

Decreased expression of ING2 gene and its clinicopathological significance in Chinese NSCLC patients

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The inhibitor of growth 2 (ING2) is a member of ING family, involved in cell cycle regulation, DNA repair, apoptosis and senescence, and participating in chromatin remodeling and transcriptional regulation by histone modification. Recent researches suggest ING2 plays roles in carcinogenesis both as tumor suppressor gene and oncogene depending on tumor types and cell status. Here, we investigated the status of ING2 in a series of 64 Chinese non-small cell lung cancer (NSCLC) patients using immunohistochemistry (IHC) and confirmed the results with Western blotting. RT-PCR results revealed the expression level of ING2 was consistent with mRNA level. The IHC results showed that ING2 protein expression was significantly decreased in NSCLC samples compared with normal lung tissues ($P < 0.05$). ING2 expression was lost in 32.8% (21/64) NSCLC tissues, which was more frequently in adenocarcinoma (ADK) than in squamous cell carcinoma (SCC), 45.8% (11/24) and 26.3% (10/38), respectively. We also found ING2 translocation from the nucleus to the cytoplasm, which may be a critical event for carcinogenesis. And the status of ING2 in SCC was significantly associated with lymph node metastasis status and TNM stage. After sequencing ING2 gene, we found no heterozygosity or mutation. Taken together, these results indicated that the aberrantly expression of ING2 may contribute to NSCLC tumorigenesis.

Key words: inhibitor of growth 2, non-small cell lung cancer, pathology

Lung cancer remains the leading cause of cancer-related deaths worldwide [1]. Early diagnosis with surgical resection has a 67% 5-year survival [2]. However, the total 5-year survival rate of lung cancer cases was less than 17% [1], due to the lack of sensitive screening tests for early detection of lung cancer and ineffective treatment for advanced and metastatic disease [3]. Non-small-cell lung carcinoma (NSCLC) accounts for approximately 80% to 85% of all lung cancers. The development of NSCLC is a complex process associated with multiple factors and stages, including accumulation of activation of oncogenes and inactivation of tumor suppressor genes [4]. Although several molecular predictors are being identified involving in lung tumorigenesis, others remain to be discovered [5]. Further researches on suppressor genes, apoptosis-related genes, and autophagy-related genes in the tumor progression of NSCLC will contribute to new strategies for early diagnosis, prognostic indicators and treatment of NSCLC [6].

The inhibitor of growth family member 2 (ING2) was identified as a candidate of tumor suppressor by computational

homology search with ING1 in 1998, mapped to chromosome 4q35.1 and localized to the subtelomeric region of chromosome 4 [7]. ING2 is comprised of three exons, exon 1a, 1b and 2, which encodes two isoforms, ING2a and ING2b, resulting from an alternative splicing between exons 1a and 1b [8]. The expression level of ING2b mRNA is much lower than ING2a, and ING2b protein has never been detected [9]. ING2a also called ING2 and ING1L, encodes a 33kDa protein shares the highest amino acid homology with ING1 among the ING protein family members (ING1 to ING5) [10]. ING2a protein is characterized by 6 highly conserved regions, including a leucine zipper-like (LZL) motif at the N-terminal end, a potential chromatin regulatory domain (PCR), a nuclear localization sequence (NLS) containing 3 nuclear targeting sequences (NTS), a phosphorylation-dependent interacting motif (PDIM), a plant homeodomain (PHD) zinc-finger in C-terminal region and a protein-interacting motif (PIM) [11]. ING2a promoter possesses regulatory elements such as two p53 binding sites and one NF- κ B binding site that control its transcription. Moreover,

ING2a protein expression level has reported to be regulated by ubiquitylation interacting with SMURF1 (Smad Ubiquitination Regulating Factor 1) [12]. ING2 has also been shown to interact with several other proteins such as p300, the mSin3A-HDAC complex, the Brg-based SWI-SNF complex, BRMS1, trimethylated histone H3 lysine 4 (H3K4me3) and phosphatidylinositol 5-phosphate [10, 13-17]. These interactions are important for the functions of ING2 in cell cycle regulation, DNA repair, apoptosis, senescence, chromatin remodeling and transcriptional regulation [18]. ING2 proteins were mainly localized in the nucleus [17] and the expression of ING2 is relatively low in most normal tissues, except in testis [19]. High expression of ING2 was observed in testis and moderate expression was observed in ovary, skeletal muscle, and pancreas [7, 8].

The expression of ING genes is frequently lost or down-regulated in human tumors [20]. Most studies on ING1 addressed a significant decrease or loss of ING1 at mRNA and protein level, including lung cancer [21]. Although ING2 has been considered to be a candidate tumor suppressor gene for more than a decade, recent evidences suggest it also play roles as a tumor oncogene [22]. Since the complicated roles of ING2 played in various types of cancers, we investigated the expression of ING2 protein in Chinese NSCLC patients by immunohistochemistry and Western blotting, and then analyzed the associations between the protein expression status of ING2 and clinico-pathological features.

Materials and methods

Patients and tissue specimens. NSCLC tumor lesions were obtained from 64 Chinese patients (48 men and 16 women, age range from 45 to 76 years with a median age of 57.5 years) who were diagnosed and treated consecutively in Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical College between January 2005 and August 2010. They consisted of 24 adenocarcinoma (ADK), 38 squamous cell carcinoma (SCC) and 2 adenosquamous carcinoma (ASC). And 28 cases of paired adjacent normal tissue specimens were also included. The adjacent normal tissues were from outside the margin of the tumors (>3cm from border of the tumor), and had no cancer cell invasion as demonstrated by hematoxylin and eosin (H&E) staining. Tissue samples were taken at the time of surgical resection of lung cancer and were immediately frozen in liquid nitrogen and stored at -80°C until use. Histological diagnosis and tumor grade were determined in accordance with the World Health Organization International Histological Classification of Lung Tumors criteria. Patients' data collected included age, gender, histological diagnosis, differentiation, size, lymph node metastasis, location and TNM stage. This study was performed following an Institutional Ethics Review Board approved protocol to investigate molecular markers relevant to lung cancer pathogenesis and informed consent was signed by all patients.

Immunohistochemistry. Sections (4µm) of the paraffin-embedded tissue blocks were cut and mounted on polylysine coated slides. Then they were dewaxed in xylene and re-hydrated in

graded ethanols. After deparaffinization, antigen retrieval treatment was performed at 120°C (autoclave) for 5 min in a 10nmol/L sodium citrate buffer (pH 6.0). After inhibition of endogenous peroxidase activity using a 0.3% hydrogen peroxide solution at room temperature for 15 min, sections were incubated with anti-ING2 poly (Sigma-Aldrich, USA, 1:90) overnight at 4°C. Binding sites of the primary antibody were visualized using a Dako EnVision kit (Dako, Glostrup, Denmark) in accordance with the manufacturer's instructions. Finally, sections were counterstained with hematoxylin and mounted with glycerol gelatin.

ING2 staining in NSCLC tissues was determined by two pathologists. The pathologists were blinded to any clinical details related to the patients. Percentage of ING2+ cells was determined by each observer, and the average of three scores was calculated. Normal testis tissues used as ING2 positive control and tissue sections immunoreacted without anti-ING2 antibody under the same experimental conditions as negative control. Evaluation of the ING2 expression level was classified into four groups: -, 0%; +, 1-25%; ++, 25-50% and +++, >50%, respectively. The percentage of positive cells was assigned a value based on the presence or absence of ING2 staining, irrespective of staining intensity. The group - labeled as negative, the other groups labeled as positive.

Western blot analysis. ING2 expression in part of these NSCLC samples was confirmed by Western blot with 10 case-matched fresh lesions. After homogenized in nitrogen liquid, 100mg tissues were weighted and treated with 400µl lysis buffer (pH7.4, 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 1% Triton X-100, PMSF 1mM), and then centrifugation at 12,000g at 4°C for 30min after homogenized with ultrasonic. Cell lysate aliquots were separated in 10% SDS-PAGE gel and then electro-blotted onto PVDF membranes (Millipore, Bedford, MA, USA) and blocked by incubating with PBS containing 5% non-fat dry milk for 4hrs. After blocking, membranes were washed in PBS containing 0.2% Tween-20 for three times and then probed with the primary polyclonal antibody against ING2 (Sigma-Aldrich, USA) at 1:250 dilution, and β-actin (Sigma-Aldrich, USA) at 1:2000 dilution overnight at 4°C. After incubation, membranes were washed in PBS containing 0.2% Tween-20 for another three times. The membranes were subsequently incubated for 30min at room temperature with Peroxidase/DAB+ Rabbit/Mouse (Dako), and developed with Dako REAL™ EnVision™ Detection System (Dako) for 1-3min. The density of ING2 concentration was determined with Quant one analysis image software (Bio-Rad, USA) and normalized with β-actin.

RNA isolation and RT-PCR. Total RNA was extracted using TrizolReagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration and quantity were assessed by absorbency at 260nm using a DNA/Protein Analyzer (Eppendorf, Germany). 2µg of total RNA was reverse transcribed into first-stand cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen, NY, USA) and oligo(dT)12-18 primers (Invitrogen, NY, USA) in a 20µl reaction. Subsequently, PCR amplification was performed on 500ng

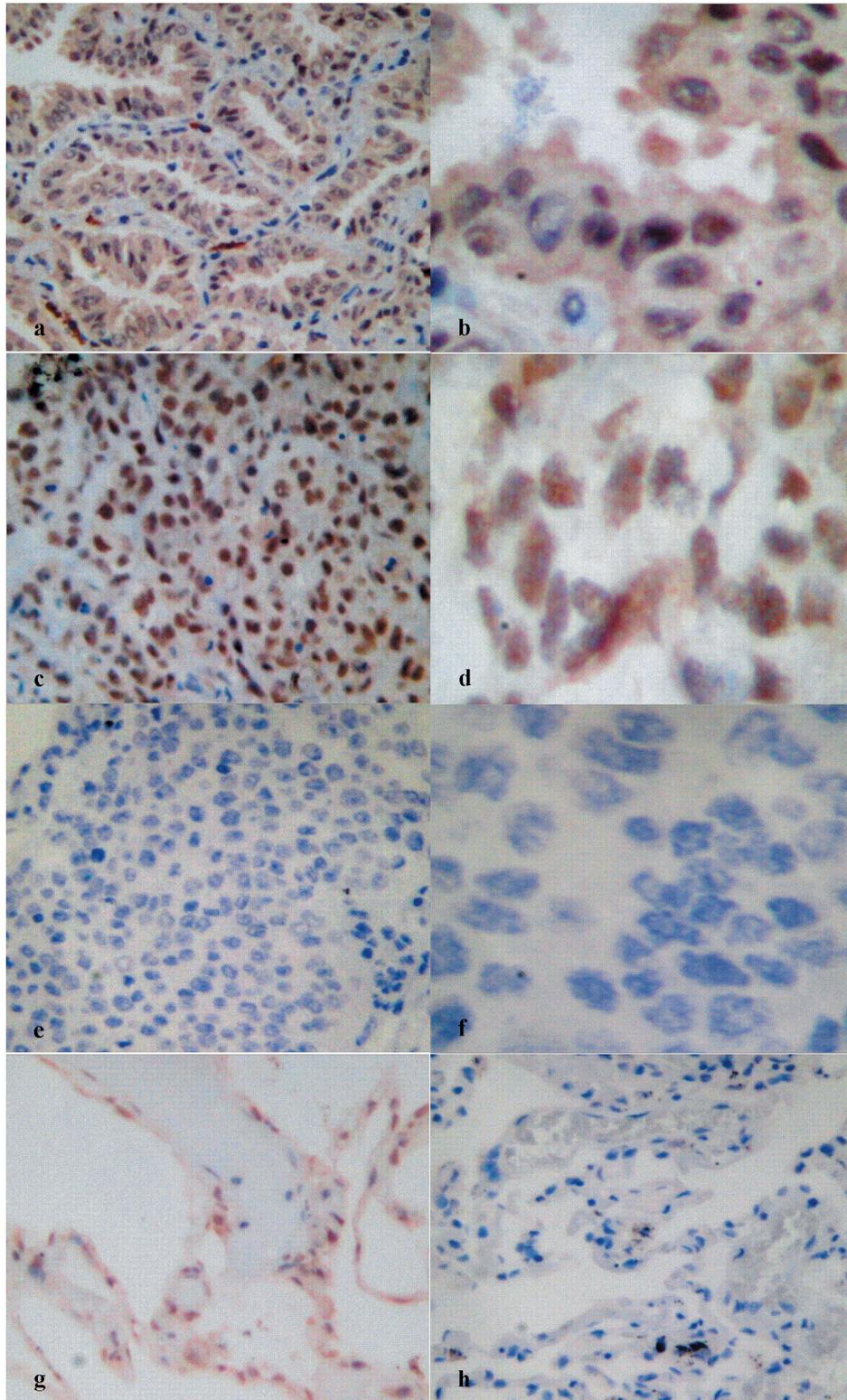


Figure 1. Immunohistochemical staining of NSCLC tissue lesions and surrounding non-cancerous tissues use an anti-ING2 antibody. (a and b) Positive staining with both cytoplasm and nuclear in NSCLC lesions at two different magnification (100 \times) and (400 \times), respectively. (c and d) Positive staining with nuclear in NSCLC lesions at two different magnification (100 \times) and (400 \times), respectively. (e and f) Negative staining of NSCLC lesions at two different magnification (100 \times) and (400 \times), respectively. (g) Positive staining of surrounding non-cancerous tissues (100 \times). (h) Negative staining of surrounding non-cancerous tissues (100 \times).

cDNA template with the following condition: 2.5µl 10×PCR buffer, 0.5µl 10mM 2'-deoxyribonucleotide 5'-triphosphate (dNTP) mix, 20pmol of each primer, 1.5mM MgCl₂, and 0.5U Taq polymerase (Roche, Indianapolis, USA) in a final volume of 25µl. RT-PCR was performed with GAPDH as an internal control. The primers used were listed as follows: ING2 (sense: 5'-AAC GAG CCT CAG ATA AAG CA-3', antisense: 5'-GAT CAT CAC AGT CTT CAA TCC C-3'), GAPDH (sense: 5'-GAA ATC CCA TCA CCA TCT TCC AGG-3', antisense: 5'-GAG CCC CAG CCT TCT CCA TG-3'), and the corresponding PCR products are 132bp and 120bp, respectively. PCR amplification was programmed for 1 cycle at 95°C for 4min, 30 cycles of 95°C for 30s, 58°C for 45s and 72°C for 30s, followed by a final extension of 7min at 72°C. The amplification products were electrophoresed through 1.5%(w/v) low melting point (LMP) agarose gels and visualized with ethidium bromide (Sigma, St. Louis, MO, USA) on top of a UV light illuminator. The density of ING2 concentration was performed using Quant one analysis image software and normalized with GAPDH.

Genomic DNA extraction and sequencing. Genomic DNA from frozen tumors and normal lung tissues samples were digested with proteinase K and extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The primers specific for ING2 exon1 were: sense 5'-CGG ATC GGC AGG ATG TTA GG-3' and anti-sense 5'- TGT CAC GGG AGA AAG GGA AG-3', while for ING2 exon2 were: sense 5'- TGA GTT CTA ATT TCA ATT CTG T- 3' and antisense 5'-TTA CCC TAA AAC ATT TTC TGA-3', respectively. PCR products were purified and cycle sequencing was performed using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA).

Statistical analysis. All statistical analyses were performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL). Differences in ING2 expression between NSCLC tissues and adjacent tissues were analyzed by non-parametric Mann-Whitney *U* test. Correlations between the ING2 expression status and clinical parameters were calculated with Pearson chi-square test. *P* values <0.05 was considered statistically significant.

Results

ING2 expression in NSCLC and adjacent normal tissues. The clinicopathological features of 64 cases of NSCLC were summarized in Table 1. To investigate whether ING2 protein was dysregulated in NSCLC, the expression of ING2 in NSCLC (n=64) and paired adjacent non-tumor tissues (n=28) were analyzed in this study. The expression level of ING2 was valued as the way described in the section of immunohistochemistry. The expression of ING2 protein was located both in the cytoplasm and nucleus in several NSCLC tissues. However, in the non-tumor tissues, ING2 expression was mostly staining in the nucleus (Fig.1). The expression of ING2 was significantly decreased in NSCLC when compared with the adjacent non-tumor tissues (*P*<0.05) (Fig.2).

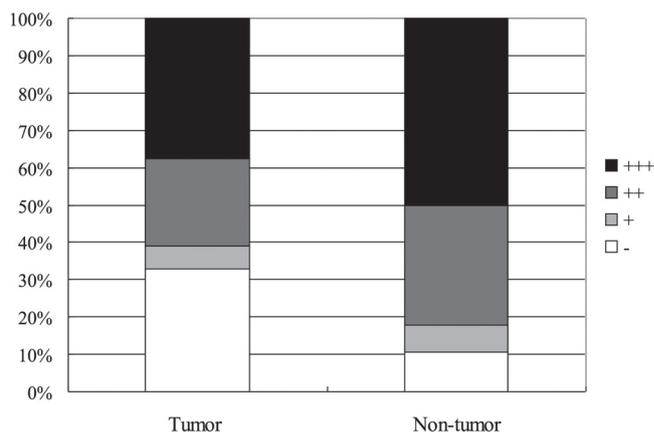


Figure 2. Summary results of ING2 protein staining in NSCLC and their matched non-malignant lung tissues with IHC (n=28). The levels of staining for ING2 expression were significantly decreased in NSCLC compared with non-tumor lung tissues (*P*<0.05).

Table 1. Correlations between ING2 expression and clinicopathologic parameters of 64 NSCLC cases

Variable	Cases in each group		ING2 expression		p-Value ^a
	n	%	Negative	Positive	
Age					0.247
≤57.5	33	51.6	13	20	
>57.5	31	48.4	8	23	
Gender					0.192
Male	49	76.6	14	35	
Female	15	23.4	7	8	
NSCLC subtype					0.114
ADK	24	37.5	11	13	
SCC	38	59.4	10	28	
Differentiation					0.750
Poor and undifferentiated	17	30.9	4	13	
Well and moderate	38	69.1	12	26	
Size					0.365
≤5cm	34	61.8	9	25	
>5cm	21	38.2	8	13	
Lymph node metastasis					0.653
Without	30	46.9	9	21	
With	34	53.1	12	22	
Tumor location					0.179
Left	29	45.3	7	22	
Right	35	54.7	14	21	
TNM stage					0.232
I	17	26.6	3	14	
II	34	53.1	12	22	
III/IV	13	20.3	6	7	

^a Comparison of ING2 expression status between or among each variable using the Pearson chi-square test.

To further investigate if the expression of ING2 is decreased at protein level in NSCLC, we performed Western blot on NSCLC specimens and corresponding adjacent non-cancerous lung tissues (n=10). To determine the mechanisms by which ING2 decrease of expression occurs in lung tumors, we investigate the

mRNA expression level of paired samples by semiquantitative RT-PCR and the possibility of the mutation occurs in DNA sequences. Our data showed that immunohistochemistry results were highly consistent with that of the Western blot analysis and RT-PCR analysis (Fig.3A, B and C), and ING2 protein expres-

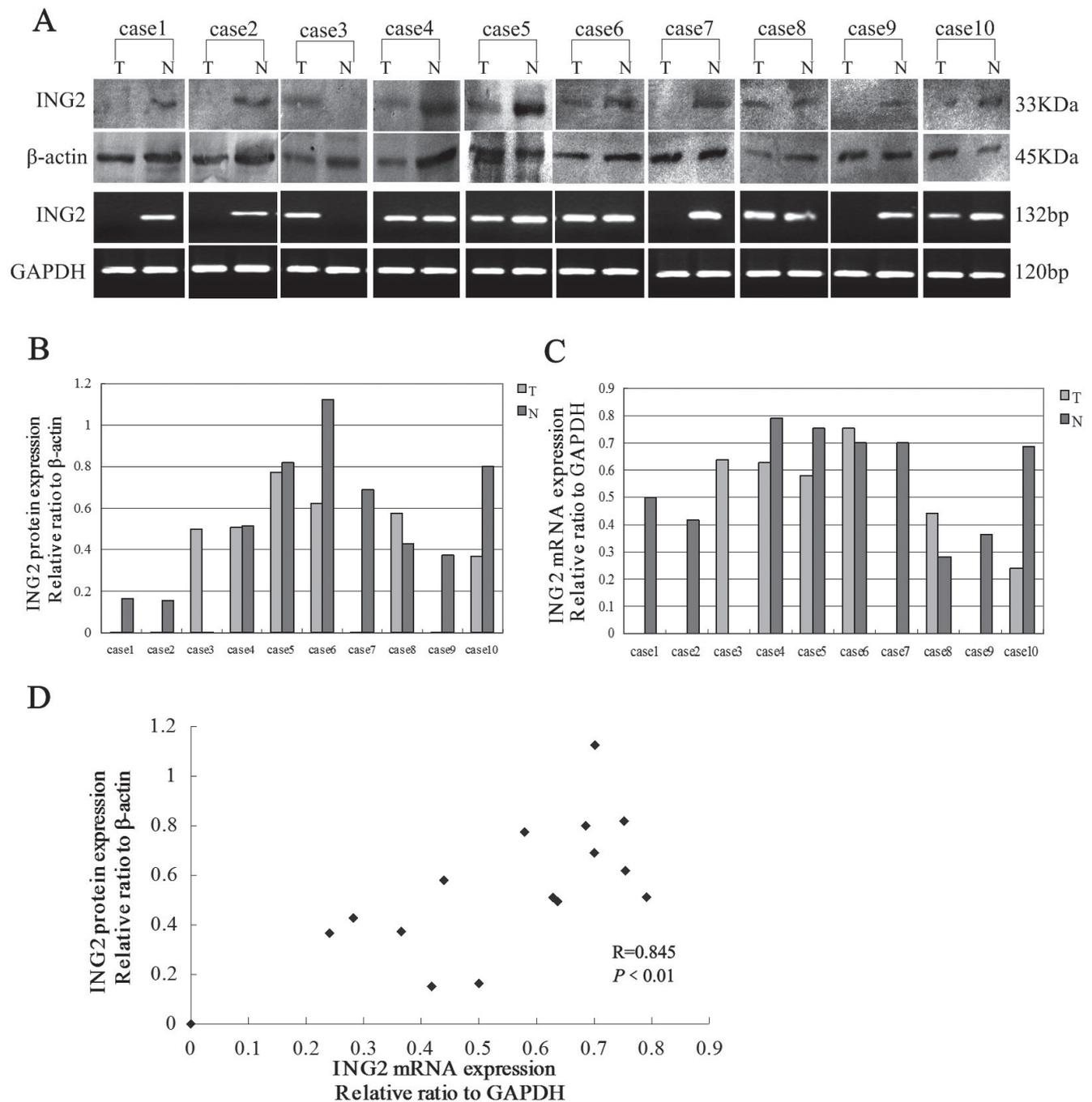


Figure 3. Expression pattern of ING2 in NSCLC and corresponding non-tumors specimens. (A, B and C) ING2 protein expression and mRNA expression in NSCLC and matched adjacent non-malignant tissues were examined using Western bolt with β -actin as loading control and RT-PCR with GAPDH as internal control respectively (n=10). (D) ING2 protein expression was correlated with ING2 mRNA expression. Person's correlation analysis was performed ($R=0.845$, $P < 0.01$). T: Tumor tissue, N: Non-tumor tissue.

sion was positively correlated with the ING2 mRNA (Fig.3D, $P<0.01$). Mutation analysis of ING2 was performed in genomic DNA from 10 patients with NSCLC by PCR-direct sequencing and no mutation was identified in these cases.

Relationship between ING2 protein expression and clinicopathological characters of NSCLC. ING2 expression was lost in 32.8% (21/64) NSCLC tissues, which was more frequently in ADK, 45.8% (11/24), than in SCC, 26.3% (10/38). ING2 expression in NSCLC lesions was not significantly associated with clinical parameters such as patient age, gender, tumor subtype, differentiation, size, lymph node metastasis location and TNM stage (Table 1).

However, considering the different proportions of ING2 loss in two subtypes of NSCLC, ADK and SCC, we analyzed the relationships between expression status of ING2 and ADK or SCC. ING2 expression in SCC lesions was associated with lymph node metastasis status (χ^2 -test, $P=0.008$) and TNM stage (χ^2 -test, $P=0.036$). However, ING2 expression in ADK and SCC was not significantly associated with other clinical parameters (Table 2).

Discussion

ING2, a member of ING family, has important functions in senescence, apoptosis, gene transcription, chromatin modification and DNA damage repair [23]. Previous studies also indicated that ING2 could interact with the transforming growth factor (TGF)- β signaling pathways and enhance the transcription of target genes and cell cycle arrest [24].

A few studies have focused on the relationship between human ING2 and cancers. Two studies on basal cell carcinomas [25] and head and neck squamous cell carcinoma [26] revealed a LOH in the region of 4q35, which includes ING2, underlying a potential role of ING2 in both carcinomas. Other studies provided further evidence that ING2 was involved in tumorigenesis. Two studies by Okano [27] and Ythier D [28] described the reduced expression of ING2 in lung cancer cell lines and NSCLC respectively. In another study by Lu F [29], a reduced nuclear expression of ING2 was observed in human melanomas when compared to dysplastic nevi, while no correlation between ING2 nuclear expression and tumor growth phase or tumor stage was established. This data indicated that the reduced ING2 expression may involved in the initiation rather than progression of melanoma. The study of Zhang H K [30] revealed that the lost of ING2 expression in hepatocellular carcinoma (HCC) could be involved in the progression of HCC and associated with poor prognosis. More recently, a study from Larrieu found a novel and specific function of ING2 in regulation of G1/S transition. ING2 may regulate G1/S transition by mediating H3K4me3 at p21 promoter without p53 [31, 32]. This study revealed the knockdown of ING2 may accelerate cell cycle progression, which provides evidence for the potential link between loss of ING2 and cancer development. Another report by Saito M provides an animal model to study idiopathic and iatrogenic infertility in

Table 2. Correlations between ING2 expression and clinicopathologic parameters of 24 ADK cases and 38 SCC cases

Variable	Cases in each group		ING2 expression		p-Value ^a
	n	%	Negative	Positive	
ADK Age					0.217
≤57.5	11	45.8	7	4	
>57.5	13	54.2	4	9	
SCC Age					1.000
≤57.5	21	55.3	6	15	
>57.5	17	44.7	4	13	
ADK Gender					1.000
Male	13	54.2	6	7	
Female	11	45.8	5	6	
SCC Gender					0.164
Male	35	92.1	8	27	
Female	3	7.9	2	1	
ADK Differentiation					0.509
Poor and undifferentiated	2	10.5	0	2	
Well and moderate	17	89.5	7	10	
SCC Differentiation					1.000
Poor and undifferentiated	14	40.0	4	10	
Well and moderate	21	60.0	5	16	
ADK Size					0.214
≤5cm	14	60.9	5	9	
>5cm	9	39.1	6	3	
SCC Size					1.000
≤5cm	18	60	4	14	
>5cm	12	30	2	10	
ADK Lymph node metastasis					0.105
Without	15	62.5	9	6	
With	9	37.5	2	7	
SCC Lymph node metastasis					0.008
Without	13	34.2	0	13	
With	25	65.8	10	15	
ADK Tumor location					1.000
Left	6	25.0	3	3	
Right	18	75.0	8	10	
SCC Tumor location					0.267
Left	22	57.9	4	18	
Right	16	42.1	6	10	
ADK TNM stage					0.596
I-II	20	83.3	10	10	
III-IV	4	16.7	1	3	
SCC TNM stage					0.036
I-II	29	76.3	5	24	
III-IV	9	23.7	5	4	

^a Comparison of ING2 expression status between or among each variable using the Pearson chi-square test.

men found the function of ING2 in spermatogenesis and also demonstrated an evidence of ING2 as a tumor suppressor by increased incidence of soft-tissue sarcomas in *Ing2*^{-/-} mice [33]. Interestingly, a research on colon cancer suggest ING2

also play roles as a tumor oncogene, which demonstrated ING2 was upregulated and increased invasion by enhanced MMP-13 [22]. In addition, a putative NF- κ B binding site was found in the promoter of ING2 and the activation of NF- κ B could positively regulate ING2 expression.

To our knowledge, there are no reports of ING2 in human NSCLC of Chinese population. In our study, we investigated the expression pattern of ING2 in Chinese NSCLC patients to evaluate its potential clinical relevance. Our findings demonstrate the expression of ING2 protein was significantly downregulated in NSCLC compared with paired normal lung tissues and the lost frequency of ADK was more than SCC, which is in accordance with the observations of Ythier D [34]. In our study ING2 expression was lost in 32.8% NSCLC tissues. However, Ythier D reported ING2 was lost in more than 50% NSCLC tissues. According to the results of Western blot and RT-PCR, we found that the dysregulation of ING2 in NSCLC occurred in both transcription and translation. Furthermore, in IHC results we investigated if the ING2 expression was translocated from nucleus to cytoplasm in several NSCLC tissues, especially in ING2 high staining samples, in contrast to normal tissues. In agreement with our results, the aberrantly localization of ING2 was also reported in some other tumors, including HCC [35] and colon cancer [22]. Moreover, study on ING1 and ING3, homologs of ING2, also found similar event in brain tumor [36] and HCC [37]. So we presumed that the mislocalization of ING2 may trigger the degradation and loss of its function in nucleus, which is similar to the lost of ING2 expression in NSCLC. The precise mechanism of the aberrantly localization of ING2 remains elusive and needs further research.

In the correlation analysis of ING2 and clinical characteristics, we found no relationship between ING2 staining and any variable in NSCLC. However, we found that ING2 expression in SCC lesions was negatively associated with lymph node metastasis status and TNM stage, which was not found in Ythier D's study. These findings demonstrated that the downregulation of ING2 may play a continued role in the processing of SCC advancement and development.

In addition, the sequencing data for ING2 exon1 and exon2 in this study showed that different expression levels of ING2 in NSCLC tissues was not relevant with loss of heterozygosity or mutations in ING2 DNA. This result is also in accordance with the observations of Okano, that no mutation was detected in 31 cancer cell lines and 30 lung cancer biopsies [27], and Ythier D, that no LOH and no sense mutation was found in lung cancer samples [34]. The ING2 involvement in tumors seems to be linked to the expression level rather than its mutation status [38].

Conclusively, our research suggested that the dysregulation of ING2 frequently occurred in NSCLC, including downregulation, lost expression and aberrantly localization, which may be related with the progression of NSCLC, particularly in SCC. Dysregulation of ING2 in NSCLC occurred in both transcription and translation levels, and it is a candidate of tumor suppressor gene for NSCLC.

More studies, especially on the role of the molecular mechanism of ING2 in carcinogenesis for NSCLC and other tumors, including cellular localization, crosstalk of ING2 with other ING family members need to be elucidated, which will help a better characterization of ING2 diagnostic and therapeutic potential for NSCLC and other tumors.

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