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Down-regulation of *ING4* is associated with initiation and progression of lung cancer

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Abstract

Aims—Tumour suppressor *ING4* is one of *ING* family genes, which are involved in cell cycle arrest, gene transcription regulation, DNA repair and apoptosis. *ING4* inhibition has been reported in various tumours, including gliomas, breast tumours, and stomach adenocarcinoma. The aim was to evaluate *ING4* expression in lung cancers.

Method and results—By immunohistochemistry of 246 lung tumour tissues, reduced ING4 nuclear and cytoplasmic expression were both revealed in lung cancer and associated with tumour grade. Interestingly, compared with normal tissues, we found more tumours with ING4 expression in the cytoplasm higher than in the nucleus. Nuclear ING4 inhibition correlated with the tumour stage and lymph node metastasis. Consistent with these findings, semiquantitative reverse transcriptase-polymerase chain reaction and Western blotting demonstrated decreased *ING4* mRNA and expression in 100% (50 / 50) tumour tissues. Furthermore, ING4 expression was lower in grade III than in grades I–II tumours. Reduced *ING4* mRNA correlated with lymph node metastasis.

Conclusions—Our results indicate that overall inhibition of ING4 expression and ING4 expression higher in cytoplasm than in nucleus of tumour cells may be involved in the initiation and progression of lung cancers, and thus, analysis for ING4 expression may be useful as a clinical diagnostic and prognostic tool for lung cancer.

Keywords

ING4 expression; lung cancer; mRNA; tumour suppressor gene

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Introduction

Lung cancer is the leading cause of cancer death worldwide.[1] Approximately 80% of all lung cancers can be histologically classified as non-small cell lung cancer (NSCLC).[2] The incidence and mortality of lung cancer has markedly increased in China over the past years.[3]

The human inhibitor of growth family (*ING*) gene family has five members (*ING1–5*), which have been considered as type II tumour suppressor genes.[4–7] ING proteins possess a nuclear localization sequence (NLS) and a plant homeo domain (PHD) finger motif in their high homology C-terminus.[8,9] Studies have shown that ING proteins may enhance p53 activity and regulate various biological activities, including apoptosis, cell cycle arrest and DNA repair.[9] ING proteins are also components of histone acetylase and histone deacetylase complexes involved in chromatin-mediated transcriptional regulation.[10] Much evidence supports a tumour suppressor role of *ING* genes in different types of human cancer. For example, reduce p33ING1b expression has been detected in NSCLC.[11] p33ING1b expression inhibition and perhaps translocation from the nucleus to the cytoplasm may be involved in the development and progression of melanomas.[12] Inhibition of ING2 expression was found to be critical in the progression of lung cancers, even in those with a *p53* mutation.[13] In addition, low ING3 mRNA levels have been documented as a marker for aggressive head and neck carcinoma.[14]

ING4 is the most investigated *ING* gene in tumours after *ING1*. Studies have shown that overexpression of ING4 results in p53-dependent apoptosis.[5] For ING4 translocation into the nucleus and interaction with p53, NLS is required.[15] Evidence also indicates that ING4 could suppress the expression of hypoxia-inducible factor-1α-responsive genes through the ING4 PHD domain under hypoxic conditions.[16,17] Moreover, ING4 has been shown to interact physically with the p65 subunit of nuclear factor (NF)-κB and regulate brain tumour angiogenesis through transcriptional repression of NF-κB-responsive genes.[18] ING4 also has a role in the suppression of loss of contact inhibition induced by the proto-oncogene MYCN.[19] In addition, the tumour suppressor RNX3 has been implicated in upregulation of ING4 expression during apoptosis in the human gastric carcinoma cell line MKN-1.[20] ING4 was further found to down-regulate interleukin (IL)-6, IL-8, MMP-2 and MMP-9 expression in the human lung adenocarcinoma cell line A549.[21] Mutations in *ING4* transcripts resulting in inactive proteins have also been detected in a number of lung cancer cell lines.[19] Furthermore, inhibition of ING4 expression and locus deletion have been documented in many types of cancer, including gliomas, head and neck squamous cell carcinomas (SCCs), breast tumours, stomach adenocarcinoma and melanoma.[18,19,22–24]

However, there is no report focusing on ING4 in clinical lung cancer tissues. Therefore, the actual role of ING4 in the initiation and progression of lung cancers remains to be established. In the present study, the expression of ING4 was investigated in 246 lung cancer tissues to determine whether ING4 plays a role in the development and progression of lung cancer, and whether it may be targeted as a diagnostic and prognostic tool for lung cancer.

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Materials and methods

TISSUES

The tumour and normal lung surgical specimens from lung cancer patients and the autopsy brain specimens used in this study represented excess pathological / normal materials that were obtained in accordance with procedures approved by the Human Ethics Review Board at the Second Affiliated Hospitals of Harbin Medical University (Harbin, China). All patients provided written, informed consent. The normal and tumour states of specimens were confirmed by examination of haematoxylin and eosin-stained histology sections by pathologists. A total of 246 lung cancer patients were obtained from patients that included 157 men and 89 women. Of these patients, 211 were ≤70 years old and 35 were >70 years old. Histological tumour subtypes were assessed using the World Health Organization classification.[25] Histopathological grading of tumours into well (grade I, $n = 58$), moderate (grade II, $n = 94$) or poorly (grade III, $n = 71$) differentiated types was performed in a qualitative manner based on conventional pathological criteria (i.e. architectural and cytological atypia). The tissues examined included 99 SCCs, 129 adenocarcinomas, three large cell carcinomas, four carcinoid tumours, six adenosquamous carcinomas, one unclassified carcinoma and four small cell carcinomas. Tumours were staged using the American Joint Committee on Cancer Tumour Node Metastasis classification system and consisted of 55 stage I, 26 stage II and 52 stage III tumours.[26]

SEMIQUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

The One-step SYBR Green I-based semiquantitative reverse transcriptase-polymerase chain reaction (SQRT-PCR) was performed to detect *ING4* mRNA levels in 50 NSCLC samples (One-step SYBR RT-PCR kit; TaKaRa, Dalian, China). The specimens were frozen in liquid nitrogen immediately upon removal from the patients and subsequently stored at −80°C. Total RNA was isolated with Trizol (Invitrogen, Camarillo, CA, USA) following the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal PCR control. The primers were: *ING4*, 5′-TCGTGCTCGTTCCAAAGG-3′ and 5′-GGCAATAGGT GGGTTCGTT-3′; *GAPDH*, 5′-CGGATTTGGTCGTATTG GG-3′ and 5′-TCTCGCTCCTGGAAGATGG-3′.

The SQRT-PCR results were analysed using the Rotor-Gene Real-Time Analysis Software 6.0 (Corbett Robotics, Brisbane, Australia). T represents the mRNA levels of *ING4* in tumour specimens; N represents the levels of *ING4* mRNA in normal specimens; T / N indicates the relative mRNA levels of *ING4* in tumour specimens. Statistical analysis was performed using the *t*-test, and a *P*-value <0.05 was considered significant.

WESTERN BLOT ANALYSES

Western blot analyses were performed to detect the expression of ING4 in the 50 NSCLCs specimens also examined for mRNA. For protein extraction, tissues were first ground thoroughly in liquid nitrogen, then lysed in radio immunoprecipitation assay buffer [150 mM NaCl, 1% (v / v) Triton X-100, 0.5% (w / v) deoxycholate and 1% (w / v) sodium dodecyl sulphate (SDS) 50 mM Tris base] with a protease inhibitor cocktail, and disrupted by ultrasound three times. After incubation on ice for 1 h, the solution was centrifuged at 14

000 ***g*** for 40 min at 4°C. The supernatant was collected and the concentration of total protein determined using the Bradford assay.

Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA), and ING4 was detected using an anti-ING4 rabbit polyclonal antibody (dilution 1:1000; Invitrogen Corporation). IRDye 700DX-conjugated affinity-purified goat antirabbit immunoglobulin G (IgG) (dilution 1:7000) and IRDye 800DX-conjugated affinity-purified goat antimouse IgG (dilution 1:7000) were obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). Proteins were detected using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Immunoblot images were scanned using the Odyssey / LI-COR software (LI-COR Biosciences). The Scion imaging software (Scion Corporation, Frederick, MD, USA) was used to quantify the bands. T represents the ING4 expression in tumour specimens. N represents ING4 expression in normal specimens and T / N indicates relative ING4 expression in tumour specimens. Statistical analysis was performed using the *t*-test, and a *P*-value <0.05 was considered significant.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry analyses were performed to detect ING4 protein expression in 246 human lung cancer tissues. Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was performed by autoclave sterilization in ethylenediamine tetraaceticacid buffer for 2 min. Slides were then incubated with 10% normal goat serum solution for 20 min to reduce background non-specific staining. A rabbit polyclonal antibody against ING4 (Invitrogen Corporation) was applied at a concentration of 1:500 and incubated at 4°C overnight. The Polink-1 Horseradish Peroxidase Detection System for Rabbit Antibody [for diaminobenzidine (DAB); Golden Bridge Int., Inc., Mukilteo, WA, USA) was used according to the manufacturer's instructions. The slides were then incubated with DAB (Golden Bridge Int.) to visualize ING4 expression. Formalinfixed paraffin-embedded human brain tissues, which express ING4 in glial cells, were used as internal positive controls. Negative staining controls were obtained by omitting the primary antibody.

The slides were reviewed by two pathologists who were unaware of the clinical status of the patients. The intensity of ING4 immunoreactivity in the cytoplasm and nucleus was scored separately and designated 0–3 (0, negative; 1, weak; 2, moderate; and 3, strong). The percentage of cells with both cytoplasmic and nuclear ING4 reactivity was also scored into four categories: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). In cases with discrepancy between duplicate scores, the higher score was taken as the final score. The level of ING4 reactivity was further evaluated by immunoreactive score (IRS), which is calculated by multiplying the scores of intensity of reactivity and the percentage of positive cells.[24,27] Based on IRS, the ING4 pattern of immunoreactivity was defined as: negative (Ne; IRS 0), weak (We; IRS 1–4), moderate (Mo; IRS 6–8) and strong (St; IRS 9–12). Statistical analysis was performed using the chi squared test, and a *P*-value <0.05 was considered significant.

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Results

SQRT-PCR ANALYSIS OF *ING4* MRNA IN LUNG CANCER TISSUES

ING4 expression has previously been analysed in various tumour tissues but not in lung cancer tissues. Initially, we assessed *ING4* mRNA level in 50 paired tumour and adjacent normal tissues by SQRT-PCR. Reduced ING4 mRNA expression was found in 100% of tumour tissues examined ($n = 50$). Figure 1A shows that *ING4* mRNA levels of the tumour tissues examined were, on average, about three-quarters of those in adjacent normal tissues ($P = 0.000$).

We then sought to determine whether *ING4* mRNA levels correlated with the age and gender of the patients, grade, lymph node metastasis, tumour stage and type of NSCLC. As shown in Figure 1B, although the relative *ING4* mRNA level (T / N) seemed to be lower in grade II–III tumours than in grade I tumours, and seemed to be lower in stage II–III tumours than in stage I tumours, the results were not statistically significant. However, the relative *ING4* mRNA level (T / N) in tumour tissues with lymph node metastasis N0 and N1 was significantly less than in tumour tissues with lymph node metastasis N2 ($P = 0.020$). The relative *ING4* mRNA levels in tumour tissues did not significantly correlate with other clinical parameters.

WESTERN BLOT ANALYSIS OF ING4 EXPRESSION IN LUNG CANCER TISSUES

ING4 protein expression was also screened by Western blotting in the same 50 lung cancer tissues and adjacent normal tissues that we examined for mRNA levels. Figure 2A shows the general pattern of reduced ING4 expression in tumour tissues compared with adjacent normal tissues. Down-regulation of ING4 expression was observed in all tumour tissue examined ($n = 50$). Figure 2B shows that the average ING4 level of tumour tissues was less than half of that in adjacent normal tissues ($P = 0.000$). Thus, consistent with reduced levels of *ING4* mRNA in lung cancer tissues, ING4 protein levels were also reduced in these tissues.

We then performed statistical analysis of the age and gender of the patient, differentiation extent, lymph node metastasis, tumour stage and type of the lung cancers. Figure 2C shows that the relative ING4 levels (T / N) in tumour tissues were significantly lower in grade III than in grade I–II tumours ($P = 0.046$). Although no statistically significantly so, the more advanced stage tumours had lower ING4 levels. Furthermore, the ING4 level in tumour tissues with N0 and N1 lymph node metastasis was higher than tumour tissues with N2 lymph node metastasis. No significant correlation was observed between relative ING4 expression and other clinical parameters.

IMMUNOHISTOCHEMISTRY ANALYSIS OF ING4 EXPRESSION IN LUNG CANCER TISSUES

Following analysis of ING4 mRNA and protein levels in 50 lung cancer tissues by quantitative RT-PCR and Western blotting, we examined ING4 expression in 246 lung cancer tissues (including 45 of the 50 tissues originally examined) and 69 normal lung tissues (from the 246 patients) by immunohistochemistry. For this study, we used autopsy brain specimens as positive control showing nuclear immunoreactivity (Figure 3A).

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Negative control lung normal tissues were stained without the use of the primary antibody (Figure 3B). As shown in Figure 3C, normal lung tissues showed both nuclear and cytoplasmic immunoreactivity for ING4, with stronger reactivity in the nucleus than in the cytoplasm. On the other hand, lung cancer tissues showed nuclear reactivity alone, both nuclear and cytoplasmic reactivity, or cytoplasmic reactivity alone. Figure 3D is a representative tumour tissue with cytoplasmic reactivity alone. To determine how ING4 affects lung tumours, we examined the relationship between (i) lung cancer, patient age and gender, extent of differentiation, lymph node metastasis, and tumour stage, and (ii) ING4 expression in (a) cytoplasm, (b) nucleus, and (c) both cytoplasm and nucleus.

First, we analysed ING4 cytoplasmic immunoreactivity in lung cancer tissues compared with normal tissues. As shown in Table 1, ING4 cytoplasmic reactivity was weaker in lung cancer than in normal tissue ($P = 0.000$). Especially in tumour tissues, 28.0% (69 / 246) showed immunonegativity and 53.7% (132 / 246) showed weak reactivity, while in normal tissues only 1.4% (1 / 69) showed immunonegativity and 49.3% (34 / 69) showed weak reactivity (Table 1). We also observed that ING4 cytoplasmic expression was less in grade III than in grade I–II tumour tissues ($P = 0.013$, Table 2). In addition, 40.8% (29 / 71) of grade III tumour tissues showed ING4 cytoplasmic immunonegativity while only 21.1% (32 / 152) of grade I–II tumour tissues showed ING4 cytoplasmic negativity (Table 2). No significant difference in reactivity was observed among SCCs, adenocarcinomas, large cell carcinomas, carcinoid tumours, adenosquamous carcinomas, unclassified carcinoma or small cell carcinomas. However, three small cell lung cancers (SCLCs) showed cytoplasmic immunonegativity and one showed weak cytoplasmic reactivity. Therefore, cytoplasmic reactivity appears to be a little weaker in SCLC than in NSCLCs ($P = 0.055$, Table 2). Among 45 of the 50 paired lung cancers and normal tissues originally examined for ING4 mRNA and protein (by Western blotting), cytoplasmic expression of ING4 was significantly weaker in tumour tissues than in their paired normal tissues ($P = 0.001$). ING4 cytoplasmic reactivity did not correlate with gender or age of the patient, stage, or lymph node metastasis of the tumours.

Second, we analysed ING4 nuclear immunoreactivity in the 246 lung cancer tissues. We found moderate and strong ING4 nuclear reactivity in 65.2% (45 / 69) of normal lung tissues but in only 11.9% (29 / 246) of lung cancer tissues (Table 1). Overall, ING4 nuclear expression was lower in tumour tissues than in normal lung tissues ($P = 0.000$, Table 1). Analysis of tissues from 45 of the 50 patients examined for ING4 expression by SQRT-PCR and Western blotting also revealed weaker nuclear immunoreactivity for ING4 in the tumour tissues compared with their paired normal tissues ($P = 0.000$). We then analysed ING4 nuclear reactivity relative to age and gender, extent of differentiation, lymph node metastasis, and tumour stage. We found that nuclear reactivity in grade III tumour tissues was significantly weaker than in grade I–II tumour tissues ($P = 0.000$, Table 2). Figure 3 shows representative grade I lung adenocarcinoma (Figure 3E) and squamous carcinoma (Figure 3F), grade II lung adenocarcinoma (Figure 3G) and squamous carcinoma (Figure 3H), grade III lung adenocarcinoma (Figure 3I) and squamous carcinoma (Figure 3J). We also found that ING4 nuclear reactivity of tumours with N0 and N1 lymph node metastasis was stronger than in tumours with N2 lymph node metastasis ($P = 0.010$, Table 2). In addition, ING4 nuclear expression in stage I and stage II tumours was greater than in stage

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III tumours (*P* = 0.038, Table 2). We found no correlation between ING4 nuclear immunoreactivity and patient age and gender, or histology.

Finally, we analysed the localization of ING4 in both the cytoplasm and the nucleus relative to lung cancer, and patient and clinical factors. There were 44.7% (110 / 246) of tumour specimens versus 13.0% (9 / 69) of normal specimens with ING4 cytoplasmic reactivity only or ING4 cytoplasmic reactivity observed stronger than nuclear reactivity. Analysis for nuclear reactivity showed that 28 / 246 (11.4%) tumour specimens and 19 / 69 (27.5%) normal specimens had ING4 nuclear reactivity only or ING4 nuclear reactivity stronger than cytoplasmic reactivity. Therefore, compared with normal tissues, a higher number of tumour tissues showed stronger reactivity in the cytoplasm than in the nucleus. In contrast, the number of specimens with nuclear reactivity stronger than cytoplasmic reactivity was smaller in tumour tissues than in normal tissues (*P* = 0.000, Table 3). Furthermore, analysis of the relationship between ING4 location and patient clinical factors showed that a modestly higher number of grade III compared with grade I–II tissues had stronger cytoplasmic reactivity than nuclear reactivity (*P* = 0.084, Table 4). Compared with NSCLC, SCLC cells had greater nuclear ING4 reactivity (*P* = 0.065, Table 4). SCLC cells also had significantly less cytoplasmic (*P* = 0.000) and nuclear (*P* = 0.025) ING4 reactivity compared with normal lung tissues. ING4 subcellular localization did not correlate with patient gender or age, tumour stage, or lymph node metastasis.

Discussion

The ING proteins play a critical role in various cellular processes that are involved in tumorigenesis, including cell cycle arrest, senescence, apoptosis, migration and contact inhibition, transcriptional regulation, post-translational modifications, DNA repair and genomic stability.[8,9,28] Previous reports have implicated ING in lung cancer. For example, 42% of NSCLCs have been found to have reduced p33ING1b expression associated with reduced levels of p53 effector genes, p21 and bax.[11] ING2 mRNA expression has also been found to be reduced in six out of seven lung cancer cell lines.[13] However, no ING1 or ING2 mutation was detected in 31 human lung cancer cell lines and 30 lung cancer biopsy specimens examined.[11–13] In the present study, we have demonstrated for the first time that *ING4* mRNA and protein expression is significantly reduced in lung cancer tissues. We have also shown that reduced ING4 expression and partly cytoplasmic ING4 correlate with the initiation and progression of lung cancer.

A recent study has reported that *ING4* is involved in apoptosis, cell-cycle arrest and the tumour invasion pathway in the human lung adenocarcinoma cell line A549.[21] These findings, together with our observation that ING4 expression is reduced in lung cancer, and that ING1 and ING2 expression is likewise reduced in lung cancers, suggest that the ING family of tumour suppressor proteins plays important roles in the development of lung cancer. This is further consistent with previous reports of ING4 involvement in other types of human cancer. In head and neck SCC, the *ING4* mRNA level is reduced in 76% of primary tumours examined.[22] *ING4* mRNA was also found to be significantly reduced in gliomas.[18] Moreover, ING4 expression was reduced in malignant melanoma, although reduced ING4 expression was not associated with the prognosis of patients with primary

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melanomas. [23] Our previous study further demonstrated decreased ING4 expression in stomach adenocarcinoma. [24] In addition, hybridization deletion of the ING4 locus was found in primary breast tumours and in 66% of head and neck SCCs.[19,22] Together, these studies indicate that ING4 serves as a suppressor of various tumours, including lung tumours.

Consistent with previous reports, we found that ING4 localizes in the nucleus of normal brain tissues.[18,24,29] ING4 nuclear immunoreactivity stronger than cytoplasmic reactivity is detected in lung normal cells. This ING4 pattern of reactivity is in agreement with our previous observation in stomach adenocarcinoma tissues. [24] Recently, ING4 nuclear reactivity stronger than cytoplasmic reactivity was also seen in mouse lung normal tissues.[30] However, we also noted that some lung cancer tissues lack cytoplasmic ING4, yet some show greater ING4 reactivity in the cytoplasm than in the nucleus. The cytoplasmic localization of ING4 in lung cancer may be related to the possible expression of ING4-variant (V) 2. Indeed, ING4-V2, a variant of ING4, lacks the full NLS, resulting in its increased cytoplasmic localization.[31] However, ING4 also tends to be distributed in the cytoplasm under different conditions, and ING4 localization and stability differ among cell types such as in COS-7 and AZ-521 cells.[32]

Our finding that ING4 cytoplasmic and nuclear immunoreactivity are both lower in grade III compared with grade I–II tumours suggests a correlation between ING4 expression inhibition and tumour grades. This can be noted in protein analysis by Western blotting as well. Furthermore, the pattern of ING4 expression in normal lung tissues, grade I–II lung tumours and grade III lung tumours is the same as that reported in normal brain tissue, low-grade gliomas and glioblastomas, respectively. [24] In our previous study, we found that reduced ING4 mRNA level correlated with the stage of stomach adenocarcinoma.[24] ING4 levels in advanced tumour stages are lower than those in earlier tumour stages. This is also similar to what we observed in lung cancer.

ING4 has been reported to regulate tumour cell spreading and migration.[31,32] Recently, ING4 was demonstrated to have anti-invasive and anti-metastatic activities by down-regulation of the expression of MMP-2 and MMP-9 in A549 human lung carcinoma cells.[21] This may be related to our finding that ING4 nuclear expression, and reduced ING4 mRNA in lung tumours, correlate with lymph node metastasis. Past reports have indicated that cytoplasmic ING4 regulates cell spreading and migration by interaction with liprin A1, and may prevent invasion and metastasis through its anti-angiogenic function.[18,32] Once again, these are consistent with our results showing lack of ING4 in the cytoplasm of some lung cancer cells, and generally weaker ING4 cytoplasmic reactivity in the advanced stage of lung cancer, further implicating cytoplasmic ING4 in a tumour suppressor role. These findings have shed new light on our knowledge of ING4 in lung cancer, and possibly other types of cancer as well.

ING4 contains a potential bipartite NLS involved in nuclear localization and binding to p53.[15,31] In addition to shifting ING4 localization, mutations in the NLS may affect p53 function by disrupting ING4–p53 interaction *in vivo*.[15,31] Alternative splice variants of *ING4*, *ING4*-V2, *ING4*-V3 and *ING4*-V4 have been shown to attenuate ING4 function in cytoplasm due to the lack of complete NLS.[31] Another ING4 variant, ING4-DEx6A, retains

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the critical part of NLS and thus localizes in the nucleus and has the ability to interact with p53.[33] To recapitulate our findings: (i) ING4 cytoplasmic reactivity is generally reduced in tumour tissues, but (ii) more cases in tumour tissues compared with normal tissues have cytoplasmic ING4, and (iii) have cytoplasmic ING4 higher than its nuclear ING4; furthermore, (iv) the number of cases with cytoplasmic ING4 higher than its nuclear ING4 is greater in grade III than in grade I–II tumours. Thus, it appears that cytoplasmic ING4, which may include the aberrantly spliced forms of ING4, is involved in the initiation and progression of lung cancer. It is interesting that cytoplasmic mislocalization of ING1 has also been reported in brain tumours and invasive breast carcinomas. [34] A nuclear to cytoplasmic compartment shift of the p33ING1b has been associated with malignancy in melanocytic lesions.[12] In addition, nuclear ING3 expression was found to be remarkably reduced in malignant melanomas, and significantly correlated with the increased ING3 level in the cytoplasm.[35] Moreover, splicing variants of ING4 that lack the complete NLS domain or PHD domain were found to have aberrant ING4 function, suggesting wobble splicing influences subcellular localization and tumour suppressor role of ING4.[24,31,33,34] The balance of the ING4 variants and their subcellular localization may further modulate the development of lung cancers. It is also important to note that our observation that nuclear ING4 is greater in SCLC compared with that in NSCLC indicates the molecular differences between SCLC and NSCLC tumorigenesis.[36]

The differential reactivity of ING4 in lung cancer of various grades, stages, and extent of lymph node metastasis indicates that ING4 may be used as a novel tool in the diagnosis and prognosis of lung cancer.

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Abbreviations

DAB

diaminobenzidine

GAPDH

glyceraldehyde 3-phosphate dehydrogenase

IgG

immunoglobulin G

IL

interleukin

ING

inhibitor of growth family

ING4-V1, -v2, -v3 and -V4

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variant 1, 2, 3 and 4 of inhibitor of growth family, member 4

IRS
immunoreactive score

N
normal

NF-κB
nuclear factor of κ light polypeptide gene enhancer in B cells

NLS
nuclear localization

NSCLC
non-small cell lung cancer

PHD
plant homeo domain

SCC
squamous cell carcinoma

SCLC
small cell lung cancer

SDS
sodium dodecyl sulphate

SQRT-PCR
semiquantitative reverse transcriptase-polymerase chain reaction

T
tumour

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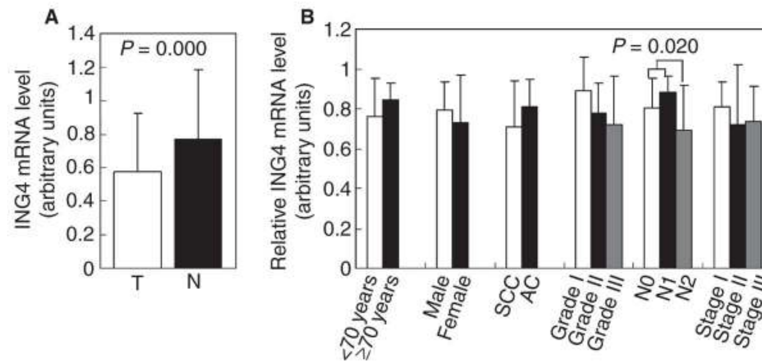

Figure 1.

ING4 mRNA is significantly reduced in lung cancer tissues compared with normal tissues. **A**, Fifty paired tumour and adjacent normal tissues were analysed for ING4 mRNA level by real-time reverse transcriptase-polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase was used as internal loading control. Average ING4 mRNA levels in paired tumour (T) and normal (N) samples are shown. **B**, Analyses of relative ING4 mRNA level (T / N) according to age of patient (≤70 years, $n = 44$; >70 years, $n = 6$), gender of patient (30 male, 20 female), tumour type [squamous cell carcinoma (SCC), $n = 20$; adenocarcinoma (AC), $n = 30$], grade (grade I, $n = 4$; grade II, $n = 31$; grade III, $n = 15$), lymph node metastasis (N0, $n = 27$; N1, $n = 4$; N2, $n = 16$), and stage (stage I, $n = 24$; stage II, $n = 10$; stage III, $n = 16$) were made. Statistical analysis was performed by *t*-test.

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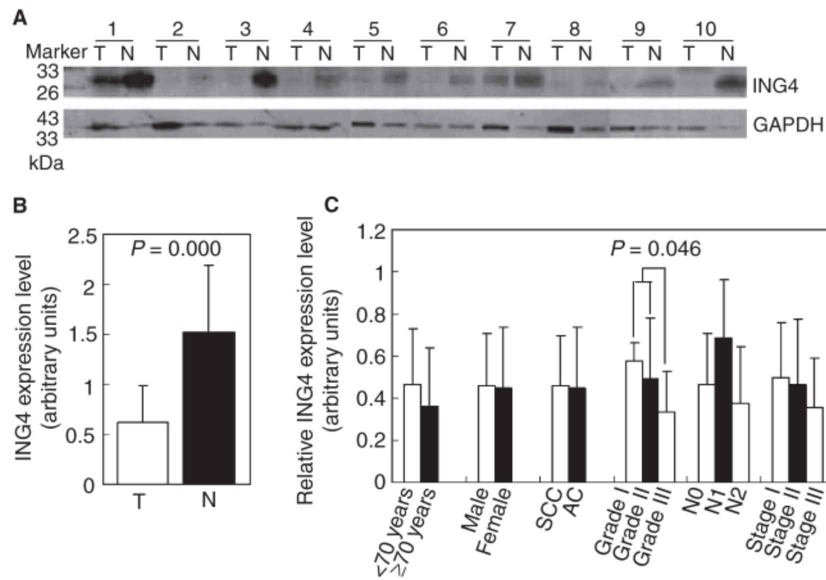

Figure 2.

ING4 expression is significantly reduced in lung cancer tissues compared with normal tissues. **A**, Fifty paired tumour and adjacent normal tissues were analysed for ING4 expression by Western blotting. Ten representative paired tumour (T) and normal (N) tissues are shown. Glyceraldehyde 3-phosphate dehydrogenase was used as internal loading control. The 29-kDa ING4 band detected is consistent with the possibility that the epitope recognized by the ING4 antibody lies in an internal region of ING4-V1 and -V2 but is absent or masked in the aberrantly spliced variant forms. **B**, Average ING4 expression in 50 paired tumour (T) and normal (N) samples are shown. **C**, Analyses of relative ING4 expression level (T / N) according to age of patient (≤70 years, $n = 44$; >70 years, $n = 6$), gender of patient (30 male, 20 female), tumour type [squamous cell carcinoma (SCC), $n = 20$; adenocarcinoma (AC), $n = 30$], grade (grade I, $n = 4$; grade II, $n = 31$; grade III, $n = 15$), lymph node metastasis (N0, $n = 27$; N1, $n = 4$; N2, $n = 16$), and stage (stage I, $n = 24$; stage II, $n = 10$; stage III, $n = 16$) were made. Statistical analysis was performed by *t*-test.

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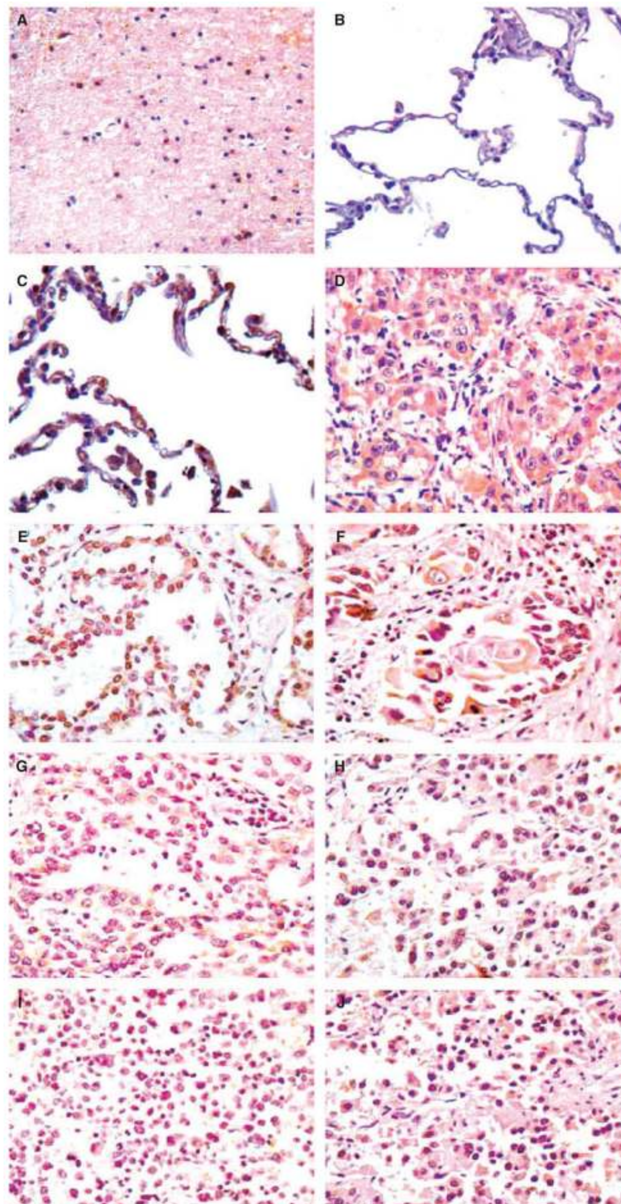

Figure 3.

ING4 protein expression is reduced in lung cancer cells compared with that of normal lung cells. ING4 expression in a positive brain tissue (**A**) and a negative lung tissue omitted by antibody (**B**) is shown in the first row. Normal lung tissue (**C**) with strong nuclear immunoreactivity and tumor tissue with cytoplasmic reactivity but not nuclear reactivity (**D**) are shown in the second row. Following, ING4 expression in a representative grade I (**E**), grade II (**G**) and grade III (**I**) adenocarcinoma (AC) is shown in the left lane. ING4 expression in a representative grade I (**F**), grade II (**H**) and grade III (**J**) squamous cell carcinoma (SCC) is shown in the right lane.

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Table 1

Analysis of *ING4* expression in lung normal and tumour tissues

		Cytoplasmic ING4					Nuclear ING4				
Tissues	**Nu**	**Ne**	**We**	**Mo**	**St**	***P***	**Ne**	**We**	**Mo**	**St**	***P***
Tumour (%)	246	69 (28.0)	132 (53.7)	43 (17.5)	2 (0.8)	0.000*	162 (65.9)	55 (22.4)	21 (8.5)	8 (3.3)	0.000*
Normal (%)	69	1 (1.4)	34 (49.3)	23 (33.3)	11 (15.9)		5 (7.2)	19 (27.5)	33 (47.8)	12 (17.4)	

Statistical analysis was performed using the chi squared test; a *P*-value <0.05 was considered significant.

Nu, Number; Ne, negative; We, weaker; Mo, moderate; St, strong; %, frequency.

* *P*-value <0.05.

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Table 2

Correlations of cytoplasmic and nuclear ING4 with clinicopathological factors of lung cancers

Variable	Nu	Cytoplasmic *ING4* Ne	We	Mo	St	*P*	Nuclear *ING4* Ne	We	Mo	St	*P*
Gender											
Male (%)	157	49 (31.2)	79 (50.3)	29 (18.5)	0 (0)	0.101	108 (68.8)	33 (21.0)	11 (7.0)	5 (3.2)	0.549
Female (%)	89	20 (22.5)	53 (59.6)	14 (15.7)	2 (2.2)		54 (60.7)	22 (24.7)	10 (11.2)	3 (3.4)	
Age											
≤70 (%)	211	55 (26.1)	121 (57.3)	33 (15.6)	2 (0.9)	0.291	143 (67.8)	45 (21.3)	15 (7.1)	8 (3.8)	0.094
>70 (%)	35	8 (22.9)	17 (48.6)	10 (28.6)	0 (0)		19 (54.3)	10 (28.6)	6 (17.1)	0 (0)	
Grade											
I / II (%)	152	32 (21.1)	87 (57.2)	32 (21.1)	1 (0.7)	0.013 *	82 (53.9)	46 (30.3)	19 (12.5)	5 (3.3)	0.000
III (%)	71	29 (40.8)	33 (46.5)	8 (11.3)	1 (1.4)		63 (88.7)	6 (8.5)	2 (2.8)	0 (0)	
Histology											
NSCC (%)	242	66 (27.3)	131 (54.1)	43 (17.8)	2 (0.8)	0.055	160 (66.1)	54 (22.3)	21 (8.7)	7 (2.9)	0.093
SCC (%)	4	3 (75.0)	1 (25.0)	0 (0)	0 (0)		2 (50.0)	1 (25.0)	0 (0)	1 (25.0)	
Stage											
I / II (%)	81	28 (34.6)	37 (45.7)	16 (19.8)	0 (0)	0.206	66 (81.5)	10 (12.3)	5 (6.2)	0 (0)	0.038 *
III (%)	52	24 (46.2)	23 (44.2)	5 (9.6)	0 (0)		50 (96.2)	2 (3.8)	0 (.0)	0 (0)	
Node											
N0 / N1 (%)	88	26 (32.1)	40 (49.4)	13 (16.0)	2 (2.5)	0.160	65 (80.2)	11 (13.6)	5 (6.2)	0 (0)	0.010 *
N2 (%)	52	26 (50.0)	20 (38.5)	6 (11.5)	0 (0)		51 (98.1)	1 (1.9)	0 (0)	0 (0)	

Statistical analysis was performed using the chi squared test; a *P*-value <0.05 was considered significant.

Nu, number; Ne, negative; We, weaker; Mo, moderate; St, strong; %, frequency.

* *P*-value <0.05.

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Table 3

Analysis of ING4 locations in lung normal and tumour tissues

		ING4 staining cytoplasm > nucleus			ING4 staining cytoplasm < nucleus		
Tissues	**Nu**	**With**	**Without**	***P***	**Without**	**With**	***P***
Tumour (%)	246	110 (44.7)	136 (55.3)	0.000*	218 (88.6)	28 (11.4)	0.002*
No tumour (%)	69	9 (13.0)	60 (87.0)		50 (72.5)	19 (27.5)	

Statistical analysis was performed using the chi squared test; a *P*-value <0.05 was considered significant.

Nu, number; %, frequency.

* *P*-value <0.05.

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Table 4

Correlations of *ING4* locations with clinicopathological factors of lung cancers

Variable	Nu	ING4 staining cytoplasm > nucleus			ING4 staining cytoplasm < nucleus		
		With	Without	*P*	Without	With	*P*
Gender							
Male (%)	157	70 (44.6)	87 (55.4)	1.000	139 (88.5)	18 (11.5)	1.000
Female (%)	89	40 (44.9)	49 (55.1)		79 (88.8)	10 (11.2)	
Age							
≤70 (%)	211	94 (44.5)	117 (55.5)	1.000	188 (89.1)	23 (10.9)	0.567
>70 (%)	35	16 (45.7)	19 (54.3)		30 (85.7)	5 (14.3)	
Grade							
I / II (%)	152	62 (40.8)	90 (59.2)	0.084	132 (86.8)	20 (13.2)	0.108
III (%)	71	38 (53.5)	33 (46.5)		67 (94.4)	4 (5.6)	
Histology							
NSCLC (%)	136	110 (45.5)	132 (54.5)	0.130	216 (83.9)	26 (10.7)	0.065
SCLC (%)	2	0 (0.0)	4 (100.0)		2 (50.00)	2 (50.0)	
Stage							
I / II (%)	81	43 (53.1)	38 (46.9)	1.000	75 (92.6)	6 (7.4)	0.246
III (%)	52	27 (51.9)	25 (48.1)		51 (98.1)	1 (1.9)	
Node							
N0 / N1 (%)	81	44 (54.3)	37 (45.7)	0.722	75 (92.6)	6 (7.4)	0.246
N2 (%)	52	26 (50.0)	26 (50.0)		51 (98.1)	1 (1.9)	

Statistical analysis was performed using the chi squared test; a *P*-value <0.05 was considered significant.

Nu, Number; %, frequency.

* *P*-value <0.05.