

Expression profiles of inhibitor of growth protein 2 in normal and cancer tissues: An immunohistochemical screening analysis

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Abstract. Inhibitor of growth protein 2 (ING2) has an important role in the regulation of chromatin remodeling, cell proliferation, cell-cycle arrest, senescence and apoptosis. The present study performed an immunohistochemical analysis for expression profiling of ING2 protein in an array of tissues comprising normal mouse and human tissues, as well as human hepatocellular (n=62), renal clear cell (n=62), pancreatic (n=62), esophageal squamous cell (n=45), cervical squamous cell (n=31), breast (n=144), gastric (n=196), colorectal (n=96), ovarian (n=208), endometrial (n=96) and lung (n=192) carcinoma tissues. In mouse tissues, ING2 was detected in the nuclei and cytoplasm of the glandular epithelium of breast, hepatocytes, intestine, bronchium and alveoli, as well as the squamous epithelium of skin and glomeruli, and in myocardial cells, while it was located in the cytoplasm of renal tubules and striated muscle cells. ING2 protein was scattered in the brain and spleen. In human tissues, ING2 protein was principally distributed in the cytoplasm, while in it was present in the cytoplasm and nuclei in the stomach, intestine, cervix, endometrium trachea, breast and pancreas. The nuclear location of ING2 in the stomach was more prominent than that in the cytoplasm. High ING2 immunoreactivity was detected in the tongue, stomach, skin, pancreas, cervix and breast, whereas weakly in the brain stem, thymus, thyroid, lung, striated muscle, testis, bladder and ovary. In total, 617 out of 1,194 of the tested cancer tissues (51.7%) were ING2-positive. In most cases, ING2 expression was found to be restricted to the cytoplasm of all cancer tissues, while in certain cancer types, including renal clear cell, ovarian and colorectal carci-

noma, it was occasionally present in the nuclei. Among the cancer tissues examined, ING2 was most frequently expressed in breast cancer (67.4%) and gynecological cancer types, including ovarian cancer (61.5%) and endometrial cancer (57.3%). Compared with that in the respective normal tissues, ING2 expression in breast cancer tissues was decreased, while that in cervical cancer was upregulated in the nuclei as well as the cytoplasm. In endometrial cancer, expression of ING2 was increased in the nuclei and declined in the cytoplasm compared with that in the normal endometrium. ING2-positive cases were less frequent for renal clear cell carcinoma (17.7%). The results of the present study suggested that ING2 may be involved in the repair and regeneration of organs or tissues and is associated with breast and gynecological carcinogenesis.

Introduction

The gene encoding inhibitor of growth protein 2 (ING2) is made up of three exons (1a, 1b and 2) and encodes two transcript isoforms (ING2a and ING2b) by alternative splicing. The ING2 C-terminal domain contains a plant homeodomain (PHD) with a zinc-binding motif, which has a high affinity for the histone 3 tri-methylated on lysine 4 and a nuclear localization sequence (NLS) (1). Initially, ING2a was shown to regulate cell proliferation, cell-cycle arrest, senescence and apoptosis due to enhancing p53 transcription and acetylation on lysine 382 by interaction with p300 acetyltransferase, which promotes p53 target genes (eg. p21 and Bcl-2-associated X protein) (2-4). In response to exogenous stresses, the p38 kinase pathway is activated to regulate phosphorylation of phosphatidylinositol 5-phosphate, resulting in an induction of ING2 accumulation to the chromatin and subsequent modulation of p53 acetylation and ING2 targets (3-7). ING2 protein recruits and stabilizes the mSin3a/higherhistone deacetylase 1 (HDAC)1-2/Sin3a-associated protein complex, and regulates target gene expression (1), which has been enhanced by phosphorylation-dependent ING2a sumoylation by small ubiquitin-like modifier 1 on lysine 195 (8), while ubiquitin ligase SMAD-specific E3 ubiquitin protein ligase 1 targets the tumor suppressor ING2 for ubiquitination and degradation (9). In addition, ING2a binds to Ski-like oncogene (SnoN), a SMAD-interacting transcriptional modulator, through its PHD domain to finally

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suppress cell proliferation, suggesting that ING2 collaborates with SnoN to mediate transforming growth factor β -induced SMAD-dependent transcription and cellular responses (10).

ING2a mRNA is highly expressed in skeletal muscle, lung, thymus, pancreas and testis (11,12). Although human ING2a and 2b have a molecular weight of 33 and 28 kDa, respectively, ING2b protein has never been experimentally detected (1,12,13). Saito *et al* (12) have reported that ING2^{-/-} mice develop soft-tissue sarcomas with an incidence of 46%, among which histiocytic sarcomas were most frequent (28%). The ING2^{-/-} male mice appeared infertile because of deficient spermatogenesis, small testes, seminiferous tubule degeneration, abnormal spermatozoal motility and morphology from the age of eight weeks. A high frequency of loss of heterozygosity (LOH) of the ING2 chromosomal region 4q32-35.1 has been found in basal cell carcinomas (14), head and neck squamous cell carcinomas (15,16), and hepatocellular carcinomas (HCC) (17). LOH of ING2 is associated with advanced tumor stages in head and neck squamous cell carcinomas (15). ING2 expression is downregulated in various cancer types, including melanoma and HCC (17), non-small cell lung carcinomas (18) and melanoma (19). The expression levels of ING2 protein were negatively correlated with tumor size, histopathological classification and serum alpha-fetoprotein in HCC, but may be employed as an independent prognostic factor to indicate favorable prognosis (17). By contrast, Kumamoto *et al* (20) demonstrated that ING2 mRNA overexpression was associated with colon carcinogenesis by upregulating the expression of matrix metalloproteinase 13 via the formation of an ING2-HDAC1-mSin3A complex.

Identification of normal tissues or cell types which express ING2 may contribute towards clarifying the physiological function of ING2, while the elucidation of its expression pattern as well as its heterogeneity among tumor cases or between tumor and normal tissues may lead to its identification as a target for gene therapy, which may be evaluated using animal models of conditional ING2 knockout (1). A previous study employed intermittent microwave irradiation for immunohistochemical analysis of ING2, during which >2.4 billion vibrations per minute enhanced the probability of specific antibody-antigen recognition (21). The present study adopted this technique to perform immunohistochemical expression profiling of ING2 protein in normal mouse and human tissues as well as in human cancer tissues.

Materials and methods

Specimens. Three male and three female C57BL/6 mice (eight weeks old; 25 g) were sacrificed under sodium pentobarbital anesthesia and brain, heart, liver, spleen, lung, kidney, breast, stomach and intestinal tissue samples were excised. All tissues were fixed in 10% neutral formalin and embedded in paraffin. An array of normal human tissues (cerebrum, cerebellum, brain stem, aorta, tongue, thyroid, esophagus, stomach, intestine, liver, pancreas, lung, trachea, appendix, smooth muscle, skeletal muscle, heart, testis, bladder and prostate) and cancer tissues (62 hepatocellular carcinomas, 62 renal clear cell carcinomas, 62 pancreatic carcinomas, 45 esophageal squamous cell carcinomas and 31 cervical squamous cell carcinomas) were purchased from Shanghai Outdo Biotech

Co., Ltd. (Shanghai, China). Normal human cervical, endometrial, ovary and breast tissues, as well as breast (n=144), gastric (n=196), colorectal (n=96), ovarian (n=208), endometrial (n=96) and lung carcinoma (n=192) tissues were obtained at The First Affiliated Hospital of Liaoning Medical University (Jinzhou, China). The normal mouse and human tissues, and the cancer tissues were also subjected to a tissue microarray using a tissue microarray apparatus (KIN-1; Azumaya, Tokyo, Japan). No patients with cancer had undergone chemotherapy, radiotherapy, or adjuvant treatment prior to surgery. The patients or their relatives provided written consent for the use of tumor tissues for clinical research, and the research protocol was approved by the Ethical and Animal Experimentation Committees of Liaoning Medical University (Jinzhou, China).

Immunohistochemistry. Consecutive sections were de-waxed with xylene, debenzolization with ethanol in water and subjected to antigen retrieval by irradiation in target retrieval solution (DAKO, Glostrup, Denmark) in a microwave oven for 15 min (500 W; Oriental Rotor Ltd Co., Tokyo, Japan). Sections were then blocked in 5% bovine serum albumin (Shanghai Chemical Technology Co., Ltd., Shanghai, China) for 5 min to prevent non-specific antibody binding. The sections were incubated with rabbit anti-ING2 antibody (cat no. 11560-1-AP; Proteintech, Chicago, IL USA; 1:50 dilution) for 15 min, followed by incubation with anti-rabbit secondary antibody conjugated to horseradish peroxidase (DAKO) for 15 min. All of the incubations were cross-linked in a microwave oven at 37°C in order to allow intermittent irradiation as previously described (21). After each treatment, the slides were washed with Tris-buffered saline containing Tween 20 (3x1 min). Bound antibodies were then stained using 3,3'-diaminobenzidine (Sangon Biotech Co., Ltd., Shanghai, China). After counterstaining with Mayer's hematoxylin, the sections were de-hydrated, cleared and mounted. Normal mouse immunoglobulin G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used instead of the primary antibody as a negative control.

Immunostaining evaluation. As indicated in Figs. 1-3, ING2 protein was localized to the cytoplasm or/and nuclei. Initially, the high-expression field was selected at low magnification and one hundred cells were randomly counted from five different representative fields at a high magnification by two independent examiners (Miss S Zhao and Professor HC Zheng). In the case of conflicting data, the two examiners continued until a final agreement was reached. The percentages of counted cells were scored as follows: 0-10%, negative (-); and 11-100%, positive (+). The density of immunostaining was assessed using ImagePro Plus software, version 16.0 (Media Cybernetics, Rockville, MD, USA). The percentage of ING2-positive cells was graded semi-quantitatively according to a four-tier scoring system: (-), negative; (+), weakly positive; (++) moderately positive; and (+++) strongly positive.

Results

As shown in Fig. 1, the sub-cellular location of ING2 was in the nuclei, cytoplasm or nucleocytoplasm in the mouse tissues in either sporadic or localized patterns, although expression levels

Table I. Immunohistochemical detection of inhibitor of growth protein 2 expression in normal mouse tissues.

Tissue source	Tissue type	Localization
Brain		Scattered
Heart	Myocardial fibroblasts	Nuclei and cytoplasm
Lung	Bronchial glandular epithelial cells	Nuclei and cytoplasm
	Alveolar glandular epithelial cells	Nuclei and cytoplasm
Kidney	Renal tubules	Cytoplasm
	Glomeruli	Nuclei and cytoplasm
Intestine	Glandular epithelium	Nuclei and cytoplasm
Spleen endothelium		Scattered
Skin	Squamous epithelium	Nuclei and cytoplasm
Striated muscle	Striated muscle cells	Cytoplasm
Liver	Glandular epithelium	Nuclei and cytoplasm
Breast	Glandular epithelium	Nuclei and cytoplasm

Table II. Immunohistochemical detection of ING2 expression and localization in normal human tissues.

Tissue type	ING2 expression	
	Nucleus	Cytoplasm
Cerebrum	-	++
Cerebellum	-	++
Brain stem	-	+
Thymus	-	+
Skeletal muscle	-	++
Aorta	-	++
Tongue	+++	+++
Thyroid	-	+
Esophagus	++	++
Stomach	+++	++
Intestine	++	++
Liver	-	++
Pancreas	+	+++
Lung	-	+
Trachea	-	++
Skin	++	+++
Appendix	+	++
Muscle	-	+
Striated muscle	-	+
Smooth muscle	-	++
Testis	-	+
Bladder	-	+
Prostate	-	++
Cervix	++	+++
Endometrium	+++	++
Ovary	-	+
Breast	+++	++

Scoring system: (-), negative; (+), weakly positive; (++) moderately positive; (+++), strongly positive. ING2, inhibitor of growth protein 2.

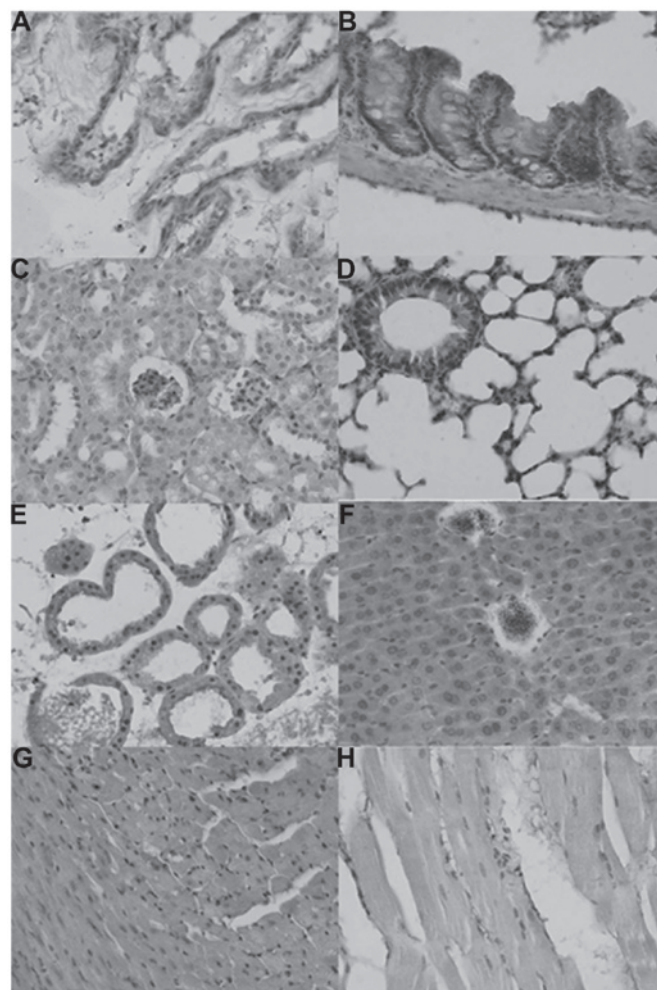


Figure 1. Immunohistochemical detection of inhibitor of growth protein 2 expression in normal mouse tissues. (A) Skin; (B) intestine; (C) kidney; (D) lung and trachea; (E) breast; (F) liver; (G) myocardium; (H) striated muscle.

differed among tissues and cell populations. ING2 reactivity was detectable in the nuclei as well as the cytoplasm of the glandular epithelium of the breast, liver, intestine, bronchium

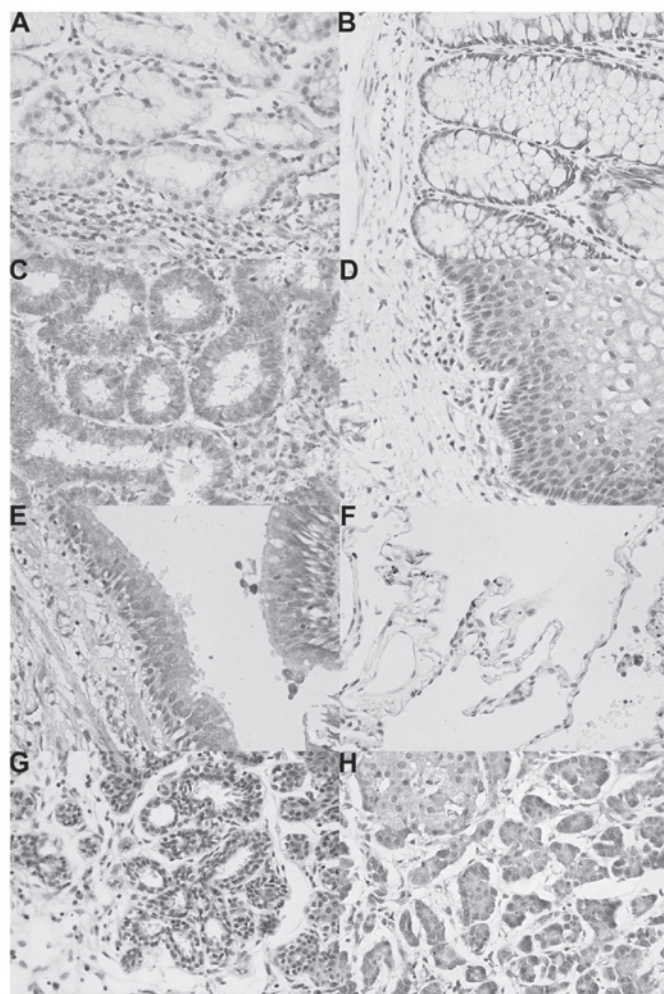


Figure 2. Immunohistochemical detection of inhibitor of growth protein 2 expression and localization in normal human tissues. (A) Stomach; (B) intestine; (C) endometrium; (D) cervix; (E) trachea; (F) lung; (G) breast; (H) pancreas.

and alveolus, the squamous epithelium of the skin, glomeruli, and in myocardial cells, while it was located in the cytoplasm of renal tubules and striated muscle cells. ING2 protein was scattered in the brain and spleen (Table I).

In human tissues, ING2 protein was primarily distributed in the cytoplasm; however, it was located in the cytoplasm and nuclei of tissues from the stomach, intestine, cervix, trachea, breast and pancreas. In the stomach, the nuclear location of ING2 was more prominent than that in the cytoplasm (Fig. 2). According to the density of the immunostaining, ING2 was highly expressed in the tongue, stomach, skin, pancreas, cervix and breast, whereas it was weakly expressed in the brain stem, thymus, thyroid, lung, striated muscle, testis, bladder and ovary (Table II).

In total, 617 out of 1,194 cancer tissues assessed in the present study were ING2-positive (51.7%), with a homogeneity in their expression pattern (Fig. 3; Table III). ING2 expression was detected in the cytoplasm of all cancer types, whereas it was present in the nuclei of certain cancer tissues, including lung, breast and endometrial cancers. Among them, ING2 was more frequently expressed in breast cancer (67.4%; 97/144) and gynecological cancers, including ovarian cancer (61.5%; 128/208) and endometrial cancer (57.3%; 55/96). Compared

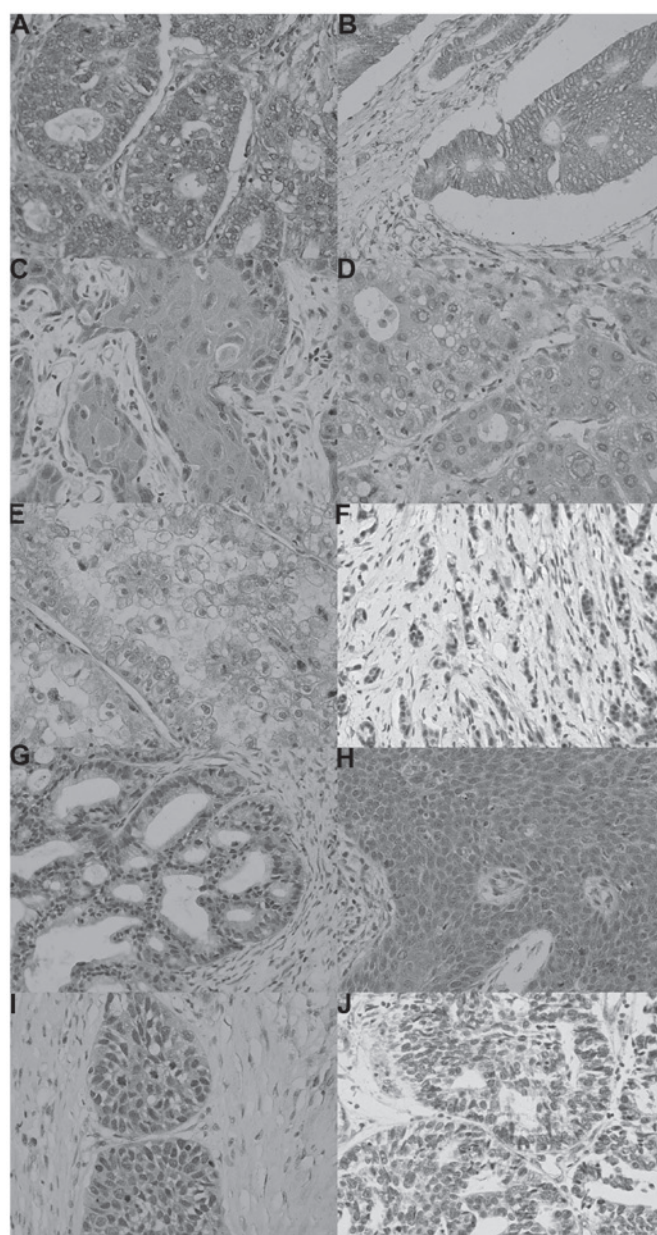


Figure 3. Immunohistochemical detection of inhibitor of growth protein 2 expression and localization in human cancers. (A) Gastric carcinoma; (B) colorectal cancer; (C) esophageal carcinoma; (D) hepatocellular carcinoma; (E) renal clear cell carcinoma; (F) breast cancer; (G) ovarian cancer; (H) cervical carcinoma; (I) lung carcinoma; (J) endometrial carcinoma.

with their respective normal tissues, ING2 expression in breast cancer tissues was decreased, while it was upregulated in the nuclei as well as in the cytoplasm of cervical cancer tissues, whereas ING2 was increased in the nuclei and declined in the cytoplasm of endometrial cancer tissues (Fig. 4). By contrast ING2-positive cases were less frequent in renal clear cell carcinoma (17.7%; 11/62).

Discussion

ING2 has been identified and characterized as a Type-II tumor suppressor gene as its involvement in cancer appears to be linked to its level of expression rather than its mutational status (1,18). ING2 protein contains NLS and PHD

Table III. Immunohistochemical detection of ING2 expression in human cancer tissues.

Cancer type	n	Positive cases (n)	PR (%)	ING2 expression	
				Nucleus	Cytoplasm
Hepatocellular carcinoma	62	25	40.3	-	+
Renal cell carcinoma	62	11	17.7	-	+
Pancreatic cancer	62	20	32.3	-	+
Esophageal carcinoma	45	19	42.2	+	+
Cervical carcinoma	31	15	48.4	+	+
Breast cancer	144	97	67.4	+	+
Gastric carcinoma	196	85	43.4	+	+
Colorectal carcinoma	96	56	58.3	+	+
Ovarian cancer	208	128	61.5	+	+
Endometrial carcinoma	96	55	57.3	+	+
Lung carcinoma	192	106	55.2	+	+

Scoring system: (-), negative, 0-10%; (+), positive, 11-100%. ING2, inhibitor of growth protein 2; PR, positive rate.

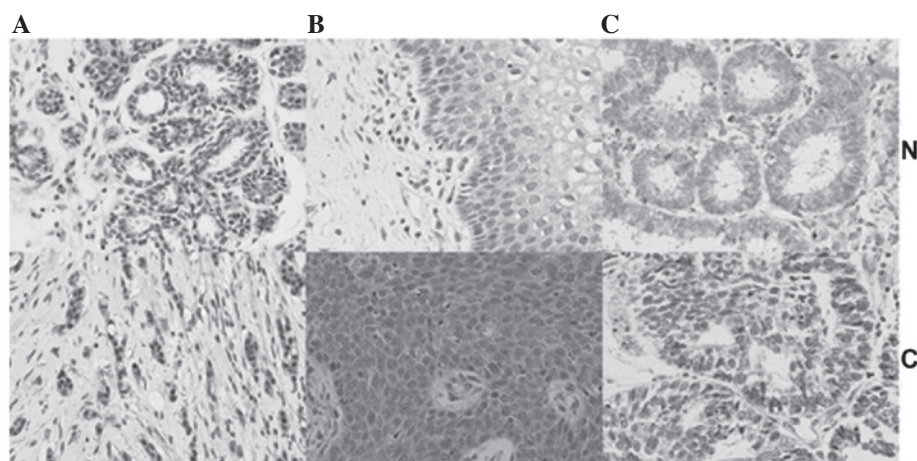


Figure 4. Immunohistochemical detection of inhibitor of growth protein 2 expression and localization in human cancers. (A) Breast; (B) cervix; (C) endometrium. N, normal tissue; C, cancer tissue.

finger motifs in its C-terminus (1). In the present study, the expression levels and cellular localization of ING2 protein were determined in a tissue array comprising normal mouse and human tissues, as well as human cancer tissues. Positive expression of ING2 was observed in the cytoplasm of normal mouse and human tissues as well as human cancer tissues; however, ING2 was present in the cytoplasm as well as in the nuclei in certain tissue types. It has been reported that LOH of the ING2 chromosomal region or the ING2 gene may result in the downregulation of its expression (14-17). Cen^{zig} *et al* (22) found that the mutation or deletion of ING5 NLS is responsible for the nucleocytoplasmic translocation. Zhang *et al* (17) demonstrated that immunostaining for ING2 was mostly located to the cytoplasm, while weak nuclear staining was also observed in HCC tissues and normal hepatocytes, in line with a study by Gozani *et al* (5), who reported that phosphatidylinositol 5-phosphate 4-kinase type II β expression decreased endogenous nuclear ING2 in HT1080 fibrosarcoma cells. It has been reported that ING1

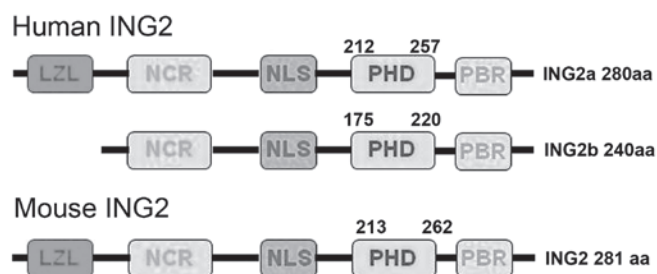


Figure 5. Schematic diagram of human and mouse ING2 protein structure. ING2, inhibitor of growth protein 2; LZL, leucine zipper-like motif; NCR, novel conserved region; NLS, nuclear location sequence; PHD, plant homeodomain; PBR, poly basic region.

phosphorylation by 14-3-3 family members (23) or Src (24) caused its cytoplasmic re-localization leading to apoptotic induction. Future studies should investigate the purpose of ING2 restoration in the cytoplasm as well as its function.

Amino acid sequence alignment has revealed a high similarity between human ING2a and mouse ING2 (96% identity) (Fig. 5) (1); this may explain for the findings of the present study, which revealed no substantial differences in the patterns of ING2 expression between mouse and human samples, with the exception of sub-cellular location in certain cell types. In human tissues, high expression of ING2 protein was detected in the tongue, stomach, skin, pancreas, cervix and breast, while its expression was low in the brain stem, thymus, thyroid, lung, striated muscle, testis, bladder and ovary, suggesting the functional involvement of ING2 in the functions of specific cell types or in cells in a specific functional state. In order to further investigate the roles of ING2, our group has inhibited ING2 using a cell-specific promoter in order to establish an animal model of ING2-negative cancer. Previous studies have highlighted the anti-proliferative and apoptotic functions of ING2 (4,8). ING2 overexpression in the stomach, tongue, skin, pancreas, cervix and breast may indicate its association with regenerative processes regardless of the identity of the cells being glandular or squamous epithelium, which is supported by the finding of relatively low expression in organs with a decreased capacity for cell repair and renewal, including the brain stem, thymus, thyroid, skeletal muscle and testis. Of note, ING2 has been involved in muscle differentiation by regulating myogenin transcription (25), providing an explanation for ING2 overexpression in muscle cells.

ING2 is a candidate tumor suppressor gene, whose expression is frequently lost in tumors. The present study focused on common epithelial cell-derived tumors and demonstrated that breast cancer and gynecological cancer types, including ovarian and endometrial carcinoma, showed a high incidence of ING2 expression, indicating that ING2 protein may be closely linked to estrogen production. By contrast, the positive rate for ING2 expression in renal clear cell carcinoma was <20%. This finding may indicate that ING2 may be used as a therapeutic target in renal clear cell carcinoma. ING2 was reported to interact with proliferating cell nuclear antigen and regulates its quantity of the chromatin fraction, thereby directly maintaining DNA replication and integrity (26,27). The ING2 C-terminus recruits histone methyltransferase activity and trimethylates histone H3 at lysine 9 (28). The leucine zipper-like motif of ING2 is critical for the proper functioning of ING2 in DNA repair, apoptosis and p53-dependent chromatin remodeling by acting as a scaffold protein to mediate the interaction between p53 and p300 (3-7). The profiling of ING2 expression performed in the present study provided useful hints regarding the disruption of DNA replication, proliferation and apoptosis in various epithelial cancer types.

In conclusion, the present study not only elucidated the abundant expression of ING2 in normal mouse and human tissues as well as human cancer tissues, but also demonstrated the differential expression and/or sub-cellular location of ING2 among various tissues, cell types and single cells, suggesting differential functional involvement. According to the results of the present study, it is hypothesized that ING2 may be involved in the repair and regeneration of organs or tissues and have an important role in gynecological carcinogenesis.

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