Overexpression of Id-1 protein is a marker in colorectal cancer progression

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Abstract. The inhibitor of differentiation/DNA binding 1 (Id-1), a negative regulator of basic helix-loop-helix transcription factors, plays an important role in the regulation of cell proliferation and differentiation. We examined the Id-1 expression by immunohistochemistry in 9 adenomas, 79 primary colorectal adenocarcinomas matched with 40 adjacent normal mucosa specimens and its relationship with clinicopathological factors. The Id-1 expression was increased in the carcinoma compared to the adjacent normal mucosa either in the unmatched and matched samples or to the adenoma. There was no significant difference in the Id-1 expression between normal mucosa and adenoma. The Id-1 expression of carcinoma was increased from Dukes' stages A to B, to C and to D. The cases with lymph node metastasis had a higher rate of a stronger Id-1 expression than those without lymph node metastasis. In conclusion, Id-1 overexpression plays an important role in colorectal cancer progression.

Introduction

The helix-loop-helix (HLH) transcription factor has a highly conserved HLH structural domain consisting of two α helices separated by a loop region of variable sequences and length (1). The HLH structural motif of these proteins mediates dimerization and the N-terminal basic domain is responsible for DNA binding and gene activation (1). Hererodimers regulate the expression of cell cycle regulatory proteins and tissue-specific genes formed between tissue-specific and ubiquitous basic HLH (bHLH) transcription factor binding to a so-called 'E-box' DNA sequence (2). The inhibitor of differentiation/DNA binding (Id), a member of the HLH family, functions by dimerization with other bHLH proteins.

Four members of the Id family, Id1-4, have already been identified and the genes are located on 20q11, 2p25, 1p36.1 and 6p21-22, respectively, which encode proteins ranging from 13 to 20 kDa. Id proteins act as dominant negative regulators of bHLH DNA binding proteins by preventing DNA binding and gene transcription (3). Id-1 has been reported as an oncogene that regulates cellular senescence, growth and survival in the development of tumors (4,5). Moreover, Id-1 overexpression was associated with tumor invasion, advanced stage and poor prognosis in prostate (6), breast (7), endometrial (8) and cervical cancers (9). The abrogation of Id1-3 functions, by an antisense oligonucleotide blockade or by the enforced expression of the bHLH protein, led to growth arrest in human colon cancer cell lines (10).

Our study examined the Id-1 expression by immunohistochemistry in adjacent normal mucosa, adenoma and adenocarcinoma and investigated the correlation between the Id-1 expression and clinicopathological factors in cancer patients.

Materials and methods

Patients. Adenocarcinomas were collected from 79 patients with colorectal adenocarcinoma. Among them, 40 cases had adjacent normal mucosa, i.e., normal mucosa adjacent to primary adenocarcinoma on the same slides, histologically free from pre-tumor and tumor. The study also included 9 adenomas from additional patients with adenoma. The patients were diagnosed at the Department of Pathology, Tangshan Gongren Hospital, China, between 2004 and 2005.

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None of the patients received preoperative radiotherapy and chemotherapy. The patient gender, age, tumor site, size, gross status, invasive depth, growth pattern, Dukes' stage, lymph node status, histological type, grade of differentiation, necrosis, inflammatory reaction and fibrosis were obtained from surgical and pathological records. The mean age of cancer patients at the time of surgery was 58 years (range, 27-83 years). Right-sided tumors were found in 22 cases including caecum, ascending and transverse colon, left-sided ones were found in 16 cases including descending and sigmoid colon and rectal ones were found in 41 cases. The mean diameter of carcinomas was 4.7 cm (range, 1.5-13.0 cm). The growth pattern was divided into expansive- or infiltrative-type based on the pattern of the tumor cell growth and invasiveness. Differentiation was graded as good, moderate and poor. All the slides including adenomas and adenocarcinomas along with adjacent normal mucosa were confirmed by two pathologists (Zhang ZY and Zhang H).

Immunohistochemical staining. The sections (5 μm) from paraffin-embedded tissue blocks were incubated at 60˚C overnight, deparaffinized in xylene, re-hydrated in graded ethanol and washed in distilled water. To retrieve the masked epitope, the sections were boiled in 0.01 M citrate buffer (pH 6.0) for 2 min in a high-pressure cooker. After cooling at room temperature for 30 min and washing in phosphate-buffered saline (PBS, pH 7.4) buffer, the endogenous peroxidase was blocked with 0.3% H_2O_2 containing methanol for 20 min and rinsed with PBS. Then, the sections were incubated overnight at 4˚C with a polyclonal rabbit antibody against Id-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and diluted at 1:50 with PBS. After washing with PBS, the slides were incubated with polymer enhancer for 20 min at 37˚C and then with polymerized horseradish peroxidase anti-mouse/rabbit IgG for 30 min [Elivision™ plus polyer horseradish peroxidase (mouse/rabbit) IHC kit, Fuzhou Maixin Biology Technology Limited Company, Fuzhou, China]. The peroxidase reaction was performed by the use of 3,3'-diaminobenzididine for 2 min. The sections were finally counterstained with haematoxylin and mounted.

The sections known to stain positively were included as negative and positive controls. For negative controls, the sections incubated with PBS instead of the primary antibody were not stained, unlike the positive controls.

The slides were microscopically examined and scored independently by the two pathologists without any clinical or histopathological information. To avoid artefacts, areas with poor morphology, margins of the sections and necrosis were not considered. The Id-1 expression was predominantly present in the cytoplasm of tumor and normal epithelial cells, as well as fibroblast and muscles of blood vessels in the tumor-associated stroma. Thus, the Id-1 expression in the tumor and epithelial cells, and stroma was respectively classified as negative, weak, moderate or strong. In the sections showing a heterogeneous staining pattern, if the stronger staining was more than one third of the sections the staining was considered for scoring. Among the 128 sections evaluated, there were discountenances in 22 sections in the first round of the evaluation. These sections were re-read by the two pathologists, individually and matched. The final 6 discrepant sections were re-examined by dual-microscope and a concurrent score was achieved.

Statistical analysis. The Chi-square test, Fisher exact test or McNemar's method was used to test the relationship of the Id-1 expression in normal mucosa, adenoma and carcinoma, as well as the relationship between the Id-1 expression and clinicopathological variables. Spearman's coefficient of correlation was used to test the correlation between the Id-1 expression in tumor and stroma. All P-values mentioned are two-sided and P-values <0.05 were judged as statistically significant.

Results

The Id-1 expression in adjacent normal mucosa, adenoma and carcinoma. The Id-1 expression was examined in 40 adjacent normal mucosa samples, 9 adenomas and 79 carcinomas and was predominantly present in the cytoplasm of epithelial cells of normal mucosa and tumor cells (Fig. 1). Only 2 carcinomas showed weak nuclear staining, besides cytoplasmic staining. A strong Id-1 expression was also seen in tumor embolus in the vessels (Fig. 1). Fig. 2 presents the frequency of the Id-1 expression in normal mucosa, adenoma and carcinoma. In the normal mucosa samples, 1 (2.5%) sample showed strong staining, 10 (25.0%) showed moderate, 19 (47.5%) showed weak and 10 (25.0%) were negative. In the adenomas, 1 (11.1%) case had strong staining, 2 (22.2%) had moderate and 6 (66.7%) had a weak expression. In the carcinomas, 23 (29.1%) had strong, 43 (54.4%) had moderate and 13 (16.5%) had weak staining. There was no negative case in the adenomas and carcinomas.

According to the similarity of clinicopathological features, in further statistical analyses, cases with an Id-1 negative and weak expression were considered as a weaker staining group while moderate and strong-stained cases as a stronger staining group. The Id-1 stronger expression was significantly increased in carcinomas when compared to the adjacent normal mucosa either in the unmatched cases ($P<0.0001$) or matched cases ($P<0.0001$) and was also significantly increased in carcinomas when compared to adenomas ($P=0.003$). However, there was no significant difference of the expression between the adjacent normal mucosa and adenoma ($P=0.73$).

The Id-1 expression in carcinomas in relation to clinico-pathological variables. We analyzed the relationship between the Id-1 expression in carcinomas and clinico-pathological variables. There was a significant difference of the Id-1 expression among Dukes' stages ($P=0.03$, Table I). Further analysis showed that the frequency of the Id-1 stronger expression was significantly increased from Dukes' A to C (50.0% vs. 88.5%, $P=0.04$) and tended to be increased from A to D (50.0% vs.100.0%, $P=0.07$). There were no differences between Dukes' stages A and B (50.0% vs. 71.4%, $P=0.61$), B and C (71.4% vs. 88.5%, $P=0.11$), as well as C and D ($P=0.15$). The frequency of the Id-1 stronger expression was higher in carcinomas with lymph node metastasis compared to those without metastasis (89.5% vs. 68.2%, $P=0.02$, Table I). There was no significant association

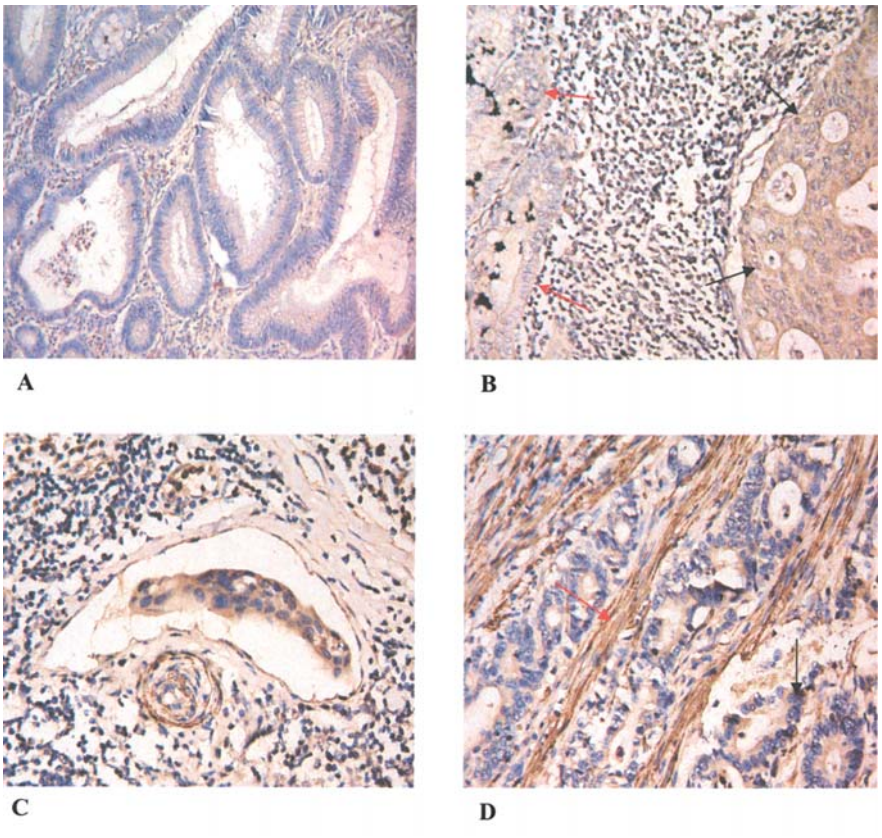

Figure 1. Id-1 immunohistochemistry in adjacent normal mucosa, adenoma and adenocarcinoma of colorectum. (A) Weak expression in adenoma. (B) A weak expression in adjacent normal mucosa (red arrow) and a strong expression in carcinoma (black arrow). (C) Strong expression in tumor embolus. (D) A strong expression in stroma (red arrow) and weak in carcinoma (black arrow).

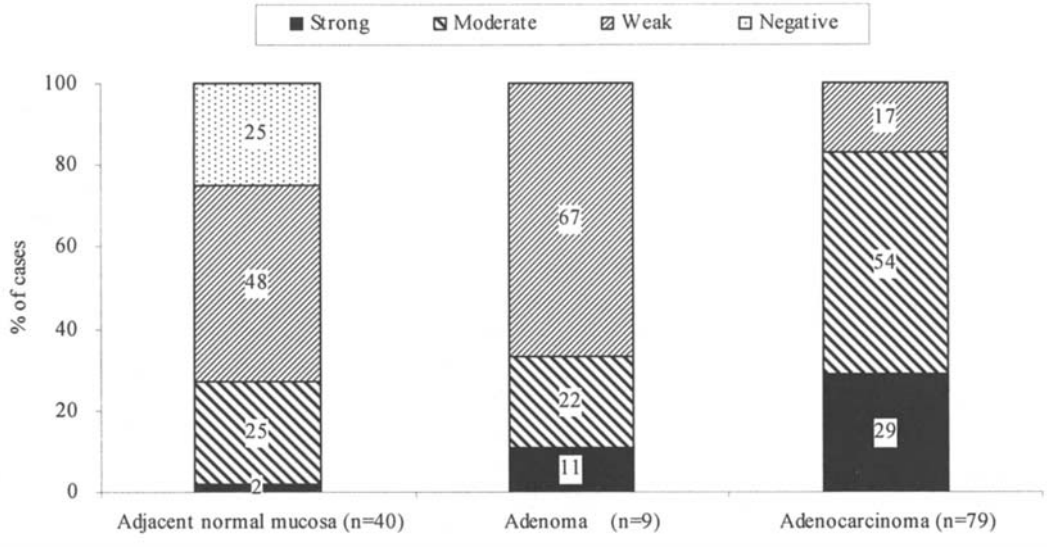

Figure 2. The Id-1 expression in adjacent normal mucosa, adenoma and adenocarcinoma of the colorectum.

of the Id-1 expression with other clinicopathological variables including gender, age, tumor site, size, gross status, invasive depth, growth pattern, histological type, grade of differentiation, necrosis, inflammatory reaction and fibrosis (P>0.05, Table I).

Id-1 was also present in the cytoplasm of the fibroblast and the muscle of the blood vessels in the tumor-associated stroma (Fig. 1) and there were 60 (75.9%) cases in the stronger group (including 27 moderate staining) and 19 (24.1%) in the weaker group (including 1 negative case). We further examined the Id-1 expression in the stroma of carcinomas in relation to clinicopathological variables and found that men had a higher rate of the Id-1 stronger expression than women (85.7% vs. 64.9%, P=0.03). There was no significant

Table I. The Id-1 expression in relation to clinicopathological variables in colorectal adenocarcinoma.

Variables	n	Weaker		Stronger		P-value[a]
		n	(%)	n	(%)	
Gender						0.58
Men	42	6	(14.3)	36	(85.7)	
Women	37	7	(18.9)	30	(81.1)	
Age (years)						0.80
≤58	40	7	(17.5)	33	(82.5)	
>58	39	6	(15.4)	33	(84.6)	
Tumor site						0.40
Right	22	5	(22.7)	17	(77.3)	
Left	16	1	(6.3)	15	(93.7)	
Rectum	41	7	(17.1)	34	(82.9)	
Tumor size (cm)						0.28
≤4.7	44	9	(20.5)	35	(79.5)	
>4.7	35	4	(11.4)	31	(88.6)	
Gross status						0.72
Polyploid	15	2	(13.3)	13	(86.6)	
Ulcerative	64	11	(17.2)	53	(82.8)	
Invasive depth						0.35
Intra-bowel wall	13	1	(7.7)	12	(92.3)	
Ultra-bowel wall	66	12	(18.2)	54	(81.8)	
Growth pattern						0.73
Expansive	33	6	(18.2)	27	(81.8)	
Infiltrative	46	7	(15.2)	39	(84.8)	
Dukes' stage						0.03
A	6	3	(50.0)	3	(50.0)	
B	14	4	(28.6)	10	(71.4)	
C	52	6	(11.5)	46	(88.5)	
D	7	0	(0.0)	7	(100.0)	
Lymph node status						0.02
Non-metastasis	22	7	(31.8)	15	(68.2)	
Metastasis	57	6	(10.5)	51	(89.5)	
Histological type						0.16
Non-mucinous	61	12	(19.7)	49	(80.3)	
Mucinous/signet-ring cell	18	1	(5.6)	17	(94.4)	
Grade of differentiation						0.95
Good	17	3	(17.6)	14	(82.3)	
Moderate	54	9	(16.7)	45	(83.3)	
Poor	8	1	(12.5)	7	(87.5)	
Necrosis						0.26
≤30%	73	13	(17.8)	60	(82.2)	
>30%	6	0	(0.0)	6	(100.0)	
Inflammatory reaction						0.63
Weak	63	11	(17.5)	52	(82.5)	
Strong	16	2	(12.5)	14	(87.5)	

Table I. Continued.

Variables	n	Weaker		Stronger		P-value[a]
		n	(%)	n	(%)	
Fibrosis						0.79
Weak	46	8	(17.4)	38	(82.6)	
Strong	33	5	(15.2)	28	(84.8)	

[a]Chi-square or Fisher exact test.

association of the Id-1 expression in the stroma with other clinicopathological variables (P>0.05, data not shown). Spearman's coefficient of correlation revealed a significant correlation of the Id-1 expression between the tumor cells and stroma (r=0.39; P=0.0004).

Discussion

The Id-1 expression was up-regulated in several types of human cancers including colorectal (10), gastric (11), ovarian (12) and medullary thyroid cancer (13) when compared to the corresponding normal tissues. In the present study, the Id-1 expression was significantly increased in colorectal carcinoma compared to the adjacent normal mucosa or the adenoma. These results suggest that Id-1 played a role in the development of tumors. Moreover, we found that the adenoma did not differ from normal mucosa as regards the Id-1 expression, which was in line with a previous study where the Id-1 expression did not increase in the adenoma compared to the normal mucosa in the colorectum (10). Together with this study, we propose that the increased expression of Id-1 may be a relatively late event during colorectal tumorigenesis. However, the expression of Id-1 was up-regulated in C-cell hyperplastic foci in the medullary thyroid cancer (13), dysplastic/metaplatic ducts in chronic pancreatitis (14) and cirrhosis of the liver with a high risk of hepatocellular carcinoma development (15). These results indicated that Id-1 may be an early marker of pancreatic, thyroid and hepatocellular malignant transformation (13-15). These inconsistent results may be partly due to the different types of tumors.

Id-1 has been found to be involved in the invasion and metastasis of gastric (11), oral (16), breast (17) and prostate cancers (18). In the present study, the frequency of the Id-1 stronger expression was significantly increased in the advanced Dukes' stage and the cases with lymph node metastasis. However, Wilson *et al* (10) did not find an association of Id-1 with Jass stages in colorectal cancer although they found a relationship of the Id-2 expression with the advanced Jass stage. This may depend on the stage system, number of cases, etc. As a negative regulator of transcription, Id-1 may be responsible for changes in gene expression that led to an increased growth and invasion of tumor cells. The constitutive expression of Id-1 converted the non-aggressive prostate cancer cells into more proliferative, invasive and migratory phenotype cells (18). Human metastastic breast cancer cells became significantly less invasive *in vitro* and less metastasis *in vivo* after a down-regulated Id-1 expression. A systemically targeted Id-1 expression *in vivo* may reduce metastases of breast cancer cells in tumor-bearing mice (17). The evidence supports our results that the Id-1 protein was involved in tumor invasion and metastasis and may play a pivotal role in the progression of colorectal cancer.

Tumor invasion is a multi-step process and many of the stages require degradation of the extracellular matrix (ECM) (19). Matrix metalloproteinases (MMPs) are a major family regulating the degradation of the basement membrane and ECM. The overexpression of MMPs was involved in tumor initiation, invasion and metastasis. During the involution of the mammary gland, the Id-1 expression resulted in the up-regulation of a novel 120 kDa MMPs protein, a type IV collagenase MMP family member and directly correlated with the invasiveness of breast cancer cell lines and thus degraded gelatine (20). Breast cancer cells transfected with Id-1 dissociated and invaded the basement membrane and resumed proliferation (20). The high expression of Id-1 also induced an increased secretion of MMP-2 in prostate cancer (18). In the present study, we first examined the Id-1 expression in the stroma and further found that the stromal Id-1 expression was significantly correlated with the tumor cellular Id-1 expression. It may be that there was an interaction between the stromal and tumor cellular Id-1 in tumor progress. We could not prove whether Id-1 was involved in tumor progression by interacting with ECM, especially through the up-regulation of MMPs.

We found that male patients had a higher rate of a stronger Id-1 expression in the stroma than female patients. Epidemiological and molecular studies showed a gender difference in several aspects. For example, a higher incidence was seen in males but proximal cancers were more common in females. A reduced risk of colon cancer was demonstrated with the use of hormone replace treatment (21). Moreover, the effect of the sex steroid on cell growth could be partially substituted or stimulated by the Id-1 expression in breast and prostate cancer cell lines (7,22). Id-1 may contribute to the regulation of multiple pathways for hormone stages in colorectal cancer.

In conclusion, our results suggest that Id-1 protein was involved in the development of colorectal cancers and its overexpression may be a marker in tumor progression.

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