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RESEARCH

# Downregulated Expression of Inhibitor of Growth 4 (ING4) in Advanced Colorectal Cancers: A Non-Randomized Experimental Study

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**Abstract** Colorectal cancer has a high cure rate if it can be detected early. Identifying and understanding the genes involved may enable early diagnosis and reduce mortality. The aim of our study was to investigate the correlation between the expression of ING4 and the pathological features in patients with colorectal cancer. We assayed ING4 protein expression levels in tumor samples from 97 patients diagnosed with colorectal cancer between January 2001 and January 2002. The patients received no other treatments except surgery. ING4 protein expression was downregulated in adenoma relative to normal mucosa and further reduced in colorectal cancer tissues. Furthermore, the suppression of ING4 expression was also related to the more advanced Dukes' stages. We observed that ING4 expression levels in patients with lymphatic metastasis were lower than those without metastasis. Together, our results indicate that ING4 play a role in colorectal carcinoma progression.

**Keywords** Colorectal carcinoma · Tumor suppressor gene · ING4 · Immunohistochemistry

**Abbreviations**

| | |
|---|---|
| ING4 | inhibitor of growth 4 |
| PHD | plant homeodomain |
| HIF-1 | hypoxia-inducible factor 1 |
| IL | interleukin |
| COX2 | cyclooxygenase 2 |
| DAB | diazoaminobenzene |
| ANOVA | analysis of variance |
| SKP2 | S-phase kinase-associated protein 2 |
| H & E staining | hematoxylin and eosin staining |

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## Introduction

Colorectal carcinoma is one of the most prevalent cancers worldwide. In the United States, it is among the top 3 causes of cancer-related deaths, with approximately 150,000 new cases in 2006 [1]. The prognosis of advanced colorectal cancer remains poor, with an estimated 5-year survival rate of less than 10% [2]. Early detection of colorectal carcinoma is associated with very high cure rates, making early diagnosis a very important goal in the management of this disease. A majority of colorectal carcinomas (up to 80%) are sporadic in nature, indicating that the environment plays a more important role in the development of this cancer than heredity [3, 4]. Understanding the mechanism of colorectal carcinoma progression is therefore an important goal and identification of genes that play a role in this progression may contribute significantly to early diagnosis and help reduce cancer related deaths.

Inhibitor of growth 4 (ING4) is a tumor suppressor gene frequently mutated in human cancers [5, 6]. ING4 has been shown to function in a number of cellular processes, including the repression of cell proliferation [7–9], cell cycle [7, 9], tumor growth [5, 11, 12], loss of contact inhibition [6, 13, 14], and angiogenesis [8, 10]. ING4 is a member of a family of proteins characterized by a highly conserved C-terminal plant homeodomain (PHD)-like zinc-

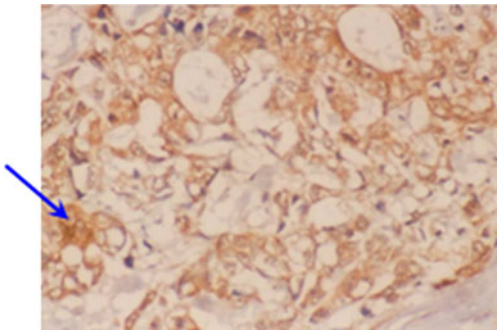

**Fig. 1** ING4 expression in moderately differentiated colorectal carcinoma tissue. (×460 magnification)

finger domain. This protein family has been implicated in a number of processes including oncogenesis, apoptosis, DNA repair, chromatin remodeling, and cell cycle control [15, 16]. The ING4 gene itself spans a 13 kb region on chromosome 12p13-31 and consists of 8 exons and 7 introns [17]. Its 1380 bp cDNA encodes a 249 amino acid protein [7]. ING4 protein has been localized to the nucleus [8, 18, 19] and is detected in all types of human tissues [14].

Although the exact mechanism of action of ING4 protein is not completely understood, it appears to have regulatory effects on an array of cellular targets. ING4 inhibits cell contact inhibition induced by overexpression of MYCN or MYC genes by modulating the transcription of MYC downstream target genes [14]. It has been shown to inhibit angiogenesis and tumor growth via negative regulation of hypoxia-inducible factor 1 (HIF-1) [12, 20] and repression of angiogenesis related genes including IL6, IL-8, and Cox2 [21]. ING4 is known to interact with p300, a component of the histone acetylase complex [7] and has also been shown to acetylate p53 and modulate its transcriptional activity [17, 22]. Overexpression of ING4 inhibits cell growth, induces p27 expression, blocks Wnt-1/β-signal induction, and down-regulates the cell cycle related proteins cyclin D1, SKP2, and COX2 [10].

The onset and progression of colorectal carcinoma is thought to be controlled by multiple processes. In this study, we compared ING4 expression levels in colorectal carcinoma tissues and normal mucosa tissues and investigated the correlation between ING4 expression and the onset, progression, metastasis, and clinical profiles of patients with colorectal carcinoma.

## Materials and Methods

### Materials

Patients presenting with colorectal cancer at the Tumor Hospital of Harbin Medical University between January 2001 to January 2002, who had only received surgery and had not undergone any chemotherapy or adjuvant therapy, were included in this study. A total of 97 patients met these criteria; 58 were male and 39 were female (ratio of 1.48:1) with an average age of 57.5 years. Among these, 35 patients were diagnosed with colon cancer and 62 with rectal cancer, with these diagnoses confirmed by a pathologist. There were 21 cases with highly differentiated cancers, 60 displayed moderate differentiation, 7 displayed low differentiations, and there were 9 cases of mucoid adenocarcinoma. Examination of the extent of tumor invasion indicated that 20 patients displayed tumor invasion to the muscular layer, 35 to the serosa, and 42 patients displayed tumor invasion outside of the serosa. Furthermore, 51 patients (52.6%) displayed metastasis to the lymph nodes and 24 patients (24.7%) displayed metastasis to the liver. In addition to the 97 primary colorectal carcinoma tissue samples collected from these patients, we also collected 20 adenoma and 20 normal mucosa tissue samples from matched patients for immunohistochemical analysis.

### Methods

ING4 protein expression was assayed in tissue samples from primary colorectal carcinoma, adenoma, normal colon mucosa from matched patients, metastasized lymph nodes, and metastasized liver. Rabbit anti-human ING4 polyclonal antibody against human ING4 transcript variant 1 mRNA (cDNA clone MGC:12557, IMAGE:4309278) was purchased from Proteintech Group, USA and used at a dilution of 1:50. Immunohistochemistry was performed using a HistostainTM-Plus Kit (SP-9001) and DAB color reagent (Pekin Zhongqiao Biotechnology) according to the manufacturer's instructions.

**Table 1** ING4 expressions in normal mucosa, adenoma, and colorectal carcinoma tissues

| Tissues | N | ING4 expression | | $\chi^2$ analysis |
|---|---|---|---|---|
| | | # Positive | % | |
| Primary colorectal carcinoma | 97 | 72 | 74.23% | |
| Adenoma | 20 | 17 | 85% | 0.0419* |
| Normal mucosa | 20 | 20 | 100% | 0.0105* |

*$P<0.05$ relative to expression in primary colorectal carcinoma

**Table 2** Summary of clinical pathological features relative to the level of expression of ING4

| Variables | Total | Level of ING4 expression | | | | *P*-value |
|---|---|---|---|---|---|---|
| | | Negative (*N*=25) | Weak positive (*N*=29) | Positive (*N*=25) | Strong positive (*N*=18) | |
| Gender | | | | | | |
| Male | 58(59.8%) | 15(60.0%) | 18(62.1%) | 16(64.0%) | 9(50.0%) | 0.810 |
| Female | 39(40.2%) | 10(40.0%) | 11(37.9%) | 9(36.0%) | 9(50.0%) | |
| Age (years) | | | | | | |
| Mean ± SD | 57.5±11.1 | 56.6±10.6 | 56.1±11.6 | 59.5±11.5 | 58.4±10.9 | 0.680 |
| Tumor Site | | | | | | |
| Colon | 35(36.1%) | 12(48.0%) | 10(34.5%) | 7(28.0%) | 6(33.3%) | 0.505 |
| Rectum | 62(63.9%) | 13(52.0%) | 19(65.5%) | 18(72.0%) | 12(66.7%) | |
| Pathology Type | | | | | | |
| Protruded type | 48(49.5%) | 12(48.0%) | 13(44.8%) | 16(64.0%) | 7(38.9%) | 0.191 |
| Infiltration type | 17(17.5%) | 4(16.0%) | 9(31.0%) | 1(4.0%) | 3(16.7%) | |
| Ulcerative type | 32(33.0%) | 9(36.0%) | 7(24.1%) | 8(32.0%) | 8(44.4%) | |
| Tissue type | | | | | | |
| Adenocarcinoma | 88(90.7%) | 23(92.0%) | 26(89.7%) | 23(92.0%) | 16(88.9%) | 1.000 |
| Mucinous Adenocarcinoma | 9(9.3%) | 2(8.0%) | 3(10.3%) | 2(8.0%) | 2(11.1%) | |
| Infiltration level | | | | | | |
| Intra-mura | 20(20.6%) | 3(12.0%) | 5(17.2%) | 10(40.0%) | 2(11.1%) | 0.187 |
| Intra-serosa | 35(36.1%) | 11(44.0%) | 12(41.4%) | 7(28.0%) | 5(27.8%) | |
| Sub-serosa | 42(43.3%) | 11(44.0%) | 12(41.4%) | 8(32.0%) | 11(61.1%) | |
| Tumor differentiation | | | | | | |
| High | 24(24.7%) | 9(36.0%) | 7(24.1%) | 7(28.0%) | 15(83.3%) | 0.016* |
| Middle | 65(67.0%) | 11(44.0%) | 22(75.9%) | 17(68.0%) | 15(83.3%) | |
| Low | 8(8.2%) | 5(20.0%) | 0(0%) | 1(4.0%) | 2(11.1%) | |
| Dukes' Stage | | | | | | |
| A | 16(16.5%) | 2(8.0%) | 3(10.3%) | 9(36.0%) | 2(11.1%) | 0.036* |
| B | 23(23.7%) | 3(12.0%) | 10(34.5%) | 3(12.0%) | 7(38.9%) | |
| C | 35(36.1%) | 12(48.0%) | 7(24.1%) | 9(36.0%) | 7(38.9%) | |
| D | 23(23.7%) | 8(32.0%) | 9(31.0%) | 4(16.0%) | 2(11.1%) | |
| Lymph metastases | | | | | | |
| Yes | 51(52.6%) | 19(76.0%) | 13(44.8%) | 12(48.0%) | 7(38.9%) | 0.051 |
| No | 46(47.4%) | 6(24.0%) | 16(55.2%) | 13(52.0%) | 11(61.1%) | |
| Liver metastases | | | | | | |
| Yes | 24(24.7%) | 8(32.0%) | 10(34.5%) | 4(16.0%) | 2(11.1%) | 0.183 |
| No | 73(75.3%) | 17(68.0%) | 19(65.5%) | 21(84.0%) | 16(88.9%) | |

* *P*-values less than 0.05 were considered statically significant

ING4 staining was semi-quantitatively assessed and graded based on both ING4 positive rate and ING4 expression intensity. Intensities were categorized as negative, weak (light yellow), medium (yellow-brown), or strong (brown), and scored as 0, 1, 2, and 3, respectively; positive rates were graded as 0, 1–5%, 6–50%, or >50%, and scored as 0, 1, 2, and 3, respectively. The sum of both values was used to grade expression levels; 3: weak positive (+), 4–5: positive (++), and 6: strong positive (+++). When the positive rate was <5%, a negative (−) grade was assigned regardless of the staining intensity. Known ING4-positive tissue slides were used as positive controls, and PBS was used as a negative control for antibody staining. The staining results were examined independently by two pathologists double blinded to the procedures.

## Statistics Analysis

Categorical variables were presented as frequency with percentages, while continuous variables were presented as mean ± standard deviation. For comparison among the four levels of ING4 expression, analysis of variance (ANOVA)

was performed. A Chi-square or Fisher's exact test was performed for categorical variables. $P<0.05$ was considered statistically significant.

## Results

### ING4 Expression was Detected in Normal Mucosa, Adenoma, and Colorectal Carcinoma Tissues

Immunohistochemistry using an anti-human ING4 antibody demonstrated that ING4 expression was mainly observed in the nucleus, with a speckled staining pattern and a variety of intensities. It was predominately expressed by epithelial cells in normal intestinal mucosa, adenoma, and colorectal carcinoma (Fig. 1). ING4 expression was inversely related to disease progression, with a positive rate of 100%, 85%, and 74.23% in normal mucosa, adenoma, and colorectal carcinoma, respectively. Colorectal carcinoma tissue had significantly lower levels of ING4 when compared with normal mucosa or adenoma (Table 1).

### The Relationship Between ING4 Expression in Colorectal Carcinoma and Patients' Clinical Pathology

Table 2 shows a summary of the pathological features relative to the level of ING4 expression.

Quantitative analysis of ING4 expression indicated that 25 patients (25.8%) were identified to be negative (−), 29 patients (29.9%) were weakly positive (+), 25 patients (25.8%) were positive (++), and 18 patients (18.6%) were strongly positive (+++). Analysis of variance indicated that only the distribution of tumor differentiation and Dukes' stage significantly correlated with the different levels of ING4 expression (see Table 2). Specifically, higher tumor differentiation correlated significantly with decreased levels of ING4 expression ($P=0.016$), and a more advanced Dukes' stage correlated significantly with decreased levels of ING4 expression ($P=0.036$). We analyzed the data for patients with colon cancer ($n=35$) and patients with rectal cancer ($n=62$) separately and did not find a statistically significant difference between the two groups (data not shown).

### The Relationship Between ING4 Expression and Survival Rate

Table 3 presents the effect of different ING4 levels on the risk of death based on Cox-regression model analysis and shows that although the hazard ratio (HR) indicated a decreased risk of death relative to increased ING4 expression levels (strong-positive was set as reference group), the change in the HR was not statistically significant (HR[95%CI]=1.23 [0.41, 3.67], $P$ value=0.712 for positive, 1.11 [0.37,3.32], $P$ value=0.848 for weak positive, and 0.98 [0.31, 3.10], $P$ value=0.976 for negative levels of ING4 expression).

**Table 3** Effect of different ING4 levels on the risk of death based on Cox-regression model analysis. ($N=97$)

| Level of ING4 | B | Standard error | Wald[a] | Sig[b]. | HR[c] | LB[c] | UB[c] |
|---|---|---|---|---|---|---|---|
| Negative | −0.02 | 0.59 | 0.001 | 0.976 | 0.98 | 0.31 | 3.10 |
| Weak-positive | 0.11 | 0.56 | 0.037 | 0.848 | 1.11 | 0.37 | 3.32 |
| Positive | 0.21 | 0.56 | 0.137 | 0.712 | 1.23 | 0.41 | 3.67 |
| Strong-positive (Reference) | – | – | – | – | – | – | – |

[a] Wald: test statistic from the data

[b] Sig: $p$-value indicating whether the variable was associated with a risk of death

[c] HR: equivalent to Exp(B), hazard ratio against negative level of ING4 expression; LB: lower bound of 95% confidence interval for HR; UB: upper bound of 95% confidence interval for HR

## Discussion

In this study, we investigated the association between the expression profiles of ING4 protein and the pathological features among patients with colorectal cancer. Quantitative analysis of ING4 expression indicated that protein levels were inversely related to disease progression, with the lowest expression observed in more advanced carcinomas relative to adenoma and normal mucosa tissues. Specifically, moderately and highly differentiated adenomas had significantly higher levels of ING4 expression when compared with poorly differentiated adenomas. We also observed that a more advanced Dukes' stage correlated significantly with decreased levels of ING4 expression. Together, these findings indicated that ING4 expression levels were closely associated with colorectal carcinoma progression.

Our observation that ING4 expression was downregulated in colorectal carcinomas is consistent with a number of previous studies that indicated lower ING4 expression in a number of different cancers. While ING4 is widely expressed in normal cell lines, it is downregulated in glioblastoma [23, 24] and melanoma [25] cell lines as well as in head and neck squamous cell carcinomas [17].

ING4 was previously shown [20] to suppress the transcriptional activity of hypoxia inducible factor (HIF). Upregulation of HIF is a hallmark of most tumors, where tumor hypoxia leads to stabilization and increased HIF expression along with increased transcription of HIF target genes such as VEGF. These changes result in increased tumor angiogenesis. ING4 was shown to function as a chromatin-dependent adaptor protein and physically interact with prolyl hydroxylase to recruit other unidentified chro-

matin remodeling factors to suppress HIF activity [12, 20]. These results are very important in the context of our data, which show that ING4 is downregulated in poorly differentiated, advanced colorectal carcinoma. It will be interesting to study the correlation between ING4 expression and the expression of some important HIF target genes such as VEGF, in patients with advanced colorectal carcinoma.

We did not find a significant relationship between ING4 expression and risk of death based on the Cox regression model analysis, suggesting that ING4 is not an independent risk factor affecting the survival of patients with colorectal carcinoma. The occurrence and development of colorectal cancer is a multi-gene, multi-stage process, involving the activation of some proto-oncogenes and inactivation of some tumor suppressor genes. A single gene or protein cannot be used as a standard of diagnosis or prognosis. For example, although tumor markers CEA and CA199 have no diagnostic significance individually, the combination of the two and others can predict prognosis and survival time in colorectal cancer and guide the selection of treatment plan [26].

Interestingly, our data demonstrated that primary colorectal carcinoma patients with lymph node metastasis had significantly lower levels of ING4 expression when compared to patients without metastasis. Although this may suggest that lower ING4 expression reflects an increased likelihood of metastasis, we did not observe a similar trend in patients with metastasis to liver. Further studies exploring ING4 expression and metastasis rates may illuminate this potentially important relationship.

## Conclusions

In this study, we demonstrated that ING4 is downregulated in colorectal cancer. Patients with the most advanced disease exhibited the lowest levels of ING4 protein. We aim to conduct further experiments with larger patient populations to confirm our data, and to Explore the mechanism underlying the modulation of ING4 expression levels.

**Competing interests** The author(s) declare that they have no competing interests.

## References


1. United States Cancer Statistics (USCS), 1999–2005 Cancer Incidence and Mortality Data, 2007; http://apps.nccd.cdc.gov/uscs/
2. Hawk ET, Levin B (2005) Colorectal cancer prevention. J Clin Oncol 23:378–391
3. Cheah PY (1990) Hypotheses for the etiology of colorectal cancer—an overview. Nutr Cancer 14:5–13
4. Lichtenstein P et al (2000) Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark and Finland. N Eng J Med 343:78–85
5. Zhien M (1995) Expression and clinical significance of gene mutation in human colon tumor tissues. Chin J Onc 17:332–334
6. Kim S, Chin K, Gray JW, Bishop JM (2004) A screen for genes that suppress loss of contact inhibition: identification of ING4 as a candidate tumor suppressor gene in human cancer. Proc Natl Acad Sci USA 101:16251–16256
7. Shiseki M et al (2003) p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. Cancer Res 63:2373–2378
8. Shen JC et al (2007) Inhibitor of growth 4 suppresses cell spreading and cell migration by interacting with a novel binding partner, liprin alpha1. Cancer Res 67:2552–2558
9. Zhang X et al (2004) ING4 induces G2/M cell cycle arrest and enhances the chemosensitivity to DNA-damage agents in HepG2 cells. FEBS Lett 570:7–12
10. Li X et al (2008) ING4 induces cell growth inhibition in human lung adenocarcinoma A549 cells by means of Wnt-1/beta-catenin signaling pathway. Anat Rec 291:593–600
11. Garkavtsev I et al (2004) The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. Nature 428:328–332
12. Ozer A, Wu LC, Bruick RK (2005) The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). Proc Natl Acad Sci USA 102:7481–7486
13. Kim S (2005) HuntING4 new tumor suppressors. Cell Cycle 4:516–517
14. Xu L, Li T (2007) The effect of ING4 gene in tumor progression. Int J Genetics 30:147–149
15. Aasland R, Gibson TJ, Stewart AF (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem Sci 20:56–59
16. Campos EI, Chin MY, Kuo WH, Li G (2004) Biological functions of the ING family tumor suppressors. Cell Mol Life Sci 61:2597–2613
17. Gunduz M et al (2005) Frequent deletion and down-regulation of ING4, a candidate tumor suppressor gene at 12p13, in head and neck squamous cell carcinomas. Gene 356:109–117
18. Zhang X et al (2005) Nuclear localization signal of ING4 plays a key role in its binding to p53. Biochem Biophys Res Commun 331:1032–1038
19. Brat DJ, Bellail AC, VanMeir EG (2005) The role of interleukin 8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro Oncol 7:122–133
20. Ozer A, Bruick RK (2005) Regulation of HIF by prolyl hydroxylases: recruitment of the candidate tumor suppressor protein ING4. Cell Cycle 4:1153–1156
21. Colla S et al (2007) The new tumor-suppressor gene inhibitor of growth family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 alpha (HIF-1 alpha) activity: involvement in myeloma induced angiogenesis. Blood 110:4464–4475
22. Doyon Y et al (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol Cell 21:51–64
23. Hassler M et al (2006) Diversity of cytogenetic and pathohistologic profiles in glioblastoma. Cancer Genet Cytogenet 166:46–55
24. Liu L, Chang H, Yang JC (2007) Candidate tumor suppressor gene ING4: research progress. Chin J Mod Drug Appl 1:69–72
25. Li J, Martinka M, Li G (2008) Role of ING4 in human melanoma cell migration, invasion and patient survival. Carcinogenesis 29:1373–1379
26. Yu JK, Yang MQ, Jiang TJ, Zheng S (2004) The optimal combination of serum tumor markers with bioinformatics in diagnosis of colorectal carcinoma. Zhejiang Da Xue Xue Bao Yi Xue Ban 33(5):407–410