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Role of ING Family Genes in Head and Neck Cancer and Their Possible Applications in Cancer Diagnosis and Treatment

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1. Introduction

Cancer is one of the most common diseases, which treat human life. It produces huge psychological, economical and social burdens. Enormous preventive, diagnostic and therapeutic research efforts have been done to eradicate this deadly disease. Though some success have been obtained in terms of disease control for some of the neoplasms such as some of the lymphoma types, thyroid cancer and for some of the other solid tumors such as breast cancer in case of early detection, most still are left as a deadly disease. On the other hand current treatment modalities including surgery or/and chemoradiotherapy bring a huge hand local damage to the tissues and thus decrease the quality of life yet most shows recurrence and metastasis, which also questions the efficiency of these treatments.

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequent cancers that lead to death, making it a major health problem in the world. HNSCC includes oral, oro/nasopharyngeal and laryngeal cancers and accounts for more than 644,000 new cases worldwide, with a mortality of 0.53 and a male predominance of 3:1 [1,2]. Despite advanced technology in the detection and treatment of HNSCC, it continues to pose a great threat for human life. Most patients suffering from this malignancy are at an advanced stage upon diagnosis in which 51% present regional metastasis and 10% with distant metastasis. The 5-year relative survival rate with regional metastasis is about 51% and with distant metastases is 28% [1,3].

Though much development has been obtained in surgical techniques, chemoradiation protocols, little progress was shown in terms of long-term survivals during several decades.

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Moreover, surgery now one of the main therapeutic options in these cancers gives rise to extensive local damage. Many patients lose partially or completely the organs in head and neck area, which have important functions such as speaking, eating, swallowing and respiration. Thus these patients finally fall into huge psychological and functional burden. On the other hand, last 2-3 decades provided great developments and progress in human genome technology, which warranted development novel diagnostic and therapeutic methods, which are finally supposed to be more effective and to preserve functional properties.

As for most cancer types, head and neck cancer is basically a genetic associated disease. Cumulative alterations of various genes are responsible for development of this cancer type. Environmental and personal factors such as smoking, drinking, dirty air, exposure to carcinogenic chemicals through working environment or foods finally influence on genes. These affected genes are altered either as genetically or epigenetically and mutated nonfunctional or deficient products then result in cancer development.

Our current knowledge on human genome showed that two major groups of tumor-associated genes, oncogenes and tumor suppressor genes (TSG) have been implicated in the carcinogenic process (**Figure 1**). In normal cell, a critical balance between TSG and oncogenes is necessary for physiological survival of the cell. In normal cell, product of oncogene is needed for its proliferation, survival and growth. On the other hand, TSG is necessary for balancing and suppressing excessive growth of the cell and entering into carcinogenic process. Most oncogenes are growth factors or their receptors, as well as various molecules of signaling pathways, proapoptotic genes, cell cycle proteins and transcription factors. These proteins finally induce cell cycle, proliferation and growth. As same for most genes, oncogenes have two alleles in nuclear genome. Role of oncogenes in human cancer is mediated through enforcement of protein function upon activated mutation of one of the alleles in its genomic location. The rest allele is usually not affected and final output is more cell proliferation, growth and cancer when other factors such inactivation of tumor suppressors, inability of immunological as well as apoptotic mechanisms are added.

On the other hand, tumor suppressors have been defined as genetic elements whose inactivation allows cell to display one or more phenotypes of neoplastic growth [4]. Products of TSG usually include inhibitors of cell cycle, chromatin remodeling factors, anti-apoptotic genes and some genes regulating gene expression at genetic and epigenetic levels. Similar to oncogenes, TSG also consist of two copies at their chromosomal loci. Inactivation of TSG leads to carcinogenic process. Until recently, Knudson two-hit hypothesis has been known as an explanation of inactivation of TSG during carcinogenesis [5]. According to this hypothesis, one of the alleles of a TSG is lost through carcinogenic effect, while the rest allele is usually inactivated through mutation of the gene (**Figure 2**). However, a novel class of TSG with haploid insufficiency, in which one allele is lost and the remaining allele is haplo-insufficient, has been described recently, and the patients with these hemizygous TSG in their genome are accepted as carriers for deficient allele of a TSG and they show a tumor-prone phenotype especially when challenged with carcinogens such as smoking, alcohol, x-ray, chemicals etc (Class II TSG) [6-10].

Functional balance/imbalance of Oncogene and Tumor suppressor gene (TSG) in normal and cancer Cells

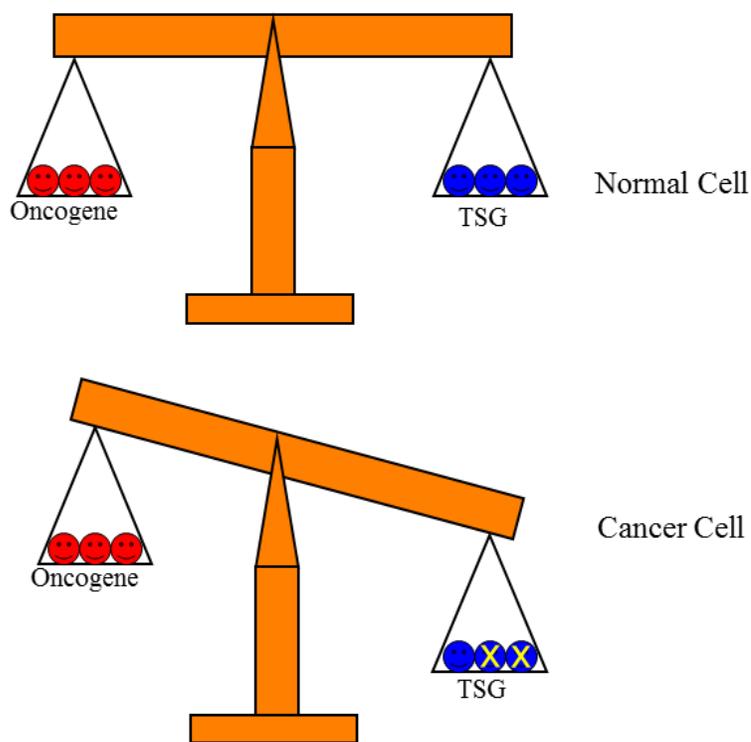


Fig. 1. Schematic representation of two major groups of genes on cell growth and cancer development.

Inactivation mechanism of tumor suppressor gene

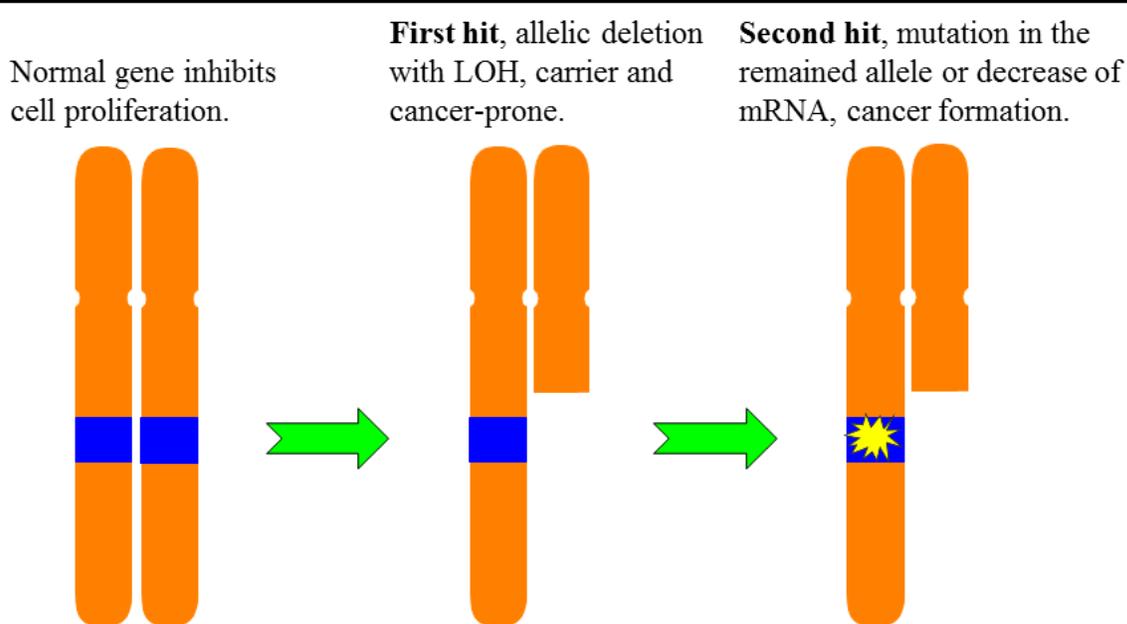


Fig. 2. Schematic representation of two-hit mechanism for inactivation of tumor suppressor gene

In the current review, we will focus on a recently identified TSG group, ING family tumor suppressors. Thus this review will mainly include alterations of ING family tumor suppressor in human cancer and their possible applications in molecular diagnosis and therapy of cancer. The five members of the ING family were recently identified by our group and other researchers in the field [11-17]. All proteins of ING family genes contain a highly conserved plant homeodomain (PHD) finger motif in the carboxy (C)-terminal end that is commonly detected in various chromatin remodeling proteins [18,19]. The N-terminal part of each ING protein seems to be unique, which determines the structure and different functions of various ING genes [11,12,16]. Although exact functions of ING family genes have not been clarified, the gene products have been reported to be involved in transcriptional regulation, apoptosis, cell cycle, angiogenesis and DNA repair through p53-dependent and -independent pathways. Moreover, ING family genes have also been known to constitute complexes with histone acetyltransferases (HAT) and histone deacetylases (HDAC) [11, 12, 16,20].

Within the members, ING1 is the founding member and thus most information about the family genes comes from the researches on ING1. ING1 was first isolated using subtractive hybridization between short segments of cDNAs from normal and a number of breast cancer cell lines [21]. These randomly fragmented cDNAs interfered with the activity of tumor suppressors by either blocking protein production through anti-sense sequences or abrogating function in a dominant-negative fashion through truncated sense fragments [21]. The other four members of ING gene family have been identified through sequence homologies with ING1, followed by functional in-vitro and then in-vivo cancer patient tissue analysis [11,12,19,22,23].

We characterized the genomic structure of ING1 gene and showed that ING1 gene produced at least 4 mRNA variants from 3 different promoters for the first time. Two of these variants, p33ING1b consisting of exons 1a and 2, and p24ING1c consisting of a truncated p47ING1a message including the first ATG codon in exon 2, are expressed majorly, while p47ING1a consisting exon 1b and exon 2, was not detected in head and neck tissues [13]. Our continuous efforts led to identification of ING3 [14]. Following these works, other groups and our group published investigations on other members of ING family including ING2, ING4 and ING5 [15,16,24-32]. Although almost all of the ING family members are known to be negative regulator of the cell growth, recent studies also demonstrated some of the members or splicing variants also functioning as oncogene, thus complicating the role of these genes in human carcinogenesis [31,32].

2. Disorders of ING family genes in human tumors

Rearrangement of ING1 gene locus was demonstrated in one neuroblastoma cell line and reduced expression in primary cancers and cell lines in early clinical studies at the time of ING1 cloning [11-17,21]. Following ING1 cDNA cloning, we identified the genomic structure of the human ING1 gene and showed its tumor suppressor character for the first time by finding its chromosomal deletion at the 13q34 locus and tumor-specific mutations in a number of head and neck squamous cell carcinoma (HNSCC) samples [13].

Regarding with the mRNA expression status of ING family genes, only a few studies exist in the literature. Toyama et al. detected 2-10-fold decreases in ING1 mRNA expression in 44%

of breast cancer and in all of 10 breast cancer cell lines examined [33]. Interestingly, the majority of breast cancers showing decreased ING1 expression had metastasized to regional lymph nodes, whereas only a small subset of cancers with elevated ING1 expression compared to adjacent normal tissues were metastatic. Another study also revealed reduced expression of breast cancer samples [34]. Down-regulation of ING1 mRNA has also been demonstrated in various other cancer types, including lymphoid malignancies, gastric tumors, brain tumors, lung cancer, ovarian cancer and esophagogastric carcinomas, though no comprehensive clinical correlation was performed [11,12,17,20,35-43]. Uncommon missense mutations and reduced protein expression of ING1 have also been detected in esophageal carcinomas [44], and colon cancer cell lines [36] while no mutation was detected in leukemia [37,45], oral cancers [46] and lymphoid malignancies [35].

For loss of ING gene and their protein functions, loss of heterozygosity (LOH), promoter CpG hypermethylation and nucleo-cytoplasmic protein mislocalization have been proposed [11,12,17,20]. Using methylation-specific PCR, the p33ING1b promoter was methylated and silenced in almost a quarter of all cases in primary ovarian tumors [42]. No differences or increased expression of ING1 were observed in recent studies of myeloid leukemia or melanoma [45,47].

Recently reduced expression of ING2 mRNA as well as protein was observed in hepatocellular carcinoma (HCC) [48]. Decreased ING2 expression (but not ING2 mutation) has been observed in lung cancer [49]. Decrease of nuclear ING2 protein was observed in melanoma [50]. On the other hand increased expression of ING2 mRNA was shown in colon cancer [51]. Moreover, ING2 may play a role in melanoma initiation, since reduction of nuclear ING2 has been reported in radial as well as vertical growth phases in metastatic melanoma as compared to dysplastic nevi [52]. On the other hand, reduced ING2 expression was associated with tumor progression and shortened survival time in HCC [48]. These epidemiological studies suggest that ING2 loss or reduction may be important for tumor initiation and/or progression [11,12,17,20].

As shown for ING2, decreased nuclear ING3 protein expression was associated with a poor survival rate. The survival rate was 93% for the patients with strong nuclear ING3 staining, whereas it declined to 44% for the patients with negative-to-moderate nuclear staining [52]. In a recent study, we also demonstrated frequent deletion of chromosomal locus of each of ING family member including ING3 in ameloblastomas [53].

ING4 mRNA was decreased in glioblastoma and associated with tumor progression [54]. Decreased ING4 has been associated with increased expression of IL-8 and osteopontin (OPN) in myeloma [11,55]. In both reports, decreased ING4 expression was associated with higher tumor grade and increased tumor angiogenesis. In myeloma, it was also associated with increased expression of interleukin-8 and osteopontin [11,55]. Expression of ING4 was decreased in malignant melanoma as compared to dysplastic nevi, and was found to be an independent poor prognostic factor for the patients [56]. ING4 was found to suppress the loss of contact inhibition and growth. Moreover some mutation and deletion were detected in cell lines derived from human cancers such as breast and lung [57].

Significant reduced expression of ING4 was detected in gliomas as compared with normal human brain tissue, and the extent of reduction correlated with the progression from

lower to higher grades of tumours [54]. Klironomos et al. investigated immunohistochemically the expression pattern of ING4, NF-kappaB and the NF-kappaB downstream targets MMP-2, MMP-9 and u-PA in human astrocytomas from 101 patients. They found that ING-4 expression was significantly reduced in astrocytomas, and it was associated with tumor grade progression. Expression of a NF-kappaB subunit p65 was significantly higher in grade IV than in grade III and grade I/II tumors, and a statistical significant negative correlation between expression of ING4 and expression of nuclear p65 was noticed [58].

Recently Nagahama et al. reported up-regulation of ING4 in a human gastric carcinoma cell line (MKN-1) by promoting mitochondria-mediated apoptosis via the activation of p53 [59]. Both mRNA and protein of ING4 expression were down regulated in hepatocellular carcinoma tissues. ING4 expression level correlated with prognosis and metastatic potential of hepatocellular carcinoma [60]. In another recent study, ING4 mRNA and protein expression were examined in gastric adenocarcinoma tissues and human gastric adenocarcinoma cell lines by RT-PCR, real-time RT-PCR, tissue microarray immunohistochemistry, and western blot analysis [61]. Their data showed that ING4 mRNA and protein were dramatically reduced in stomach adenocarcinoma cell lines and tissues, and significantly less in female than in male patients. Decrease of ING4 mRNA expression was found to correlate with the stage of the tumour [61]. Wang et al. examined ING4 protein expression in 246 lung cancer samples and overall reduced ING4 expression and higher ING4 expression in cytoplasm than in nucleus of tumour cells were detected, suggesting its involvement in the initiation and progression of lung cancers [62].

Examination of ING4 protein expression levels in colorectal cancer samples from 97 patients showed that ING4 protein was down regulated in adenoma relative to normal mucosa and further reduced in colorectal cancer tissues. Decrease of ING4 protein expression was also related to the more advanced Dukes' stages and ING4 expression levels in patients with lymphatic metastasis were lower than those without metastasis, suggesting that ING4 play a role in colorectal carcinoma progression [63].

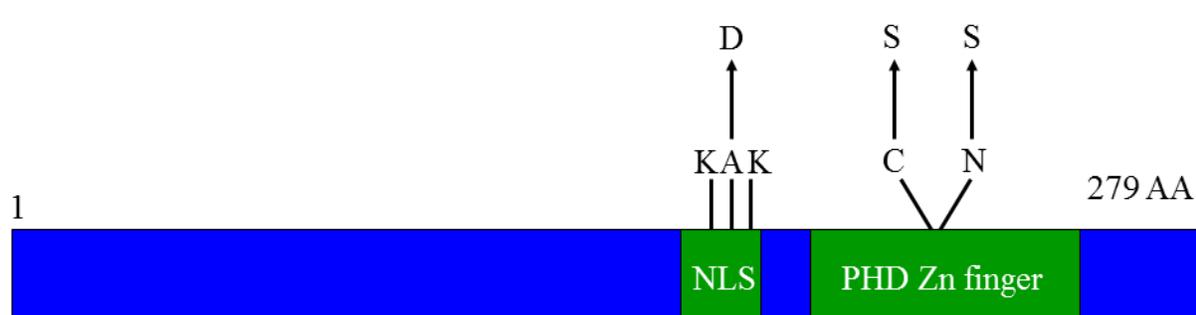
Xing et al. analyzed ING5 expression in gastric carcinoma tissues and cell lines (MKN28, MKN45, AGS, GT-3 TKB, and KATO-III) by Western blot and reverse transcriptase-polymerase chain reaction. An increased expression of ING5 messenger RNA was found in gastric carcinoma in comparison with paired mucosa and lower expression of nuclear ING5 protein and cytoplasmic translocation was detected in gastric dysplasia and carcinoma than that in nonneoplastic mucosa [64]. Nuclear ING5 expression was negatively correlated with tumor size, depth of invasion, lymph node metastasis, and clinicopathologic staging, whereas cytoplasmic ING5 was positively associated with depth of invasion, venous invasion, lymph node metastasis, and clinicopathologic staging in colorectal carcinomas [65].

3. Abnormalities of ING family genes in head and neck cancer

At the time of ING1 cloning, deletion of chromosome 13q34 was shown in head and neck cancer but ING1 gene was not known to be responsible for this deletion. Later in a comprehensive study, our group demonstrated tumor specific missense mutations in ING1

gene and frequent deletion at long arm of chromosome 13 for the first time in a human cancer [13]. Of 34 informative cases of head and neck squamous cell carcinoma, 68% of tumors showed loss of heterozygosity at chromosome 13q33-34, where the ING1 gene is located. By this study, ING1 has been recognized to be an important TSG at least in head and neck cancer. These mutations were found in the PHD zinc finger domain and putative nuclear localization signal, which may abrogate the normal function of ING1 protein (Figure 3). Following this study, our group led most of the researches for ING family genes in head and neck cancer.

Aminoacid substitutions in mutant cases of ING1



ING1 protein structure

Fig. 3. Mutations detected at PHD zinc finger domain and nuclear localization signal (NLS) of ING1 protein, which may abrogate its function

On the other hand, examination of 71 indian oral cancer cases demonstrated only polymorphic changes but not somatic possible function effective alterations [66]. However, analysis of esophageal cancer, which display some similarities especially for hypopharyngeal cancer, also demonstrated somatic mutations in ING1, supporting our results [44].

We recently demonstrated that frequent deletion of ING2 locus at 4q35.1 associated with advanced tumor stage in HNSCC [67]. LOH was detected in about 55% of the informative samples and high LOH frequency was statistically associated with advanced T stage, suggesting that ING2 LOH might occur in late stages during HNSCC progression. On the other hand, positive node status (N) appeared to be the only independent prognostic factor for both overall and disease free survivals.

We showed frequent allelic loss of ING3 in HNSCC [14]. We analysed LOH at 7q31 region in 49 HNSCC by using six polymorphic microsatellite markers and found allelic deletion in 48% (22/46) of the informative cases. We detected two preferentially deleted regions, one is around D7S643 and the other around D7S486. When we redefined the map of 7q31 region according to the contiguous sequences, a recently identified gene, ING3, was found in the proximity of D7S643. But ING3 mutation was very rare in our study (a sole missense mutation of ING3 at codon 20). In another recent our study using a large study population, about half of the 71 tumor samples demonstrated downregulation of ING3 compared to their matched normal

counterparts. We revealed that down-regulation of ING3 was more evident in late-stage tumors as compared with early stage patients, and patients with low ING3 mRNA expression demonstrated worse survival rates as compared to the patients with normal-high ING3 expression [68]. We also examined p53 mutation status and investigated its relationship with ING3, as well its clinicopathological characteristics. Although most clinicopathological variables were not significantly related to ING3 downregulation or p53 mutation status, a significant relationship was detected in terms of overall survival between the cases with low and normal to high ING3 expression. At 5 years follow up, approximately 60% of the patients with normal to high ING3 expression survived, whereas this was 35% in the patients with low ING3 expression. Multivariate analysis also showed downregulation of ING3 as an independent prognostic factor for poor overall survival. These results reveal that ING3 would function as a potential tumor suppressor molecule and that low levels of ING3 may indicate an aggressive nature of head and neck cancer.

We analyzed loss of heterozygosity at 12p12-13 region in 50 head and neck squamous cell carcinomas by using six highly polymorphic microsatellite markers and found allelic loss in 66% of the informative cases. To identify ING4 function, mutation analysis was performed. Though mutation of the ING4 gene was not found in head and neck cancers, the mRNA expression level examined by quantitative real-time RT-PCR analysis demonstrated decreased expression of ING4 mRNA in 76% of primary tumors as compared to matched normal samples. Since p53 dependent pathways of other ING family members have been shown, we examined p53 mutation status and compared with ING4 mRNA expression in tumor samples. However, no such direct relationship has been detected. In conclusion, frequent deletion and decreased mRNA expression of ING4 suggested it as a class two tumor suppressor gene and may play an important role in head and neck cancer [15].

In a recent study, nuclear expression of ING4 was found to gradually decrease from non-cancerous epithelium and dysplasia to HNSCC and was negatively correlated with a poorly-differentiated status, T staging, and TNM staging in HNSCC. On the other hand, cytoplasmic expression of ING4 was significantly enhanced in HNSCC and was significantly associated with lymph node metastasis and 14-3-3 η expression. Moreover, nuclear expression of ING4 was positively correlated with p21 and p300 expression and with the apoptotic index. Their results suggested that the decreases in nuclear ING4 and cytoplasmic translocation of ING4 protein play important roles in tumorigenesis, progression and tumor differentiation in HNSCC [69].

Our group reported the first study linking ING5 chromosome locus to a human cancer. We demonstrated a high ratio of LOH in oral cancer using 16 microsatellite markers on the long arm of chromosome 2q21-37.3 [24]. ING5 appeared to be a strong candidate tumor suppressor in this study though several other candidate TSGs including ILKAP, HDAC4, PPP1R7, DTYMK, STK25, BOK are also localized at the area, where frequent deletion has been detected [11,12,24]. Moreover, our recent study revealed decreased expression of ING5 mRNA and mutations in oral cancer samples as compared to their corresponding normal controls, suggesting its tumor suppressive role in cancer [25]. Examination of 172 cases of HNSCC for ING5 protein by immunohistochemistry using tissue microarray, and in 3 oral SCC cell lines by immunohistochemistry and Western blot showed that a decrease in nuclear ING5 localization and cytoplasmic translocation were detected, supporting the

previous studies and strong involvement of ING5 in tumorigenesis and tumor differentiation in HNSCC [70].

4. Possible applications of ING family genes in molecular diagnosis and therapy of cancer

So far most of the studies for possible applications of ING family genes in molecular diagnosis and therapy of cancer include cancer types other than head and neck cancer. However, ING family genes express ubiquitously and are involved in carcinogenesis of many cancer types especially in head and neck carcinogenesis. Thus the following section of the review is added as a model for possible application of ING family genes as diagnostic and therapeutic target.

4.1 Use of ING family genes for prediction of cancer behavior

4.1.1 Sub-cellular localization of ING proteins as a biomarker

Most tumor suppressors contain nuclear transport signals that facilitate their shuttling between the nucleus and the cytoplasm. This type of dynamic intracellular movement not only regulates protein localization, but also often impacts on function. Shuttling of tumor suppressor proteins between nucleus and cytoplasm has been reported to be involved in the regulation of cell cycle and proliferation. Deregulation of the nucleocytoplasmic cargo system results in the mislocalization of TSG proteins, which then alter function of TSG proteins [71]. The mistargeting of tumor suppressors can finally reveal direct cellular consequences and potentially lead to the initiation and progression of cancer. Abnormalities in nucleocytoplasmic cargo system leading the mislocalization of tumor suppressors were reported for p53, BRCA1, APC, VHL, BRG1 and ING1, and these abnormalities driven by genetic and epigenetic alterations in the tumor suppressor or their partners generally occur during the carcinogenic process [72-76]. For ING1, 2 of 3 different tumor specific somatic mutations that we detected in head and neck cancer were located at or near nuclear targeting domain, which could possibly abolish its functions through accumulation of the protein in the cytoplasm instead of in the nucleus [13].

In a recent study, Nouman et al. reported that translocation of p33ING1b from the nucleus into the cytoplasm of melanocytes may have an important role in the development and progression of melanomas [77]. Immunostaining with new monoclonal antibodies (MAb) of GN1 and GN2 showed that ING1b product, a nuclear protein, was accumulated in the cytoplasm and was closely associated with malignant melanoma development. The authors suggested that detection of this subcellular mobilization with MAb ING1b may be an early indicator and could be of value in diagnostic approach.

In another study of Nouman et al. nuclear expression of p33 (ING1b) was decreased in breast cancer cells, both in intensity and proportion of the cells stained. Reduction in nuclear expression of ING1 protein was associated with enhanced cytoplasmic p33 (ING1b) expression in a considerable number of cases. Those cases, which show p33 (ING1b) protein mislocalization, were also associated with more poorly differentiated tumors. Thus the authors suggested that p33 (ING1b) expression could be used as a marker of differentiation in invasive breast cancer. These results support the view that loss of p33 (ING1b) in the

nucleus may be an important molecular event in the differentiation and pathogenesis of invasive breast cancer [78].

Similarly loss of nuclear expression of p33 (ING1b) was detected in 78% of cases of acute lymphoblastic leukemia (ALL). This loss in nuclear expression was associated with increased cytoplasmic expression of the protein. Kaplan Meier survival analysis demonstrated a trend towards a better prognosis for patients with tumors that had lost nuclear p33 (ING1b), suggesting that the loss of nuclear p33 (ING1b) expression may be an important molecular event in the pathogenesis of childhood ALL and can be used as a biomarker for prognosis [79].

In another similar study, Vieyra et al. demonstrated that sub-cellular mislocalization of p33ING1b is a commonly seen in gliomas and glioblastomas [80]. Overexpression and aberrant localization of ING1b into the cytoplasm were observed in all of the 29 brain tumors. p33 (ING1b) normally contains a nuclear targeting sequence [11,12,16]. It has been previously demonstrated that altered sub-cellular localization of p33 (ING1b) abrogates its proapoptotic functions [81]. Loss of targeting domains that ensure the proper intracellular localization of p33 (ING1b) or physical association of ING with p53 could account for the abnormal localization of p33 (ING1b) in cancer. Recent experimental observations, including post-translational stabilization of p53 by p33 (ING1b) [82], and the discovery of the p53 associated a parkin-like cytoplasmic-anchoring protein, PARC [83] and its p53-regulatory role support the possibility that association of ING proteins with p53 could account for the abnormal localization. Further studies in this field will clarify this point.

For a normal function of ING1, the protein should be in the nucleus. ING1b protein phosphorylated on serine residue at position 199 has been reported to bind 14-3-3 proteins and subsequently be exported from the nucleus [84]. It has recently been shown that ING1 also binds karyopherin proteins and that disruption of this interaction affects subcellular localization and activity of the ING as a transcriptional regulator [84].

For ING1, few studies exist regarding with its subcellular localization. However for other member of ING family proteins, it mostly remains unknown and only few studies exist for subcellular alterations during carcinogenesis. Similar to the study of Nouman et al. [77] nuclear ING3 expression was found to be remarkably reduced in malignant melanomas compared with dysplastic nevi, which was significantly correlated with the increased ING3 level in cytoplasm. Moreover the reduced nuclear ING3 expression was significantly correlated with a poorer disease-specific 5-year survival of the patients with primary melanoma, especially for the high-risk melanomas with the survival rate reducing from 93% for patients with strong nuclear ING3 staining in their tumor biopsies to 44% for those with negative-to-moderate nuclear ING3 staining. Interestingly, the multivariate Cox regression analysis revealed that reduced nuclear ING3 expression is an independent prognostic factor to predict patient outcome in primary melanomas [85].

By using tissue microarray technology and immunohistochemistry, ING2 expression in human nevi and melanoma biopsies was examined. The data showed that nuclear ING2 expression was significantly reduced in radial and vertical growth phases, and metastatic melanomas compared with dysplastic nevi. Reduced ING2 has been suggested as an important indicator in the initiation of melanoma development [86].

In a recent study, the subcellular localization of ING4 has been shown to be modulated by two wobble-splicing events at the exon 4-5 boundary, causing displacement from the nucleolus to the nucleus. The authors provided evidence that ING4 was degraded through the ubiquitin-proteasome pathway and that it is subjected to N-terminal ubiquitination. It has also been demonstrated that nucleolar accumulation of ING4 prolongs its half-life, but lack of nucleolar targeting potentially increases ING4 degradation. Taken together, data of this work suggested that the two wobble-splicing events at the exon 4-5 boundary influenced subnuclear localization and degradation of ING4 [87].

ING4 has been reported to interact with a novel binding partner, liprin alpha 1, which results in suppression of the cell spreading and migration [88]. Liprin α 1/PPFIA1 (protein tyrosine phosphatase, receptor type f polypeptide) is known to be a cytoplasmic protein necessary for focal adhesion formation and axon guidance. Cytoplasmic ING4 may regulate cell migration through interacting with liprin α 1, and with its known anti-angiogenic function, may prevent invasion and metastasis. This interaction could explain the specific property of ING4 from other ING proteins.

In summary, sub-cellular localization of ING proteins or their interaction partners could be detected with various molecular and immunohistopathological methods and may be used as a biomarker for the behavior of the tumor and prediction of the disease progress.

4.1.2 Genetic and epigenetic alterations of TSG as prognostic biomarker

Alterations in allelic status, expression of mRNA and/or protein of the ING family genes provide potential usage of these genes as biomarkers in human cancer. Regarding with relation between genetic alterations of various genes and clinical outcome has recently been investigated. Since only few studies regarding with ING family genes exist in the literature, we will first give examples, which has been reported for other genes and summarize those published for ING tumor suppressors. In such a research, FHIT gene methylation has been found as a prognostic marker for progressive disease of early lung cancer [89]. Methylation and LOH analysis of FHIT gene showed that loss or reduced FHIT expression was significantly associated with squamous cell carcinoma type and smokers. Also methylation in normally appearing lung mucosa was related with an increased risk for progression into lung cancer, suggesting that FHIT can be used as a biomarker for this cancer type. In another report, allelic loss at 3p and 9p21 was related with elevated risk of malignant transformation of the premalignant lesions in head and neck cancer [90]. Similarly LOH at 8p was a predictor for long-term survival in hepatocellular carcinoma [91].

Another study highlighted the prognostic role of p16 in predicting the recurrence-free probability in patients affected by low-grade urothelial bladder by using p16 expression and LOH at 9p21 and proved the fact that the method is likely to be used in everyday urologic clinical practice to better describe the natural history of urothelial bladder carcinomas [92]. LOH at 16q23.2 was shown to be a predictor of disease-free survival in prostate cancer [93]. Our group has recently demonstrated that deletion at chromosome 14q was associated with poor prognosis in head and neck squamous cell carcinomas [1]. We also showed that frequent deletion of ING2 locus at 4q35.1 associates with advanced tumor stage in head and neck squamous cell carcinoma [67]. Interestingly, in our study, deletion at Dickkopf (dkk)-3 locus (11p15.2) was detected to be related with lower lymph node metastasis and better

prognosis in head and neck squamous cell carcinomas, suggesting the different nature of this gene, yet its potential use as a prognostic biomarker [94].

Detection of a gradual increase of mRNA expression of the DNA replication-initiation proteins from epithelial dysplasia (from mild through severe) to squamous cell carcinoma of the tongue has been used as biomarker to distinguish precancerous dysplasia from SCC and is useful for early detection and diagnosis of SCC as an adjunct to clinicopathological parameters [95]. In a recent work, we demonstrated downregulation of TESTIN and its association with cancer history and a tendency toward poor survival in head and neck squamous cell carcinoma [96]. The increased serum midkine concentrations were strongly associated with poor survival in early-stage oral squamous cell carcinoma, suggesting it as a useful marker not only for cancer screening but also for predicting prognosis of OSCC patients [97].

Information on human genome project provided that many gene including cancer-associated genes show alternative splicing. In such a study, deregulation of survivin splicing isoforms has been shown to influence significant implications in tumor aggressiveness and prognosis [98]. In ING family genes, some of the members also have splicing variants. Although we don't have detail study for these variants, their deregulation may have an impact for carcinogenesis. In our work, two major variants of ING1 (p33ING1 and p24ING1) revealed different expression patterns. Our researches indicated alternative splicing variants for ING1, ING3, ING4 and ING5 [13-15,25]. For ING2, a recent study reported 2 splicing variants [31,32]. Though both of them showed decreased expression in head and neck cancer tissues as compared to the normal counterparts, methylation analysis demonstrated that only p33ING1 variant was associated with methylation (Gunduz *et al.* unpublished data).

Not only single gene alterations associated with clinical outcome but also genome-wide or microarray studies were also examined. In such as study, genome-wide transcriptomic profiles obtained for 53 primary oral cancer and 22 matching normal tissues exhibited up-regulated genes and down-regulated genes. In conclusion, this study provided a transcriptomic signature for oral cancer that may lead to a diagnosis or screen tool [99]. In a recent study, the expression levels of ITGA3, ITGB4, and ITGB5 with functional normalization by desmosomal or cytoskeletal molecule genes were shown as candidate biomarkers for cervical lymph node metastasis or for the outcome of death in oral cancer [100].

Another recent study identified allelic deletion of ING1 as a novel genomic marker as related progression to glioblastoma by using comparative genomic hybridization and DNA microarray [101]. In another study, low levels of ING1 mRNA have been reported to be significantly associated with poor prognosis in neuroblastoma [102]. The expression level of ING1 was also closely related to survival. These results suggest that decreased level of ING1 mRNA and/or protein expression could be an indicator of poor prognosis in advanced stages and/or poor survival of various human tumors. On the other hand, an association between p33ING1b protein expression and clinical outcome in colorectal cancer demonstrated that although patients with decreased p33ING1b protein expression in the tumor have a shorter overall and metastasis-free survival rate as compared with patients with normal p33ING1b protein expression, no statistical significance was achieved [103].

However a significant association between p53 mutation status and overall and metastasis-free survival has been found.

Regarding with ING2 gene, its reduced mRNA as well as protein expressions were shown to be associated with tumor progression and shortened survival time in HCC [48]. Recently, our group reported that high LOH frequency in ING2 locus at 4q35.1 was significantly associated with advanced tumor stage in HNSCC, suggesting that ING2 LOH might occur in later stages during HNSCC progression [67]. Hence, the relevance of ING2 in HNSCC carcinogenesis and the potential prognostic significance of ING2 are promising results for future studies.

Several recent studies examined correlation between ING3 protein expression and clinicopathological variables [52,54]. Interestingly, significant reduction of nuclear ING3 was detected in human malignant melanoma, indicating the status of ING3 as a prognostic and therapeutic marker for melanoma [52]. As it has been shown for ING2, decreased nuclear ING3 protein expression was also associated with a poorer 5-year survival rate. The survival rate was 93% for the patients with strong nuclear ING3 staining, whereas it decreased to 44% for the patients with negative-to-moderate nuclear staining. We have recently reported mRNA expression of ING3 in HNSCC and compared the clinicopathological characteristics to evaluate its prognostic value as a biomarker [14,68]. This study revealed that down-regulation of ING3 was more evident in late-stage tumors as compared with early stage cases. Analyses have also showed that down-regulation of ING3 could be used as an independent prognostic factor for poor overall survival and low levels of ING3 may indicate an aggressive nature of HNSCC.

Recently the correlation of the ING4 with patient survival and metastasis was revealed to be as a potential prognostic marker in melanoma [56]. It has been found that ING4 expression was significantly decreased in malignant melanoma compared with dysplastic nevi, and overexpression of ING4 inhibited melanoma cell invasion compared with the control.

4.1.3 ING genes as chemosensitivity marker

Overall survival of head and neck squamous cell carcinoma patients has not improved in the decades. Currently treatment strategies for this cancer are based on the tumor-node-metastasis (TNM) classification. However, due to the extreme biological heterogeneity of the cancer cells, treatment planning especially for chemoradiotherapy is quite difficult and chemotherapy is an important therapeutic modality for cancer, and identification of the genes that predict the response of cancer cells to these agents is critical to treat the patients more efficiently. Although clinical determinants such as TNM classification will be still important, it is now becoming possible, by molecular markers, to elucidate biological information about host and tumor, to break through the molecular heterogeneity and eventually to optimize the choice of treatment [104].

In a recent analysis for prediction of chemosensitivity, it has been reported that examining the TP to DPD ratio of their tumors could identify HNSCC patients, who would most benefit from capecitabine-based chemotherapy. Moreover, the potential role of TP gene therapy in TP to DPD ratio manipulation to optimize the tumoricidal effect of capecitabine has been demonstrated [105]. In another similar study, acquired (10-fold) resistance of

Cal27, a tongue cancer cell line, against cisplatin has been shown to be associated with decreased DKK1 expression and this resistance could partially be reversed by DKK1 overexpression, thus suggesting DKK1 and the WNT signaling pathway as a marker and target for cisplatin chemosensitivity [106].

Recent findings suggest that the ING genes might also have a role in regulating the response of cancer cells to chemotherapeutic agents. In an osteosarcoma cell line, U2OS cells, one of the ING1 splicing variant p33ING1b, prominently enhanced etoposide-induced apoptosis through p53-dependent pathways [107]. In another study of the authors, ectopic expression of p33ING1b was shown to upregulate p53, p21WAF1 and bax protein levels and activate caspase-3 in taxol-treated U2OS cells. Thus the study demonstrated that p33ING1b increased taxol-induced apoptosis through p53-dependent pathway in human osteosarcoma cells, suggesting that p33ING1b may be an important marker and/or therapeutic target in the prevention and treatment of osteosarcoma [108].

Tallen et al. [39] questioned whether p33ING1 mRNA expression correlates with the chemosensitivity of brain tumor cells. They found that, unlike other tumor types, ING1 levels were higher in glioma cell lines than in normal control cells. Medulloblastoma cells revealed the lowest ING1 expression of the lines tested. Comparing all cell lines, p33ING1 gene expression significantly correlated with resistance to vincristine, suggesting that p33ING1 mRNA levels may be used to predict the chemosensitivity of brain tumor cells to vincristine.

The tumor suppressor ING1 shares many biological functions with p53 including cell cycle arrest, DNA repair, apoptosis, and chemosensitivity. To investigate if the p33ING1 isoform is also involved in chemosensitivity, Cheung et al. overexpressed p33ING1 in melanoma cells and examined for cell death after treatment with camptothecin. Results from the survival assay and flow cytometry analysis showed no significant difference among cells transfected with vector, p33ING1, and antisense p33ING1, indicating that p33ING1 does not enhance camptothecin-induced cell death in melanoma cells. Moreover, co-transfection of the p33ING1 and p53 constructs had also no effect on the frequency of cell death. Thus influence of ING1 expression for chemosensitivity may have different depending on the cancer type [109]. In another work, down-regulation of ING1 in the p53-deficient glioblastoma cell line, LN229, increased apoptosis following treatment with cisplatin, indicating that reduced ING1 expression may predict the sensitivity of cancer cells to chemotherapy independent of their p53 status [110]. Although most studies reported that expression of ING genes results in an increase in chemosensitivity, various conditions in different tumors should be tested to predict exact chemotherapy response. These differences could also be related with expression variations of the alternative splicing forms of ING genes.

Another member of ING family, ING4 negatively regulated the cell growth with significant G2/M arrest of cell cycle. Besides overexpression of ING4 enhanced the cell apoptosis triggered by serum starvation in HepG2 cells. Furthermore, the exogenous ING4 upregulated endogenous p21 and Bax in HepG2 cells, but not in p53-deficient Saos-2 cells, suggesting that G2/M arrest induced by ING4 could be mediated by the increased p21 expression in a p53-dependent manner, although there is no significant increase of p53 expression in HepG2 cells. Moreover, HepG2 cells with exogenous ING4 could significantly

increase cell death, as exposed to some DNA-damage agents, such as etoposide and doxorubicin, implying that ING4 could enhance chemosensitivity to certain DNA-damage agents in HepG2 cells [111]. In another study, chemopreventive agent curcumin (diferuloyl methane) induced ING4 expression during the cell cycle arrest by a p53-dependent manner in glioma cells (U251) [112]. Therefore ING4 has been suggested for a possible role in the signaling pathways of the chemotherapeutic agents.

4.2 Applications of ING family for gene therapy

Cancer still poses a great threat to human life and classical treatment modalities have still failed to eradicate it. Developments in human genome technology and progress in knowledge of the genes provided us alternative methods such as gene therapy to cure this fatal disease. Currently researchers are working on several basic methods to treat cancer using gene therapy. Some of these methods target healthy cells i.e. immune system cells to enhance their ability to fight cancer. Other approaches directly involve cancer cells, to destroy them or at least to stop their growth. The later method usually involves restoration of the tumor suppressor genes. In tumor cells, ING transcript levels are now known to be often downregulated though mutations are very rare. However, as explained in the above sections, it has been now known that the inactivation of ING family genes at genetic and epigenetic levels has a major role in the carcinogenesis of various neoplasms. Considering involvement of ING tumor suppressors in many cancer types, it can be thought that ING family genes may be of potential target for molecular therapy in human cancer. However, only few preclinical studies exist to evaluate this potential. Thus this section will only give an image and possible speculations for using these genes in cancer therapy.

Regarding the gene therapy of ING family genes in the literature, a few *in vitro* studies have been reported. In 1999, the introduction of ING1 gene using virus vectors was reported as a pioneer and a promising approach for the treatment of brain tumors [113]. Although adenovirus-mediated introduction of isolated ING1 transcript has inhibited the growth of glioblastoma cells, combined transduction of p33ING1 and p53 synergistically enhanced the apoptosis in these cells [113], suggesting that ING1 may function as a proapoptotic factor as well as enhancing the effect of p53.

Another study has shown similar findings and supported the cooperative role of ING1 and p53 in esophageal cancer [114]. Co-introduction of ING1 and p53 induced more cell death as compared to single use of each gene transcript in esophageal carcinoma cells. Thus, the synergistic effect between p33ING1 and p53 for induction of apoptosis has been suggested for 2 different human cancers, i.e. esophageal carcinoma and glioblastoma. Considering these two *in vitro* studies, combined gene therapy of one or more ING family members with/without p53 emerged as a promising alternative therapy in those cases with the failure of single use of p53 gene therapy.

Another method in gene therapy could be potentialisation of the introduced gene. In fact, a study showed that one of the ING1 splicing isoforms, p47ING1a, was differentially up-regulated in response to cisplatin in human glioblastoma cells (LN229) that express ING1 proteins and harbor mutated TP53, which might represent a response to protect DNA from this DNA-damaging agent. Thus ING1 down-regulation may sensitize glioblastoma cells

with deficient p53 to treatment with cisplatin. It was concluded that the status of p53-independent-ING expression level might predict the relative sensitivity to treatment with cisplatin and HDAC inhibitors in glioblastoma. These studies suggest that molecular therapy of ING1 could be combined with chemotherapeutics in a subset of human cancer.

Interestingly, some of ING family members have additional functions for suppressing the tumor growth such as anti-angiogenesis for ING4. Thus future studies using other members alone or in combination for gene therapy will provide more successful and promising results. In fact, it has been shown that ING4 gene therapy may be effective in human lung carcinoma as a novel anti-invasive and anti-metastatic agent [115]. Adenovirus-mediated ING4 expression suppressed the tumor growth and cell invasiveness in A549 lung cancer cells, suggesting that ING4, as a potent tumor-suppressing agent, present great therapeutic potential. Another interesting study displayed that ING4 inhibited MMP-2 and MMP-9 expressions in melanoma cells, which may contribute to the suppression of melanoma cell invasion [56]. This study demonstrated that overexpression of ING4 significantly decreased melanoma cell invasion by 43% and suppressed cell migration by 63%. Since degradation of basement membrane and extracellular matrix (ECM) is the first step in the invasion and metastasis of malignant tumors, down-regulation of the MMP-2 and MMP-9 expressions with Ad-ING4 may be a potential method in suppressing degradation components of the ECM and basement membrane and thus metastasis. In this respect, association of the ING4 with the MMP pathway may open a new avenue and offer novel opportunities for molecular therapy of cancer. In another recent study, Xie et al. [116] demonstrated that Ad-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, induced apoptosis, and downregulated interleukin (IL)-6 and IL-8 expression of transfected tumor cells. In athymic mice bearing the PANC-1 human pancreatic tumors, intratumoral injections of Ad-ING4 suppressed the tumor growth, downregulated CD34 expression, and reduced the tumor microvessel formation. Therefore, this study provided a framework for future clinical application of Ad-ING4 in human pancreatic and other carcinoma gene therapies.

In conclusion, these reports suggest that the transfer or forced expression of ING4 into cancer cells by gene therapy also targets its related molecules such as MMPs. Thus combination of ING4 gene therapy with chemicals, which inhibit MMPs, could be a promising treatment method in various cancer types. In this respect, possible applications each member of ING family genes for gene therapy should be tested. A summary of ING gene alterations and their use as possible biomarkers and consequences of the gene restoration in cancer are shown in **Figure 4**.

5. Future aspects

Over a decade of research on the ING family genes has revealed that ING genes are involved in various functions from chromatin remodeling to cell cycle suppression and apoptosis. Moreover ING family genes also cooperate with major tumor suppressor p53 and make complexes with HAT and HDAC. Alterations of these genes occur commonly in many cancer types. Recent studies also suggest that allelic deletion or down-regulation of mRNA

Alterations of ING family genes and their use as biomarkers as well as therapy in cancer

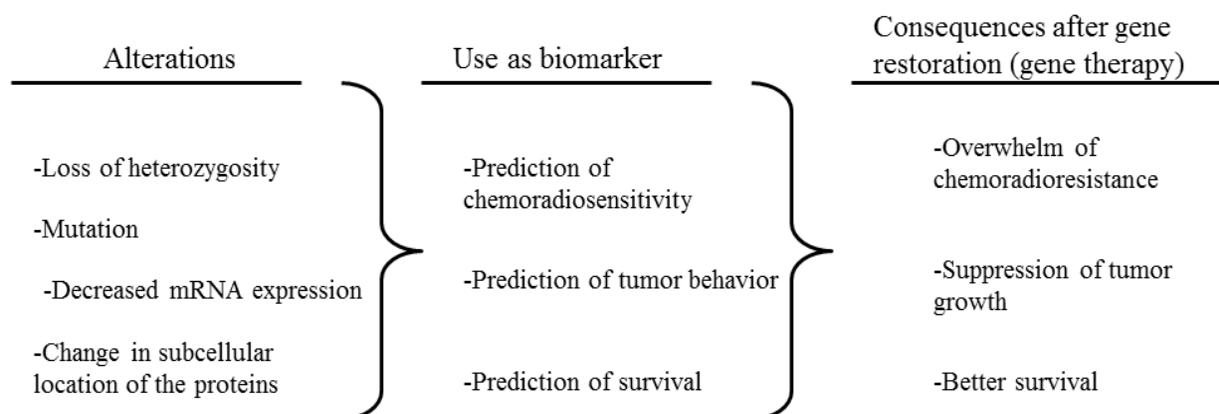


Fig. 4. Alterations of ING family genes and their possible use as biomarker for diagnosis and gene therapy in cancer

expressions as well as change of subcellular localization of proteins of the genes are likely to be used as a prognostic or predictive marker in human cancer. Today many cancer types including head and neck are treated based on clinical staging and findings such as lymph node involvement, TNM stage. However these clinical markers are most time not enough to follow tumor behavior or patients response to the therapy. Thus new methods are warranted to overcome these shortcomings to get a better response to the therapy or to predict which therapeutic method is best for each patient. At this point, involvement of ING genes in p53 tumor suppressor pathways and crosstalk between the variant of a single ING gene need to be clarified for focusing on INGs as diagnostic biomarkers. Progress on the knowledge of functions of ING family genes as well as the relationship with p53 and other unknown molecules will elucidate their roles in the development of human cancers, which will result in their uses in cancer diagnostics as well as therapy.

Cancer today is still one of the most dangerous diseases for human life. So far for treatment of cancer, surgery and chemoradiotherapy are the major therapies. Major difficulty for treatment of the cancer is inefficiency of chemoradiotherapy since each person gives different response to the therapy. So far clinical staging or findings is used to plan for treatment of cancer. However, this is not enough since many patients are resistant to these therapies and there is currently no way to understand efficiency of these methods. Moreover these treatment modalities are not specific and demonstrate high toxicities. Recent developments in human genome and technology provide novel methods for prediction of therapy or tumor behavior as well as tumor-targeted specific therapeutic methods. Thus although development of many molecular biomarkers for prediction of tumor behavior are tested and genetic therapy trials are ongoing, five-year later it is likely to see some of these methods as routine clinical use. For example, LOH of some TSG loci or expression profiles of single or multiple genes or mutation status could direct our therapy and we can have a nearly 100% success for each patient since the treatment will be individualized based on use of multiple molecular biomarkers. Some of these markers could be developed based on the

studies on ING family genes. Furthermore, current gene therapies using mostly p53 gene as a tumor suppressor could be expanded to members of ING family genes or combined of various tumor suppressors. Besides gene therapy can also be considered to combine with chemoradiotherapy.

6. Key points

ING family genes are recently identified major tumor suppressor involved in many cancer types.

ING family tumor suppressors have wide functions from cell growth, cell cycle suppression, DNA repair, chromatin remodeling to apoptosis.

ING family genes also cooperate with other tumor suppressors such as p53 and HAT and HDAC.

Alterations of ING genes at genetic and epigenetic level as well as their proteins showed promising results for their use as a molecular biomarker.

These genes are also likely to be used for gene therapy as a single agent or combined with other tumor suppressors.

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8. References

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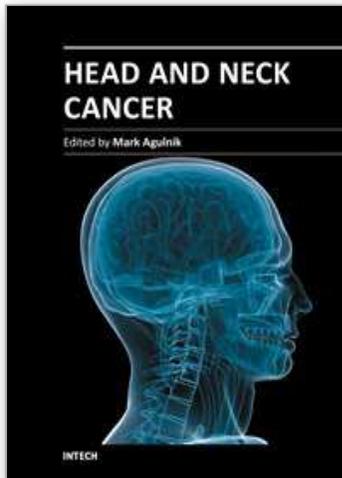
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Head and Neck Cancer provides an interesting and comprehensive overview of all aspects of head and neck cancer including overviews of the disease, basic science aspects pertaining to the disease, diagnosis, treatment and outcomes for patients with this disease. The chapters written by world renowned experts cover the entire discipline of head and neck oncology and include discussions of regional disparity is, advances in basic science understanding, advances in her radiotherapy, chemotherapy and targeted agents as well as a focus on reconstruction, prostheses, and aspects of quality of life and health outcomes. The book is designed to be both practical and comprehensive for every physician treating his complex disease.

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