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Expression and prognostic value of *ING3* in advanced laryngeal squamous cell carcinoma

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ABSTRACT

Objectives: Laryngeal squamous cell carcinomas (LSCC) is one of the most common aggressive neoplasms of the head and neck region. There is a significant need for identification of successful and accurate prognostic markers to better estimate the clinical outcomes for LSCC patients. In this study, we aimed at analyzing the differential expressions of inhibitor growth *(ING)* family members and to evaluate the prognostic values of deregulated *ING* genes in LSCC.

Methods: We investigated the relative expressions of *ING* genes in laryngeal tumor-normal tissue pairs at mRNA level using quantitative real-time polymerase chain reaction and relative expression of *ING3* in the protein level using Western Blot analysis.

Results: The rate of genetic alterations of *ING3* was relatively higher in head and neck cancers including LSCC. *ING3* expression was significantly upregulated in LSCC tissue samples at both mRNA and protein level. Higher expression of *ING3* was also correlated with poor disease-free survival of patients with head and neck cancer.

Conclusions: Our findings assigned an oncogenic feature for *ING3* in laryngeal cancer with a significant upregulation detected in advanced cases and suggested a vital prognostic potential for *ING3*.

Keywords: Larynx cancer, *ING* gene family, oncogene, prognosis

Laryngeal squamous cell carcinomas (LSCC) is one of the most common aggressive neoplasms of the head and neck region, accounting for approximately 85 to 90% of all malignant laryngeal tumors [1]. Multiple factors including smoking, alcohol consumption, air pollution, diet, HPV infection, and radiation are related to the occurrence of LSCC, which is a complex process where the imbalance between the expressions of multiple tumor suppressor genes and oncogenes, deregulation of vital signaling pathways and defects of immune system are observed [2, 3]. Despite considerable advances have been achieved in radiotherapy, chemotherapy, adjuvant chemotherapies, surgical treatment, and diagnosis techniques, approximately 60% of patients with LSCC progress to advanced stages, who experience dyspnea, dysphagia,

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and dysphonia leading to significant reduction in the quality of life [4, 5]. Invasion, lymphatic metastasis, and tumor recurrence, which are observed along with late diagnosis, are the main factors that contribute to poor prognosis and high mortality rates in advanced LSCC [6]. In addition, due to late diagnosis at advanced stages, the 5-year survival rates of LSCC patients remain poor that did not change over the last 30 years [7]. Therefore, there is a significant need for identification of successful and accurate prognostic markers to better estimate the clinical outcomes for LSCC patients.

The human Inhibitor of Growth *(ING)* family comprised of five conserved genes, *ING1, ING2, ING3, ING4* and *ING5*, which share between 32% and 76% DNA sequence homology [8, 9]. Their encoded proteins contain a highly conserved plant homeodomain (PHD) in the C-terminal region, a Cys4-His-Cys3 form of zinc finger that interacts directly with histone H3, a nuclear localization signal (NLS) in the middle region, and a novel conserved region (NCR) with unknown function [10-12]. Previous studies showed that ING proteins are involved in a wide variety of cellular processes including DNA repair, histone methylation and acetylation, senescence, cell growth, colony formation, apoptosis, cell cycle control, chromatin remodeling, and angiogenesis [9, 13, 14]. Mutations and altered expressions of *INGs* have been reported in different types of cancers including lung cancer, osteosarcoma, oral squamous cell carcinoma, breast cancer, prostate cancer, gastric cancer, colorectal cancer, and laryngeal squamous cell carcinoma [15-17].

ING3 gene, located on chromosome 7q31.3, is a member of the *ING* family, which consists of 12 exons encoding a 46.8 kDa protein with 418 amino acids [14, 15, 18-20]. ING3 is a part of the NuA4-Tip60 MYST-HAT multi-subunit complex core unit, which also contains EPC1, EAF6, and TIP60 as main participants, that are responsible for the acetylation of histones H2A and H4 [13, 21, 22]. So far, studies showed that *ING3* acts as a type II tumor suppressor as downregulated in many types of cancer and play significant roles in modulating transcription, cell cycle control, and apoptosis [23-25]. However, recent studies have reported that *ING3* is highly expressed in rapidly proliferating human tissues [22, 26]. In addition, in contrast to tumor suppressor role, various studies have reported that upregulation of *ING3* stimulate cell proliferation, tumor growth, and androgen receptor activation. High *ING3* levels also correlated with poor prognosis in prostate cancer suggesting an oncogenic potential for *ING3* [26, 27]. Although ING3 has been reported to be abnormally expressed in a number of cancer types, the roles of ING3 in laryngeal carcinogenesis are largely unknown. Therefore, we aimed at analyzing the differential expressions of ING family members and to evaluate the prognostic value of deregulated ING3 in LSCC.

In this study, we assigned an oncogenic potential for *ING3* in laryngeal cancer with a significant upregulation detected in advanced cases. We also demonstrated that *ING3* expression significantly correlates with its physical interactor EPC1 with oncogenic potential in head and neck cancer. Our results suggest a vital prognostic potential for *ING3* in laryngeal cancer although further functional studies are required to strengthen its potential as a prognostic marker.

METHODS

In Silico Analysis

cBioPortal web tool was used to analyze the differential expression and copy number changes of *ING* gene family members and ING3 interactors using the RNA sequencing expression data of tumor and normal samples of head and neck origin collected from the Cancer Genome Atlas Program (TCGA) [28]. Co-occurrence analysis of ING3 and its interactors was carried out using cBioPortal as well. The mean differential expression of *ING* gene family members, the relative expression of *ING3* in tumor tissue samples considering their stage and T classification, and *ING3/EPC1* correlation were presented using the data obtained from UALCAN, which is a comprehensive web resource for analyzing cancer OMICS data [29]. Differential expression of *ING3* in 11 datasets deposited at Oncomine was evaluated and Sengupta (26 nasopharyngeal tumor and 12 normal specimens) and Ginos (41 head and neck squamous cell carcinoma and 13 normal specimens) data with profound alterations in *ING3* expression were plotted [30]. The interactors of ING3 was identified using String web tool [31]. Overrepresentation analysis of ING3 and its interactors was performed using WEB-based GEne SeT

AnaLysis Toolkit.

Patients and Tissue Samples

This study was reviewed and approved by the Institutional Ethics Committee of Ataturk University, Faculty of Medicine (IRB No: B.30.2.ATA.0.01.00/555). Participants were included into the study after receiving their written informed consents. 28 laryngeal tumor tissue specimens with at least 70% tumor cell content and 28 corresponding adjacent laryngeal normal tissue samples were obtained from Department of Otorhinolaryngology, Faculty of Medicine, Atatürk University. The tumor cell content of the laryngeal tumor tissue specimens and the absence of any cancerous or dysplastic content in the corresponding adjacent laryngeal normal tissue samples was histopathologically confirmed at Department of Medical Pathology, Faculty of Medicine, Ataturk University. All tumors and matched non-tumor tissue samples were histologically analyzed by a pathologist. Histological analysis was reviewed by two independent pathologists after H&E staining. Clinical stages were categorized according to the seventh edition of the UICC-TNM classification. Fresh tissue materials were collected immediately after surgery to prevent degradation of RNA and protein contents, snap frozen, and stored at -80 ºC. Patients didn't receive radiotherapy, chemotherapy, or immunotherapy prior to the surgery. The clinico-pathological data of patients were summarized in Table 1.

RNA Isolation

Total RNA samples from equal amounts of tumor and normal laryngeal tissue specimens grounded within liquid nitrogen were extracted using TRIzol reagent (Invitrogen, San Diego, CA, United States) following the manufacturer's protocol. Total RNA concentrations and purities were measured spectrophotometrically with Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT, United States).

cDNA Synthesis and Quantitative Real-Time PCR

A total of 1µg RNA for each sample was reverse−transcribed into complementary DNA (cDNA) using "High Capacity cDNA Reverse Transcription Kit" (Thermo Fisher Scientific, Waltham, MA, United States) according to manufacturer's instructions. Relative expression levels of *ING* family members were analyzed with quantitative real time polymerase chain reactions (qRT−PCR) with 5 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis Bio-Dyne, Tartu, Estonia) using Rotor−Gene qRT−PCR (Qiagen, Düsseldorf, Germany) device with standard parameters. Data were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expressions. Primers used in qRT−PCR were listed in Supplementary Table 1. All reactions were carried out in duplicates. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. The clinico-pathological data of the patients

	LSCC Subjects
Age	
≤ 60	15
> 60	13
Gender	
Male	27
Female	1
Tumor Location	
Supraglottic	16
Subglottic	12
Size (cm)	
≤ 2	13
>2	15
T Classification	
T1 and T2	12
T3 and T4	16
Histological Grade	
I and II	10
III and IV	17
Lymphatic metastasis	
N0	17
N+	11
Neck Dissection	
No	15
Yes	13
Adjuvant Therapy	
No	8
Yes	20

Western Blot Analysis

Proteins were extracted using modified RIPA Lysis Buffer (EcoTech Biotechnology, Erzurum, Turkey) containing phenylmethanesulfonyl fluoride (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Dallas, TX, United States). Equal amounts of each protein sample mixed with 10 × Laemmli Sample Buffer (EcoTech Biotechnology, Erzurum, Turkey) in 9/1 ratio was loaded and separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (EcoTech Biotechnology, Erzurum, Turkey) using semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, United States). Membranes were blocked in 5% non-fat milk powder dissolved in 1× PBST buffer (EcoTech Biotechnology, Erzurum, Turkey) for 1 hour at room temperature, and then incubated with primary antibodies against ING3 (diluted in 1:1000; Santa Cruz Biotechnology, Cat No: sc-101245, Dallas, TX, United States), β−actin (diluted in 1:200; Santa Cruz Biotechnology, Cat No: sc-47778, Dallas, TX, United States) diluted in PBST buffer on a shaker overnight at 4°C. After washing with PBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies (1/3,000; Santa Cruz Biotechnology, Dallas, TX, United States) for 1 hour at room temperature. The Clarity Max ECL Western Blotting Substrate (BioRad, United States, Hercules, CA, Dallas, TX, United States) or *ClearBand* Western Blotting Substrate (EcoTech Biotechnology, Erzurum, Turkey) were used to visualize protein signals and quantification of bands was performed using Image J program.

Statistical Analysis

Correlation of I*NG3/EPC1* expressions was measured using Pearson's Correlation test. Association of histologic grade with the presence of genomic alteration in at least one of the *ING3* and *EPC1* in the tumor specimens was tested using Chi-squared test. $P < 0.05$ was considered as statistically significant.

RESULTS

Expressions of *ING* family genes are upregulated and these genes have genetic alterations in LSCC patients

We initially analyzed mutation characteristics and genetic alteration of *ING* family members in head and neck squamous cell carcinoma samples deposited at TCGA database (TCGA-HNSCC) using cBioPortal. As shown in Figure 1A, high mutation frequencies of *ING* family members were observed in head and neck

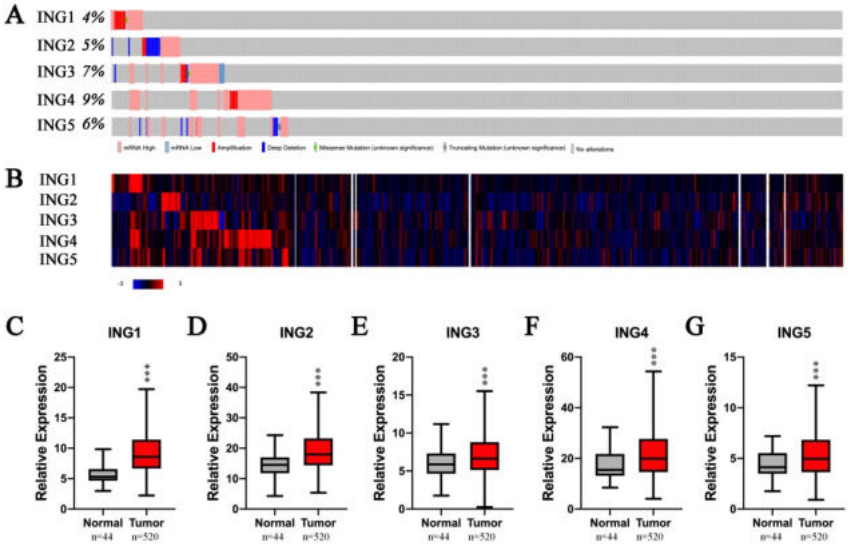


Fig. 1. ING family members have genetic alterations and are upregulated in head neck cancer patients. (A) Frequent genetic alterations observed in ING family members in head and neck cancer tissues. (B) Expression profile of ING family members in head and neck cancer tissues. Relative mean expression levels of (C) ING1, (D) ING2, (E) ING3, (F) ING4 and (G) ING5. Gene expressions were normalized to GAPDH. ***$p < 0.001$.

squamous cell carcinoma patients. *ING3, ING4*, and *ING5* were the three genes with the highest rate of sequence alterations, and their mutation rates were 7%, 9%, and 6%, respectively (Fig. 1A). Similar pattern was observed when their expressions in tumor samples were analyzed in the mRNA level using RNA sequencing data of TCGA-HNSCC (Fig. 1B). Next, the mRNA expression patterns of *ING* family members were investigated between head and neck squamous cell carcinoma and normal tissues using UALCAN web portal. The result showed that all of *ING* family members were significantly upregulated in primary head and neck squamous cell carcinoma tissues compared to normal samples (Figs. 1C-G, all $p < 0.05$).

Expression of ING3 is upregulated in LSCC tissues and significantly related to clinicopathological parameters

To explore levels of ING family members, we initially examined the mRNA expression of *ING1a, ING1b, ING2, ING3, ING4,* and I*NG5* in LSCC patients using qRT-PCR. The results revealed that the expression of ING3 was significantly increased in laryngeal cancer tissues compared with adjacent non-cancerous tissues, while no significant statistical difference was observed in the expressions of *ING1a, ING1b, ING2, ING4,* and *ING5* between cancer tissues and adjacent non-cancerous tissues (Fig. 2A). We then used Oncomine online portal further to verify the mRNA levels of *ING3*. Sengupta (26 nasopharyngeal tumor and 12 normal specimens) and Ginos (41 head and neck squamous cell carcinoma and 13 normal specimens) data showed that *ING3* was profoundly upregulated in the cancer tissues compared with adjacent non-cancerous tissues (Figs. 2B and 2C). Interestingly, *ING3* expression was not significantly altered in other datasets deposited in Oncomine, which were mostly comprised of specimens with oral cavity ori-

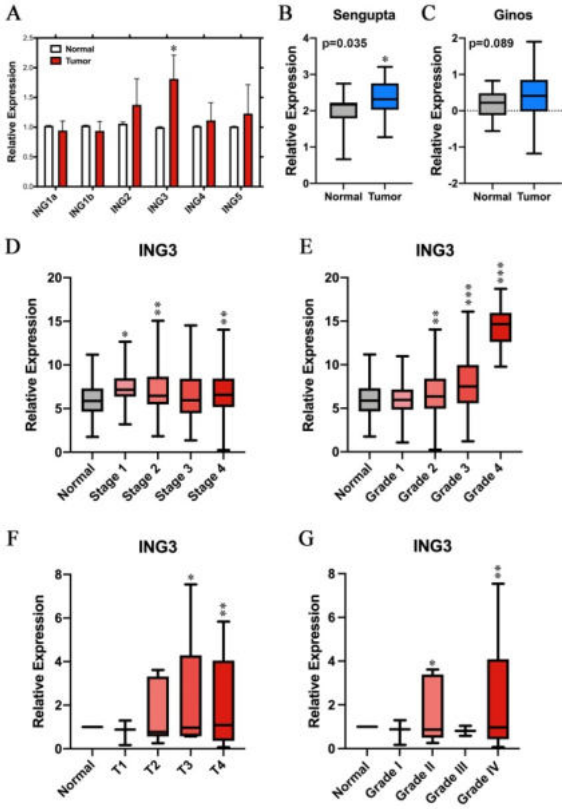


Fig. 2. Expression of ING3 is upregulated in LSCC tissues and its expression is significantly related to clinico-pathological parameters. (A) Relative mean expression levels of ING family members in LSCC tissues and adjacent non-tumor tissues. (B) Relative mRNA level of ING3 in Sengupta dataset obtained from Oncomine. (C) Relative mRNA level of ING3 in Ginos dataset obtained from Oncomine. (D) Relative ING3 level in different stage head and neck tumor tissue samples deposited in TCGA. (E) Relative ING3 expression level in different grade head and neck tumor tissue samples deposited in TCGA. (F) Relative ING3 expression level in different T stage LSCC tissue samples. (G) Relative ING3 expression level in different grade LSCC tissue samples. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

gin.

After mRNA expression of *ING3* found to be upregulated in LSCC patients, we then focused on the relationship of *ING3* mRNA expressions with clinicopathological parameters in individual head and neck squamous cell carcinoma patients using UALCAN. The results show that patients with advanced stage head and neck cancer had relatively higher *ING3* mRNA expression compared with normal tissue samples (Fig. 2D). In addition, *ING3* mRNA expression was also significantly upregulated in tumor tissue samples of head and neck cancer patients with higher grades compared with normal specimens (Fig. 2E). In line with these findings, we also observed overexpression of *ING3* in patients with advanced LSCC (Figs. 2F and 2G). Taken together, these results suggested that transcriptional levels of *ING3* is significantly correlated with different clinicopathological parameters in LSCC patients and LSCC patients with advanced stages tended to have higher levels of *ING3*.

ING3 has increased protein level in LSCC patients

ING3 mRNA level was significantly higher in LSCC tissues compared with matched non-cancerous LSCC tissues. To further confirm these results, we performed western blot analysis using 10 tumor-normal tissue pairs collected from LSCC patients (Fig. 3A). Our data revealed that, ING3 expression in protein level was significantly increased in cancer tissues compared with adjacent non-cancerous tissues (Fig. 3B), which is consistent with our qRT-PCR and *in silico* analysis. These results provided strong evidence that ING3 is upregulated in LSCC.

ING3 interacts with a hub of genes mostly related to DNA damage

After analysis of *ING3* genetic alterations, expression pattern, and its prognostic value in LSCC patients, we performed protein-protein interaction analysis using the STRING database to explore the possible functional protein network of ING3. The top 10 hub genes with the highest protein-protein interaction confident scores were located in the core shell of the network including KAT5, BRD8, MEAF6, MRGBP, EPC1, MORF4L2, DMAP1, YEATS4, MORF4L1, and RUVBL2 (Fig. 4A). Further molecular gene set enrichment analysis revealed that these proteins were involved in chromatin modification, chromatin organization, HATs acetylate histones, sensing of DNA double strand breaks, telomere extension by telomerase, regulation of TP53 activity, activation of the TFAP2 (AP-2) family of transcription factors, and resolution of D-loop structures through synthesis-dependent strand annealing (Table 2). Subsequently, the genetic information and function of these 10 genes in association with *ING3* in HNSCC patients were explored using the cBioPortal web tool. We investigated the cancer genomic alteration characteristics of these genes in HNSCC and found a significant rate of genetic alterations across a set of HNSCC samples based

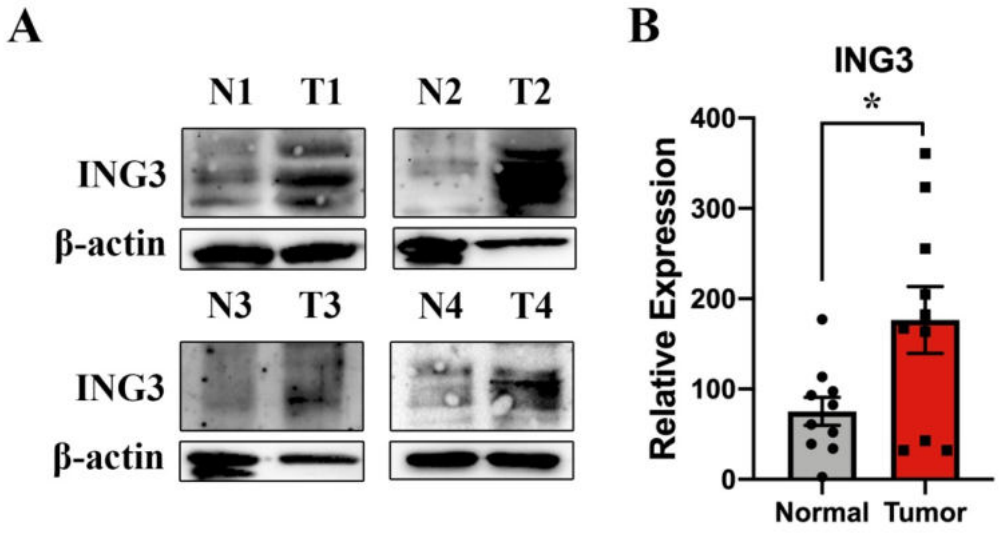


Fig. 3. ING3 have increased protein level in LSCC patients. (A) Representative images of ING3 expression in laryngeal normal and tumor tissue samples. (B) Relative mean ING3 expression in protein level in LSCC tissue samples. Protein levels were normalized to β-Actin. *$p < 0.05$; t-test.

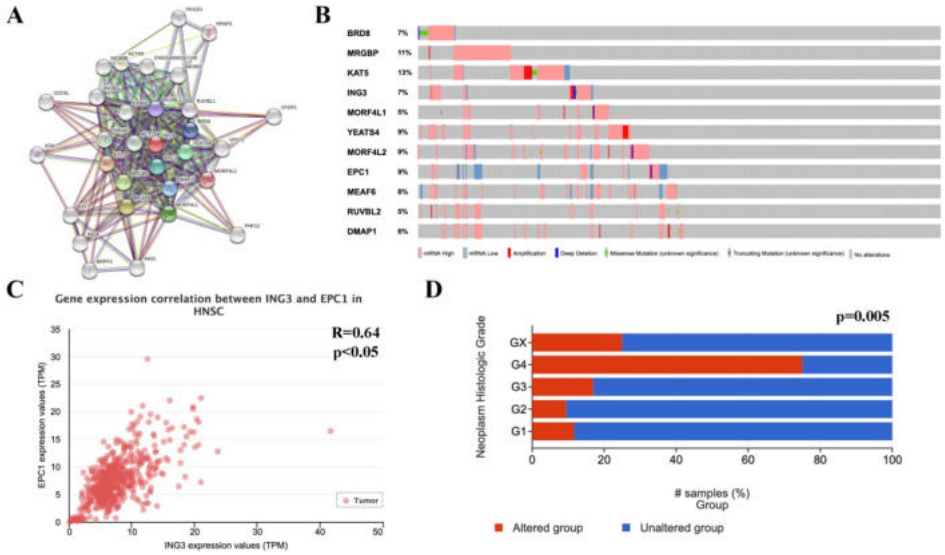


Fig. 4. Functional protein interaction network analysis of ING3 and predicted mutations and functions of hub genes. (A) Protein interaction network of ING3 depicted using STRING database. (B) Genetic alterations of ING3 related genes (C) The correlation of ING3 and EPC1 expressions. (D) Association of alterations of these two genes with advanced histological grade of head and neck cancer.

on a query of these 10 genes associated with *ING3*. As shown in Fig. 4B, they were altered in five types of genetic alterations including amplification and high mRNA expression, in the queried HNSCC samples. Among these alterations, the deep deletion and high mRNA expression accounted for the most changes in HNSCC. Then, we investigated co-occurrence of *ING3* interactors in tumor HNSCC sample. The results showed that *ING3* was associated with both *EPC1* and *MEAF6*, where only the relationship of *ING3* with *EPC1* was statistically significant (Table 3). Therefore, EPC1 was selected for further analysis. The correlation of *ING3* and *EPC1* expression was assessed using UALCAN web portal. The statistical scatter plots

Table 2. Over-representation analysis of ING3 interactors

Enriched Gene Set	Size	Expect	Ratio	*p* value
Chromatin modifying enzymes	275	0.28665	38.375	> 0.001
Chromatin organization	275	0.28665	38.375	> 0.001
HATs acetylate histones	142	0.148	74.317	> 0.001
Sensing of DNA Double Strand Breaks	6	0.0062536	159.91	0.0062388
Telomere Extension By Telomerase	6	0.0062536	159.91	0.0062388
Regulation of TP53 Activity	160	0.16676	11.993	0.011483
Activation of the TFAP2 (AP-2) family of transcription factors	12	0.012507	79.955	0.012442
Generic Transcription Pathway	1169	1.2184	3.283	0.026095
Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA)	26	0.027099	36.902	0.02678
Regulation of TP53 Activity through Acetylation	30	0.031268	31.982	0.030842

Table 3. Co-occurrence of ING3 interactors in HNSCC tumor samples

Gene A	Gene B	Neither	A Not B	B Not A	Both	Log2 Odds Ratio	*p* value	Tendency
MEAF6	**DMAP1**	194	6	0	3	> 3	**< 0.001**	Co-occurrence
MORF4L2	**MEAF6**	182	12	5	4	> 3	**0.003**	Co-occurrence
ING3	**MEAF6**	187	7	6	3	> 3	**0.006**	Co-occurrence
MRGBP	**MORF4L2**	176	11	12	4	2.415	**0.021**	Co-occurrence
ING3	**EPC1**	188	8	5	2	> 3	**0.04**	Co-occurrence

showed that *ING3* expression had a strong positive association with the expression of *EPC1* in HNSCC patients (Fig. 4C). In addition, we investigated the relationship between the alteration status of these two genes and the histological grade of neoplasm in HNSCC samples using cBioPortal web tool. Most notably, we found that the patients possessing any kind of alterations of these two genes tended to have advanced histological grade HNSCC (Fig. 4D).

DISCUSSION

LSCC is a frequent aggressive tumor type occurring in the head and neck region with a quite unsatisfactory overall 5-year survival rate for patients (50-70%) [32]. Of the cancers with head and neck origin, LSCC along with pharyngeal cancers display significant divergence from oral cavity cancers in terms of clinical outcome characteristics that might be partly due to distinct tumor biology including discrete mutation profiles [33]. Another recent study pointed a considerable dissimilarity in the methylation patterns of genes and allelic imbalances present in oral cancers and cancer of the larynx and pharynx, pointing potential differences during the carcinogenesis processes of tumors originated from distinct parts of the head and neck region [34]. These differences might be speculated to be responsible for altered chemoradiotherapy response, depicting the significance of the investigation of molecular features of each type on their own and also together to be able to figure out the underlying mechanisms more clearly.

Although there have been constant evolution in the surgical and oncological techniques, long term survival of LSCC patients still remains unfavorable [35]. The main factors leading to poor clinical outcome are considered to be frequent locoregional recurrence and metastasis [36] and the lack of reliable prognostic markers make current treatment modalities less effective, which necessitates the urgent identification of vital predictive biomarkers and the related underlying molecular mechanisms associated with acquisition of aggressive phenotype of LSCC. Unraveling those markers will help determination of patients with higher recurrence and metastasis risk after surgery and will give the opportunity to treat them with appropriate adjuvant postoperative treatment strategies.

In this study, we investigated the differential expression of *ING* family members and found *ING3* as a potential biomarker for advanced LSCC. We showed its overexpression in tumor tissues compared to corresponding adjacent normal specimens, pointing its oncogenic potential in laryngeal cancer. Our *in silico* analysis also demonstrated the significant overexpression of *ING3* in late stage and high grade head and neck tumor tissues, which is in parallel with our findings.

The members of *ING* family, comprised of five genes with varying number of isoforms, function as important transducers of cell signaling associated with DNA repair, apoptosis, senescence, cell cycle regulation, histone modifications, and transcriptional regulation through interactions with proteins or DNA [14]. Considering their frequent inactivation in cancer cells, they are mostly recognized as tumor suppressor genes [37] and reduced expression of *ING3* was reported in various cancers including head neck squamous cell carcinoma [38]. However, a recent study reported overexpression of *ING3* in rapidly proliferating tissues with high self-renewal capacity like small intestine and bone marrow, suggesting a potential role for ING3

in cellular growth [39]. Similarly, overexpression of *ING3* was found in hypertrophic cardiomyocytes, which lead to an abnormal enlargement of the heart muscle as a result of inactivation of the AMPK and activation of the p38 MAPK signaling pathways [40].

More interestingly, mice models deficient for *ING1* and *ING2* were found to develop spontaneous cancers, whereas, *ING4* deficient mice were reported to be free of spontaneous tumor formation, however, a recent study investigated *ING3* deficiency in mouse models and described homozygous *ING3* deficient mice as embryonic lethal and associated loss of *ING3* expression with growth retardation [22]. This condition was reported to be related to inhibited proliferation and induced apoptosis, which were associated with DNA damage and distortion of PI3K/AKT signaling pathway [41]. Another study demonstrated that *ING3* is overexpressed in prostate cancer cells compared to benign prostate tissues that is correlated with therapeutic resistance and poor clinical outcome [42]. Same study reported that ectopic *ING3* overexpression is sufficient on its own to transform the non-tumorigenic normal human dermal fibroblasts to cancerous state and sets up a gene expression profile that is necessary for cell proliferation [42]. In addition, suppression of *ING3* expression in DU145 prostate cancer cells resulted in inhibition of cellular migration and invasion *in vitro,* ascribing a possible oncogenic potential for *ING3* [15]. Interestingly, increased *ING3* expression was associated with poor prognosis in erythroblast transformation-specific-related gene (ERG) negative prostate cancer patients [15]. Besides, ERG-like 10-gene signature, which includes *ING3* with higher expression in castration-resistant prostate cancer tissues compared to benign or localized prostate cancer specimens, was demonstrated to better estimate patients' clinical outcome than ERG status alone [43]. Another study proposed ING3 as an androgen receptor (AR) coactivator with the function of AR nuclear translocation that lead to increased proliferation and migration of prostate cancer cells [26].

As to the head and neck squamous cell carcinoma, allelic loss and low *ING3* expression was reported in 2002 in human head and neck cancers including laryngeal cancer. However, considering the low number of laryngeal samples and lack of detection in the protein level, this study does not provide strong clues about the function of *ING3* [44]. Additional analysis of *ING3* expression in head and neck cancer tissue samples in the mRNA level conducted by the same group demonstrated downregulation of *ING3* expression in cancerous samples compared to normal head and neck tissues. However, of the 71 tissue pairs collected from head and neck cancer patients, only 13 pairs were from laryngeal cancer patients and 7 tumor samples of them were either with high or normal *ING3* expression compared to normal tissues [24]. These findings point the need for further analysis for characterization of *ING3* functions in head and neck cancers with a specific attention on laryngeal cancer. Our results demonstrated that the mean expression of *ING3* is higher in laryngeal tumor tissue samples in both mRNA and protein level compared to normal tissue specimens. On the other hand, it is of importance to mention that the cytoplasmic vs. nuclear localization of ING3 is also important for its potential to contribute to the carcinogenesis [45]. A recent study reported that cytoplasmic expression of ING3 was significantly upregulated in head and neck tumor samples compared to normal tissues, although a decreased nuclear expression of ING3 was detected [46]. Additionally, high cytoplasmic ING3 expression was significantly correlated with lymph node metastasis [46]. These findings suggest further detailed analysis of laryngeal tumor samples using immunohistochemistry to delineate into the potential involvement of differential subcellular localization of ING3 protein during laryngeal carcinogenesis.

Although named as inhibitor of growth, *ING2* was also suggested as an oncoprotein in colon cancer, where its overexpression was detected in cancerous lesions although not supported by further functional tests [47]. Standing as a potential oncogene in laryngeal cancer, *ING3* is the most distinguished member of the *ING* family with the features of less similarity in amino acid sequence to *ING1/2* and *ING4/5* and not localization within the close proximity to telomeric regions in contrast to other members of the family [13]. ING3 is involved in a histone acetyl transferase complex called NuA4-Tip60 MYST as a core member, which is responsible for H2A and H4 acetylation that is generally linked to transcriptional activation. In contrast to transcriptional repressor properties of ING1 and ING2, ING3 functions as a transcriptional activa-

tor that might be important for its putative oncogenic function during laryngeal carcinogenesis [13]. Interestingly, we found strong correlation between *ING3* and another component of the NuA4-Tip60 MYST histone acetyl transferase complex, *EPC1*, assigning a potential role for EPC1 in laryngeal cancer. Suppression of *EPC1* was reported to promote E2F1 mediated apoptotic signaling as a result of DNA damage leading to inhibition of tumor cell motility. On the other hand, *EPC1* in cooperation with *E2F1* induces a metastasis related gene expression fingerprint in especially advanced cancers [48]. Interestingly, in a study where a cancer-relevant subset of more than 500 mutant ESC lines were investigated, a gene signature involving *EPC1* was reported to be associated with response to radiotherapy [49]. In line with these findings, *Epc2* knock down in murine MLL-AF9 AML cells led to induction of apoptosis and loss of stem cell potency of leukemia cells [50]. In addition, side population of human myeloma cell lines with high tumorigenic and self-renewal potential were demonstrated to be enriched with a gene set including *EPC1* [51].

Lastly, as we found overexpression of *ING3* in advanced LSCC cases, many researches reported detection of genomic and transcriptional alterations of *ING* gene family members in advanced cancers. Borkosky *et al*. demonstrated statistically significant relation of frequent deletion of *ING2* locus at 4q35.1 with advanced T stage, proposing the occurrence of *ING2* loss of heterozygosity in advanced stages during HNSCC progression [52]. Similarly, low expression of *ING1* was found in high malignancy grades of astrocytoma [53] and its reduced expression was related to poor prognosis in advanced neuroblastomas [54]. More interestingly, decreased expression of *ING4* in colorectal cancer was reported to contribute to metastasis and poor prognosis via promoting angiogenesis and its expression was negatively correlated with lymph node metastasis, advanced TNM stage and poor overall survival [55]. In line with this report, reduced *ING4* expression was found to be associated with increased stage and histological grade of ovarian cancers and was in negative correlation with micro vessel density [56]. Low *ING4* expression was also correlated with advanced Dukes' stages of colorectal cancer, where *ING4* expression levels were lower in tumor samples of patients with lymphatic metastasis [57].

Here, we reported oncogenic potential of *ING3* in laryngeal cancer with the significant deregulation observed in advanced cases. We also found that *ING3* expression significantly correlates with its physical interactor EPC1 with oncogenic potential as well in head and neck cancer. Our results assign a crucial prognostic potential for *ING3* in laryngeal cancer although further clinical studies that are going to be performed in larger cohorts and further *in vitro* and in vivo functional tests are required to strengthen its potential as a prognostic marker.

CONCLUSION

Here, we reported oncogenic potential of ING3 in laryngeal cancer with the significant deregulation observed in advanced cases. We also found that ING3 expression significantly correlates with its physical interactor EPC1 with oncogenic potential in head and neck cancer. Our results assign a prognostic potential for ING3 in laryngeal cancer although further clinical studies that are going to be performed in larger cohorts and further *in vitro* and *in vivo* functional tests are required to strengthen its potential as a prognostic marker.

Authors' Contribution

Study Conception: GK, NB, OFK, AT, AS; Study Design: OFT, AT; Supervision: OFK; Funding: AT; Materials: N/A; Data Collection and/or Processing: NB, GK, RG, AS; Statistical Analysis and/or Data Interpretation: BG, AT, OFK; Literature Review: N/A; Manuscript Preparation: NB, GK, OFK and Critical Review: AT, AS, BG, OFK.

Conflict of interest

Neslisah Barlak, Gulnur Kusdemir, Rasim Gumus, Betul Gundogdu, Abdulkadir Sahin, and Arzu Tatar, declare that they have no conflict of interests. Omer Faruk Karatas holds stocks in EcoTech Biotechnology. The terms of this arrangement have been reviewed and approved by Erzurum Technical University in accordance with its policy on objectivity in research.

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