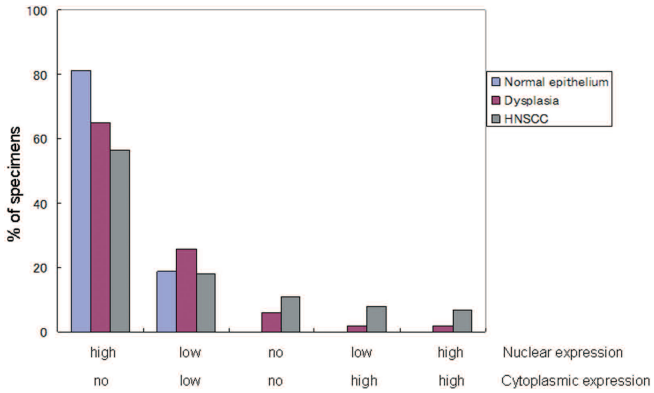

Fig. 1. p33ING1b subcellular expression in normal epithelium, dysplasia and HNSCC. The percentage of positive p33ING1b expression in normal epithelium, dysplasia and HNSCC was illustrated according to different subcellular localizations. The proportion of nuclear-high and cytoplasmic- low expression was decreased stepwise from normal epithelium to dysplasia to HNSCC. In contrast, total loss of p33ING1b expression was gradually increased in dysplasia and HNSCC. High expression of cytoplasmic p33ING1b was primarily detected in HNSCC compared with normal epithelium and dysplasia.

The relationship between p33ING1b expression and clinicopathological features in HNSCC

As summarized in Table 3, no significant correlation was identified between high nuclear expression of $p33^{ING1b}$ and any clinicopathological variable. By contrast, high expression of cytoplasmic $p33^{ING1b}$ in HNSCC was significantly correlated with poor differentiation (p=0.001), T staging (p=0.013), lymph node metastasis (p=0.003), and TNM staging (p=0.020), but not with the other variables.

Correlation between p33ING1b expression and expression of other molecular markers associated with cancer development and prognosis

Expression of 14-3-3η that may be involved in cytoplasmic retention of $p33^{ING1b}$ (Gong et al., 2006) was primarily observed in the cytoplasm (Fig. 2e), while p300, p21 and PCNA, functional partners of $p33^{ING1b}$, were detected in the nuclei of tumor cells (Fig. 2f-h). The PCNA labeling index (LI) was 43.54±25.33%

Table 2. Relationship of $p33^{ING1b}$ expression in normal epithelium, dysplasia and HNSCC.

Groups	N	Nuclear [n]			Cytoplasmic [n]		
		Low	High	HR (%)	Low	High	HR (%)
Normal epithelium	48	9	39	81.25	48	0	0
Dysplasia	60	20	40	66.67	58	2	3.33
HNSCC	214	79	135	63.08*	183	31	14.49**

HR: high expression rate; *: in comparison to Normal epithelium (p=0.045); **: in comparison to Normal epithelium (p=0.002) and Dysplasia (p=0.014)

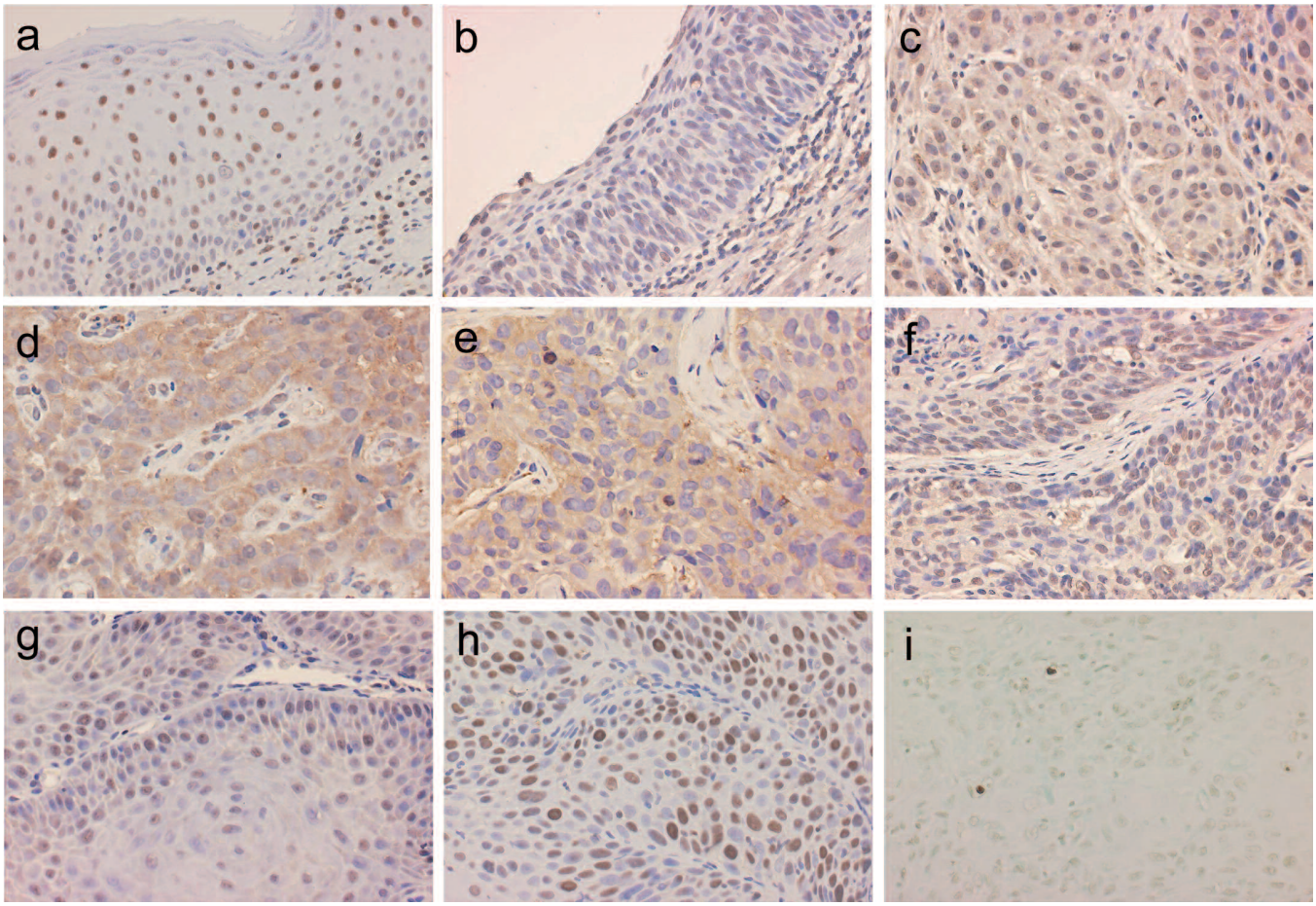

Fig. 2. Immunohistochemical staining of $p33^{ING1b}$ and other molecular markers in HNSCC tissue samples. High nuclear expression of $p33^{ING1b}$ was detected in normal epithelia **(a)**, but was downregulated in dysplastic epithelia **(b).** Decreased nuclear expression and increased cytoplasmic expression of $p33^{ING1b}$ was found in some HNSCC cases **(c)**. Only high cytoplasmic expression of $p33^{ING1b}$ was also detected in a few HNSCC cases **(d)**. High cytoplasmic expression of 14-3-3η was detected in some HNSCC cases **(e)**. p300 **(f)**, p21 **(g)** and PCNA **(h)** were observed in the nuclei of tumor cells. Apoptotic cells were identified by the TUNEL assay and exhibit brown nuclear staining **(i).** Note: Slides were stained with DAB and counterstained with Mayer's haematoxylin. x 400

(mean ± SD), and the AI, assessed by the TUNEL assay (Fig. 2i), was 2.23±2.25% (mean ± SD) in HNSCC samples.

High expression of nuclear p33ING1b was positively correlated with expression of p300, p21 and the AI (p<0.001), but was inversely correlated with cytoplasmic p33ING1b expression (p=0.025, Table 4). In contrast, high cytoplasmic p33ING1b expression was positively correlated with 14-3-3η expression (p=0.009) and the PCNA LI (p<0.001), but was inversely correlated with

Table 3. Relationship between p33ING1b expression and clinicopathological features in HNSCC.

Clinicopathological features	N	Nuclear [n (%)]			Cytoplasmic [n (%)]		
		Low	High	p	Low	High	p
Age				0.940			0.850
<65	86	31(36.0)	55(64.0)		73(84.9)	13(15.1)	
≥65	128	48(37.5)	80(62.5)		110(85.9)	18(14.1)	
Sex				0.822			0.585
male	177	66(37.3)	111(62.7)		150(84.7)	27(15.3)	
Female	37	13(35.1)	24(64.9)		33(89.2)	4(10.8)	
Location				0.164			0.332
larynx	128	41(32.0)	87(68.0)		112(87.5)	16(12.5)	
pharynx	45	23(51.1)	22(48.9)		37(82.2)	8(17.8)	
tongue	41	15(36.6)	26(63.4)		34(82.9)	7(17.1)	
Differentiation				0.137			0.001*
well	121	40(33.0)	81(67.0)		110(90.9)	11(9.1)	
moderate	61	25(40.4)	36(59.6)		52(85.2)	9(14.8)	
poor	32	14(43.7)	18(56.3)		21(65.6)	11(34.4)	
T staging				0.302			0.013*
Tis~T1	21	7(33.3)	14(66.7)		21(100)	0(0)	
T2~T4	62	29(46.8)	33(53.2)		48(77.4)	14(22.6)	
Lymph node metastasis				0.814			0.003*
-	54	23(42.6)	31(57.4)		51(90.7)	5(9.3)	
+	29	13(44.8)	16(55.2)		22(62.1)	11(37.9)	
TNM staging				0.378			0.020*
0~I	19	7(36.8)	12(63.2)		19(100)	0(0)	
II~IV	64	29(45.3)	35(54.7)		50(78.1)	14(21.9)	

*: Statistically significant (p<0.05).

Table 4. Relationship between expression of p33ING1b and tumor-related molecular markers in HNSCC.

Tumor-related molecular markers	N	Nuclear [n (%)]			Cytoplasmic [n (%)]		
		Low	High	p	Low	High	p
14-3-3η				0.944			0.009*
-	104	38(36.5)	66(63.5)		96(92.3)	8(7.7)	
+	110	41(37.3)	69(62.7)		87(79.1)	23(20.9)	
p300				<0.001*			0.576
-	106	53(50.0)	53(50.0)		88(83.0)	18(17.0)	
+	108	26(24.1)	82(75.9)		95(88.0)	13(12.0)	
p21				<0.001*			<0.001*
-	140	67(47.9)	73(52.1)		111(79.3)	29(20.7)	
+	74	12(16.2)	62(83.8)		72(97.3)	2(2.7)	
PCNA LI				0.200			<0.001*
<50%	132	53(40.2)	79(59.8)		124(93.9)	8(6.1)	
≥50%	82	26(31.7)	56(68.3)		59(72.0)	23(28.0)	
Apoptotic index (AI)				<0.001*			0.985
<2%	125	58(46.4)	67(53.6)		107(85.6)	18(14.4)	
≥2%	89	21(23.6)	68(76.4)		76(85.4)	13(14.6)	
Cytoplasmic p33ING1b				0.025*			
Low expression	183	62(33.9)	121(66.1)				
High expression	31	17(54.8)	14(45.2)				

*: Statistically significant (p < 0.05).

p21 expression (p<0.001, Table 4). In addition, expression of p21 was negatively correlated with the PCNA LI (p=0.020) (data not shown).

Double immunofluorescence labeling of p33ING1b with 14-3-3η, p300, and p21

Double immunofluorescence staining revealed that cytoplasmic p33ING1b was primarily coexpressed with 14-3-3η, but nuclear p33ING1b was frequently coexpressed with p300, and p21 in HNSCCs. A large number of tumor cells exhibited orange staining, indicating colocalization of the two proteins assessed (Fig. 3).

Analysis of expression and subcellular localization of p33ING1b in oral SCC cell lines

We investigated whether HNSCC-derived cells lines exhibit similar patterns of expression of p33ING1b with those in HNSCC tissues. We first made RT-PCR analysis of p33ING1b mRNA for expression and detection of the mutations. SAS cells with the most undifferentiated phenotype contained the lowest level of the p33ING1b mRNA (Fig. 4a). Direct sequencing of the amplified products identified no mutation in the NLS and PHD domain finger (Fig. 4a). Western blot analyses detected p33ING1b in both nuclear and cytoplasmic fractions in all three cell lines, consistent with the observations with HNSCC tissues. SAS cells contained similar levels of nuclear p33ING1b and a significantly higher amount of cytoplasmic p33ING1b compared to HSC-2 and B88 cells (Fig. 4b). Also, SAS cells exhibited lower nuclear expression of p300 and p21 compared to HSC-2 cells derived from highly-differentiated SCC and higher cytoplasmic expression of 14-3-3η compared with the other cell lines. The same tendency was observed in immunohistochemistory of the cells (Fig. 5). Thus, despite the decrease in the amount of mRNA, a significant portion of SAS cells retained nuclear expression of p33ING1b and contained a higher amount of cytoplasmic p33ING1b, suggesting that expression of p33ING1b is posttranslationally regulated at least in part. In addition, all cell lines exhibited high PCNA

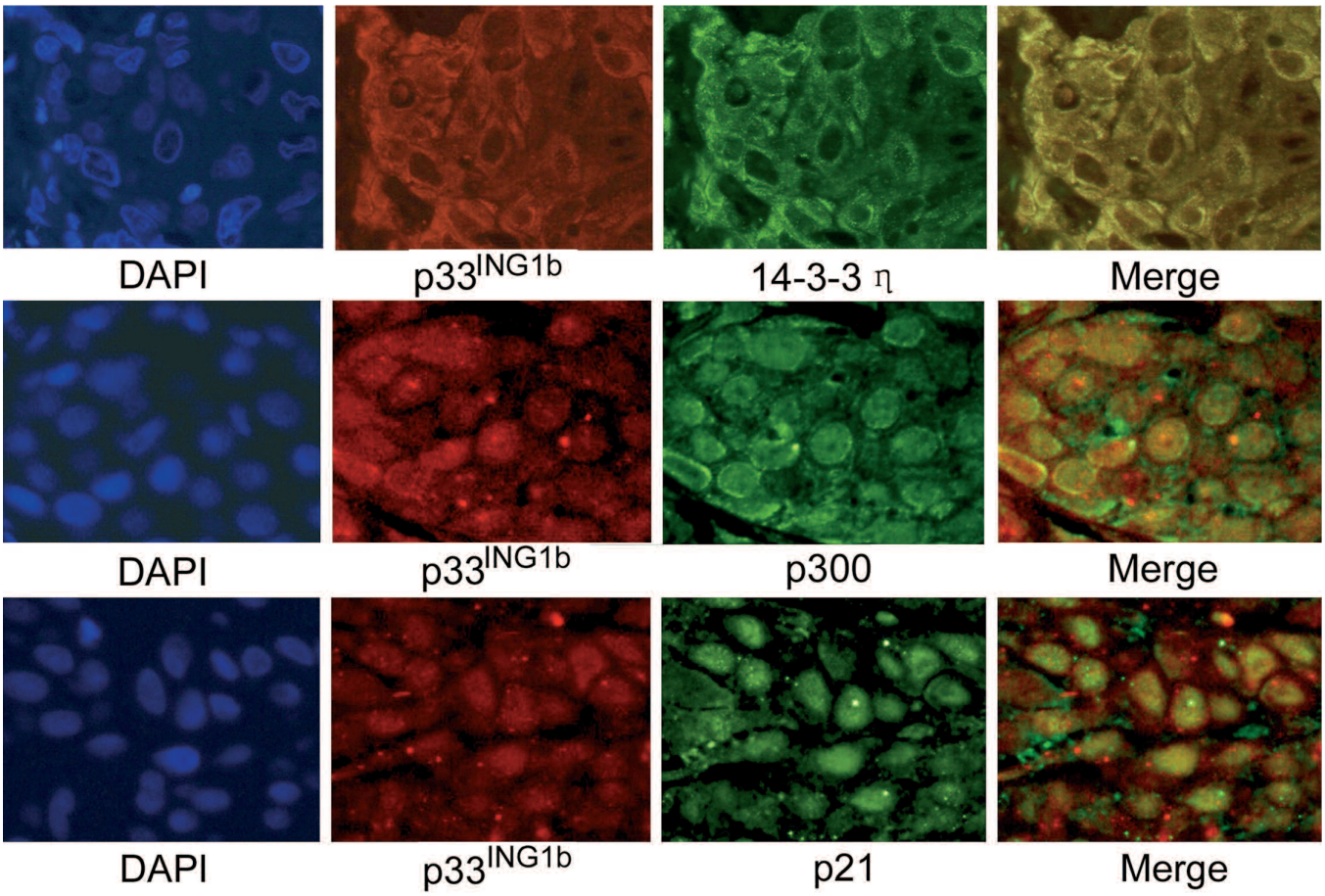

Fig. 3. Double immunofluorescence staining of p33ING1b with 14-3-3η, p300, and p21 in HNSCC. Staining of p33ING1b is shown in red, and staining of 14-3-3η, p300 and p21 in green. Areas of colocalization of both proteins appear orange in the merged images. x 400

expression (82.2%~86.5%) and low apoptotic indices (0~0.01%) (data not shown).

Discussion

In the present study, we found that nuclear expression of p33ING1b was significantly downregulated in HNSCC samples compared to normal epithelium. A previous study found 68% LOH for *ING1* in HNSCC (Gunduz et al., 2000). In addition, a 7% to 50% decrease in expression of the p33ING1b mRNA was shown in oral SCC by RT-PCR (Hoque et al., 2002; Tachibana et al., 2002). Loss of p33ING1b expression was also demonstrated in 75% of 49 oral SCCs using IHC (Zhang et al., 2008). These results imply that the *ING1* gene functions as a tumor suppressor in HNSCC. However, mutations in the *ING1* appear to be a rather rare event (Sanchez-Cespedes et al., 2000) and some other factors could be involved in the loss of tumor suppressor activity of p33ING1b in HNSCC cases where expression of p33ING1b is retained.

p33ING1b has been found to form complexes with several nuclear proteins involved in cell cycle regulation, DNA repair and chromatin remodeling (Coles and Jones, 2009). For example, p33ING1b associates with p53 and inhibits cell growth in a functional p53 dependent-manner (Garkavtsev et al., 1998). Also, p33ING1b was proposed to compete with MDM2 for the same binding site on p53, leading to an increase in the stability and activity of p53 (Leung et al., 2002), suggesting p33ING1b may participate in the regulation of p53 signaling pathway. Therefore, the nuclear localization of p33ING1b may be crucial for its functions for tumor suppression and, in addition to the downregulation or loss of p33ING1b expression and loss-of-function mutations in the gene. Mislocalization of the protein may lead to dysregulation of the cell growth and/or resistance to apoptosis, contributing to a malignant transformation of the cells. Our observations in this study suggest that decrease or loss of p33ING1b nuclear expression may participate in tumorigenesis of HNSCC.

A previous study demonstrated that decreases of nuclear expression of p33ING1b correlate with poor differentiation in breast cancers (Nouman et al., 2003). Expression of p33ING1b was reduced in hepatocellular carcinoma (HCC), especially in moderately and poorly differentiated HCCs (Ohgi et al., 2002). In this study, we examined 214 cases of HNSCC and found total loss of p33ING1b expression in 25 cases (12%). The rest of HNSCC expressed p33ING1b to levels comparable with non-cancerous tissues. However, differently from non-cancerous tissues, p33ING1b in HNSCC localized to the cytoplasm in addition to the nuclei. Cytoplasmic expression of p33ING1b was increased in accordance with malignant grading and was positively correlated with poor differentiation and tumor progression. Because

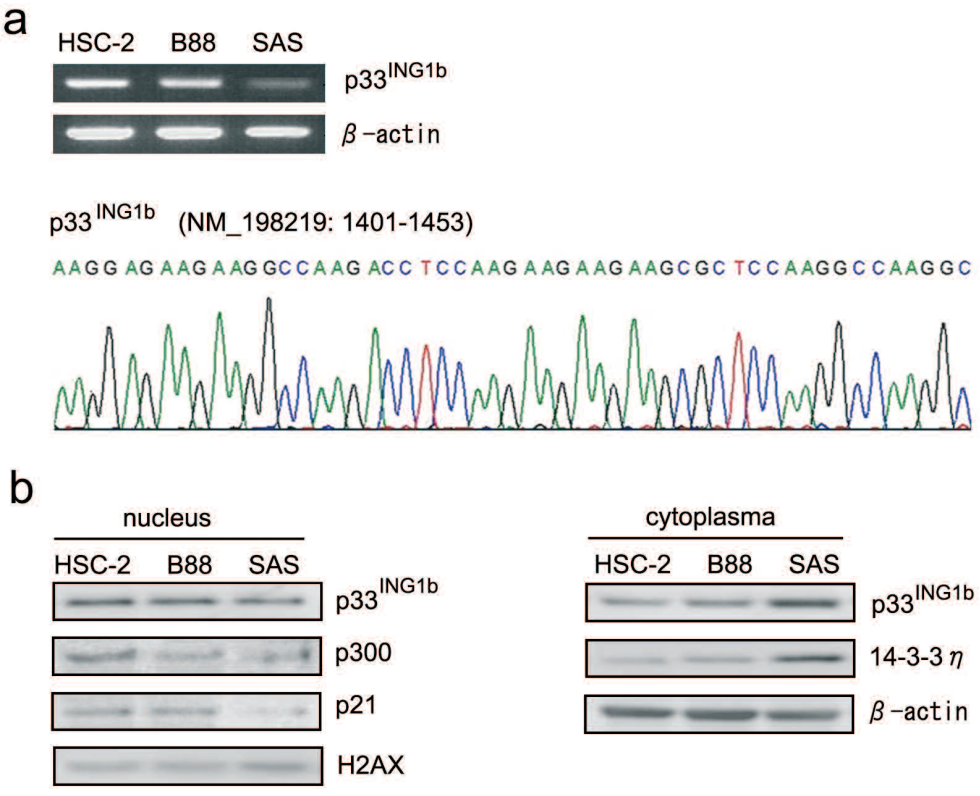

Fig. 4. Expression of p33ING1b and other molecular markers in oral SCC cell lines. **a.** RT-PCR of p33ING1b mRNA expression in the three cell lines and sequence analysis of PCR products. **b.** Western blot analysis of protein levels of p33ING1b, 14-3-3η, p300 and p21 in extracts from the nucleus and cytoplasm. H2AX was used to control for equal loading of nuclear proteins, and ß-actin was used to control for equal loading of cytoplasmic protein.

expression of p33^{ING1b} was retained in many cases of HNSCC (Table 2) and high cytoplasmic expression of p33^{ING1b} accompanied low nuclear expression in HNSCC, increases in cytoplasmic p33^{ING1b} may be due to mislocalization. Similar observations were reported in melanoma (Nouman et al., 2002b), oral SCC (Zhang et al., 2008), breast cancers (Nouman et al., 2003), brain tumors (Vieyra et al., 2003) and lymphoblastic leukemia (Nouman et al., 2002a). Our recent study also demonstrated that cytoplasmic mislocalization of ING5, another member of the *ING* gene family, is related to tumorigenesis of HNSCC (Li et al., 2010a). Our results, together with the previous studies, suggest that changes in the localization of p33^{ING1b} from the nucleus to the cytoplasm are involved in tumorigenesis and progression of HNSCC where expression of p33^{ING1b} is retained.

The deletion or mutations in the NLS of p33^{ING1b} could be responsible for the cytoplasmic accumulation of the protein. However, they appear to be a rather rare event in HNSCC (Gunduz et al., 2000). Also, no mutation was found in the NLS and PHD domain of three oral SCC cell lines haboring cytoplasmic p33^{ING1b} in this study. Notably, cytoplasmic p33^{ING1b} expression was found to significantly correlate with the cytoplasmic levels of 14-3-3η in HNSCC tissues (Table 4) as well as in cultured SCC cells (Fig. 4b, SAS cells) and these two proteins appear to coexpress in the cytoplasm (Fig. 3). 14-3-3 family proteins primarily reside in the cytoplasm

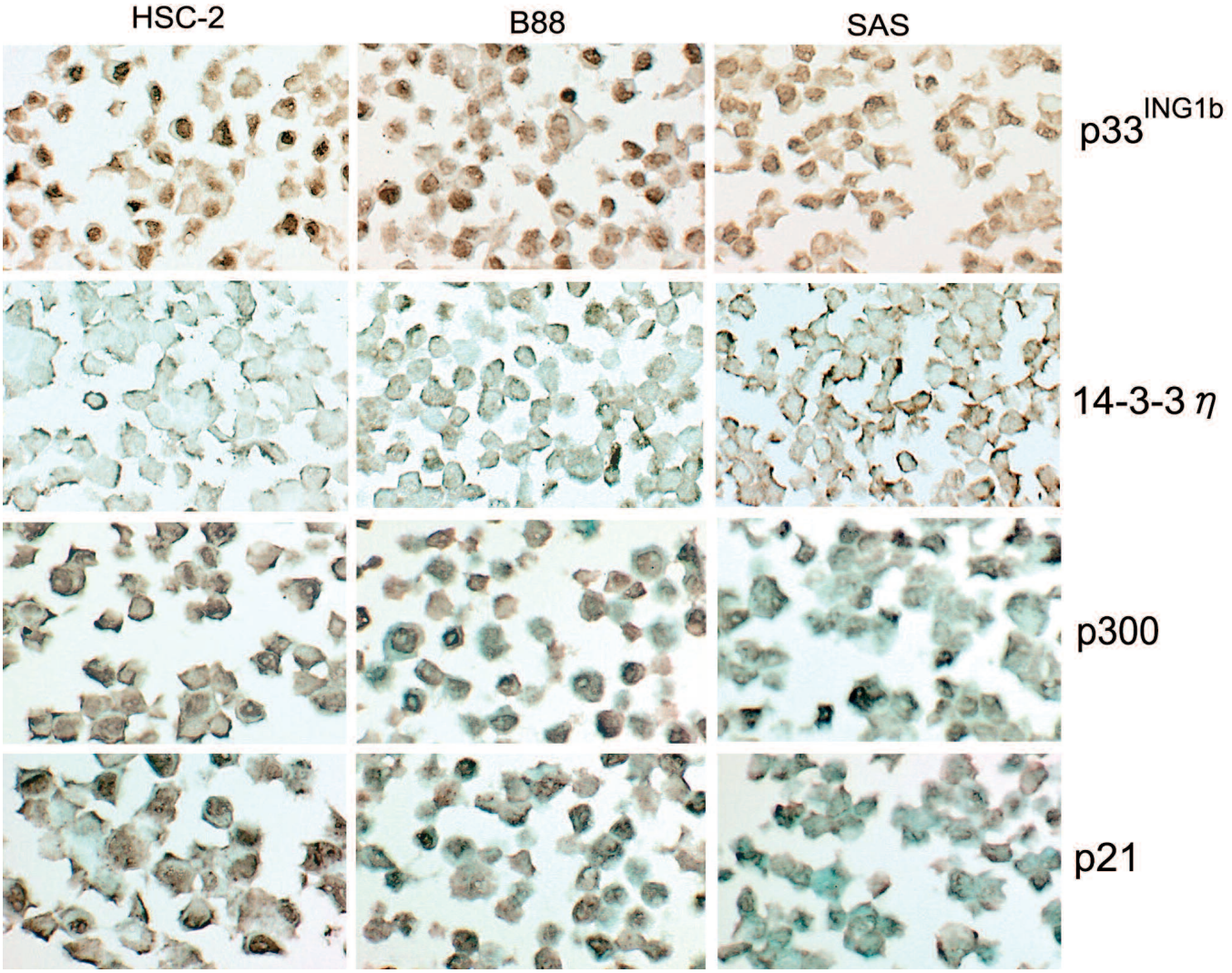

Fig. 5. Immunohistochemical staining of p33^{ING1b} and other molecular markers in oral SCC cell lines. HSC-2, B88 and SAS oral SCC cell lines were subjected to immunohistochemical staining for p33^{ING1b} and the following related molecular markers; 14-3-3η, p300 and p21. Note: Slides were stained with DAB and counterstained with methyl green. x 400

where they associate with phosphorylated ligands involved in many cellular processes, including regulation of the cell cycle and DNA damage checkpoints (Hermeking and Benzinger 2006). A recent study has demonstrated that p33ING1b, phosphorylated at serine residue 199 is bound by the 14-3-3 family-proteins and that this interaction may tether p33ING1b in the cytoplasm (Gong et al., 2006). Our observations are in line with this notion. At present, however, we should not exclude the possibility that dysfunction of karyopherins α2 and ß1 that mediate nuclear import of p33ING1b (Russel et al., 2008) in HNSCC results in the cytoplasmic accumulation of p33ING1b.

The present study showed the positive correlation between nuclear expression of p33ING1b and p300 (Table 4) and their colocalization in the nuclei (Fig. 3). p300 is implicated in chromatin remodeling at DNA lesion sites to facilitate PCNA function in DNA repair (Hasan et al., 2001). ING1 proteins present in complexes containing p300 and PCNA carrying HAT activity. Overexpression of p33ING1b, but not p47ING1a, is shown to induce hyper-acetylation of histones H3 and H4 *in vitro* and *in vivo* (Vieyra et al., 2002). Therefore, nuclear p33ING1b may be involved in chromatin remodeling through the interaction with p300, but it remains to be determined whether or not this interaction contributes to tumor suppression in HNSCC. Expression of p21WAF1/Cip1 was also found to be positively correlated with the nuclear expression of p33ING1b (Table 4), consistent with the previous report that p33ING1b can induce expression of p21 (Waga et al., 1994). An immunofluorescence study showed the possible association of p21 and p33ING1b in nuclei (Fig.2), which has not been reported so far. The levels of nuclear p33ING1b positively correlated with p21 and AI (Table 4). Moreover, p21 expression was inversely correlated with PCNA labelling index. As p21 can suppress cell growth and promote apoptosis in certain conditions (Abbas and Dutta, 2009), the association of p33ING1b with p21 could strengthen p21 mediated suppression of cell growth and/or promotion of apoptosis in HNSCC. It is currently under investigation whether p33ING1b binds with p21.

In conclusion, decreases in nuclear expression of p33ING1b, either through downregulation or relocation of the protein from the nucleus to the cytoplasm, may be related to tumorigenesis and progression of HNSCC. The p33ING1b protein may accumulate in the cytoplasm partly through an interaction with 14-3-3η. p33ING1b in nuclei associates with p300 and p21, implying its roles in regulation of chromatin remodeling, growth inhibition and apoptosis.

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