



mRNA and protein of p33ING1 in normal and cancer tissues

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Background: Inhibitor growth protein 1 (ING1) is a tumor suppressor, and its down-regulation is involved in the progression and aggressive phenotypes of human malignancies through its interactions with the H3K4me3 and p53.

Methods: We collected datasets to analyze the relationship between ING1b mRNA expression and accumulative survival rate, and carried out immunohistochemistry analyses to determine the expression profiles of the p33ING1 protein on the mouse, normal human, and human cancer tissue microarrays.

Results: Compared with normal tissues, the ING1b mRNA was highly expressed in various types of cancer tissues, including, colorectal, lung, and breast cancers, and was positively correlated with the overall survival rate of gastric cancer patients. In mouse tissues, the subcellular location of p33ING1 was frequently nuclear; however, it was occasionally cytoplasmic or nucleocytoplasmic. There was a positive detection in the neuron body, a part of glial cells, the glandular epithelium of the stomach, intestines, breast, hepatocytes, heart, skeletal muscle cells, the bronchial and alveolar epithelium, and nephric tubules. In human tissues, the p33ING1 protein, apart from its cytoplasmic distribution, was distributed in the nuclei of the tongue, esophagus, stomach, intestine, lung, trachea, skin, appendix, cervix, endometrium, ovary, and breast. p33ING1 immunoreactivity was strongly detected in the stomach, trachea, skin, cervix, and breast, while it was weak in the other tissues. The positive rate of p33ING1 was 41.0% in the tested cancer entities (489/1,194). In general, p33ING1 expression was restricted to only the cytoplasm for all cancers, whereas it was found in the nucleus of renal clear cells, ovarian and colorectal cancers. Among them, p33ING1 was expressed in more than half of squamous cell carcinomas derived from the esophagus and cervix, while it was rarely expressed in hepatocellular (21.0%) and renal clear cell carcinoma (19.4%).

Conclusions: The findings suggest that p33ING1 might be participated in the repair and regeneration of organs or tissues the repair and regeneration of organs or tissue, and the carcinogenesis of the highly proliferative epithelium.

Keywords: Inhibitor growth protein 1 (ING1); expression profile; immunohistochemistry; bioinformatics; mouse; human; cancer

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Introduction

The inhibitor of growth (ING) family, class II tumor suppressors, consists of 5 members, and function as the reader and writer of the histones epigenetic code, regulating DNA damage repair, chromatin remodeling, cellular

senescence, the cell cycle, apoptosis, and cell differentiation. The human *ING1* gene has 4 exons (1a, 1b, 1c, and 2) and 2 introns. In the literature, it has been reported that there are multiple alternatively spliced transcript variants encoding distinct isoforms from 3 different promoter

regions, which include the p24ING1c (Isoform B: 210aa and E:262aa), p33ING1b (Isoform A:279aa), p47ING1a (Isoform D:422aa), and ING1d (Isoform C: 235aa), which share an identical C terminus with the conserved plant homeodomain (PHD) finger motif (1,2). In recent years, many researchers have become interested in p33ING1b because of its function as a tumor suppressor.

The p33ING1 protein has nuclear localization sequences (NLS, aa142-192) and physically interacts with p53 in the nucleus, whose complex is speculated to bind to Bax and p21 promoter and up-regulate their expression (3). p33ING1 interacts with proliferating cell nuclear antigens (PCNA) via the PCNA-binding protein domain to maximally induce apoptosis in a stress-induced manner (4). The cellular senescence-inhibited gene (CSIG) protein was identified as a binding partner for p33ING1 in the nucleolus (5). The p33ING1 protein binds to and regulates the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) chromatin-remodeling complexes, which are responsible for the modulation of gene expressions in response to a variety of stresses, like angiopoietin (6,7). In contrast, p33ING1 interacts with members of the 14-3-3 family through Ser199 phosphorylation, resulting in its nucleus to plasmic translocalization, such as in mitochondria where the ING1 protein colocalizes and interacts with Bax to induce apoptosis (8). The tyrosine kinase Src can physically associate with phosphorylating ING1 (tyrosine 55 and 212), causes nuclear-to-cytoplasmic relocation of ING1 and reduces stability of ING1 (9).

ING1 mRNA was found to be universally expressed in various human tissues as 2 major bands at 2.2 and 2.5 kb by Northern blot (10). Most tissues were found to show various degrees of expression of *ING1* band p33ING1c, but not *ING1a* (11). Coles *et al.* (12) found that *ING1* deletion in mice reveals a p53-independent role of *ING1* for the suppression of cell proliferation and tumorigenesis, and apoptotic induction by up-regulating Bax expression. Kichina *et al.* (13) reported that mouse *ING1* deletion results in decreased body size, hypersensitivity to radiation, and increased lymphomas incidence rates. A significant decrease in *ING1* expression was also observed in gastric (14) and hepatocellular carcinoma (15), esophageal squamous cell carcinoma (16) tissues, when it was compared with the corresponding normal tissues. Rare mutations of *ING1* in these tumors suggest that other mechanisms may be contributed to down-regulating *ING1* expression in colorectal (17) and breast (18) cancers, head neck squamous cell carcinoma (19), lung cancer (20), basal cell carcinoma (21),

and brain tumors (22).

Identification of the normal tissue or cell types that express p33ING1 would contribute to the clarification of its physiological functions, while the observation of its expression patterns and heterogeneity between tumor cases will benefit targeted gene therapies and the establishment of an animal model of conditional *p33ING1* knockout. In the present study, the intermittent microwave irradiation was employed for immunohistochemistry of p33ING1, during which microwaving causes minute vibrations of more than 2.4 billion times/s, and enhances specificity (23). The protein expression of p33ING1 has been detected in normal mouse and human tissues, and human cancer tissues. Additionally, *ING1b* mRNA was investigated using the TCGA and Kaplan-Meier databases.

Methods

Specimen and tissue microarray

Three male and female C57BL/6 mice (8 weeks old) were sacrificed under sodium pentobarbital anesthesia, and the brain, lung, heart, breast, stomach, liver, spleen, kidney, and intestine were collected. Ten percent neutral formalin soaked all tissues for 48 hours. Then all tissues were embedded in paraffin, and cut into 4 μ m sections. The human normal and cancer tissue arrays were purchased from Shanghai Outdo Biotech (Co., Ltd., Shanghai, China). The human normal tissues included cerebellum, brain stem, tongue, heart, lung, aorta, thyroid, esophagus, stomach, intestine, pancreas, liver, trachea, appendix, smooth muscle, skeletal muscle, testis, bladder, and prostate; and the cancer tissues included 62 renal clear cell carcinomas, 62 hepatocellular carcinomas, 45 esophageal squamous cell carcinomas, 62 pancreatic carcinomas, 31 cervical squamous cell carcinomas. The human breast, cervix, endometrium, and ovary tissues were sampled from surgical samples in our hospital. One hundred and ninety-six gastric cancer, 96 colorectal cancer, 208 ovarian cancer, 96 endometrial cancer, 144 breast cancer and 192 lung cancer were collected from our hospital. Tissues were subjected to the performance of tissue microarray using Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan). The cancer patients did not receive chemotherapy, radiation therapy, or adjuvant therapy before the operation. The patients or their relatives provided written consent for the use of tumor tissue for clinical research, and the research protocol was approved by the Ethical and Animal Experimentation Committees of Shengjing Hospital of China Medical University.

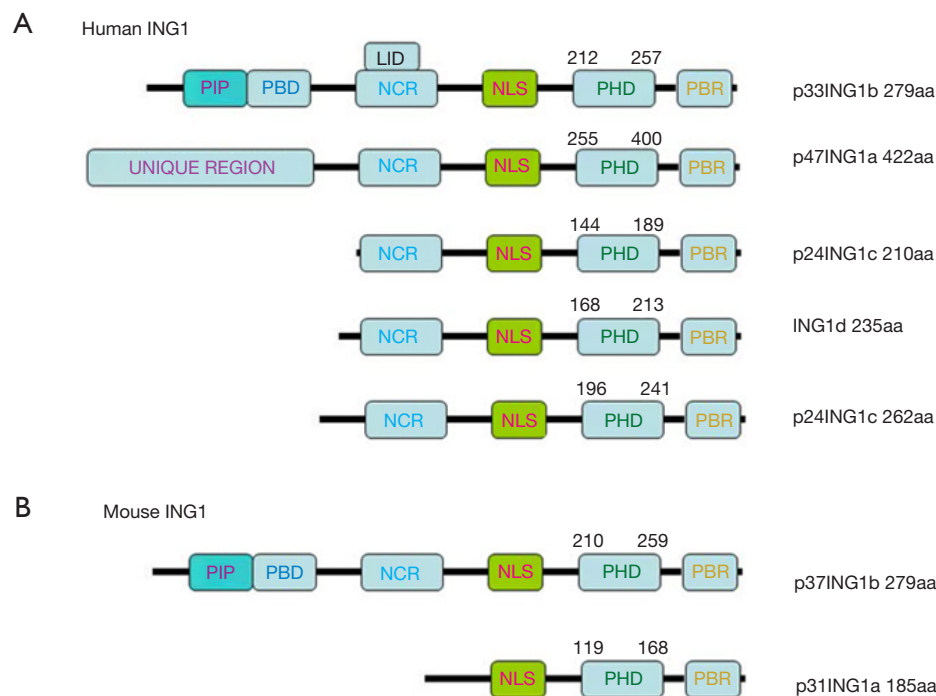


Figure 1 The schematic diagram of human and mouse ING1 protein structure. (A) Human ING1 protein; (B) mouse ING1 protein. ING1, inhibitor growth protein 1.

Immunohistochemistry

Consecutive sections were dewaxed with xylene and rehydrated with alcohol. Sections were then soaked in target retrieval solution (TRS, Dako, USA) with a microwave oven for 20 minutes (Oriental Rotor Ltd. Co., Tokyo, Japan). Sections were blocked in 5% bovine serum albumin for 30 minutes and incubated with mouse anti-p33ING1 antibody (Cab3, sc-21728, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 15 minutes, followed by incubation with anti-mouse secondary antibody conjugated to horseradish peroxidase (ready for use, Dako, USA) for 15 minutes. All incubations were put into a microwave oven to allow intermittent irradiation (23). The slides were washed with TBST (3×1 minute) after each treatment. The p33ING1 protein was visualized using 3, 3'-diaminobenzidine. After being counterstained with Mayer's hematoxylin, the sections were dehydrated, cleared, and mounted. Instead of the primary antibody, normal mouse IgG as a negative control.

Immunostaining evaluation

As indicated in *Figures 1-3*, p33ING1 protein was localized

to the cytoplasm and/or nuclei. Firstly, we (Zhao S and Zheng HC) selected the strong expression field under the low magnification, and randomly counted 100 cells from 5 different representative fields of each section. Secondly, the inconsistent data were confirmed by both persons. The percentages of counted cells were scored as follows: 0–10%, negative (-); 11–100%, positive (+).

Database analysis

p33ING1 mRNA expression and prognostic significance were analyzed in the Oncomine (www.oncomine.org) and Kaplan-Meier plotter (<http://kmplot.com>) databases.

Results

The schematic diagram of human and mouse ING1 protein structure

As *Figure 1A* shows the humanING1 protein includes 5 isoforms: p33ING1b (279aa), p47ING1a (422aa), p24ING1c (210aa and 262aa), and ING1d (235aa), which have an identical C terminus with a conserved PHD finger motif. The mouse ING1 protein includes 2 isoforms:

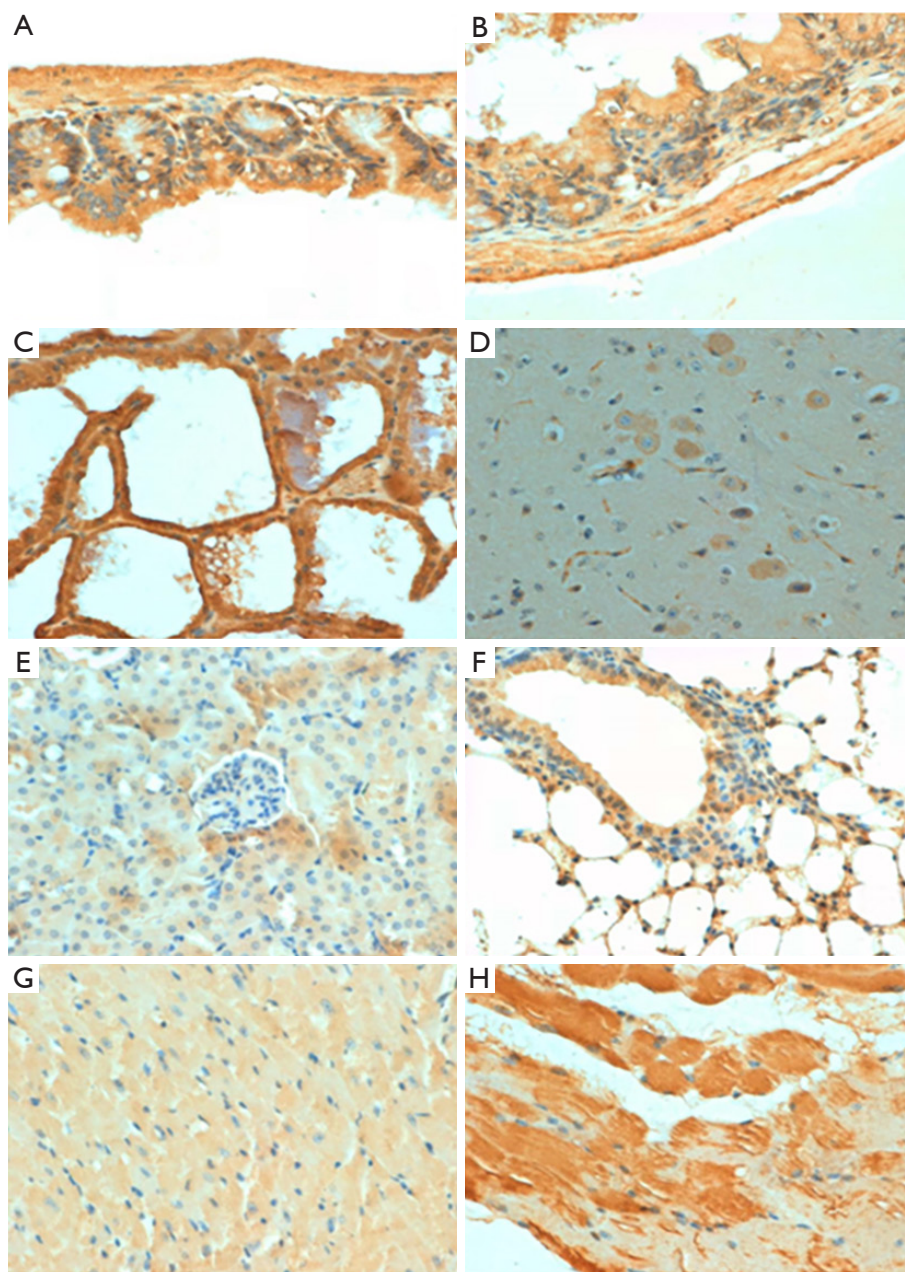


Figure 2 Immunohistochemical reactivity of normal mouse tissues with anti-p33ING1. (A) stomach; (B) intestine; (C) breast; (D) brain; (E) liver; (F) lung and bronchi; (G) heart muscle; (H) striated muscle. All the pictures were captured by a 200× microscope.

p37ING1b (279aa) and p31ING1a (185aa) (Figure 1B).

p33ING1b protein expression in normal mouse tissues

As Figure 2 shows, the subcellular location of p33ING1 was often nuclear but occasionally cytoplasmic or nucleus to plasmic in the mouse tissues with either a sporadic

or local pattern, although expression levels differed among tissues and cell populations. The distribution of p33ING1 expression is summarized in Table 1. p33ING1 protein was sporadically localized in the skin and spleen. In other organs, it was positively detected in the neuron body, part of the glial cells, the glandular epithelium of the stomach, the intestine and breast, hepatocytes, the heart and

Table 1 p33ING1 expression in normal mouse tissues

Cell types	Tissue
Glandular epithelium	Stomach, intestine, breast, lung
Neurons cells	Brain
Glial cells	Brain
Cardiocytes	Heart
Nephric tubules	Kidney (weak)
Striated muscle cells	Muscle
Hepatocytes	Liver (weak)
Sporadic	Spleen, skin

striated muscle cells, the bronchial and alveolar epithelium, and nephric tubules.

p33ING1b protein expression in normal human tissues

In human tissues, p33ING1 protein was detected in the nucleus of the lung, tongue, esophagus, stomach, intestine, trachea, skin, appendix, breast, cervix, endometrium, and ovary (*Figure 3*) although its cytoplasmic localization was observed in all tissues. p33ING1 immunoreactivity was strongly detected in the stomach, trachea, skin, cervix, and breast, while it was weakly expressed in the cerebrum,

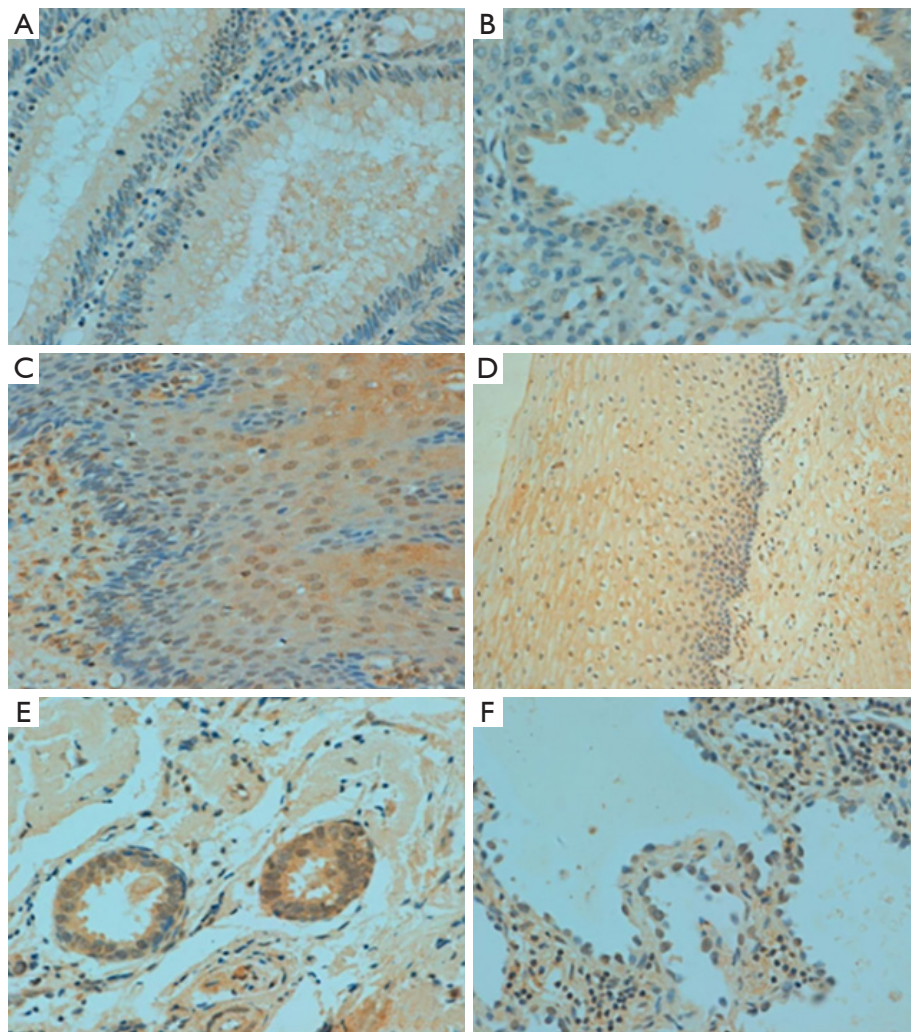


Figure 3 Immunohistochemical reactivity of normal human tissues with anti-p33ING1. (A) Intestine; (B) endometrium; (C) esophagus; (D) cervix; (E) breast; (F) lung and bronchi; (G) heart. All the pictures were captured by a 200× microscope.

Table 2 p33ING1 expression in normal human tissues

p33ING1 expression	Tissue
Nucleus	–
Cytoplasm	Prostate, bladder, testis, heart, skeletal muscle, smooth muscle, pancreas, liver, thyroid, aorta, brain stem, cerebellum, cerebrum
Nucleus and cytoplasm	Breast, ovary, endometrium, cervix, appendix, trachea, lung, intestine, stomach, esophagus, tongue

cerebellum, brain stem, thymus, thyroid, pancreas, skeletal muscle, testis, and bladder (Table 2).

***p33ING1b* protein expression in human cancer tissues**

p33ING1-positive specimens were found in 489 of 1,194 evaluated cancer entities (41.0%), with homogeneity in the expression pattern (Figure 4 and Table 3). In general, p33ING1 expression was found to be restricted to the cytoplasm of all cancers, and occasionally in the nucleus of some cancer tissues, like in renal clear cell carcinoma, ovarian carcinoma, and colorectal carcinoma. p33ING1 was expressed in more than half of squamous cell carcinomas derived from the esophagus and cervix, although the highest rate was detected in breast cancer (57.6%, 83/144). p33ING1-positive cases were rare in hepatocellular carcinoma (21.0%, 13/62) and renal clear cell carcinoma (19.4%, 12/62).

***ING1b* mRNA expression in human cancers tissues**

In the TCGA data, *ING1b* mRNA expression was higher in colorectal cancer, lung cancer, breast cancer than in the normal tissues (Figure 5A,B,C, $P < 0.05$). But *ING1b* mRNA expression was no significant difference between gastric cancer and gastric normal tissues (Figure 5D, $P > 0.05$). *ING1b* mRNA expression was also no significant difference between liver cancer and liver normal tissues (Figure 5E, $P > 0.05$).

The relationship between ING1b mRNA expression and accumulated survival rate in human tumors

In the Kaplan-Meier plotter data, *ING1b* mRNA was positively correlated with the overall survival rates in gastric and lung cancer patients (Figure 6A,B, $P = 0.0036$). There was no correlation between *ING1b* mRNA expression in liver cancer (Figure 6C, $P = 0.061$). However, there was a lack of survival data for colorectal cancer. In addition, *ING1b* mRNA was positively related to progression-free survival rates in breast cancer patients (Figure 6D, $P = 0.061$).

Conclusions

In a study by Nouman *et al.* (24), two monoclonal antibodies were produced by a standard murine hybridoma technique, including MAb GN1 recognizing p33ING1bN-terminal (aa 1–32) and GN2 recognizing its C-terminal (aa 33–279). The immunostaining results showed that the immunoreactivity of GN1 was principally distributed to the nucleus and cytoplasm, although MAb GN2 only demonstrated cytoplasmic positivity. The nuclear localization signal (NLS) of ING1 and phosphorylation-dependent sites for nucleus to plasmic translocation were localized in the C-terminal. In combination with a rare mutation of ING1, we believe that both antibodies recognize different epitopes, which are distinctly exposed to different antigen retrieval solutions or approaches. In the present study, we examined a wide range of tissue samples and found that the positive signal of p33ING1b was detected in the cytoplasm of normal mouse and human tissues and human cancer tissues, but haphazard in both the cytoplasm and nucleus, which is in consistent with the study mentioned above (24). The discrepancy might be attributed to the different antibody and immunostaining methods. Also, we speculated that Santa Cruz p33ING1b might react with the C-terminal of p33ING1b. Finally, we think that the non-specific reaction of antigen-antibody might give another explanation for the differences between these findings.

Due to the amino acid sequence alignment being revealed to have a high degree of similarity between mouse ING1b and human ING1b since they are 89% identity (2), we also did not find differences in patterns of p33ING1 expression between mouse and human tissues, except for the subcellular location in several cell types. In human tissues, p33ING1b protein was strongly detected in the stomach, trachea, skin, cervix, and breast, but weakly in the cerebrum, cerebellum, brain stem, thymus, thyroid, pancreas, skeletal muscle, testis, and bladder, indicating the functional involvement of p33ING1b in specific cell types and a specific functional status. Subsequently, we conditionally

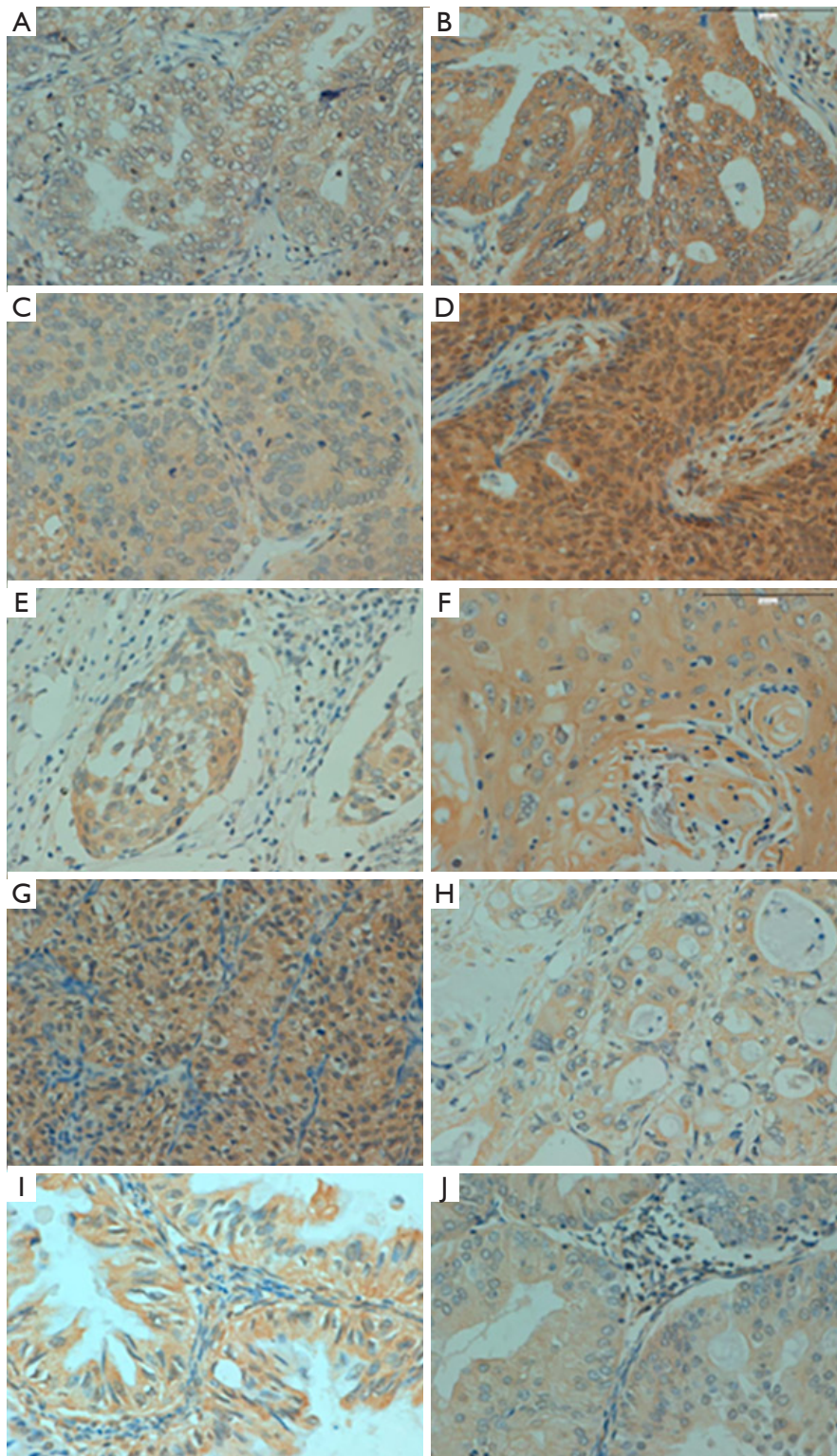
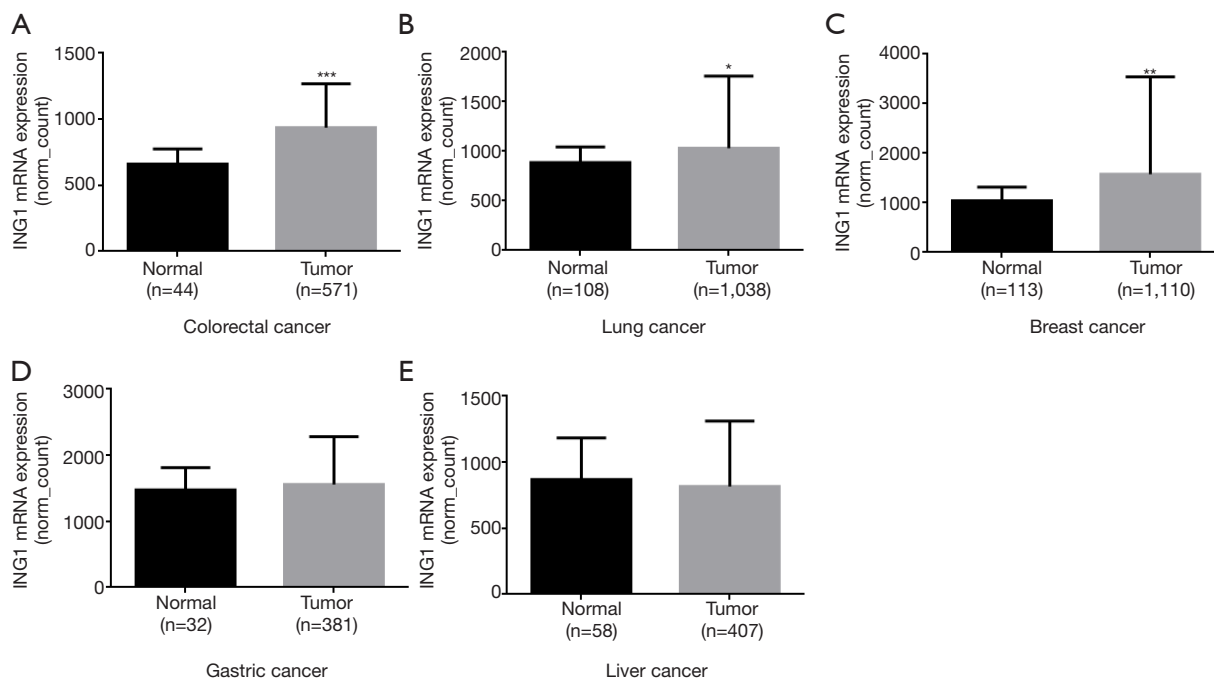


Figure 4 p33ING1 protein expression in human cancer by immunohistochemistry. (A) Gastric carcinoma; (B) colorectal carcinoma; (C) hepatocellular carcinoma; (D) breast carcinoma; (E) cervical carcinoma; (F) esophageal carcinoma; (G) renal carcinoma; (H) pancreatic carcinoma; (I) ovarian carcinoma; (J) endometrial carcinoma. All the pictures were captured by a 200× microscope.

Table 3 p33ING1 expression in various human carcinomas

Cancer type	Total cases	Positive cases	Positive rate (%)	p33ING1 expression	
				Nucleus	Cytoplasm
Hepatocellular carcinoma	62	13	21.0	-	+
Renal clear cell carcinoma	62	12	19.4	+	+
Pancreatic carcinoma	62	24	38.7	-	+
Esophageal carcinoma	45	23	51.1	-	+
Cervical carcinoma	31	17	54.8	-	+
Breast carcinoma	144	83	57.6	+	+
Gastric carcinoma	196	57	29.1	-	+
Colorectal carcinoma	96	51	53.1	+	+
Ovarian carcinoma	208	96	46.2	+	+
Endometrial carcinoma	96	44	45.8	-	+
Lung carcinoma	192	69	35.9	-	+

**Figure 5** ING1 mRNA expression in human cancer. (A) Colorectal carcinoma; (B) lung carcinoma; (C) breast carcinoma; (D) gastric carcinoma; (E) liver carcinoma. ING1, inhibitor growth protein 1.

deleted p33ING1b using the tissue-specific promoter to construct the animal model of cancer. In the articles, the antiproliferative and apoptotic function of p33ING1b has been emphasized (4,8). The p33ING1b overexpression

in the stomach, trachea, skin, cervix, and breast might be closely linked to the higher regenerative ability regardless of either the glandular or squamous epithelium, which is confirmed by the lower expression in the organs with weak

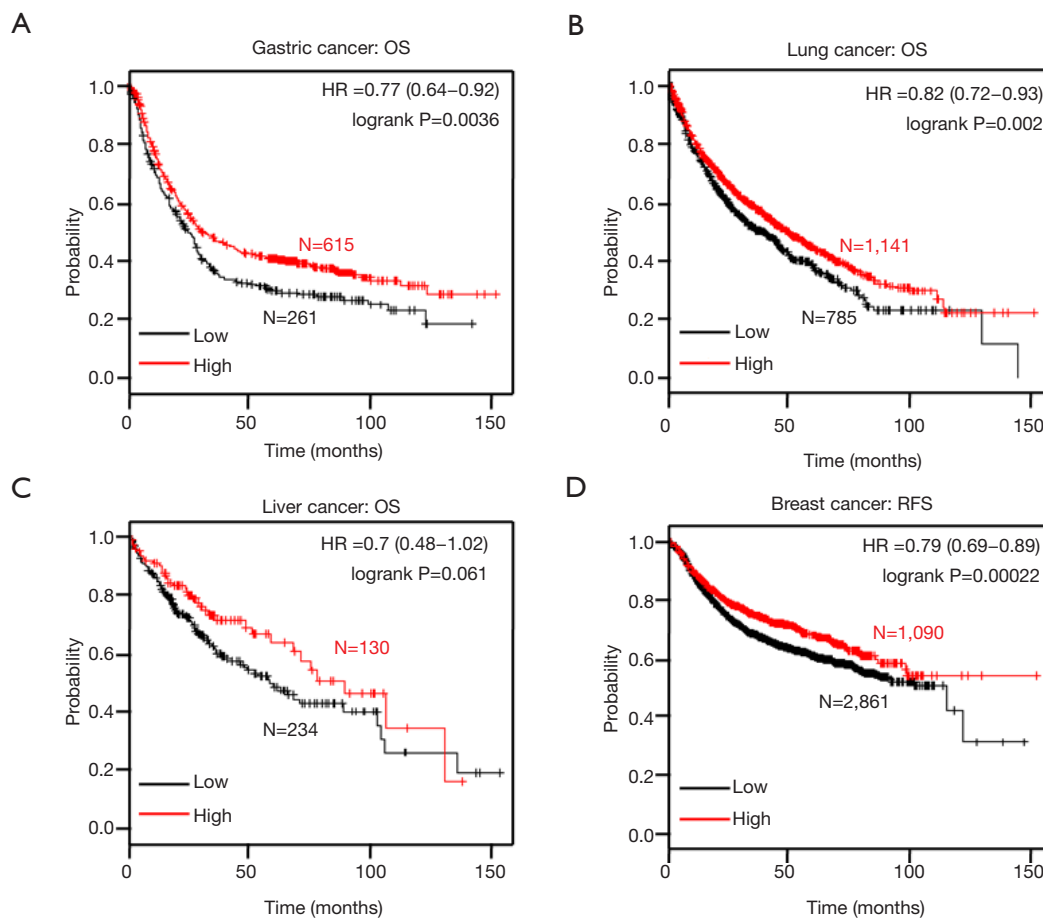


Figure 6 Relationship between *ING1* mRNA expression and accumulate survival rate in human tumors. (A) gastric carcinoma; (B) lung carcinoma; (C) hepatocellular carcinoma; (D) breast carcinoma. HR, hazard ratio.

repair and renewal, like the brain, testis, and spleen.

Here, we investigated the more frequent epithelial tumors and demonstrated that breast and cervical cancers had a high positive rate of p33ING1b expression, which is in agreement with the data in normal human tissues. In contrast, hepatocellular and renal clear cell carcinomas were shown to have less p33ING1b expression at a positive rate of 20% or so. This would significantly advance the understanding of cancer patients who could potentially benefit from p33ING1b-targeting therapy. Previously, deletion of nuclear p33ING1b was observed in melanoma, seminoma, papillary thyroid carcinoma, ductal breast carcinoma, and acute lymphoblastic leukemia, while cytoplasmic p33ING1b was more restricted, being detected in around 30% of neoplastic tissues. These findings suggest that p33ING1b function may be changed by its translocation to the cytoplasm in human cancer cells (11).

Vieyra *et al.* (25) found that *ING1* protein aberrantly localized to the cytoplasm, and slightly lower than to the nucleus of glioma cells, which is in line with our findings. A high frequency of loss of heterozygosity (LOH) in the *ING1* chromosomal region, 13q34, has been found in non-small cell lung carcinoma, head and neck carcinoma, and esophageal squamous cell carcinoma (16,20,26), but its mutation is very rare, suggesting that LOH might result in down-regulated *ING1b* expression in human malignancies.

Interestingly, the results of expression analysis showed that human *ING1* was regulated at a transcriptional level. Thus, *ING1b* mRNA is ubiquitously expressed in all tissues and here was analyzed in gastric, colorectal, liver, breast, and lung cancer. There is a clear link between the survival rate of cancer patients and *ING1b* mRNA expression. This observation may indicate that the survival rate is dependent on the human *ING1* gene, while the biological significance

of this transcriptional regulation is not yet clear. Previous studies found decreased ING1 expression in several human cancers, including in cervical cancer, renal carcinoma, acute lymphocytic leukemia (8,27,28), and other cancers. We noticed that most of the current studies referred to tumors of the embryo, gynecological urinary tract, and nervous system, and our results were related to gastrointestinal carcinoma. Therefore, we supposed that the different types of carcinoma might serve as a reason for this controversial result.

In summary, our research clarifies the variable expression of p33ING1b in normal mouse and human tissues, and human cancer tissues, and demonstrates the differential expression and/or subcellular location of p33ING1b among tissues, suggesting its differential functional involvement. According to our data, we hypothesize that ING1 might be participated in the repair and regeneration of tissues and may contribute to the carcinogenesis of the highly proliferative epithelium.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.04.28>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The human tissue collection was approved by the Ethics Committee of Shengjing Hospital of China Medical University (Shenyang, Liaoning province). All patients consented to participate in this research.

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