

## Review Article

# ING Genes Work as Tumor Suppressor Genes in the Carcinogenesis of Head and Neck Squamous Cell Carcinoma

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. The evolution and progression of HNSCC are considered to result from multiple stepwise alterations of cellular and molecular pathways in squamous epithelium. Recently, inhibitor of growth gene (*ING*) family consisting of five genes, *ING1* to *ING5*, was identified as a new tumor suppressor gene family that was implicated in the downregulation of cell cycle and chromatin remodeling. In contrast, it has been shown that *ING1* and *ING2* play an oncogenic role in some cancers, this situation being similar to TGF- $\beta$ . In HNSCC, the *ING* family has been reported to be downregulated, and *ING* translocation from the nucleus to the cytoplasm may be a critical event for carcinogenesis. In this paper, we describe our recent results and briefly summarize current knowledge regarding the biologic functions of *ING* in HNSCC.

## 1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. More than 500,000 new cases and the over 50% mortality rate annually indicate a major health problem worldwide [1]. HNSCC is a broad term that represents squamous cell carcinomas that arise in the upper aero- and digestive tract, including the larynx, the pharynx, and the oral cavity. These sites form a functional and anatomic unit and share exposure to the same etiological factors in carcinogenesis [1]. It is well known that smoking and alcohol abuse are major risk factors for HNSCC. Additionally, human papillomavirus (HPV) infection is another implicated risk factor, in particular for oropharyngeal SCC [2, 3].

The evolution and progression of HNSCC are considered to result from multiple stepwise alterations of cellular and molecular pathways in the squamous epithelium [4]. Although lifestyle factors account for the majority of HNSCCs, genetic alterations will cause some individuals to be more sensitive to these environmental factors. Therefore, screening for reliable genetic changes can provide a possible opportunity to predict the risk of malignant transformation.

Tumor suppressor genes (TSGs) are often referred to as “gatekeepers” because they prevent cancer development by direct control of cell growth through genes such as *p53* and *p16*, the inactivation of which has been reported in many tumors. The alterations of TSG, including mutation, loss of heterozygosity (LOH), and microsatellite instability, are considered to increase genetic susceptibility for malignant transformation. Previous studies have identified that alterations of *p53* and *p16* are associated with the development and progression of HNSCC [5–8]. Inhibitor of growth gene (*ING*) family, a new candidate TSG class, is implicated in cell cycle control, senescence, apoptosis, DNA repair, and chromatin modeling. The loss or downregulation of *ING* expression has been observed in HNSCC. In this paper, we summarized current knowledge on the biological function of *ING* family members and their status in the tumorigenesis of HNSCC.

## 2. ING Gene Family

*ING1*, the first member of the *ING* family, was discovered through a subtractive hybridization assay between normal mammary epithelium and breast cancer cell lines and was

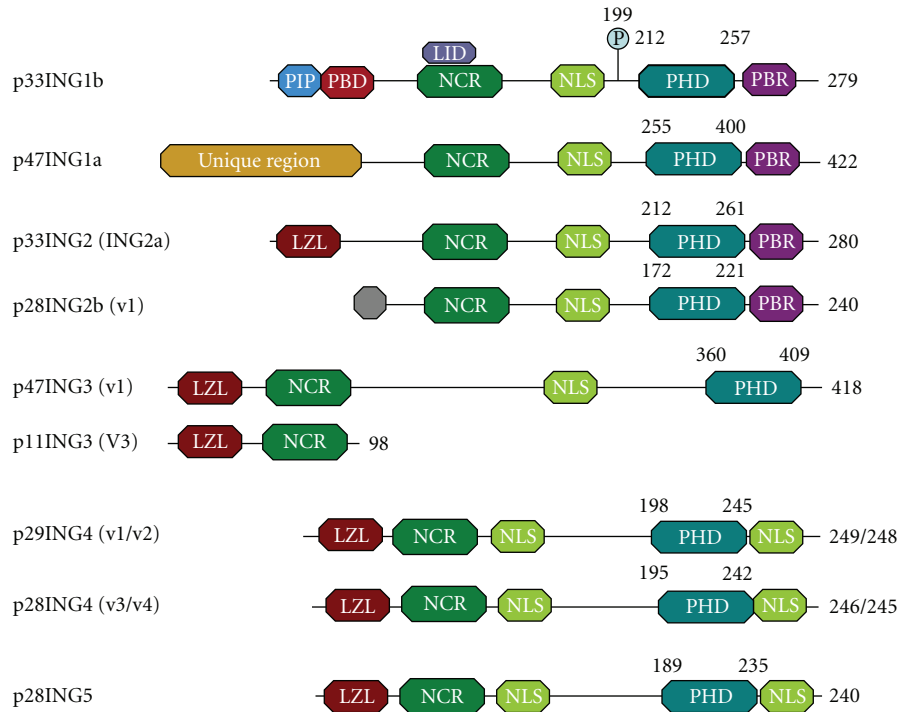


FIGURE 1: Structure of ING proteins in *Homo sapiens*. Each ING protein with its name and encoding major variants is listed on the left. The characterized domain composition, approximate location, is shown on the right. All ING proteins contain three conserved regions, a PHD (plant homeodomain), NLS (nuclear localization signal), and NCR (novel conserved region) from C-terminal region to N-terminal region. An LZL (leucine zipper-like domain) is present in ING2-5. p33<sup>ING1b</sup> also have a PIP (PCNA-Interacting Protein Motif) domain through which it binds to PCNA following UV irradiation, a PBD (partial bromodomain) which commonly found in chromatin-associated protein, and an LID (Lamin Interaction domain). p33<sup>ING1b</sup> binds to lamin A/HDAC complexes via LID to maintain its levels and biological function in nucleus. Additionally, phosphorylation sites were found at serine 199 of p33<sup>ING1b</sup>. 14-3-3 bind to phosphorylated serine 199 result in translocation of p33<sup>ING1b</sup> from the nucleus to the cytoplasm.

shown to play a role in neoplastic transformation [9]. Subsequently, four other members of *ING* family, *ING2*, *ING3*, *ING4*, and *ING5*, were identified by computer homology searches and were shown to have 32 to 76% DNA sequence homology with *ING1* [10–13]. The *ING* genes each mapped to independent chromosomes: 13q34, 4q35, 7q31, 12p13.3, and 2q37.3. All of the *ING* genes except *ING3* localize to the subtelomeric region of their respective chromosomes [14]. In addition, phylogenetic analysis identified that *ING* genes are conserved in many species, including humans, mice, rats, and yeast [15]. 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 [16]. These data suggest that *ING* genes play important roles in biological processes central to life.

Most *ING* genes, excluding *ING5*, encode variants due to different promoters, exons, and alternative splice variants. *ING1* encodes four isoforms, p47<sup>ING1a</sup>, p33<sup>ING1b</sup>, p24<sup>ING1c</sup>, and p27<sup>ING1d</sup>, which vary in mass between 24 and 47 kDa. Among these isoforms, p33<sup>ING1b</sup> is the most widely expressed in normal tissues [17]. *ING2* encodes two isoforms. *ING2a*, also called *ING2* and *ING1L*, encodes a 280-aa protein (p33<sup>ING2</sup>) that shares 58.9% similarity with p33<sup>ING1b</sup> [10, 11]. Recently, *ING2b* was identified and shown to be transcribed

from the middle of intron 1 of *ING2a* [18]. In addition, *ING3* encodes two isoforms, p47<sup>ING3</sup> and p11<sup>ING3</sup> [12]. *ING4* encodes eight splice variants: *ING4.v1*, *v2*, *v3*, *v4*, *ING4ΔEx2*, *ΔEx3*, *ΔEx6A*, and *ΔEx6B* [19, 20]. Only *ING5* encodes a unique 240-aa protein (p28<sup>ING5</sup>) [13]. Splice variants of *ING* proteins may compensate or compete with each other and create more diversity in *ING* functions.

### 3. The Structure and Function of ING Proteins

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) (Figure 1). The N-terminal region of each *ING* protein is unique, which determines the differential structures of *ING* proteins [21]. The PHD domain, a zinc finger domain that binds histone H3 in a methylation-sensitive manner, has been implicated in chromatin remodeling [22]. Localization of *ING* proteins in the nucleus is critical to their function [23]. The NLS targets *ING1* or other *ING* proteins to different chromatin domains in the nucleus and nucleolus in response to UV-induced DNA damage [24]. Moreover, a leucine zipper-like (LZL) domain is present in *ING2*–5 and has the potential to form a

hydrophobic face near the N-terminus of ING proteins [25]. Regarding its function, the LZL domain may be linked to nucleotide excision repair and induction of apoptosis [26]. p33<sup>ING1b</sup> also carries three other domains. A proliferating cell nuclear antigen-(PCNA-) interacting protein motif (PIP) domain binds with PCNA following UV irradiation. A partial bromodomain (PBD) is commonly found in chromatin-associated protein. A lamin interaction domain (LID) binds with lamin A/HDAC complexes to maintain its levels and biological function in the nucleus [27]. Recently, a phosphorylation site was found at serine 199 of p33<sup>ING1b</sup>. 14-3-3 family proteins can bind to phosphorylated serine 199, resulting in translocation of p33<sup>ING1b</sup> from the nucleus to the cytoplasm [28].

#### 4. PHD Domain and Epigenetic Control

Although the *ING1* gene was cloned as a candidate gene for tumor suppression, 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 [29]. Therefore, we first described the functions of ING family proteins in the epigenetic control of gene transcription and DNA replication, details of which are now going to be elucidated.

Epigenetic control of gene transcription is attained partly by modulation of covalent modifications such as acetylation, methylation, and/or phosphorylation of nucleosomal histones within gene promoters [30]. ING proteins are known to be a component of either histone acetylase (HAT) complexes or histone deacetylase (HDAC) complexes that activate and inactivate gene transcription, respectively. p33<sup>ING1b</sup> interacts with the mSin3/HDAC complex and also with proteins associated with HAT activity such as p300, inducing hyperacetylation of histones H3 and H4 [31–33]. Similarly, ING2 complex with p300 also serves as a component of the mSin3/HDAC complex [34–36]. ING3 associates with the hNuA4/Tip60 HAT complex (nucleosome acetyltransferase of H4 and Tat interactive protein, respectively; Tip60 is the human homolog of yeast Esa1 HAT) that is responsible for acetylation of histone H4 and H2A [36, 37]. Both ING4 and ING5 bind to p300 [13], but they also associate with different HAT complexes. ING4 is identified as a component of a four-subunit HAT complex containing HBO1 (histone acetyltransferase binding to origin recognition complex-1). HAT and its cofactors JADE1/2/3 preferentially acetylate histone H4 [36]. ING5 associates with MOZ (monocytic leukemic zinc-finger protein)/MORF (MOZ related factor) HAT and its cofactor BRPF (bromodomain-PHD finger protein) 1/2/3, resulting in increased specificity for acetylation of histone H3 lysine 14 [36].

In turn, the PHD domains of ING family proteins were recently shown to recognize trimethylated lysine 4 of histone H3 (H3K4me3) that in many cases associates with active gene transcription [30]. ING2 was firstly shown to bind H3K4me3 via its PHD domain and stabilize the mSin3-HDAC complex, resulting in repression of DNA damage-

induced transcription of cyclin D1 gene [22, 38]. ING1 also binds H3K4me3, and this binding is somehow necessary for ING1-mediated DNA repair upon UV irradiation as well as doxorubicin-mediated induction of apoptosis in HT1080 fibrosarcoma cells [39]. Intriguingly, cancer-associated mutations in the ING1 PHD domain impaired the binding of ING1 to H3K4me3 with concomitant loss of functions in DNA repair and apoptosis, implying that the binding of ING1 to H3K4me3 underlies its tumor-suppressive functions [39]. Binding of ING4 to H3K4me3 and its biological outcomes were extensively studied [40–42]. Promoters bound by ING4 in response to DNA damage were identified using a chromatin immunoprecipitation technique followed by whole genome promoter tiling arrays [42]. ING4 was recruited to its target promoters upon interaction with H3K4me3 and increased the acetylation of histone H3 lysine 9, leading to activation of gene transcription and sensitization to cell death or inhibition of anchorage-independent cell growth [42].

These compiled lines of evidence indicate that one basic function of ING family proteins is to translate H3K4me3 markings on the nucleosomes into activation or inactivation of gene transcription, DNA replication, or DNA repair through the associated HAT or HDAC complex. Based on this simple framework, further questions as follows may be posed for the elucidation of the substance of “cellular contexts” as described above, the same family member of ING can complex with either HAT or HDAC. (1) What determines the combination of ING proteins and HAT or HDAC complexes and the final outcomes? (2) What modulates the inducible or constitutive binding of ING proteins to H3K4me3 within gene promoters? (3) Do ING family members compete for the binding to the same H3K4me3 within a promoter or have some specificity for it? To answer these questions, identification of the genes modulated by ING family proteins and side-by-side analyses of transcriptional response of the gene and factors that associate with ING proteins as made in [42] may be helpful.

#### 5. ING and DNA Repair

The balance between cell growth and cell death is characterized in tissue development and homeostasis. In response to slightly stressful stimuli, cells usually start a cellular stress response including DNA repair to ensure survival. However, when irreversible damage accumulates, cells can permanently arrest the cell cycle (cellular senescence) or trigger a cell death program (apoptosis) [43].

*ING1* is the founding member of the ING family and the most well studied. Paul et al. confirmed that ING1 interacts specifically with three proteins, p38MAPK, mammalian JNK/p38MAP kinase (MEKK4), and RAD50, by utilizing a cross-species (yeast, fly, and human) bioinformatics-based approach. Both p38MAPK and MEKK4 participate in a well-defined stress response pathway. These novel ING-interacting proteins further link ING proteins to cellular stress and DNA damage signaling [44]. Nucleotide excision repair (NER) is a crucial stress response mechanism for

maintaining genomic stability. Overexpression of p33<sup>ING1b</sup> can enhance NER of both UV-damaged genomic DNA and exogenous plasmid DNA in a host-cell-reactivation assay. Moreover, p33<sup>ING1b</sup> requires the participation of functional p53 in DNA repair and may be a crucial component in the GADD45-mediated NER pathway [45]. Conversely, missense mutations in the SAP30-interacting domain and PHD finger motif of *ING1* abrogated the enhancement of NER in a host-cell-reactivation assay and a radioimmunoassay [46]. In addition, PCNA is an essential processivity factor for DNA polymerases and functions in both eukaryotic chromosomal DNA replication and NER. p33<sup>ING1b</sup> contains a PIP motif within its N-terminus. By competitively binding PCNA through its PIP domain, p33<sup>ING1b</sup> may contribute to regulating the switch from DNA replication to DNA repair [47]. In both normal human epithelial keratinocytes (NHEKs) and a keratinocyte cell line, HaCaT, the expression levels of p33<sup>ING1b</sup> were elevated by UV induction independent of p53 status, thus suggesting that *ING1* may participate in the cellular stress response and skin carcinogenesis [48]. In addition, *ING2* interacted with certain HAT or HDAC proteins through its LZL domain, instead of the PHD region, to regulate histone H4 acetylation, chromatin decondensation, and NER [35]. Therefore, *ING* proteins may participate in DNA repair through the regulation of the NER pathway in response to cellular stress and DNA damage.

## 6. *ING* Proteins and Cell Cycle

Loss of proper control of the cell cycle is a major cause of cell transformation. Cellular senescence refers to the arrest in the G0 phase of the cell cycle [49]. p33<sup>ING1b</sup> was upregulated in senescent human fibroblasts, and antisense p33<sup>ING1b</sup> extends the proliferative lifespan of normal human fibroblasts [50]. Moreover, ectopic expression of *ING1* in diploid human fibroblasts resulted in cell cycle arrest with some features of cellular senescence [51]. Chromatin immunoprecipitation analysis indicated that the chromatin binding affinity of p33<sup>ING1b</sup> was higher in senescent cells compared with young cells, thus suggesting that *ING1*-mediated functions may be subject to age-dependent mechanisms of control directed to prevent induction of apoptosis in senescent but not in young cells [52]. *ING2* enhanced the interaction between p53 and p300 and acted as a cofactor for p300-mediated p53 acetylation. Overexpression of *ING2* induced senescence in young fibroblasts in a p53-dependent manner. Conversely, the downregulation of *ING2* expression by siRNA transfection led to delaying the onset of senescence [53].

Previous research has demonstrated that overexpression of p33<sup>ING1b</sup> increased the number of human diploid fibroblasts in the G0/G1 phase. Conversely, antisense p33<sup>ING1b</sup> permitted these cells to enter S phase [9]. Cyclin E is a member of the cyclin family and binds to Cdk2 in the G1 phase, which is required for the transition from G1 to S phase. Expression of p33<sup>ING1b</sup> in human hepatocellular carcinoma (HCC) was inversely correlated with cyclin E kinase activity by autoradiography [54], thus implicating that the reduction of p33<sup>ING1b</sup> expression may contribute

to the process of malignant transformation of HCC via an increase of cyclin E kinase activity. Another study indicated that ectopic expression of *ING1b* in H1299 cells sensitized the cells to short-term G2/M cell cycle delay [55]. In addition, adenovirus-mediated overexpression of *ING1* in mouse mammary epithelial cells resulted in the downregulation of cyclin B1, which accumulates during the G2-M phase of the cell cycle [55]. Moreover, adenovirus-*ING4*-mediated transfection of PANC-1 human pancreatic carcinoma cells inhibited cell growth, altered the cell cycle with S-phase reduction and G2/M phase arrest, and induced apoptosis [56]. These findings suggest that *ING* may regulate cell senescence and cell cycle via the G1/S and the G2/M cell cycle checkpoints.

## 7. *ING* and Apoptosis

Apoptosis plays important roles in normal development and removal of the cells carrying severe DNA-damages induced by DNA damaging agents. In cancer cells, activation of pathways that favor cell survival instead of apoptosis may contribute to tumorigenesis. Many different agents and growth environmental factors can be used to induce apoptosis, such as cytotoxic drugs, irradiation, and serum starvation. Expression of *ING1* increased upon the induction of apoptosis in P19 mouse teratocarcinoma cells by serum deprivation. Elevated expression of *ING1* cooperated with c-myc gene expression to enhance the extent of apoptosis in P19 and rodent fibroblast cells [57]. Ectopic expression of p33<sup>ING1b</sup> also sensitized cells to apoptosis induced by etoposide, taxol, and doxorubicin [24, 52, 55]. Ectopic expression of p33<sup>ING1b</sup>, but not p47<sup>ING1a</sup>, significantly enhanced UV- or hydrogen peroxide-induced apoptosis in young (low passage) but not senescent Hs68 cells. Moreover, cotransfection of p33<sup>ING1b</sup> and p53 increased the percentage of apoptotic cells compared to transfection of either of these two proteins alone [52]. Conversely, expression of p33<sup>ING1b</sup> antisense constructs protects cells against apoptosis [57] and promotes neoplastic transformation [9]. p33<sup>ING1b</sup> activates transcription of the p21/WAF1 promoter, a key mechanism required for p53-mediated cell growth control [58]. Adenovirus-mediated transfer of p33<sup>ING1b</sup> with p53 suggested an additive or synergistic effect on apoptosis in immortal human cancer cells [59]. In addition, p33<sup>ING1b</sup> was demonstrated to influence tumor necrosis factor (TNF)- $\alpha$ -mediated apoptosis in Hs68 cell by upregulation of HSP70 expression and enhancement of the ability of TNF- $\alpha$  [60]. All *ING* proteins tested to date show the ability to regulate apoptosis in varying degrees through similar or different signal pathways. For example, the *ING2* PHD finger interacts with phosphatidylinositol 5-phosphate (PtdIns5P) *in vivo*, and their interaction regulates the ability of *ING2* to activate p53 and p53-dependent apoptotic pathways [61]. Increased *ING2* expression was also found to increase Bax expression and enhance UVB-induced apoptosis in human melanoma cells [62]. Additionally, overexpression of *ING3* significantly promoted UV-induced apoptosis through the activation of the Fas/caspase-8 pathway, and knockdown of *ING3*



remarkably decreased UV-induced apoptosis [63]. These results suggested that ING might induce apoptosis through varied pathways in response to different agents.

## 8. ING and p53

p53 is an important TSG that is inactivated in many cancers. p53 assimilates disparate input signals, including oncogene activation, DNA damage, mitotic impairment, and oxidative stress, to initiate appropriate outputs such as initiation of DNA repair, cell cycle arrest, senescence, or apoptosis [64]. The physical and functional interactions between ING and p53 have been investigated widely, but the conclusions are not consistent. In overexpression experiments, all ING proteins except ING3 have been observed to coimmunoprecipitate with p53. Moreover, ING-induced cell cycle arrest and apoptosis were compromised in p53-deficient cultured cells [25, 65, 66]. Functional p53 is required for p33<sup>ING1b</sup>-mediated inhibition of cell growth in cultured cells. Furthermore, p33<sup>ING1b</sup> was proposed to compete with murine double minute 2 (MDM2), an important negative regulator of p53, for the same binding site on p53, leading to an increase in the stability and activity of p53 [67]. ING2 may modulate p53-dependent chromatin remodeling, apoptosis, and DNA repair by functioning as a scaffold protein to mediate the interaction between p53 and p300 [35]. Additionally, overexpression of ING4 or ING5 leads to a reduction in colony-forming efficiency, inhibition of S-phase, and induction of apoptosis in a p53-dependent manner. ING4 and ING5 may stabilize p53 and enhance p53-mediated cellular responses to genotoxic stresses and apoptotic stimuli through ING4/5-mediated acetylation of p53 [13]. These results implicated that ING proteins may be significant modulators of p53 function. However, it was worth noting that the experiments of *ING1* knockout mice and knockout cells indicated that ING1 functions were mostly independent from the p53 signaling pathway in physiological conditions [68, 69]. In mice, the *Ing1* gene decodes three spliced isoforms. *Ing1a* and *Ing1c* encode a 31 kDa protein, and *Ing1b* encodes a 37 kDa protein (p37<sup>Ing1b</sup> in human). Loss of p37<sup>Ing1</sup> induced BAX expression and increased DNA damage-induced apoptosis in primary cells and mice irrespective of p53 status. Moreover, p53 functions are unperturbed in p37<sup>Ing1</sup>-deficient cells. Moreover, p37<sup>Ing1</sup> suppressed the formation of spontaneous follicular B-cell lymphomas in mice. Therefore, p37<sup>Ing1</sup> can negatively regulate cell growth, apoptosis, and tumorigenesis in a p53-independent manner [69]. Previous studies also demonstrated that the expression of ING might be independent on p53 status in some tumor tissues. Decreased ING1 expression may play important roles in tumorigenesis of the specimens with expression of the wild-type p53 gene in gastric carcinoma [70] and nonsmall cell lung carcinoma (NSCLC) [71].

In addition, ING proteins are found to function in a p53-independent manner. One major p53-independent function of ING proteins may be negative regulation of NF- $\kappa$ B. p33<sup>ING1b</sup> and ING2 proteins were found to suppress expression of NF- $\kappa$ B by upregulating HSP70 gene expression

and augment TNF- $\alpha$ -induced apoptosis [60]. ING4 is shown to directly interact with p65(RelA) in glioma cells to inhibit transcriptional activity of NF- $\kappa$ B. Correspondingly, the expression of NF- $\kappa$ B-responsive genes is shown to be significantly increased in ING4 knockdown cells [72]. Another study showed that ING4 suppresses NF- $\kappa$ B-regulated promoters by binding with both of p65 and H3K4me3 on the promoter [73]. This recruitment of ING4 accompanies the reduction of p65 phosphorylation and concomitant change of complex formation of p65 with p300 (HAT) to HDAC1 resulting in the decrease of acetylated histones and H3K4me3 within the promoter [73]. Additionally, ING4 was found to affect the stability of hypoxia inducible factor (HIF) and mediate HIF activity [72].

Based on these findings, ING and p53 may function independently in apoptosis pathways, but they can influence the activity of each other in tumorigenesis [15]. As epigenetic regulators of chromatin structure, ING proteins may amplify the effects of p53 on gene expression and also directly affect DNA repair and apoptosis independently of p53 by altering chromatin structure.

## 9. ING Genes and Tumorigenesis

Previous studies have implicated members of the *ING* family as candidate type II TSGs that are involved in a variety of processes, including DNA repair, cell cycle control, senescence, apoptosis, and chromatin remodeling, which are critical points for genomic integrity and stability (Figure 2). Thus, loss or decrease of ING expression may be a potential key point in tumorigenesis. Knockout experiments demonstrated that *ing1*-deficient mice were more sensitive to total body gamma radiation, and loss of *ing1* was associated with earlier onset and higher incidence of lymphomas [68].

Loss of nuclear p33<sup>ING1b</sup> was observed in melanoma, seminoma, papillary thyroid carcinoma, ductal breast carcinoma, and acute lymphoblastic leukemia by comparing these neoplastic tissues with normal cells and tissues [74]. Until now, inactivation and reduced expression of *ING* genes has been reported in cancers of lung [71], breast [75], stomach [70, 76], esophagus [77], blood [78], brain [79], and HNSCC [80]. Interestingly, ING gene mutation is uncommon in cancer. In fact, translocation of ING proteins from the nucleus to the cytoplasm has been observed in some types of cancer, such as the tumors of the breast [75] and brain [79], melanoma [74], and lymphoblastic leukemia [78]. Therefore, the ING cellular compartment shift from the nucleus to the cytoplasm may cause loss of normal cellular function and may play a central role in tumorigenesis and progression.

Like other ING genes, nonphysiological overexpression of ING2 induces apoptosis and cell cycle arrest via p53 modification [10], and decreased ING2 expression was found in cutaneous cancer [81] and HNSCC [82]. However, expression of ING2 was upregulated in colorectal cancer [83], Burkitt's lymphoma, and cervical cancer [29]. Moreover, ING2 may bind to the RPB1-mSin3A-HDAC complex on the

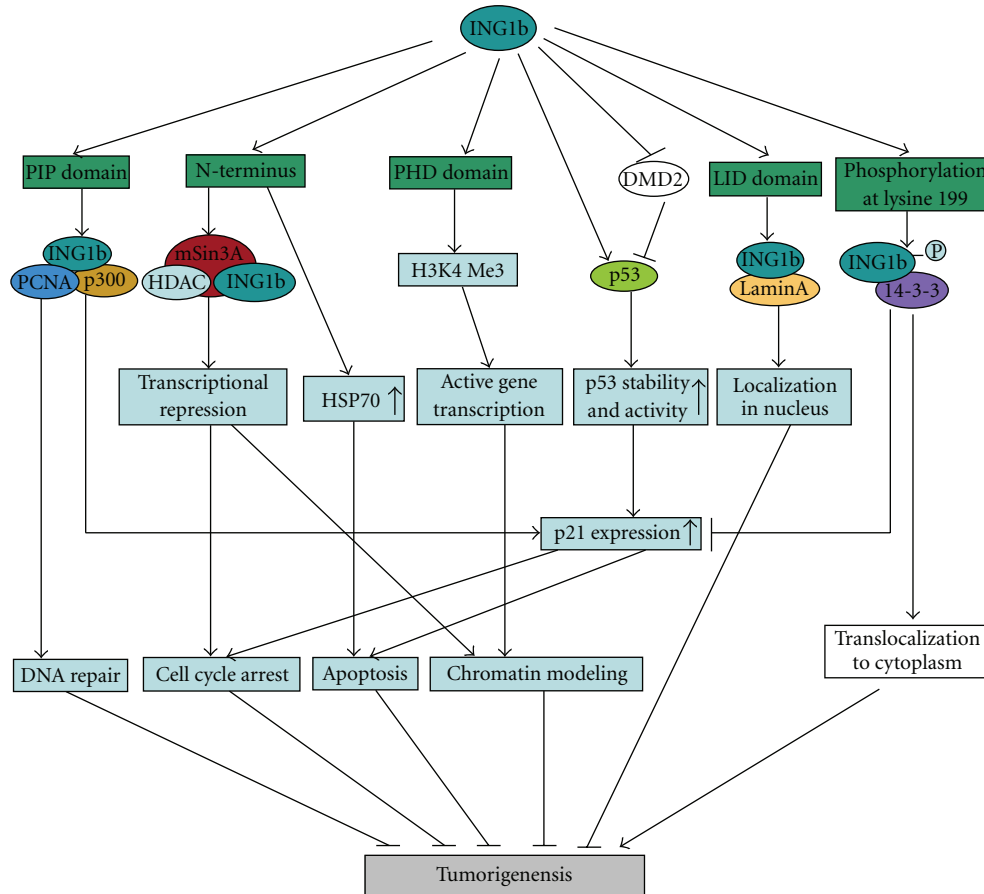


FIGURE 2: The role of p33<sup>ING1b</sup> protein in tumor suppression. p33<sup>ING1b</sup> could recognize trimethylated lysine 4 of histone H3 (H3K4me3) by PHD domain and has been implicated in chromatin remodeling and activation of some genes transcription. This binding is somehow necessary for induction of DNA repair and cell death. p33<sup>ING1b</sup> also associates with the Sin3/HDAC-mediated transcriptional repression through its unique N-terminal sequence and may be involved in repression of some essential cell cycle regulator genes. Moreover, p33<sup>ING1b</sup> binds PCNA and p300 complex to promote DNA repair through a PIP motif in response to UV-irradiation and, subsequently, may trigger apoptosis by the induction of p21 expression. p33<sup>ING1b</sup> competes with murine double minute 2 (MDM2) leading to an increase in the stability and activity of p53. p21, the one of the targets of p53, is also upregulated to involve in cell cycle arrest and the induction of apoptosis. Additionally, p33<sup>ING1b</sup> could upregulate expression of HSP70 gene to induce apoptosis independently of p53 status. Furthermore, p33<sup>ING1b</sup> binds to lamin A via LID domain to stabilize its level and biological function in nucleus. Conversely, 14-3-3 can bind to p33<sup>ING1b</sup> with phosphorylated serine 199 and results in translocation of p33<sup>ING1b</sup> from the nucleus to the cytoplasm, which may involve in tumorigenesis.

*MMP13* promoter to upregulate MMP-13 expression [83]. Thus, the function of ING2 may be different depending on the cancer type. A recent study suggested that ING2 is a novel mediator of transforming growth factor (TGF)- $\beta$ -dependent responses in epithelial cells [84]. TGF- $\beta$  is considered to have tumor suppressor-like functions in normal epithelium and also have oncogenic functions in invasive metastatic cancers. Therefore, ING2 may play different roles in normal cells and cancers by mediating the TGF- $\beta$  signaling pathway.

## 10. Expression of ING Genes in HNSCC

Previous studies have demonstrated 45.5%–68% LOH of *ING* genes in HNSCC (Table 1), and 50%–76% decreases in the mRNA levels of *ING3–5*. In recent studies, we also investigated expression as well as the subcellular localization

of ING proteins in 214 cases of HNSCC by immunohistochemistry. Decreased expression of p33<sup>ING1b</sup>, ING4, and ING5 in nuclei was observed in 36.9%, 61.3%, and 36% of the HNSCC cases, respectively. These results suggest that the loss or downregulation of nuclear expression of ING proteins participates in tumorigenesis of HNSCC. By contrast, mutations of the *ING* genes are rare (0–4.3%) in HNSCC although most of the mutations are present in the domains critical for the functions of ING proteins (Table 1), suggesting that mutations are not the major cause for *ING* family inactivation. In addition, the shift of p33<sup>ING1b</sup> from the nucleus to the cytoplasm was observed in 24.5% of 49 in oral SCCs [85]. In our studies, aberrant cytoplasmic expression of p33<sup>ING1b</sup>, ING4, and ING5 was detected in 14.5%, 68.8%, and 47.7% in 214 cases of HNSCC, respectively [80, 86, 87], while no or seldom cytoplasmic expression of these ING proteins was detectable in the cases of normal mucosa.

TABLE 1: *ING* gene mutation and expression in HNSCC.

<i>ING</i>	Origin	Methods	Mutation type/expression change	Position	Frequency	Reference
<i>ING1</i>	Patient	MM	LOH	13q34	20/44(45.5%)	[88]
	Cell lines	Sequencing	No mutation		0/5	
	Patient	Sequencing	No mutation		0/20	
	Patient	MM	LOH	13q33-34	23/34(68%)	[89]
	Patient	PCR-SSCP	Missense	PHD (215)	1/23(4.3%)	
	Patient	PCR-SSCP	Missense	PHD (216)	1/23(4.3%)	
	Patient	PCR-SSCP	Missense	NLS (192)	1/23(4.3%)	
	Patient	RT-PCR	Downregulation		6/12(50%)	[90]
	Patient	IHC	Downregulation		37/49(76%)	[85]
	Cell lines	Sequencing	No mutation		0/3	[86]
	Patient	IHC	Downregulation		79/214(36.9%)	
<i>ING2</i>	Patient	MM	LOH	4q35.1	33/55(54.6%)	[82]
<i>ING3</i>	Patient	MM	LOH	7q31	22/46(48%)	[91]
	Patient	RT-PCR	Downregulation		20/40(50%)	
	Patient	PCR-SSCP	Missense	LZL(20)	1/49(2%)	
	Patient	RT-PCR	Downregulation		37/71(52.1%)	[92]
	Patient	RT-PCR	Upregulation		15/71(21%)	
<i>ING4</i>	Patient	MM	LOH	12p13	33/50(66%)	[87]
	Patient	Sequencing	No mutation		0/50	
	Patient	Q-PCR	Downregulation		38/50(76%)	
	Patient	Q-PCR	Upregulation		7/50(14%)	
	Cell lines	Sequencing	No mutation		0/3	[93]
	Patient	IHC	Downregulation		96/214(44.9%)	
<i>ING5</i>	Patient	RT-PCR	Downregulation		19/31(61.3%)	[94]
	Patient	Sequencing	Missense	LZL(33)	1/31(3.2%)	
	Patient	Sequencing	Missense	NCR(68)	1/31(3.2%)	
	Patient	Sequencing	Missense	NCR(74)	1/31(3.2%)	
	Cell lines	Sequencing	No mutation		0/3	[80]
	Patient	IHC	Downregulation		77/214(36%)	

Note: MM, Microsatellite marker; PCR-SSCP, Polymerase chain reaction-single strand conformation polymorphism; RT-PCR, Retrotranscription-polymerase chain reaction; Q-PCR, Quantitative-polymerase chain reaction; IHC, immunohistochemistry.

Nuclear localization of *ING* proteins is required for their normal function. Therefore, decreased nuclear expression of *ING* proteins, through either downregulation of nuclear expression or relocation from the nucleus to cytoplasm, may play a crucial role in the development and progression of HNSCC (Figure 3) and may be a new biomarker for the tumorigenesis of HNSCC.

The mechanism of translocation of *ING* proteins is not fully understood. Recently, a study from Riabowol's group demonstrated that p33<sup>ING1b</sup> can especially bind members of the 14-3-3 family through phosphorylation at serine residue 199 [28]. 14-3-3 family members primarily reside in the cytoplasm and are associated with phosphorylated ligands involved in many cellular processes, including regulation of the cell cycle and DNA damage

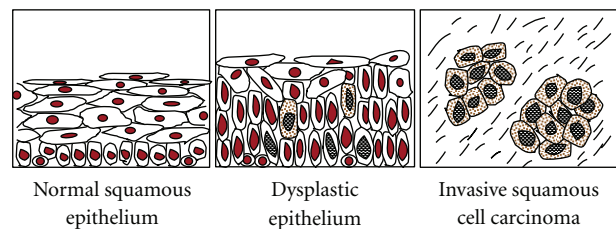


FIGURE 3: The schematic diagram of *ING* proteins expression in the malignant development of HNSCC. Nuclear expression of *ING* proteins is downregulated from normal squamous epithelium to dysplastic epithelium and invasive HNSCC. In contrast, the cytoplasmic expression of *ING* proteins in dysplastic epithelium and invasive HNSCC is gradually increased compared with normal squamous epithelium. The positive expression of *ING* proteins is shown with brown color.

checkpoints [95]. 14-3-3 binding results in tethering of significant amounts of p33<sup>ING1b</sup> in the cytoplasm [28]. Additionally, cytoplasmic p33<sup>ING1b</sup> could be imported into the nucleus through interactions between its intrinsic NLS and karyopherins  $\alpha 2$  and  $\beta 1$  [96]. In the nucleus, lamin A binds and targets ING1 and regulates ING1 levels and biological function [27]. Therefore, 14-3-3, karyopherins  $\alpha 2$  and  $\beta 1$ , and lamin A are involved in the dynamic regulation of subcellular distribution of ING1. Recently, we investigated the expression of p33<sup>ING1b</sup> and 14-3-3 $\eta$  in 214 cases of HNSCC by immunohistochemistry and found that cytoplasmic p33<sup>ING1b</sup> expression was significantly associated with 14-3-3 $\eta$  expression. Moreover, double immunofluorescence results confirmed the coexpression of p33<sup>ING1b</sup> and 14-3-3 $\eta$  (unpublished data). These data indicated that 14-3-3 $\eta$  plays an important role in the cytoplasmic accumulation of p33<sup>ING1b</sup> in HNSCC. However, the function of cytoplasmic ING is unclear and needs to be further studied.

There have been a few studies on the correlation between clinicopathological variables and expression of the *ING* genes. High LOH frequency of ING2 was statistically associated with advanced T stage, suggesting that ING2 LOH might occur at the late stage of HNSCC progression [82]. Although no clinicopathological variables were significantly related to the levels of ING3 mRNA, decreased expression of ING3 mRNA was associated with high mortality and was an independent prognostic factor for poor overall survival [92]. In our recent studies, no significant correlation was found between high nuclear expression of p33<sup>ING1b</sup> and clinicopathological variables in HNSCC, but high expression of cytoplasmic p33<sup>ING1b</sup> was significantly correlated with poor differentiation, T staging, lymph node metastasis, and TNM staging [86]. Also, high expression of nuclear ING4 in HNSCC was negatively correlated with poor differentiation, T staging, and TNM staging, while high expression of cytoplasmic ING4 in HNSCC was positively correlated with lymph node metastasis [93]. Also in the case of ING5, its nuclear expression correlated with differentiation of HNSCC, and abundant cytoplasmic expression correlated with poor differentiation [80].

## 11. Conclusions

The *ING* family genes are supposed to belong to type II TSG and are involved in multiple cellular processes including chromatin remodeling, DNA repair, cell cycle control, senescence, and apoptosis. ING proteins are expressed independently of p53 status and function in both p53-dependent and p53-independent manner. Loss or downregulation of *ING* genes expression and/or translocation of ING proteins from the nucleus to the cytoplasm may play an important role in neoplastic development of HNSCC. Thus, the *ING* gene family could be a novel p53-independent biomarker for HNSCC. Further elucidation of the functions of ING family proteins, which can be either tumor suppressive or tumorigenic, will rationalize their application for a biomarker, and it will also reveal the potentiality of ING proteins as the therapeutic target [97].

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