Reduced expression and novel splice variants of *ING4* in human gastric adenocarcinoma

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Abstract

ING4, a new member of the *ING* (inhibitor of growth) family of tumour suppressor genes, has been found to be deleted or down-regulated in gliomas, breast tumours, and head and neck squamous cell carcinomas. The goal of the present study was to investigate whether the expression and alternative splicing of *ING4* transcripts are involved in the initiation and progression of stomach adenocarcinoma. *ING4* mRNA and protein expression was 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. Alterations in *ING4* transcripts were determined through sequence analysis of *ING4* cDNA. Our 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. We also found that reduced *ING4* mRNA expression correlated with the stage of the tumour. Interestingly, by sequence analysis, we discovered five novel aberrantly spliced variant forms of *ING4 v1* and *ING4 v2*. These variants cause a codon frame-shift and, eventually, deletion of the NLS or PHD domain contributing to the mislocalization of p53 and/or HAT/HDAC complexes and, subsequently, altered gene expression

in gastric adenocarcinoma. These results suggest that attenuated and aberrant *ING4* expression may be involved in the initiation and progression of stomach adenocarcinoma.

Keywords

cancer; stomach; adenocarcinoma; tumour suppressor; mRNA expression; protein expression; splice variants

Introduction

Worldwide, stomach cancer is the third and fifth most common malignancy in males and females, respectively [1], with the highest risk populations in Asian countries [2]. The incidence rate is significantly related to age and is approximately twice as high in males than in females [1]. To aid in the development of a more effective therapy for stomach cancer, it is crucial to elucidate the molecular defects that lead to disease progression.

ING4 is a tumour suppressor protein that has been implicated in apoptosis, cell cycle arrest, gene transcription, DNA repair, and other biological events [3]. In gliomas, a significant decrease in *ING4* mRNA correlates with tumour grade [4]. ING4 protein is low in gliomas but enriched in normal brain tissues [4]. Reduced *ING4* mRNA and allelic loss are further observed in head and neck squamous cell carcinomas [5]. In addition, deletion of the *ING4* locus occurs in breast cancer cell lines and primary breast tumours [6]. Inactivating mutations in *ING4* transcripts are also found in other human cancer cell lines [6]. Furthermore, *ING4* overexpression results in reduced S-phase cells, and p53-dependent apoptosis [7]. Presumably, *ING4* also interacts with NF-κB and inhibits brain tumour angiogenesis by repressing NF-κB-responsive gene transcription [4]. *ING4* also suppresses the loss of contact inhibition induced by MYC [6].

Here we analysed the potential involvement of *ING4* in the development of gastric adenocarcinoma. For the first time, we propose that reduced *ING4* expression may be involved in the initiation and progression of gastric adenocarcinoma. We also give evidence for the existence of novel aberrantly spliced variant forms of *ING4 v1* and *ING4 v2* that possibly alter *ING4* function in gastric adenocarcinoma.

Materials and methods

Tissues and cell lines

All tumour and normal surgical (stomach) or autopsy (brain) specimens used in this study represented excess pathological/normal materials obtained in accordance with procedures approved by the Human Ethics Review Board at the First and Second Affiliated Hospitals of Harbin Medical University (Harbin, China) and Beijing Friendship Hospital, Affiliate of Capital University of Medical Sciences (Beijing, China). Surgical tissues were obtained with written consent from patients. The normal and tumour states of specimens were confirmed by examination of haematoxylin and eosin (H&E)-stained histology sections by pathologists at the same hospitals.

Gastric adenocarcinoma cell lines (poorly differentiated MGC-803 and BGC-823; moderately differentiated SGC-7901; and undifferentiated HGC-27) and human embryonic kidney (HEK) 293 cells were from Shanghai Institute of Cell Biology (Shanghai, China). Cell lines were maintained in RPMI 1640 medium containing 10% fetal calf serum.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated using Trizol (Invitrogen). cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit (Promega). β-actin was used as an internal PCR control. The primers were *ING4* : 5′-ATGGCTGCGGGGATGTATTTGGAAC-3′ and 5′-CTATTTCTTCTTCCGTTCTTGGGAGCAG-3′ [4]; β-actin: 5′-ACTCTTCCAGCCTTCCTTCC-3′ and 5′-CATACTCCTGCTTGCTGATCC-3′. The PCR products were subjected to electrophoresis and bands were visualized using the Alpha Innotech gel documentation system, utilizing a Multilmage™ Light Cabinet. Images were analysed using Alpha Innotech (version 1.2.0.1) software.

Densitometry was performed using the Scion Image program. *ING4* mRNA in tumour and normal specimens was designated as T and N, respectively (calculated as *ING*4/β-actin densitometric values). T/N indicates the relative *ING4* mRNA levels in tumour specimens. Statistical analysis was performed using the *t*-test. A *p* value of less than 0.05 was considered significant.

Real-time RT-PCR

Real-time RT-PCR was performed using Taqman TM′ technology and analysed using an ABI 7700 Sequence Detector (Gene Core Bio Technologies, Shanghai, China). Specific *ING4* primers (5′-CAAGGAATTTGGTGACGACAAG-3′ and 5′-TCCAGCCGCCGAATGT-3′) and *ING4* hybridization probes (FAM-TTTGTCCACCATCTCATAGGTCTGCATG G-TAMRA) were also synthesized by Gene Core Bio Technologies. Normalization was based on β-actin PCR products, which were also quantified by real-time RT-PCR. The real-time RT-PCR results were analysed using the Light Cycler analysis software. Real-time RT-PCR using SYBR Green was performed using a Hot Start Fluorescent PCR Core Reagent Kit (Bio Basic Inc, Markham, Ontario, Canada). Statistical analysis was performed using the *t*-test. A *p* value of less than 0.05 was considered significant.

ING4 transcript analysis

Full-length *ING4* cDNA sequences from nine gastric adenocarcinoma samples were sub-cloned into a pCR 2.1-TOPO vector (Invitrogen), transformed into *E. coli* Top10 competent cells (Invitrogen). Positive clones were selected and sequenced (Invitrogen Biological Engineering, Shanghai, China). At least five clones of each cDNA sample were sequenced.

Tissue microarray (TMA) and immunohistochemistry

The TMA and paired normal and tumour samples were taken from different patients. The TMA samples (80 tumour and 40 normal tissues) were from patients at the Beijing Friendship Hospital. The 40 paired normal and tumour tissues were from patients at the First

and Second Affiliated Hospitals of Harbin Medical University. The paired tissues were snap-frozen in liquid nitrogen immediately following collection and stored at −80 °C.

Tissue sections, including the TMA samples, were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (10 min). Antigen was retrieved by autoclaving in EDTA buffer (2 min). Tissues were then incubated in rabbit serum (20 min) to reduce non-specific staining. *ING4* goat poly-clonal antibody (Abcam) was applied overnight (4 °C). Immunoreactivity was detected using the Streptavidin/Peroxidase Histostain™ -Plus Kit (Zymed) and DAB Kit (Zhongshan Goldenbridge Biotechnology, Beijing, China). Human brain and normal stomach tissues incubated with normal goat IgG were used as positive and negative controls, respectively.

Western blot analysis

Tissues were homogenized (in 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A) and then sonicated, vortexed, and centrifuged at 12 000 g for 10 min at 4 °C. Supernatants were analysed by SDS-PAGE and western blotting using *ING4* (T-15) and β-actin antibodies (Santa Cruz Biotech). Immunoreactive bands were detected using the ECL system (New England Biolabs, Guelph, Ontario, Canada).

Results

ING4 mRNA and protein expression is reduced in gastric adenocarcinoma cell lines and tissues

ING4 expression has previously been analysed in various tumour tissues but not in gastric adenocarcinoma. Initially, we analysed 40 paired tumour and adjacent normal tissues for *ING4* expression by RT-PCR. Figure 1A shows the general pattern of reduced *ING4* mRNA expression in tumour compared with adjacent normal tissues. Reduced *ING4* mRNA expression was found in 75% (30/40) of tumour tissues examined. To confirm these findings, we performed real-time RT-PCR on 13 representative paired samples. As shown in Figure 1B, the average level of *ING4* mRNA in gastric adenocarcinoma tissues was 0.34 ± 0.07, compared with 0.71 ± 0.13 in normal adjacent tissues ($p = 0.014$), and thus, *ING4* levels of the tumour tissues were, on average, about half of those in adjacent normal tissues.

We then sought to determine whether *ING4* mRNA expression correlates with patient gender and age, lymphatic metastasis, tumour stage, and extent of differentiation of the adenocarcinoma. As shown in Figure 1C, the relative *ING4* mRNA level (T/N) in tumour tissues was significantly lower in female than in male patients ($p = 0.049$). The *ING4* mRNA level also correlated with depth of tumour penetration. In other words, *ING4* levels in tumour stages 3 and 4 (T_{3-4}) were significantly lower than those in tumour stages 1 and 2 (T_{1-2}) ($p = 0.045$). Conversely, *ING4* levels in tumour tissues did not correlate with the patient's age, lymphatic metastasis, or extent of differentiation of the adenocarcinoma. Furthermore, we did not observe significant differences in *ING4* levels in the intestinal and diffuse (0.8115 ± 0.09124 versus 0.8478 ± 0.07712, respectively; $p = 0.802$) types of

adenocarcinomas, indicating that *ING4* levels do not correlate with the type of gastric adenocarcinoma.

We then proceeded to examine ING4 protein expression by immunohistochemistry. Of the 40 patients examined, we found weak *ING4* staining (0 to +) in 72.5% (29/40) of the tumour tissues and in 20% (8/40) of the adjacent normal tissues ($p = 0.000$; data not shown). Thus, 52.5% more of the tumour tissues than the normal tissues showed weak *ING4* staining. Generally, we found stronger *ING4* staining in the nucleus than in the cytoplasm, and staining was observed in almost all normal tissues (as in Figure 2A, left panel), while reduced or lack of staining was mostly noted in tumour tissues (as in Figure 2A, right panel). Strong *ING4* staining was also seen in parietal cells (Figure 2A, left panel, arrow).

To support our immunohistochemistry data, we examined representative normal and adenocarcinoma cell lines and tissues by western blotting (Figure 2B). Consistent with our results above, densitometry of blots and analysis of the *ING4*/β-actin ratio (Figure 2C) revealed that stomach adenocarcinoma cell lines and tissues express lower ING4 protein levels compared with normal tissues.

To evaluate ING4 protein expression more thoroughly in gastric adenocarcinoma, we utilized the microarray–immunohistochemistry method to analyse 80 tumour and 40 normal tissues further. Consistent with our RT-PCR/real-time RT-PCR results, Table 1 shows that most of the tumour tissues had negligible or low *ING4* levels. In fact, out of 120 tumour tissues, 99 (82.5%) showed weak *ING4* signals. Conversely, of the 80 normal tissues, 30 (37.5%) showed weak *ING4* signals. Therefore 45% more of the tumour than normal tissues showed reduced *ING4* expression. It is striking that *ING4* was negligible in 43 (35.8%) of the tumour tissues but in only 3 (3.8%) of the normal tissues, suggesting that lack of *ING4* may indeed be involved in the initiation and progression of gastric adenocarcinoma.

We then further examined potential correlations between ING4 protein expression and patient gender or age, and extent of differentiation of the adenocarcinoma. Table 2 shows that about 80% of affected males (26/31) and females (73/89) have reduced *ING4* expression. Among them, however, 54.8% of females have negligible levels of *ING4* as opposed to 29.2% in males. Female patients are therefore almost twice as likely to lack *ING4*. Although a higher percentage of patients 60 years old and younger (48/64) seemed to show reduced *ING4* levels (0 to +) compared with patients older than 60 (31/56), the difference was statistically insignificant. Similarly, differences in *ING4* expression in poorly, moderately, and well-differentiated tumour cells were not statistically significant. Therefore, as with the mRNA levels, ING4 protein levels did not correlate with age or extent of differentiation of the stomach adenocarcinoma. In addition, no significant difference ($p = 0.112$) in ING4 protein levels was observed in the intestinal and diffuse types of stomach adenocarcinomas (data not shown).

ING4 transcript analysis revealed novel spliced variants of *ING4 v1* and *ING4 v2*

Alterations of *ING4* transcripts in gastric adenocarcinoma have not yet been elucidated. We therefore examined all the coding regions of *ING4* for potential alterations at the transcriptional level in nine representative gastric adenocarcinoma tissues and cell lines by

clone sequencing. Our data indicated that *ING4 v1, ING4 v2*, and *ING4 v4*, but not *ING4 v3*, were present in all of the adenocarcinoma samples examined and their sequence integrity was well maintained (Figure 3A). Interestingly, we discovered four novel spliced variants of *ING4 v1* (two insertions and two deletions) (Figures 3B and 3C). We also found a novel deletion in *ING4 v2*. A summary of these alterations, their consequences, and deleted domains are presented in Figures 3B and 4. Specifically, in *ING4 v1*, we found insertion at positions 38–54, reserving the head of the first intron (Figure 3C), and another insertion at positions 392–411, reserving the end of the fourth intron (Figure 3C). These insertions cause a frame-shift, deleting the bipartite nuclear localization signal (NLS) domain and the c-terminal plant homeo-domain (PHD) zinc-finger domain (Figure 4). We further found deletion at positions 501–609 in *ING4 v1* (Figure 3C), deleting the head of the sixth exon, which may cause *ING4* products to lose the PHD zinc-finger domain (Figure 4).

In *ING4 v2*, we found deletion at positions 38–276, deleting the whole second and third exons, which results in truncation of *ING4*, and deletion of both NLS and PHD zinc-finger domains (Figure 4). These four alterations were found singly or in combination (ie 38–54 insertion and 501–609 deletion) in three out of the nine tumour tissues examined. In addition, in MGC803 adenocarcinoma cells, we observed deletion at positions 110–276 in *ING4 v1*, deleting the whole third exon, which results in *ING4* truncation, and subsequently, deletion of both NLS and PHD zinc-finger domains as well (Figure 4). The *ING4* variants were also detected in normal gastric tissues but at considerably lower levels than in tumour tissues (Figure 3D). Up-regulation of *ING4* variants did not correlate with patient clinical parameters. Sequence analysis of ten adenocarcinoma tissues did not show *ING4* gene mutation (data not shown).

Discussion

The tumour suppressor *ING4* has been shown to be deleted or down-regulated in gliomas, breast tumours, and head and neck squamous cell carcinomas [5]. For example, in head and neck squamous cell carcinomas, *ING4* mRNA is reduced in 76% of primary tumours [5]. In glioblastomas, *ING4* levels are up to six times lower than in normal brain tissues [4]. Here, we found that *ING4* mRNA was likewise significantly lower in gastric adenocarcinoma tissues than in normal stomach tissues. Reduced *ING4* mRNA level correlated with depth of penetration of the tumour tissue. Thus, *ING4* levels in advanced tumour stages 3 and 4 (T_{3-4}) are lower than those in earlier tumour stages 1 and 2 (T_{1-2}), which in turn have *ING4* levels lower than those in normal tissues. Therefore, the pattern of *ING4* expression in normal and T_{1-2} and T_{3-4} gastric adenocarcinoma is consistent with that in normal, glioma, and glioblastoma tissues.

Worldwide, males are more predominantly affected than females by adenocarcinoma at any site in the stomach [8]. This has been associated with oestrogen-regulated progression of gastric adenocarcinoma. To some extent, increased lifetime exposure to oestrogen and/or progesterone may protect women from gastric cancer [9]. Indeed, there are α- and β-type oestrogen receptors (ERs) in normal and cancerous gastric mucosa [10]. Supposedly, *ING1b* stimulates oestrogen-induced ER-transcriptional activity in a dose-dependent manner, and this activation is consistent with *ING1b* function in chromatin remodelling [11]. Here, we

found stronger *ING4* staining in parietal cells than in any other cells in the normal gastric tissue. Interestingly, gastric parietal cells can synthesize and secrete oestrogen into the portal vein [12]. We then hypothesized that *ING4* might also be involved in ER signalling that possibly protects women from gastric cancer. However, we found that *ING4* levels in tumour tissues of female patients were significantly lower than those in male patients. Thus, *ING4* may not play a significant role in ER signalling, which seems to reduce gastric cancer incidence in women. Furthermore, it appears that *ING4* is not responsible for the higher incidence of male gastric adenocarcinoma.

Inactivating mutations in *ING4* transcripts have been found in various human cancer cell lines. These mutations were localized in the NLS and PHD finger domains, or cause the loss of NLS and PHD finger domains [6]. The PHD zinc-finger domain, a functionally conserved structural domain of *ING4*, can interact with histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes, which are involved in gene transcriptional regulation [13]. Conversely, the NLS domain is necessary for the effect of *ING4* on p53-mediated apoptosis [14]. Certainly, overexpression of *ING4* causes a decreased population of S-phase cells, and p53-dependent apoptosis associated with p21 transcriptional up-regulation [7]. However, *ING4* also participates in apoptosis induced by the *RUNX3* tumour suppressor in MKN-1 gastric adenocarcinoma cells [15]. Therefore, in addition to its tumour-suppressing activity, *ING4* may cooperate with other tumour suppressor proteins to induce tumour cell apoptosis in gastric cancer.

ING4, which has been mapped to chromosome 12p13.31, possesses hybridization deletion at the *ING4* locus in 10–20% of breast cancer cell lines and primary breast tumours, and in 66% of head and neck squamous cell carcinomas [5,6]. Deletion and missense mutations in *ING4* transcripts have also been detected in several tumour cell lines [6], with the most frequent mutation identified as *ING4 v4*, a variant that is also detectable in normal cell lines and tissues [4,16,17]. Initial studies showed the presence of both *ING4 v1* (*ING4*) and *ING4 v4* in the T47D breast cancer cell line [16], but the four identified variants, *ING4 v1, ING4 v2, ING4 v3*, and *ING4 v4*, are also present in both normal and tumour tissues, and the percentages of each variant are similar in adult normal tissues [17,18]. In separate studies, the splice variants, *ING4 v2, ING4 v3*, and *ING4 v4*, which lack the full NLS domain, were shown to have attenuated *ING4* function [17]. These variants were localized in the cytoplasm, supporting the important role of NLS in nuclear localization of *ING4* [17].

Here, we found that *ING4 v1, ING4 v2*, and *ING4 v4* were present in stomach adenocarcinoma tissues and that their sequence integrity was maintained (Figure 3A). However, we also discovered four novel *ING4 v1* and *ING4 v2* spliced variants (insertion and/or deletion) in these tissues. In *ING4 v1*, we found insertions at positions 38–54 and 392–411, reserving the head of the first intron and the end of the fourth intron, respectively. These insertions cause a frame-shift, deleting the bipartite NLS domain and the c-terminal PHD zinc-finger domain. *ING4 v1* deletion at positions 110–276 and 501–609 deletes the third exon and the head of the sixth exon, respectively, causing the *ING4* products to lose the PHD zinc-finger domain. We also found *ING4 v2* deletion at positions 38–276, deleting the whole second and third exons. This results in truncated *ING4* products with deleted NLS and PHD zinc-finger domains. The fact that these alterations result in frame-shift, stop

translation, and, eventually, NLS domain (which targets p53) or PHD domain (which targets HAT/HDAC complexes and subsequently regulates gene transcription) deletion suggests that these *ING4* variants could contribute to p53 mislocalization and/or HAT/HDAC complexes, and subsequently, altered gene expression in stomach adenocarcinoma.

In separate studies, newly identified *ING4* variants, designated as *ING4*-DEx2, -DEx3, and -DEx6A and -DEx6B, were found to lack exons 2, 3, and 6 (entirely or in part), respectively [16]. All variants were detected in normal human thyroid, brain, and lung tissues but *ING4*-DEx2 and -DEx6B were also detected in tumour tissues. Aberrant alternative splicing caused by such mutations, which create or disrupt splice sites, correlates with loss of tumour suppressor activity [19]. Potentially, the novel spliced *ING4 v1* and *ING4 v2* variants in gastric adenocarcinoma result from *ING4* mutations that subsequently cause altered *ING4* function. It appears that reduced *ING4* expression combined with attenuated *ING4* function due to insertions and/or deletions promotes the initiation and progression of gastric adenocarcinoma.

Acknowledgments

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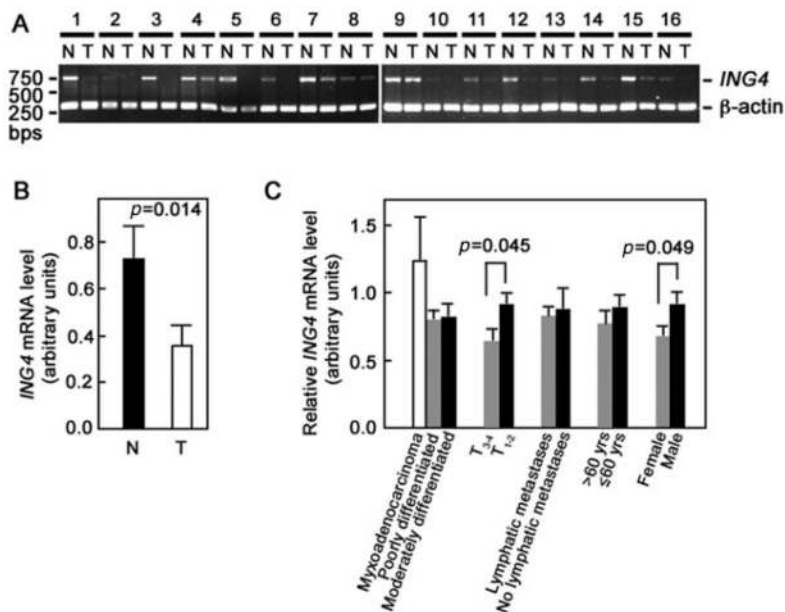
Figure 1.
ING4 mRNA is significantly reduced in gastric adenocarcinoma tissues compared with normal tissues. (A) Forty paired tumour and adjacent normal tissues were analysed for *ING4* expression by RT-PCR. Two gels showing 16 representative paired tumour (T) and normal (N) tissues are shown. β-actin was used as internal loading control. (B) Average *ING4* mRNA levels in 13 randomly picked paired tumour (T) and normal (N) samples were determined by real-time RT-PCR. (C) Analysis of relative *ING4* mRNA level (T/N) according to gender (27 males, 13 females), age of patient ($\leq$60 years, $n = 23$; $>$60 years, $n = 17$), lymphatic metastasis (lymphatic metastasis, $n = 13$; no lymphatic metastasis, $n = 27$), tumour stage (T_{1-2}, $n = 29$; T_{3-4}, $n = 11$), and extent of differentiation of the gastric adenocarcinoma (moderately differentiated, $n = 10$; poorly differentiated, $n = 26$; myxoadenocarcinoma, $n = 4$). Densitometry of PCR products was performed to analyse *ING4* levels. Statistical analysis was performed by the *t*-test. *p* value <0.05 was considered significant
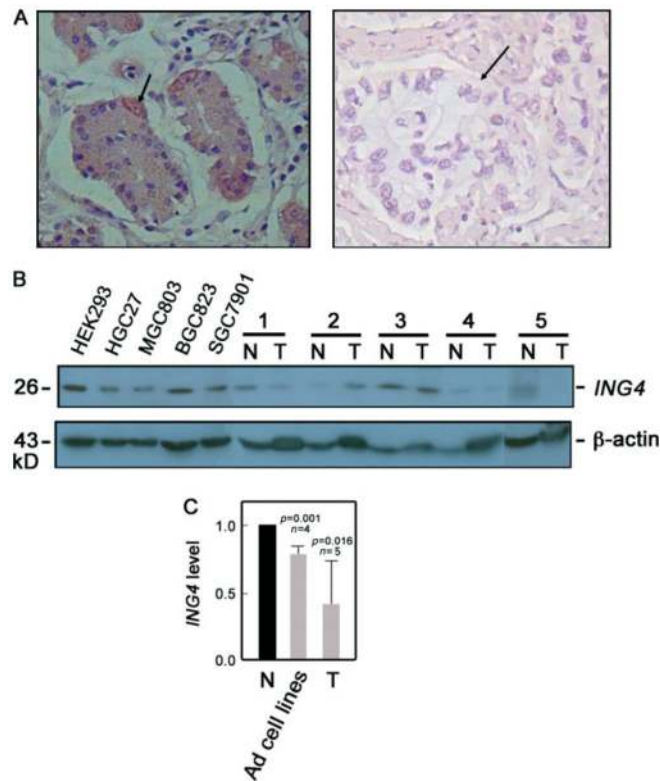
Figure 2.
ING4 protein expression is reduced in gastric adenocarcinoma cell lines and tissues. (A) *ING4* expression in representative paired tumour (right panel) and normal adjacent (left panel) tissues was analysed by immunohistochemistry. Original magnification: 400 ×. Arrows indicate a stomach parietal cell (left panel) and a stomach adenocarcinoma nest (right panel). (B) Representative adenocarcinoma cell lines (HGC27, MGC803, BGC823, and SGC7901) and paired tumour (T) and normal (N) adjacent tissues ($n = 5$) were examined for ING4 protein expression by western blot analysis. HEK293 cells served as a positive control. β-actin blot was used for internal loading control. The 29 kD ING4 band detected in gastric adenocarcinoma tissues and cell lines is consistent with the possibility that the epitope recognized by the *ING4* (T-15) antibody lies in an internal region of *ING4 v1* and *ING4 v2* but is absent or masked in the aberrantly spliced variant forms. (C) Analysis of the *ING4/β*-actin ratio following densitometric analysis of *ING4* immunoblot. Values of normal tissues were normalized to 1. Statistical analysis was performed by the *t*-test tissue
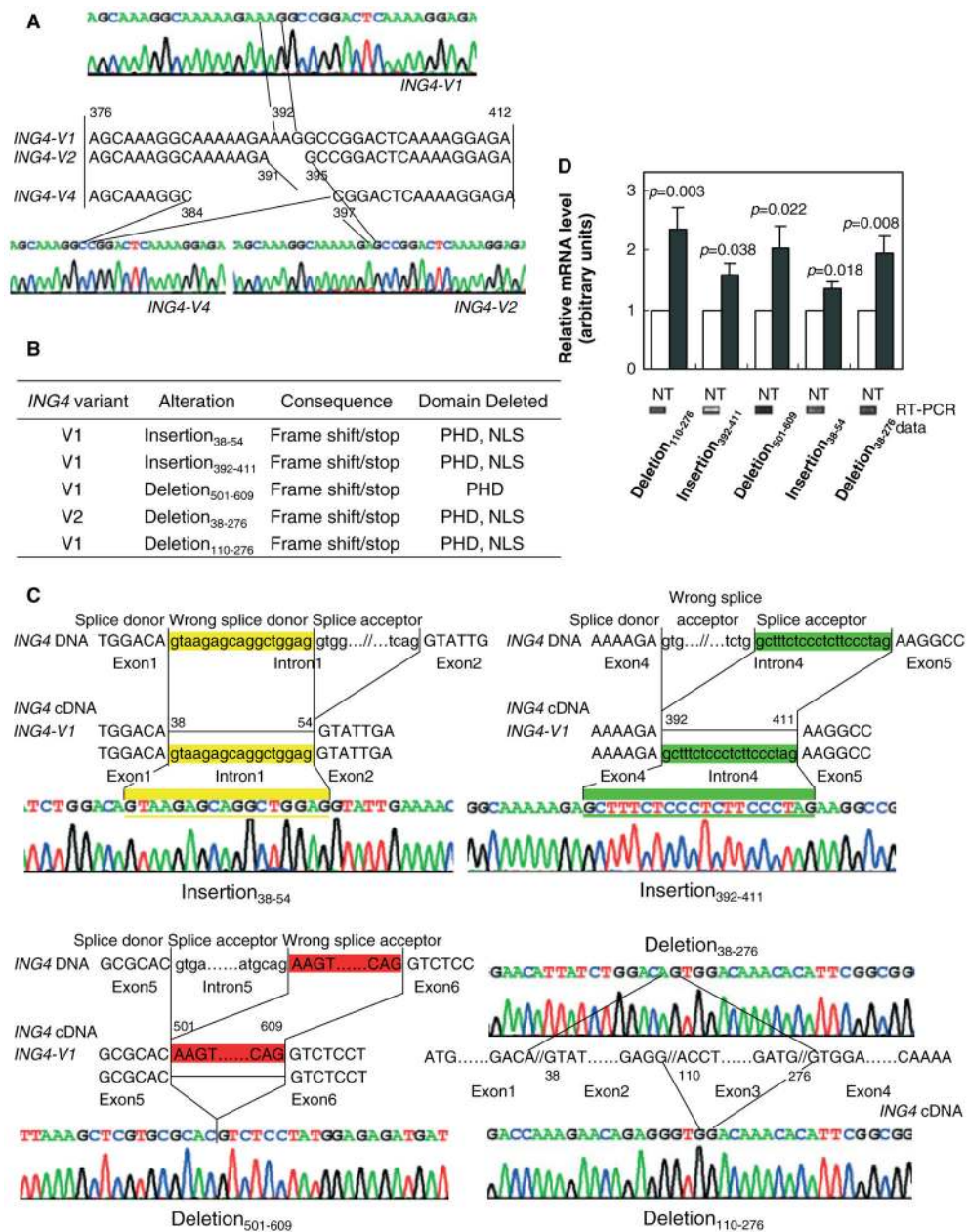
Figure 3.
Five novel aberrantly spliced variant forms of *ING4 v1* and *ING4 v2* were found in gastric adenocarcinoma tissues. (A) *ING4 v1*, *ING4 v2*, and *ING4 v4* (but not *ING4 v3*) were detected in stomach adenocarcinoma cell lines and tissues. The partial sequence maps of *ING4 v1*, *v2* and *v4*, and cDNA sequences of the variable region (exon 4) of the three *ING4*-spliced variants are shown. (B) List of the five novel *ING4 v1* and *ING4 v2* insertions and/or deletions. PHD and NLS stand for the c-terminal plant homeo-domain zinc-finger domain and the bipartite nuclear localization signal domain, respectively. (C) Schematic representations of 38–54 (yellow; upper left panel) and 392–411 (green; upper right panel) insertions, and 501–609 (red; lower left panel) deletion in *ING4 v1*. Upper- and lowercase

letters represent exon and intron sequence*s* of *ING4* genomic DNA, respectively. Splice donor, wrong splice donor causing the alteration, and splice acceptor are noted. *ING4 v1* and *ING4 v2* sequences are also shown. Schematic representations of deletion (38–276) of *ING4 v2* and deletion (110–276) of *ING4 v1* (lower right panel). Sequence maps of *ING4* exons 1, 2, 3, and 4 indicate that stomach adenocarcinoma tissues harbour deletion of nucleotides 38–276 which include the second exon and the third exon of *ING4 v2*, and deletion of nucleotides 110–276 which include the third exon of *ING4 v1* in the stomach adenocarcinoma cell line MGC-803. (D) The mRNA levels of the *ING4* variants in normal (N) and tumour (T) tissues. Deletion 110–276 (T, $n = 15$; N, $n = 13$). Insertion 392–411 (T, $n = 8$; N, $n = 11$). Deletion 501–609 (T, $n = 12$; N, $n = 11$). Insertion 38–54(N, $n = 7$; N, $n = 7$). Deletion 38–276 (N, $n = 10$; T, $n = 9$). The values for normal tissues were normalized to 1. Representative RT-PCR gels for normal tissues are also shown

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         1                                                              50
ING4_v1  MAAGMYLEHY LDSIENLPFE LQRNFQLMRD LDQRTEDLKA EIDKLATEYM
Ins₃₈-₅₄   MAAGMYLEHY LDSksrlevl ktfplnyret fss
Ins₃₉₂-₄₁₁ MAAGMYLEHY LDSIENLPFE LQRNFQLMRD LDQRTEDLKA EIDKLATEYM
Del₁₁₀-₂₇₆ MAAGMYLEHY LDSIENLPFE LQRNFQLMRD LDQRTEgqqt hsaaghrpgp
Del₅₀₁-₆₀₉ MAAGMYLEHY LDSIENLPFE LQRNFQLMRD LDQRTEDLKA EIDKLATEYM

         51                                                            100
ING4_v1  SSARSLSSEE KLALLKQIQE AYGKCKEFGD DKVQLAMQTY EMVDKHIRRL
Ins₃₈-₅₄
Ins₃₉₂-₄₁₁ SSARSLSSEE KLALLKQIQE AYGKCKEFGD DKVQLAMQTY EMVDKHIRRL
Del₁₁₀-₂₇₆ f
Del₅₀₁-₆₀₉ SSARSLSSEE KLALLKQIQE AYGKCKEFGD DKVQLAMQTY EMVDKHIRRL

         101                                                           150
ING4_v1  DTDLARFEAD LKEKQIESSD YDSSSSKGKK KGRTQKEKKA ARARSKGKNS
Ins₃₈-₅₄
Ins₃₉₂-₄₁₁ DTDLARFEAD LKEKQIESSD YDSSSSKGKK sflplp
Del₁₁₀-₂₇₆
Del₅₀₁-₆₀₉ DTDLARFEAD LKEKQIESSD YDSSSSKGKK KGRTQKEKKA ARARSKGKNS

         151                                                           200
ING4_v1  DEEAPKTAQK KLKLVRTSPE YGMPSVTFGS VHPSDVLDMP VDPNEPTYCL
Ins₃₈-₅₄
Ins₃₉₂-₄₁₁
Del₁₁₀-₂₇₆
Del₅₀₁-₆₀₉ DEEAPKTAQK KLKLVRTSPm er

         201                                                           248
ING4_v1  CHQVSYGEMI GCDNPDCSIE WFHFACVGLT TKPRGKWFCP RCSQERKKK
Ins₃₈-₅₄
Ins₃₉₂-₄₁₁
Del₁₁₀-₂₇₆
Del₅₀₁-₆₀₉
```

B

```
         1                                                              50
ING4_v2  MAAGMYLEHY LDSIENLPFE LQRNFQLMRD LDQRTEDLKA EIDKLATEYM
Del₃₈-₂₇₆  MAAGMYLEHY LDSgqthsaa ghrpgpf

         51                                                            100
ING4_v2  SSARSLSSEE KLALLKQIQE AYGKCKEFGD DKVQLAMQTY EMVDKHIRRL
Del₃₈-₂₇₆

         101                                                           150
ING4_v2  DTDLARFEAD LKEKQIESSD YDSSSSKGKK SRTQKEKKAA RARSKGKNSD
Del₃₈-₂₇₆

         151                                                           200
ING4_v2  EEAPKTAQKK LKLVRTSPEY GMPSVTFGSV HPSDVLDMPV DPNEPTYCLC
Del₃₈-₂₇₆

         201                                                           248
ING4_v2  HQVSYGEMIG CDNPDCSIEW FHFACVGLTT KPRGKWFCPR CSQERKKK
Del₃₈-₂₇₆
```

C

Figure 4.
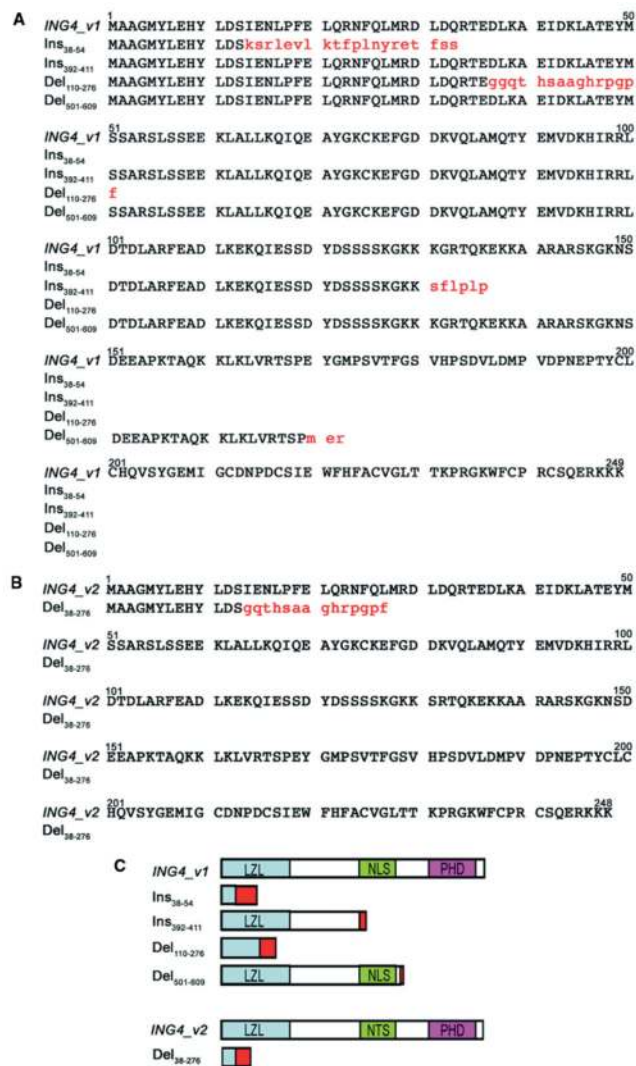Amino acid sequence alignment of the aberrantly spliced variant forms of *ING4 v1* (A) and *ING4 v2* (B) found in gastric adenocarcinoma tissues. Uppercase letters represent sequence identity, while lowercase letters in red indicate sequence variations due to a frame-shift in translation. (C) Diagrammatic representation of the aberrantly spliced variant forms of *ING4 v1* and *ING4 v2*. Different conserved domains are indicated by different coloured boxes, including a leucine zipper-like motif (LZL); the nuclear localization sequence (NLS); and the plant homeo-domain (PHD). The red box indicates the region where sequence variations occur due to a frame-shift in translation

Table 1

Analysis of *ING4* expression in normal stomach and gastric adenocarcinoma tissues

| Tissues | N | *ING4* | | | | *p* |
		0	**+**	**++**	**+++**	
Tumour	120	43	56	20	1	0.000 [*][†]
No tumour	80	3	27	29	21	

[*]
p < 0.05.

[†]
p values were calculated by the Mann–Whitney test.

Table 2

ING4 expression by gender, age, and extent of gastric adenocarcinoma differentiation by TMA analysis (*n* = 120)

Variable		N	*ING4*				*p*
			0	**+**	**++**	**+++**	
Gender	Male	31	17	9	5	0	0.043 *†
	Female	89	26	47	15	1	
Age, years	≤60	64	20	28	8	0	0.689†
	>60	56	11	20	4	3	
Differentiation	Poorly differentiated	66	29	30	6	1	0.125‡
	Moderately differentiated	37	11	17	9	0	
	Well differentiated	13	3	8	2	0	

*
p < 0.05.

†
p values were calculated by the Mann–Whitney test.

‡
p values were calculated by the Kruskal–Wallis test.