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Downregulation of nuclear ING3 expression and translocalization to cytoplasm promotes tumorigenesis and progression in head and neck squamous cell carcinoma (HNSCC)

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Summary. *ING3* (inhibitor of growth gene 3) is a member of the *ING* gene family, and is considered as a candidate tumor suppressor gene. In order to explore the roles of ING3 in tumorigenesis and cancer progression of head and neck squamous cell carcinoma (HNSCC), ING3 expression was assessed in 173 cases of HNSCC by immunohistochemistry. The expression of ING3 was also compared to clinicopathological variables, and the expression of several tumorigenic markers. Nuclear expression of ING3 in HNSCC was significantly lower than that in dysplasia and normal epithelium, and was negatively correlated with a poor-differentiated status, T staging and TNM staging. In contrast, cytoplasmic expression of ING3 was significantly increased in HNSCC, and was statistically associated with lymph node metastasis and 14-3-3η expression. In addition, nuclear expression of ING3 was positively correlated with the expression of p300, p21 and acetylated p53. In conclusion, decreases in nuclear ING3 may play important roles in tumorigenesis, progression and tumor differentiation in HNSCC. Increases in cytoplasmic ING3 may be due to 14-3-3η binding and may also be involved in malignant progression. Nuclear ING3 may modulate the transactivation of target genes, promoting apoptosis through interactions with p300 and p21. Moreover, ING3 may interact with p300 to upregulate the level of acetylation of p53, and promote p53-mediated cell cycle arrest, senescence and/or apoptosis. Therefore, ING3 may be a potential tumor suppressor and a possible therapeutic target in HNSCC.

Key words: Inhibitor of growth gene 3 (ING3), p53, HNSCC, Tumorigenesis, Prognosis

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Introduction

ING3 (inhibitor of growth gene 3), a member of the *ING* gene family, was identified through computational homology to *ING1* (Nagashima et al., 2003). Phylogenetic analysis identified that *ING* genes are conserved in many species, including humans, mice, rats and yeasts (Shah et al., 2009). Alignment data show that the human and mouse ING1 and ING3 proteins are 90% identical, whereas the human and frog ING1 and ING3 proteins are 81% and 82% identical, respectively (He et al., 2005). These data suggest that *ING* genes play important roles in biological processes central to life. The *ING3* maps to chromosome 7q31 and encodes two isoforms, $p47^{ING3}$ and $p11^{ING3}$. $p47^{ING3}$ is the main isoform, which consists of 12 exons that encode 418 amino acids. Northern blot analysis for $p47^{ING3}$ transcript expression showed it to be highly expressed in spleen, testis, skeletal muscle and heart, but less expressed in lung and brain (Nagashima et al., 2003). Splice variants of ING proteins may compensate or compete with each other and create more diversity in ING functions (He et al., 2005).

All ING proteins contain a plant homeodomain (PHD) in the C-terminal region, a nuclear localization signal (NLS), and a domain with an unknown function called the novel conserved region (NCR). The PHD domain, a zinc finger domain that binds histone H3 in a methylation-sensitive manner, has been implicated in chromatin remodeling (Peña et al., 2006). Localization of ING proteins in the nucleus is critical to their function (Ha et al., 2002). The NLS targets ING1 or other ING proteins to different chromatin domains in the nucleus and nucleolus in response to UV-induced DNA damage (Scott et al., 2001). ING3 is a stable component of the Tip60/NuA4 HAT complexes. It is required for acetylation of chromatin substrates. Tip60 and its associated proteins are important cofactors for p53-, E2F1- and nuclear-receptor-dependent transcription activation; cell response to DNA damage, apoptosis; and metastasis suppression (Doyon et al., 2004, 2006). ING3 PHD interacts with H3K4me3 by Tyr-362 and Trp-385 and enhances ING3-mediated DNA damage-dependent cell death (Kim et al., 2016). In function, INGs regulate cell proliferation, senescence, apoptosis, differentiation, angiogenesis, DNA repair, metastasis, and invasion by multiple pathways. In addition, INGs increase cancer cell sensitivity for chemotherapy and radiotherapy, while clinical observations show that INGs are frequently lost in some types of cancers (Zhang et al., 2017). The ING family of proteins are considered as tumor suppressors that physically and functionally interact with p53. The studies on the effects of overexpression or downregulation of *ING* family proteins on various cellular processes imply that the roles of the ING family genes in tumorigenesis depend on cellular contexts; they could also function as oncogenes in several aspects (Unoki et al., 2009). Until now, only few studies have focused on ING3. ING3 may function as a tumor-suppressor gene in the progression of gastric cancer (Yang et al., 2017; Zhao et al., 2018). In contrast, McClurg et al observed that the increase of ING3 expression in prostate cancer samples was correlated with poorer patient survival (McClurg et al., 2018).

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers in the world. The evolution and progression of HNSCC are considered to result from multiple stepwise alterations of cellular and molecular pathways in the squamous epithelium (Haddad and Shin, 2008). Therefore, identification of reliable molecular markers that play important roles in tumorigenesis, progression, and prognosis of HNSCC is very important, both to increase our understanding of the molecular basis of carcinogenesis and to provide possible therapeutic targets.

In previous studies, we have proved that downregulation or translocation of the nuclear expression of p33ING1b, ING4 and ING5 is involved in the tumorigenesis and progression of HNSCC (Li et al., 2010a,b, 2011a,b). To explore the roles of ING3 in tumorigenesis and cancer progression of HNSCC, we assessed ING3 expression in 173 cases of HNSCC by immunohistochemistry. Expression of ING3 was also compared to clinicopathological variables, and the expression of several tumorigenic markers.

Materials and methods

Cases

One hundred and seventy-three cases of HNSCC, consisting of 44 pharyngeal SCCs and 129 laryngeal SCCs were selected from the patient files of our hospital from 1993 to 2008. Additionally, 60 specimens of moderate to severe dysplasia and 41 specimens of non-adjacent non-cancerous epithelium, histologically adjusted to normal epithelium corresponding to each HNSCC case, were obtained from surgically resected specimens. HNSCC patient samples were from 144 men and 29 women (mean age, 66 years old; range, 33-80 years old). One hundred and forty-seven cases were well to moderately differentiated SCC, and 26 cases were poorly differentiated SCC. Forty-seven surgically resected cases were followed up by consulting case documents. Twenty-six patients had lymph node metastases, but no cases had distant metastases. None of the patients underwent chemotherapy or radiotherapy before surgery. The Ethical Committee approved the research protocol.

Immunohistochemistry

All the specimens were fixed in 10% formalin and embedded in paraffin. Typical lesions corresponding to HNSCC, dysplasia and to normal epithelium were selected according to hematoxylin and eosin (H&E) staining. Consecutive 4 μm sections were cut from the blocks. After routine deparaffinization, antigen retrieval was carried out by microwave treatment in target retrieval solution for 15 minutes. Immunohistochemistry was performed according to EnVision two-step method as previously described (Li et al., 2010a,b). Primary antibodies, sources, and working dilutions are listed in Table 1. HRP-conjugated anti-goat IgG, anti-rabbit IgG or anti-mouse IgG (1:500, DAKO, CA, USA) antibodies were used as secondary antibodies to detect the respective primary antibodies. Bound antibodies were visualized with 3,3'-diaminobenzidine (DAB). The sections were counterstained with Mayer's hematoxylin. Samples treated in parallel without the addition of the primary antibody were used as negative controls.

Immunohistochemistry evaluation

ING3 expression was detected in both nucleus and cytoplasma. Additionally, the cytoplasmic immunoreactivity of 14-3-3η and the nuclear immunoreactivities of the molecular markers mutant p53, acetylated p53, p300 and p21 were visually assessed. Each section was observed by two independent observers. The percentage of positive cells was graded

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 most stained tissues showed positive expression of ING3, the immunohistochemical results of ING3 were grouped into two categories: ING3 low expression refers to negative (–) and weak (+) scored cases, while ING3 high expression refers to moderate (++) and strong (+++) scored ones. The immunohistochemical data of 14-3-3η, mutant p53, acetylated p53, p300 and p21 were divided into two groups: negative expression (–) and positive expression (+~+++).

Double immunofluorescence staining

Double immunofluorescence staining was used to analyze the colocalization of ^{ING3} with 14-3-3η, p300 and p21 in HNSCC samples. Deparaffinized tissue sections were prepared for immunofluorescence staining as described above. The sections were firstly incubated with ING3 primary antibody at 4°C overnight. Bound antibodies were detected by incubating with Alexa Fluor 594-conjugated donkey anti-goat IgG (1:500, Invitrogen Corporation, CA, USA) for 1 hour. After each treatment, the slides were rinsed with TBST three times for 1 min. Then the slides were re-blocked in 5% BSA for 30 minutes, and were incubated with primary antibody 14-3-3η, p300 or p21 for 1 hour at room temperature, respectively. Bound antibody was detected by incubating with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500, Invitrogen Corporation, CA, USA). After each treatment, the slides were also rinsed with TBST three times for 1 min. Finally, the sections were mounted using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, CA, USA). Sections treated in parallel without primary antibodies were used as negative controls. Samples were imaged using a fluorescence microscope (Olympus AX80, Japan).

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). Associations of ING3 expression and the different types of lesions were evaluated with Pearson's Chi-squared test. Spearman correlation test was used to analyze ING3 expression compared with clinicopathological variables, and the expression of several tumorigenic markers. Kaplan-Meier survival plots were generated, and comparisons between survival curves were made using the log-rank statistic. On the basis of the results of the univariate analysis, the Cox's proportional hazards model was employed to identify factors that were independently associated with disease-specific death in HNSCC. A stepwise backward procedure was used to derive a final model of the variables that had a significant independent relationship with survival. A P-value (p)<0.05 (2-sided) was regarded as statistically significant.

Results

ING3 expression in normal epithelium, dysplasia and HNSCC

ING3 expression was observed both in nuclei and cytoplasm. ING3 expression was primarily observed in the nuclei in normal epithelium (Fig. 1A), but was found mainly in the cytoplasm in dysplasia (Fig. 1B) and HNSCCs (Fig. 1C,D). The proportion of specimens with high nuclear expression of ING3 was significantly lower in HNSCC than in dysplasia and normal epithelium. ($p<0.001$, Table. 2). However, high expression of cytoplasmic ING3 was significantly more frequent in HNSCC than in dysplasia ($p=0.002$) and normal epithelium ($p<0.001$, Table. 2). Neither nuclear nor cytoplasmic expression of ING3 in normal epithelium had statistical significance with their expression in dysplasia ($p>0.05$).

The relationship between ING3 expression and clinicopathological variables in HNSCC

Nuclear expression of ING3 in HNSCC was negatively correlated with poor differentiation ($p=0.001$), T2-T4 staging ($p=0.011$), and TNM (Tumor, Node, Metastasis) II~IV staging ($p=0.004$, Table 3), but not with age, sex, or lymph node metastasis. In contrast, high expression of cytoplasmic ING3 in HNSCC was only positively correlated with lymph node metastasis ($p=0.02$), but not with other variables.

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

Antibody	Clone	source	Company	Dilution
ING3	Polyclonal	Goat	Proteintech Group, Inc, Chicago, IL, USA	1:400
14-3-3η	Polyclonal	Rabbit	Enzo Life Sciences, Inc, PA, USA	1:600
P53(DO-7)	Monoclonal	Mouse	DAKO, Denmark	1:100
Acetylated p53	Polyclonal	Rabbit	BioLegend, CA, USA	1:100
P300 (N-15)	Polyclonal	Rabbit	Santa Cruz, Biotechnology, CA, USA	1:50
P21	Polyclonal	Rabbit	Santa Cruz, Biotechnology, CA, USA	1:50

Correlation between ING3 expression and other molecular markers associated with cancer development and prognosis

Expression of 14-3-3η (Fig. 1E) was mainly detected in the cytoplasma, but p300, p21, mutant p53 and acetylated p53 were observed in the nuclei of tumor cells (Fig. 1F-I). As shown in Table 4, high nuclear expression of ING3 in HNSCC was positively correlated with p300 expression (p<0.001, R=0.307), p21

Table 2. Relationship of ING3 expression in Normal epitheliums, dysplasias, and HNSCCs.

Groups	N	Nuclear [n(%)]			Cytoplasmic[n(%)]		
		low	High	HR (%)	Low	High	HR (%)
Normal epithelium	41	12	29	70.7	31	10	24.4
Dysplasia	60	26	34	56.7	37	23	38.3
HNSCC	173	148	25	14.5*	66	107	61.8**

HR, high expression rate; *in comparison to Normal epithelium and Dysplasia (p<0.001); ** in comparison to Normal epithelium (p<0.001) and Dysplasia (p=0.002).

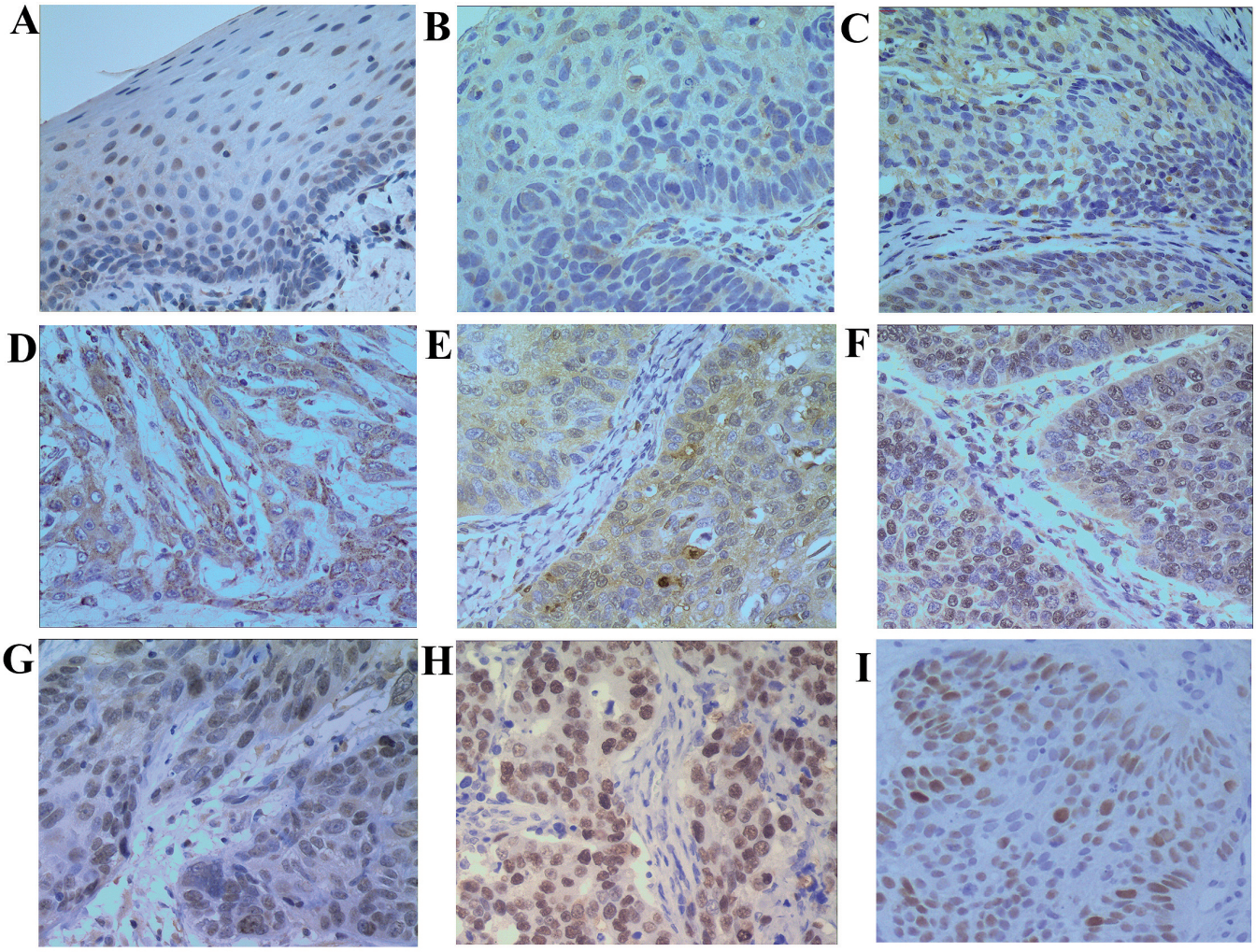

Fig. 1. Imunohistochemical staining of ING3 and other tumor-related molecular markers. High-magnification images of the fields showed that the nuclear expression of ING3 gradually decreased from the normal epithelium **(A)** to dysplastic lesion **(B)** or cancerous lesion **(C, D)**. In contract, cytoplasmic expression of ING3 was increased significantly. High cytoplasmic expression of 14-3-3η was also detected in some HNSCC cases **(E)**. Expression of p300 **(F),** p21 **(G)**, mutant p53 **(H)** and acetylated p53 **(I)**, was observed in the nuclei of tumor cells. x 400.

expression (p<0.001, R=0.432) and the acetylated p53 (p=0.009, R=0.211), but negatively correlated with cytoplasmic expression of ING3 (p=0.029, R= -0.219). High cytoplasmic expression of ING3 was positively associated with 14-3-3η expression (p<0.001, R= 0.378), but negatively correlated with p300 expression (p=0.002, R= -0.239). Neither nuclear nor cytoplasmic expression of ING3 was significantly correlated with mutant p53 expression (p>0.05, Table 4).

Double immunofluorescence labeling of ING3 with 14-3-3η, p300 and p21

Double immunofluorescence staining was used to analyze the colocalization of ING3 with 14-3-3η, p300 and p21 in HNSCC samples. In our study, ING3 expression was observed both in nuclei and cytoplasm, but primarily in the cytoplasm in HNSCCs. Furthermore, double immunofluorescence staining revealed that ING3 frequently coexpressed with 14-3-3η in cytoplasm in HNSCCs. Nuclear ING3 expression also coexpressed with p300 or p21 in some HNSCC cases. A large number of tumor cells exhibited orange staining, indicating colocalization of the two proteins assessed (Fig. 2).

Univariate and multivariate survival analysis

Follow-up information was available for 39 cases of

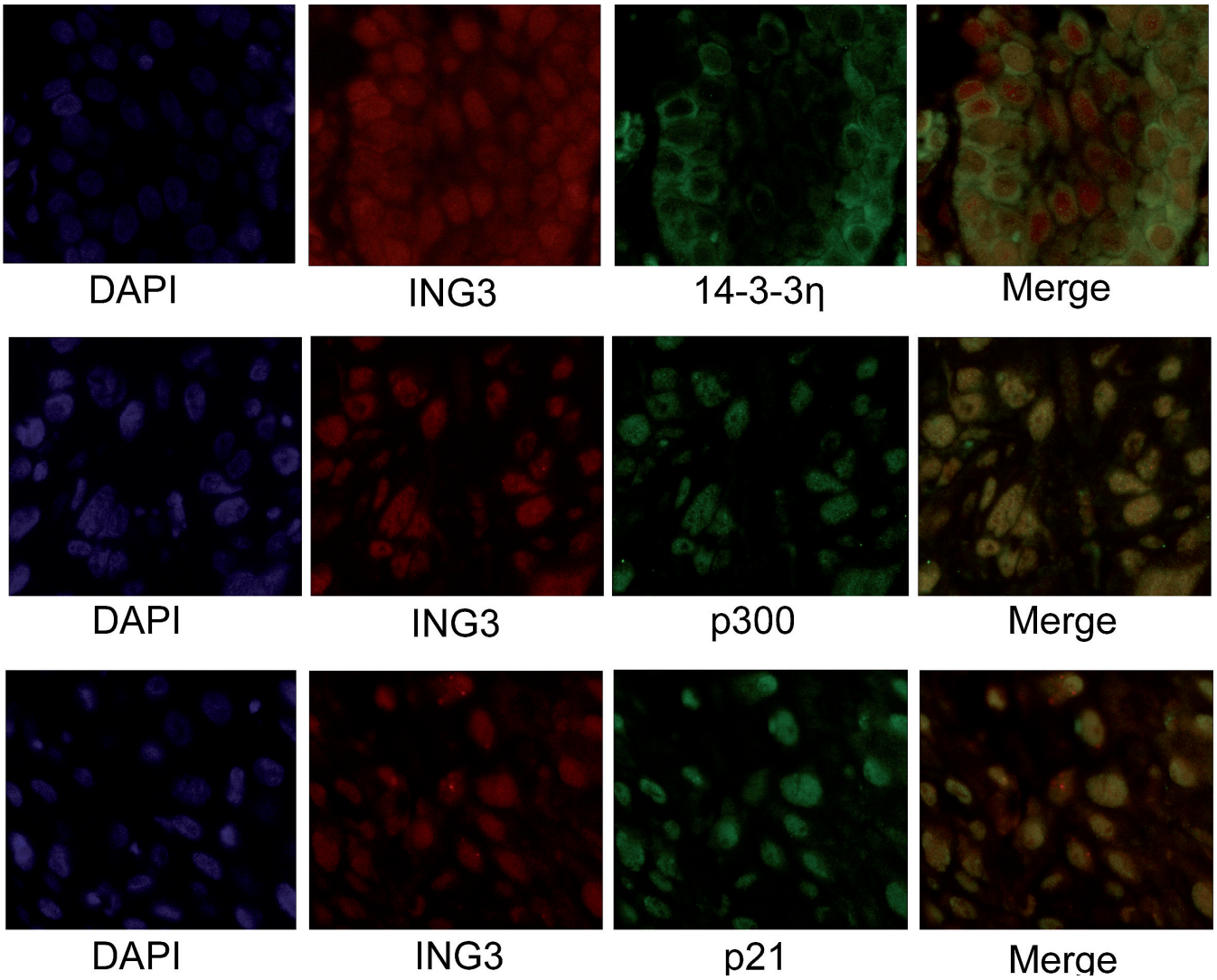

Fig. 2. Double immunofluorescence staining of ING3, 14-3-3η, P300 and P21 in HNSCC. Staining of ING3 protein is shown in red, and 14-3-3η, p300 or p21 in green. Colocalization of both proteins is shown in orange in the merged images. x 400.

HNSCC for periods ranging from one month to 9.1 years (mean=56.1 months). Fig. 3A,B showed HNSCC survival curves grouped by T staging and lymph node metastasis, respectively. But no statistical significance of cumulative survival rate was found between the patients with or without nuclear or cytoplasmic high expression (Fig. 3C,D). Univariate analysis using the Kaplan-Meier method indicated the patients with T0-T1 staging or no lymph node metastasis had higher survival rate ($p<0.05$). However, there was no significant difference in the cumulative survival rate of patients associated with either nuclear or cytoplasmic ING3 expression ($p>0.05$). In addition, multivariate analysis using Cox's proportional hazard model suggested that only T2-T4 staging ($p=0.004$) was an independent prognostic factor for overall HNSCC (Table 5).

Table 3. Relationship between ING3 expression and clinicopathological features in HNSCC.

Clinicopathological features	N	Nuclear [n(%)] low	Nuclear [n(%)] High	p	Cytoplasmic[n(%)] Low	Cytoplasmic[n(%)] High	p
Age				0.837			0.704
<65	66	56 (84.8)	10 (15.2)		24 (36.4)	42 (63.6)	
≥65	107	92 (86.0)	15 (14.0)		42 (39.3)	65 (60.7)	
Sex				0.383			0.656
male	144	125 (86.8)	19 (13.2)		56 (38.9)	88 (61.1)	
Female	29	23 (79.3)	6 (20.7)		10 (34.5)	19 (65.5)	
Differentiation				0.001			0.284
well	96	76 (79.2)	20 (20.8)		41 (42.7)	55 (57.3)	
moderate	51	47 (92.9)	43 (7.8)		16 (31.4)	35 (68.6)	
poor	26	25 (96.2)	1 (3.8)		9 (34.6)	17 (65.4)	
T				0.011			0.154
T0~TI	11	6 (54.5)	5 (45.5)		6 (54.5)	5 (45.5)	
T2~T4	36	32 (88.9)	4 (11.1)		11 (30.6)	25 (69.4)	
Lymph node metastasis				0.446			0.02
-	26	20 (76.9)	6 (23.1)		13 (50.0)	13 (50.0)	
+	21	18 (85.7)	3 (14.3)		4 (19.0)	17 (81.0)	
TNM staging				0.004			0.08
0~I	10	5 (50.0)	5 (50.0)		6 (60.0)	4 (40.0)	
II~IV	37	33 (89.2)	4 (10.8)		11 (29.7)	26 (70.3)	

Table 4. Relationship between nuclear expression of ING3 and tumor-related molecular markers in HNSCC.

Clinicopathological features	N	Nuclear [n(%)] low	Nuclear [n(%)] High	p	Cytoplasmic[n(%)] Low	Cytoplasmic[n(%)] High	p
14-3-3η				0.796			<0.001
–	95	80 (84.2)	15 (15.8)		45 (47.4)	50 (52.6)	
+	78	68 (87.2)	10 (12.8)		21 (26.9)	57 (73.1)	
P300				<0.001			0.002
–	86	83 (96.5)	3 (3.5)		23 (26.7)	63 (73.3)	
+	87	65 (74.4)	22 (25.3)		43 (49.4)	44 (50.6)	
P21				<0.001			0.09
–	111	107 (96.4)	4 (3.6)		37 (33.3)	74 (66.7)	
+	62	41 (66.1)	21 (33.9)		29 (46.8)	33 (53.2)	
mutant P53				0.388			0.836
–	90	79 (87.8)	11 (12.2)		35 (38.9)	55 (61.1)	
+	83	69 (83.1)	14 (16.9)		31 (37.3)	52 (62.7)	
Acetylated p53				0.009			0.07
–	120	108 (90.0)	12 (10.0)		40 (33.3)	80 (66.7)	
+	53	40 (75.5)	13 (24.5)		26 (49.1)	27 (50.9)	
Cytoplasmitc ING3				0.029			
Low expression	66	50 (75.8)	16 (24.2)				
High expression	107	98 (91.6)	9 (8.4)				

Discussion

In the present study, nuclear expression of ING3 was decreased in HNSCC samples in comparison to normal epithelium and dysplasia. Low nuclear expression of ING3 was positively correlated with poor differentiation, T2-T4 staging, and TNM II~IV staging in HNSCC. Previous studies have demonstrated 45.5–68% LOH of *ING* genes in HNSCC. Using six polymorphic microsatellite markers, 48% (22/46) loss of heterozygosity (LOH) at 7q31 region near the location of *ING3* was detected in HNSCC (Gunduz et al., 2002). RT-PCR analysis showed decreased or no expression of the ING3 mRNA in 50% of cases of primary HNSCC compared with normal tissues, but only one mutation was identified in the *ING3* gene. Furthermore, the decreases of ING3 mRNA or protein levels were found to be a common event in tumorigenesis in some tumors (Wang et al., 2007; Gunduz et al., 2008; Borkosky et al., 2010; Lu et al., 2012; Yang et al., 2012). Additionally,

Table 5. Multivariate survival analysis of HNSCC cohort.

Parameters	Relative risk (95%CI)	p value
Age	1.276(0.469-3.470)	0.633
Sex	0.731(0.211-2.529)	0.621
Differentiation	0.621(0.266-1.452)	0.272
T staging (T2-T4)	30.017(2.896-311.765)	0.004
Lymph node metastasis (+)	2.328(0.822-6.594)	0.112
Nuclear expression of ING3	2.790(0.786-9.902)	0.112
Cytoplasmic expression of ING3	0.547(0.217-1.377)	0.200

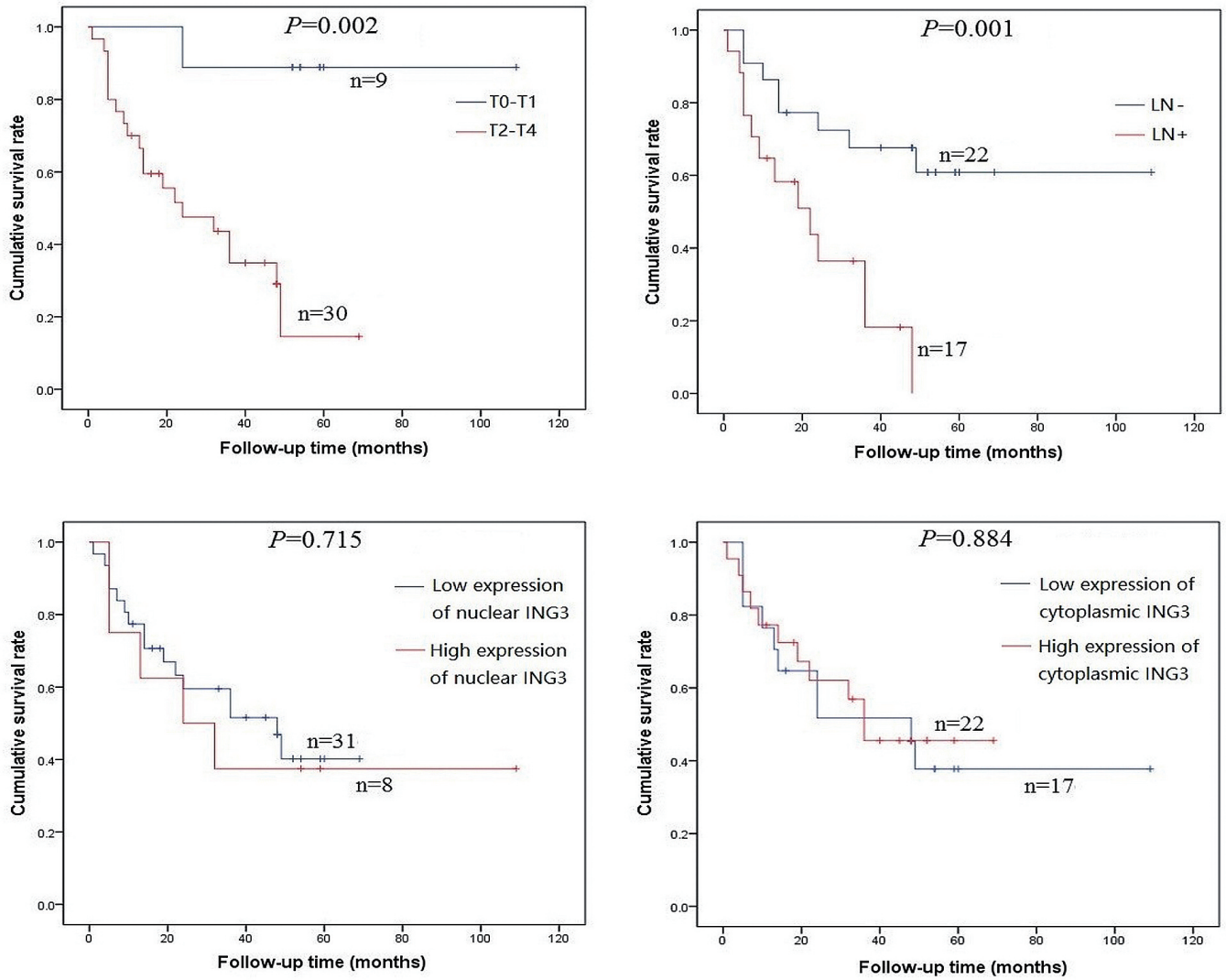

Fig. 3. Correlation between ING3 expression and prognosis of HNSCC patients. Kaplan-Meier curves show the cumulative survival rate of HNSCC patients grouped according to T staging **(A)**, lymph node metastasis **(B)**, nuclear **(C)** or cytoplasmic **(D)** ING3 expression.

ING3 represents a novel transcription target in the serine/threonin kinase RSK2-cAMP-response element-binding protein (CREB) pathway: RSK2 signaling through CREB downregulates ING3 to protect HNSCC cells from the apoptotic ''anoikis'' process, thereby contributing to cancer cell invasion and tumor metastasis (Breaux et al., 2015). These results imply that epigenetic inactivation or aberrant expression of upstream genes may cause a decrease in ING3 expression. As a subunit of the nucleosome acetyltransferase of histone4 (NuA4 HAT) complex, ING3 plays primary roles in transcription, cellular response to DNA damage, and cell cycle control (Nagashima et al., 2003). Therefore, decreased ING3 nuclear expression in HNSCC may lead to an imbalance in the control of gene expression, contributing to a malignant transformed cellular phenotype and failure to resist various genotoxic insults and stressors. Taken together, similarly to p33ING1b, ING4 and ING5 (Li et al., 2010a,b, 2011a,b), these data suggest that downregulation of nuclear ING3 expression plays important roles in tumorigenesis and progression of HNSCC.

In the present study, increased cytoplasmic immunoexpression of ING3 in primary HNSCC was positively correlated with lower nuclear ING3 expression and lymph node metastasis. It implied that the increase in cytoplasmic ING3 was likely due to a shift of ING3 from the nucleus to the cytoplasm. These results are similar to our previous studies on ING1, ING4 and ING5 in HNSCC (Li et al., 2010a,b, 2011a,b). Wang et.al also demonstrated nuclear ING3 expression was remarkably reduced in malignant melanomas compared with dysplastic nevi, which was significantly correlated with the increased ING3 level in cytoplasm (Wang et al., 2007). The mechanism of translocation of ING3 proteins is unclear. Although the NLS of ING family has been considered to be crucial for nuclear localization and biological functions of ING proteins, the effects of the small deletions in the NLS on subcellular localization are controversial (Fabbro and Henderson, 2003; Unoki et al., 2006; Tsai et al., 2008). Few mutations of ING3 gene were found. In addition, translocation of nuclear proteins can also result from alterations in other nuclear import/export signals by posttranslational modifications, including phosphorylation, ubiquitylation, glycosylation, and sumoylation (Fabbro and Henderson, 2003). Notably, cytoplasmic ING3 expression was positively correlated with 14-3-3η expression in HNSCC in the present study. Double immunofluorescence staining also confirmed that 14-3-3η was coexpressed and colocalized with cytoplasmic ING3. 14-3-3 family members primarily reside in the cytoplasmic compartment and associate with phosphorylated ligands involved in many cellular processes, including regulation of the cell cycle and DNA damage checkpoints (Hermeking and Benzinger, 2006). It was reported that p33ING1b interacts with members of the 14-3-3 family of proteins, and the interaction is regulated by the phosphorylation status of ING1. Furthermore, 14-3-3 binding resulted in significant amounts of p33(ING1b) protein being tethered in the cytoplasm (Gong et al., 2006). Although the precise mechanisms by which 14-3-3η interacts with the ING3 protein are poorly understood, our study suggests that 14-3-3η may interact with ING3 and result in its cytoplasmic retention similarly to p33ING1b. The increased expression of cytoplasmic ING3 in HNSCC samples may be an objective reflection of the absence of ING3 nuclear protein, and may be involved in malignant progression in HNSCC.

Our study also demonstrates that nuclear ING3 expression is positively associated with p300 expression in HNSCC. The p300 protein is an adenoviral E1A-binding protein that functions as a transcriptional cofactor and a HAT. p300 is involved in multiple cellular processes by acting as a bridge linking DNA-binding transcription factors. Through its function as a coactivator and an acetylase, p300 contributes to maintenance of p53 stability and is required for p53-mediated transactivation of target genes (Iyer et al., 2004). A further study demonstrated translational repression of the p53 protein by the CUGBP1-eukaryotic initiation factor 2 (eIF2) repressor complex (Harris and Levine, 2005). It suggested p300 may be a critical regulator to the p53 signaling pathway. In the present study, coexpression and colocalization of ING3 and p300 were confirmed by double immunofluorescence labeling. Therefore, we propose that ING3 may interact with p300 to modulate the transactivation of target genes, such as *p53*. Notably, our study found that nuclear expression of ING3 was positively correlated with acetylated p53 at K382, but there was no statistical significant with mutant p53 in HNSCC. p53 acts as a major defense against cancer by receiving disparate input signals, including oncogene activation and DNA damage, and initiating appropriate responses, such as DNA repair, cell cycle arrest, senescence, or apoptosis (Breaux et al., 2015). Post-translational modifications of p53 are critical in modulating its tumor suppressive functions. Acetylation has many important effects on p53. It increases p53 protein stability, and is required for its checkpoint responses to DNA damage, and so on (Reed and Quelle, 2014). In general, these modifications are mediated by two different groupings of acetyltransferases, p300/CBP/PCAF or Tip60/MOF/MOZ. ING3 is a member of the nucleosome acetyltransferase of Tip60/NuA4 HAT complex. Therefore, we speculate ING3 may interact with p300 to upregulate the level of acetylation of p53, and promote p53-mediated cell cycle arrest, senescence and/or apoptosis.

ING proteins have been reported to regulate cell growth, apoptosis, and the cell cycle in many cancers (Coles and Jones, 2009). In the present study, high expression of nuclear ING3 was significantly positively associated with p21 expression in HNSCC. The p21 protein, also known as $p21^{WAF1/Cip1}$, is a mediator of p53 activity and an inhibitor of cell cycle progression that

blocks the activity of CDK-cyclin complexes. p21 can bind to and inhibit PCNA through its carboxy-terminal domain, leading to inhibition of DNA synthesis and modulation of PCNA-dependent DNA repair pathways (Abbas and Dutta, 2009). Double immunofluorescence staining also showed that the ING3 protein was coexpressed and colocalized with the p21 protein in our study. A previous study demonstrated that ING3 overexpression resulted in a decreased population of cells in S phase, a diminished colony-forming efficiency, and induced apoptosis in RKO cells, but not in RKO-E6 cells with inactivated p53. p47ING3 activates p53-transactivated promoters, including promoters of p21/waf1 and bax (Nagashima et al., 2003). Therefore, high ING3 nuclear expression may induce apoptosis in HNSCC, partly through upregulation of p21 expression. In addition, Wang et.al found that ING3 enhanced UV-induced apoptosis through the activation of Fas/Caspase-8 pathway with independency of functional p53 in melanoma cells (Wang and Li, 2006). These results suggest that the ING3 protein may be involved in cell apoptosis progression. ING3 may dynamically regulate cell growth and apoptosis through different signaling mechanisms dependent upon the cell status. Further studies are necessary to explore the physiological roles of the ING3 protein in the regulation of cell apoptosis.

In the present study, although low nuclear expression of ING3 was positively correlated with poor differentiation, T2-T4 staging, and TNM II~IV staging in HNSCC, Kaplan–Meier survival curve analysis showed that no statistical significance of cumulative survival rate was found between the patients with or without nuclear or cytoplasmic high expression. The patients with T2-T4 staging or lymph node metastasis showed a poor prognosis. Multivariate cox regression analysis revealed that only T2-T4 staging is an independent prognostic factor to predict patient outcome in HNSCC. Gunduz's study found that approximately 60% of 67 patients with normal to high ING3 expression survived, whereas this was 35% in 67 patients with low ING3 expression at 5- year follow up. Multivariate analysis also showed downregulation of ING3 as an independent prognostic factor for poor overall survival (Gunduz et al., 2008). In addition, Kaplan-Meier curves demonstrated that the reduced nuclear ING3 expression was significantly correlated with a poorer survival of patients with primary melanoma (Wang et al., 2007) and hepatocellular carcinoma (Yang et al., 2012). Strikingly, the multivariate Cox regression analysis revealed that reduced nuclear ING3 expression is an independent prognostic factor to predict patient outcome in these tumors. Although ING3 expression was highest in benign prostate tissues compared to prostate cancer (PCa), lower ING3 levels in PCa is associated with a better survival rate compared to higher levels of ING3. The findings support a potential oncogenic role of ING3 in PCa (Almami et al., 2016). Our result is not consistent with previous studies perhaps due to difference of tumor type or the different patient groups. Valid follow-up data were available for only 39 out of 173 HNSCC patient in our study. It is necessary to analyse the significance of ING3 expression and survival time in a larger scale of HNSCC patients in future.

In conclusion, decreases in nuclear ING3 may play important roles in tumorigenesis and progression in HNSCC. The increase in cytoplasmic ING3 may be due to 14-3-3η binding and may be involved in malignant progression. Nuclear ING3 may modulate the transactivation of target genes, promoting apoptosis and cell cycle arrest through interactions with p300 and p21. ING3 may interact with p300 to upregulate the level of acetylation of p53, and promote p53-mediated cell cycle arrest, senescence and/or apoptosis. Therefore, ING3 may be a potential tumor suppressor molecule and a possible therapeutic target in HNSCC.

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