

Overexpression of Id-1 in Gastric Adenocarcinoma: Implication for a Novel Diagnostic Marker

QI WANG^{1,2}, S. W. TSAO², SONGBIN FU¹, WEICHENG XUE³, XIANGNING MENG¹,
HUICHEN FENG², Y.C WONG² and XIANGHONG WANG²

¹Laboratory of Medical Genetics, Department of Biology, Harbin Medical University, Harbin;
Departments of ²Anatomy and ³Pathology, Faculty of Medicine, The University of Hong Kong, China

Abstract. Gastric cancer is the second most common cause of cancer-related death worldwide and the highest incidence of this cancer has been reported in Asia, especially in China. Identification of early stage lesions is vital in achieving high survival rate. However, due to the lack of reliable biomarkers, the majority of gastric cancer is presented at an advanced stage. Recently, it has been reported that Id-1, a helix-loop-helix protein, may be a valuable diagnostic marker in many types of human cancer. In this study, we evaluated Id-1 protein expression in gastric cancer specimens and compared it with non-malignant tissues. In addition, to investigate whether Id-1 expression levels were associated with the aggressiveness of this disease as implicated in other cancer types, we also assessed Id-1 expression levels in primary tumours and their lymph node metastasized lesions. Our results indicate that up-regulation of Id-1 is a frequent event in gastric cancer but its expression levels are not associated with tumour metastasis. Our evidence provides a possible novel marker for the diagnosis of gastric cancer.

Gastric cancer is the second most common cause of cancer-related death worldwide. The highest incidence of gastric cancer has been reported in Asia, especially in Japan and China, while a much lower incidence has been observed in Western Europe and the United States (1). Although the incidence of this cancer has fallen significantly in developed countries, gastric cancer rates remain high in developing countries such as China (2). If detected early, for instance when the tumour is confined to the mucosa, the 5-year

survival rate can reach 90% (3). However, due to the fact that the majority of gastric cancer is detected at an advanced stage, the 5-year survival rate is very low, with overall rates of less than 25% (4). Therefore, early detection of this disease is vital in improving long-term survival.

Unlike other common cancers, such as breast and prostate cancers, research on gastric cancer, especially on the diagnostic and prognostic factors, is limited. Several biological markers that are frequently detected in gastric cancer have been reported, however, due to either small sample sizes or limited number of studies, few definitive conclusions can be drawn from the data available. For example, activation of the Her-2/neu oncogene, which shares significant homology with the epidermal growth factor receptor, has been found in gastric cancer cells and increased Her2/neu levels were associated with an unfavourable outcome (5, 6), indicating its significance in prognosis. Expression of the anti-apoptotic factor Bcl-2 is found to be higher in metastatic cancers than in the primary lesions (7), suggesting its association with invasion potential. In addition, frequent alterations of tumour suppressor genes such as p53 and PTEN are related to poor diagnosis and survival of gastric cancer patients (7-9). Recently, down-regulation of E-Cadherin, a homophilic cell-to-cell adhesion molecule which plays a crucial role in establishing the structural integrity of epithelial tissues, has been found in more than 50% of the diffused gastric cancers (10,11). Although these results provide potential new prognostic factors for gastric cancer, research on the identification of novel molecular markers with high specificity for gastric cancer is still needed.

Recently, we have identified an up-regulation of a helix-loop-helix protein, Id-1 (inhibitor of differentiation/DNA synthesis), in the development of prostate cancer (12). We and others also found that Id-1 expression was unique in many types of human cancer such as breast (13), prostate (14) and nasopharyngeal (15) cancers and that increased Id-1 expression was associated with aggressiveness of these cancers. Recently, over-expression of Id-1 has been

Correspondence to: Dr Xianghong Wang, PhD, Research Assistant Professor, Dept. of Anatomy, Laboratory Block, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, SAR, China. Tel: 852 2819 2867, Fax: 852 2817 0857, e-mail: xhwang@hkucc.hku.hk

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Table I. Clinicopathological parameters of gastric cancer specimens.

Clinicopathological parameter	Number of cases
Gender	
Male	55
Female	10
Location	
Cardia	8
Body	48
Antrum	9
Histologic type	
Moderately-differentiated	18
Moderately-poorly-differentiated	11
Poorly-differentiated	27
Poor-signet ring	1
Signet ring cell	5
Mucinous	3

suggested as an independent marker for unfavourable prognosis in early stage cervical cancer (16). These lines of evidence suggest that Id-1 expression may be a specific biomarker for both diagnosis and prognosis of human cancer. Id proteins are a group of HLH transcription factors that lack the DNA binding domain. Therefore, they act mainly as inhibitors of basic HLH proteins which positively regulate cell differentiation, resulting in inhibition of cell differentiation (17,18). Recently, the oncogenic role of Id-1 has been suggested to be regulated through inactivation of tumour suppressor pathways such as the p16^{INK4a}/RB pathway (19, 20), and activation of pathways that lead to cell proliferation and protection of apoptosis such as the Raf/MEK and NF κ B pathways (21, 22). These lines of evidence strongly indicate that Id-1 may be a common oncogene that plays an important part in the development and progression of human cancer. Since inactivation of the p16/RB pathway (23, 24) and activation of the MAPK and NF κ B pathways (25-27) are common events in gastric cancers, in this study we investigated the expression of Id-1 protein in gastric cancer specimens and studied its association with the aggressiveness of this cancer. Using 15 pairs of malignant and adjacent non-malignant tissues as well as 50 pairs of the primary and lymph node metastasized tumour samples, we studied, by immunohistochemistry, whether up-regulation of Id-1 was a frequent event in gastric cancer and its association with the progression of gastric cancer.

Materials and Methods

Gastric tissue samples. A total of 65 formalin-fixed and paraffin-embedded tumour specimens and 18 non-malignant tissues were collected from the archival tissue bank of the Department of

Pathology, the third affiliated hospital, Harbin Medical University (Harbin, China). This included 15 adenocarcinomas and adjacent non-malignant tissues, 50 pairs of primary tumour tissues and their lymph node metastasis. All of the tissues were obtained by surgical resection. For the 15 pairs of malignant and non-malignant tissues, the age distribution was between 40 and 76 years old and the male and female ratio was 2.75:1. The 50 pairs of primary and metastasized cancer tissues were collected from patients aged from 20 to 70 years and the male to female ratio was 7.3:1. The histopathology of the specimens was first examined and classified by pathologists in the Department of Pathology in the same hospital. Table I summarizes the detailed clinicopathological features of all cancer specimens used in this study. Three normal gastric tissues were collected from patients with duodenum ulcer who underwent surgical resection.

Immunohistochemistry. Detailed experimental procedures were described in our previous studies (14). Briefly, tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer-thick sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. After antigen retrieval, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min, followed by rehydration in PBS and incubation with 5% rabbit serum for 30 min to bind nonspecific antigens. The slides were then incubated overnight at 4°C with polyclonal antibodies against Id-1 (Santa Cruz Biotechnology, CA, USA) in TBS containing 2% rabbit serum and 1% bovine serum albumin. This was followed by incubation with biotinylated anti-rabbit IgG at a dilution of 1:200 for 30 min at room temperature, followed by peroxidase-conjugated avidin-biotin complexes and 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark). The sections were then counterstained with Mayer's haematoxylin and analyzed by standard light microscopy. Sections were incubated with TBS containing 2% rabbit serum and 1% bovine serum albumin without primary antibody as negative controls.

Evaluation of Id-1 immunostaining intensity. The staining intensity for Id-1 protein was scored on a scale from "+" to "++++" where the highest staining intensity was defined as "++++". For each experiment, both non-malignant and malignant specimens were included so that the relative staining intensity between these two groups could be justified and compared. All sections were evaluated by three investigators (Q.W., X.W and W.X) in a blinded manner without any information relating to clinical pathological characteristics. When there was a disagreement among the investigators regarding the scores, final results were generated after mutual agreement.

Statistical analysis. The staining intensity of each slide was scored as "-", "+", "++", "+++" or "++++", representing none, mild, moderate, or heavy staining, respectively. Specimens were then grouped according to gender of the patients, location, growth pattern, depth of invasion and histological types of the tumour. The difference between Id-1 expression intensity was analysed using the SPSS statistical software (SPSS Inc, Chicago, IL, USA). The analysis of the association between Id-1 expression and the clinical subgroups was performed by Chi-square (χ^2) test for categorical variables and by Spearman correlation test for continuous variables. A level of $p < 0.05$ was considered as statistically significant.

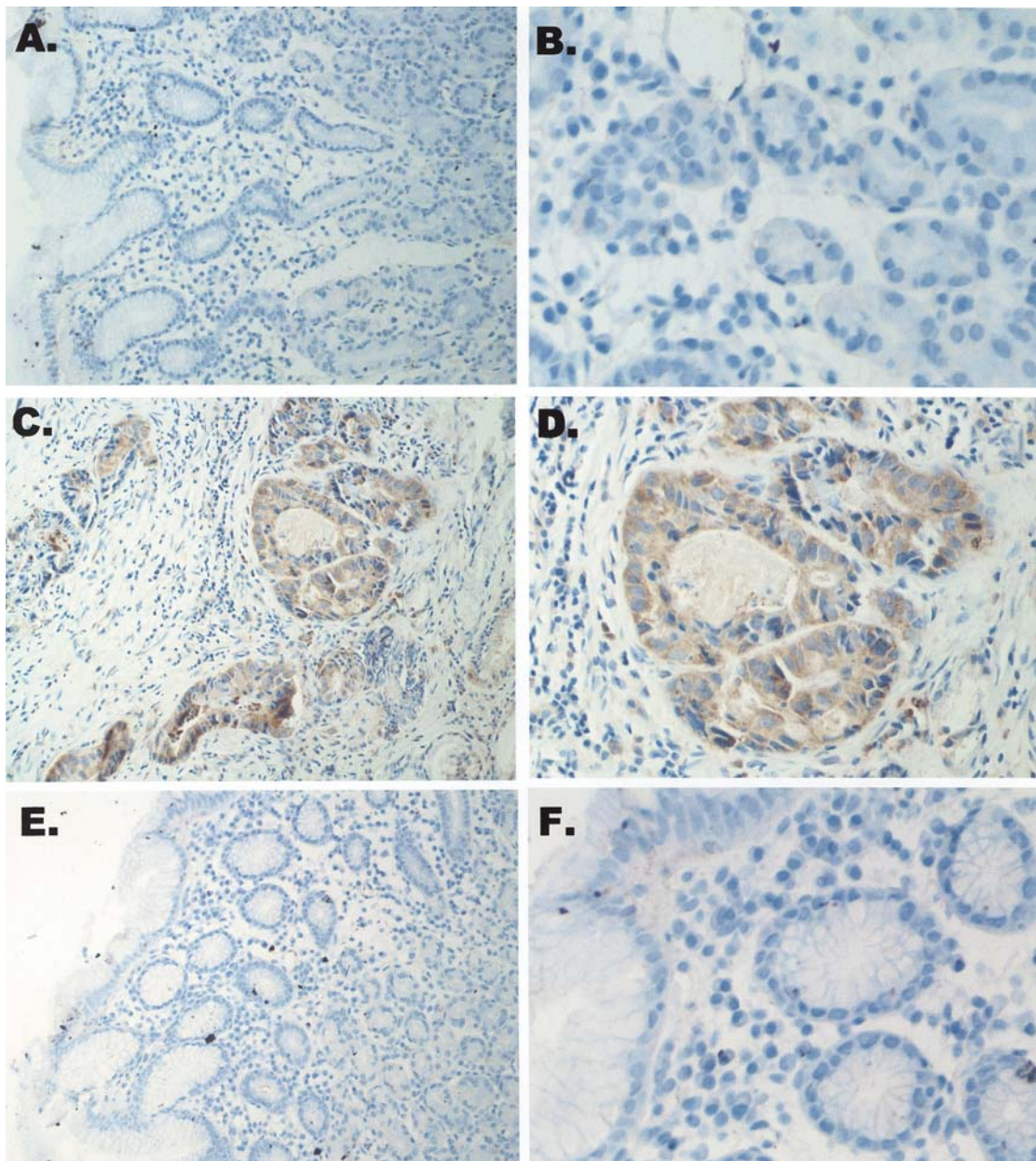


Figure 1. Immunostaining of Id-1 protein in gastric cancer and adjacent non-malignant tissues. A-B: Negative Id-1 staining is found in non-malignant gastric epithelial cells. C-D: Positive cytoplasmic Id-1 staining was observed in a case of intestinal-type gastric cancer. E-F: A case of non-malignant gastric tissue used as a negative control with omission of primary antibody. Photos were taken under 200x (A, C, E) and 400x (B, D, F) magnifications. Note that up-regulation of Id-1 is shown in malignant gastric cancer cells but absent in the non-malignant cells.

Results

Differential Id-1 expression between malignant and non-malignant gastric tissues. To investigate if there was an up-regulation of Id-1 protein in gastric cancer cells, we studied Id-1 expression on 15 primary gastric cancer specimens and their adjacent non-malignant tissues. As shown in Figure 1, while there was no evidence of Id-1 protein expression in the

non-malignant gastric epithelial cells (A and B), the adenocarcinoma cells showed strong positive cytoplasmic staining for the Id-1 protein (C and D), which agrees with our previous findings that Id-1 protein was detected in the cytoplasm of prostate and nasopharyngeal carcinoma cells but absent in non-malignant cells (14, 15). Thirteen out of 15 (86.7%) of the cancer tissues showed positive staining for Id-1, while it was undetectable in all of the non-malignant tissues

Table II. Summary of Id-1 expression in primary tumour and adjacent normal tissues.

No. of samples		Staining intensity	Fisher's exact test (Two-tailed)
Primary tumour (%)	Adjacent normal tissue (%)		
5 (33.3%)	0	++++	$P < 0.0001$
3 (20%)	0	+++	
2 (13.3%)	0	++	
2 (13.3%)	0	+	
3 (20%)	18 (100%)*	-	
Total 15	18		

Table III. Summary of Id-1 expression in primary tumours and their lymph node metastasis.

No. of samples		Staining intensity	Rank sums test
Primary tumour (%)	Lymph node metastasis (%)		
17 (34%)	14 (28%)	++++	$P < 0.05$
16 (32%)	12 (24%)	+++	
9 (18%)	5 (10%)	++	
6 (12%)	2 (4%)	+	
2 (4%)	17 (34%)	-	
Total 50	50		

(Table II). To further confirm the results on the non-malignant tissues, 3 normal gastric tissues from non-cancer patients were also studied under the same conditions and no Id-1 expression was detected. Among the Id-1-positive malignant tissues, over 50% showed high staining intensity ("+++" to "++++") (Table II). Statistical analysis indicated that Id-1 expression was significantly higher in malignant gastric cancer tissues compared to the non-malignant tissues (Table II, $p < 0.0001$).

Differential Id-1 expression between primary and lymph node metastasized gastric cancers. To further investigate the significance of Id-1 expression in gastric cancer, we next increased the sample size and studied 50 cases of primary tumour samples and their lymph node metastasis using non-malignant tissues as controls. Similar to the results observed previously (Figure 1 and Table II), the majority of the primary tumour samples (96%) stained positive for Id-1, while a high percentage (66%) had relatively high expression ("+++" to "++++") (Figure 2 and Table III). Id-1 expression in lymph node metastasized tumours was lower (Figure 2E and F), with 34% showing negative staining, compared to the primary tumours. However, over 50% of the tumour samples, regardless of being primary or metastasized, showed high staining intensity ("+++" to "++++") as observed previously (Table II). These results indicate that up-regulation of Id-1 protein is a frequent event in gastric cancer but its expression levels are not associated with metastasis.

However, we did not observe any statistically significant association between Id-1 expression levels and the gender of the patients, location, growth pattern, depth of invasion or histological type of the tumour, indicating that Id-1 expression levels may not play an essential role in the clinical characteristics of gastric cancer.

Discussion

In this study, we report an up-regulation of a helix-loop-helix protein, Id-1, in gastric cancer using 65 clinical samples. Since Id-1 expression was found in the majority of gastric cancer tissues (over 80%), our results indicate that Id-1 may provide a novel marker for gastric cancer. The fact that Id-1 expression levels were not found to be significantly associated with the metastatic potential of gastric cancer suggests that it may not be a crucial factor in metastasis.

Like other Id family members, Id-1 prevents helix-loop-helix proteins from binding to DNA, thus inhibiting transcription of a number of genes responsible for differentiation and proliferation (18). A number of recent studies have also suggested that Id-1 may function as an oncogene since its overexpression is found in many types of human cancer and its activation is able to interfere with several cell signalling pathways involved in human carcinogenesis (12,14,15, 20-22). In the present study, we provide the first evidence on the possible role of Id-1 in gastric cancer. Previously, it has been reported that inactivation of the p16/RB pathway is a common event in gastric cancer (23, 24). In addition, activation of the MAPK and NF κ B pathways are associated with gastric cancer development and progression (25-27). Since these cell signalling pathways have been shown to be regulated by Id-1 in other cell systems (14, 15, 20-22), it is possible that the increased Id-1 expression in gastric cancer may also contribute to the alteration of these pathways frequently observed in this cancer. We are currently studying the direct role of Id-1 expression in gastric cancer cell proliferation and its effect on p16/RB, MAPK and NF κ B pathways.

In addition to its role in cell proliferation, Id-1 has been indicated to promote the invasiveness of human cancer cells (12) and its expression levels were correlated with the

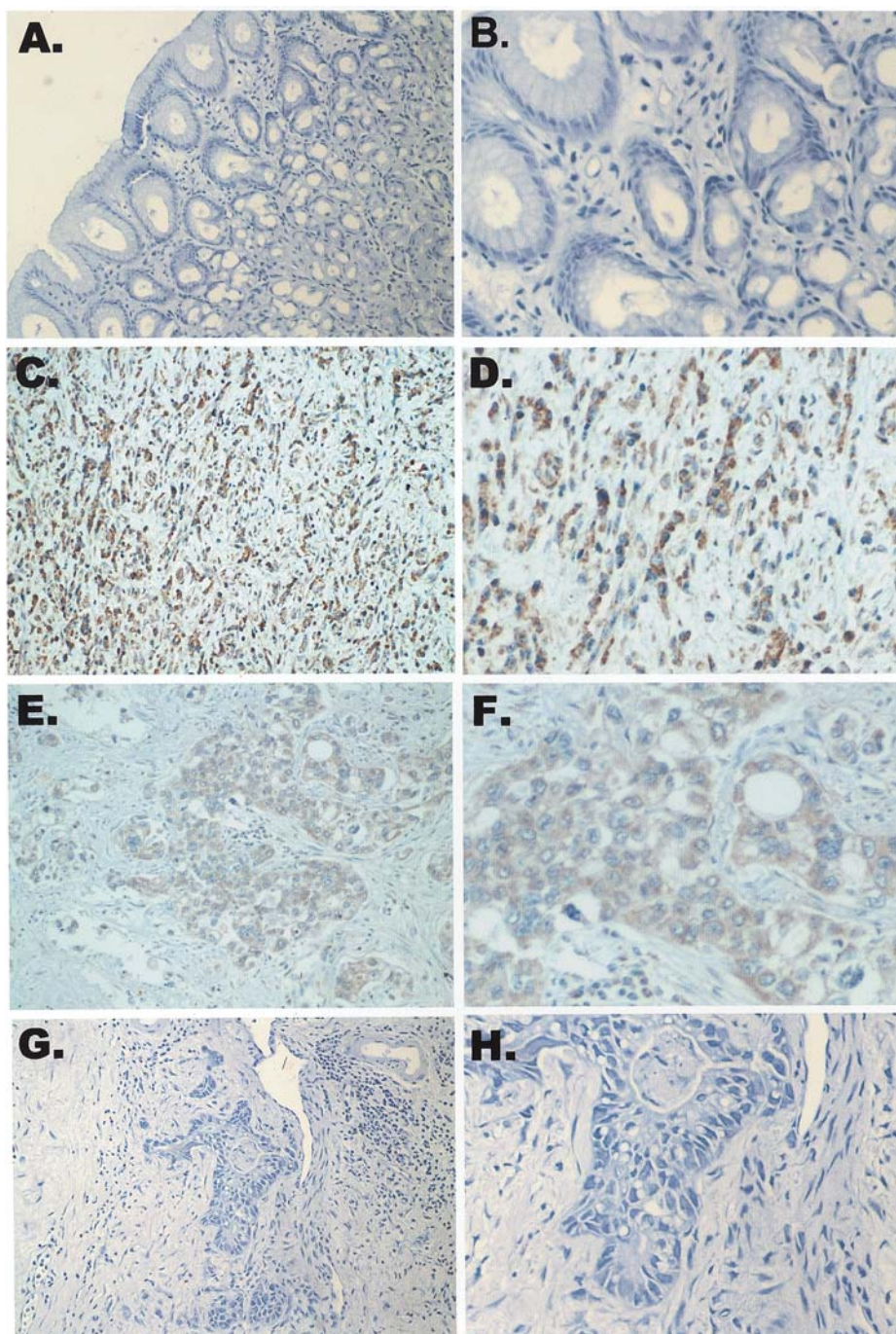


Figure 2. Immunostaining of Id-1 protein in primary gastric cancer and the lymph node metastasis. A-B: Negative Id-1 staining is found in non-malignant gastric epithelial cells. C-F: Positive cytoplasmic Id-1 staining was observed in a case of diffused-type gastric cancer (C, D) and its lymph node metastasis (E-F). G-H: A case of malignant gastric tissue used as a negative control with omission of primary antibody. Photos were taken under 200x (A, C, E, G) and 400x (B, D, F, H) magnifications. Note that Id-1 protein expression is shown in both primary gastric cancer and its metastasized lesions.

progression of several human cancers (14, 16, 28). However, using 50 primary gastric tumour samples and their lymph node metastasis, we did not observe any correlation between Id-1 expression levels and the metastatic ability of the tumours (Figure 2, Table III). In fact, the Id-1 expression levels seemed to be lower in the metastatic tumours than in the primary tumour cells (Table III). It is possible that, unlike observations in breast and ovarian cancers (28, 29),

Id-1 expression may not be essential for the progression and metastasis of gastric cancer.

In summary, we have identified an up-regulation of a novel factor, Id-1, in gastric cancer. Our results further support the role of Id-1 as an oncogene in the development of human cancer. Since the molecular pathogenesis of human gastric cancer is largely unknown, our evidence implicates a possible novel factor in the tumourigenesis of gastric cancer.

References

- 1 Neugut AI, Hayek M and Howe G: Epidemiology of gastric cancer. *Semin Oncol* 23: 281-291,1996.
- 2 Roth AD: Curative treatment of gastric cancer: towards a multi-disciplinary approach? *Crit Rev Oncol Hematol* 46: 59-100, 2003.
- 3 Hanazaki K, Wakabayashi M, Sodeyama H, Miyazawa M, Yokoyama S, Sode Y, Kawamura N, Miyazaki T and Ohtsuka M: Clinicopathologic features of submucosal carcinoma of the stomach. *J Clin Gastroenterol* 24: 150-155, 1997.
- 4 Chan AO, Wong BC and Lam SK: Gastric cancer: past, present and future. *Can J Gastroenterol* 15: 469-474, 2001.
- 5 Ross JS and McKenna BJ: The HER-2/neu oncogene in tumors of the gastrointestinal tract *Cancer Invest* 19: 554-568, 2001.
- 6 Garcia I, Vizoso F, Martin A, Sanz L, Abdel-Lah O, Raigoso P and Garcia-Muniz JL: Clinical significance of the epidermal growth factor receptor and HER2 receptor in resectable gastric cancer. *Ann Surg Oncol* 10: 234-241, 2003.
- 7 Pan CC, Chen PC, Wang LS, Lee JY and Chiang H: Expression of apoptosis-related markers and HER-2/neu in thymic epithelial tumours. *Histopathology* 43: 165-172, 2003.
- 8 Wu CW, Lin YY, Chen GD, Chi CW, Carbone DP and Chen JY: Serum anti-p53 antibodies in gastric adenocarcinoma patients are associated with poor prognosis, lymph node metastasis and poorly differentiated nuclear grade. *Br J Cancer* 80: 483-488, 1999.
- 9 Byun DS, Cho K, Ryu BK, Lee MG, Park JI, Chae KS, Kim HJ and Chi SG: Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma. *Int J Cancer* 104: 318-327, 2003.
- 10 Hirohashi S: Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 153: 333-339,1998.
- 11 Fricke E, Keller G, Becker I, Rosivatz E, Schott C, Plaschke S, Rudelius M, Hermannstadter C, Busch R, Hofler H, Becker KF and Lubber B: Relationship between E-cadherin gene mutation and p53 gene mutation, p53 accumulation, Bcl-2 expression and Ki-67 staining in diffuse-type gastric carcinoma. *Int J Cancer* 104: 60-65, 2003.
- 12 Ouyang XS, Wang X, Lee DT, Tsao SW and Wong YC: Up-regulation of TRPM-2, MMP-7 and ID-1 during sex hormone-induced prostate carcinogenesis in the Noble rat. *Carcinogenesis* 22: 965-973, 2001
- 13 Lin CQ, Singh J, Murata K, Itahana Y, Parrinello S, Liang SH, Gillett CE, Campisi J and Desprez PY: A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. *Cancer Res* 60: 1332-1340, 2000.
- 14 Ouyang XS, Wang X, Lee DT, Tsao SW and Wong YC: Overexpression of ID-1 in prostate cancer. *J Urol* 167: 2598-2602, 2002.
- 15 Wang X, Xu K, Ling MT, Wong YC, Feng HC, Nicholls J and Tsao SW: Evidence of increased Id-1 expression and its role in cell proliferation in nasopharyngeal carcinoma cells. *Mol Carcinog* 35: 42-49,2002.
- 16 Schindl M, Oberhuber G, Obermair A, Schoppmann SF, Karner B and Birner P: Overexpression of Id-1 protein is a marker for unfavourable prognosis in early-stage cervical cancer. *Cancer Res* 61: 5703-5706, 2001.
- 17 Norton JD, Deed RW, Craggs G and Sablitzky F: Id helix-loop-helix proteins in cell growth and differentiation. *Trends Cell Biol* 8: 58-65, 1998.
- 18 Norton JD: ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* 113: 3897-3905, 2000.
- 19 Ouyang XS, Wang X, Ling MT, Wong HL, Tsao SW and Wong YC: Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of p16(INK4a)/pRB pathway. *Carcinogenesis* 23: 721-725, 2002.
- 20 Alani RM, Young AZ and Shifflett CB: Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. *Proc Natl Acad Sci USA* 98: 7812-7816, 2001.
- 21 Ling MT, Wang X, Ouyang XS, Lee TK, Fan TY, Xu K, Tsao SW and Wong YC: Activation of MAPK signaling pathway is essential for Id-1 induced serum independent prostate cancer cell growth. *Oncogene* 21: 8498-505, 2002.
- 22 Ling MT, Wang X, Ouyang XS, Xu K, Tsao SW and Wong YC: Id-1 expression promotes cell survival through activation of NF-kappaB signalling pathway in prostate cancer cells. *Oncogene* 22: 4498-4508, 2003.
- 23 Tsujimoto H, Hagiwara A, Sugihara H, Hattori T and Yamagishi H: Promoter methylations of p16INK4a and p14ARF genes in early and advanced gastric cancer. Correlations of the modes of their occurrence with histologic type. *Pathol Res Pract* 198: 785-794, 2002.
- 24 Kim H, Kim YH, Kim SE, Kim NG, Noh SH and Kim H: Concerted promoter hypermethylation of hMLH1, p16INK4A, and E-cadherin in gastric carcinomas with microsatellite instability. *J Pathol* 200: 23-31,2003.
- 25 Kanai M, Konda Y, Nakajima T, Izumi Y, Kanda N, Nanakin A, Kubohara Y and Chiba T: Differentiation-inducing factor-1 (DIF-1) inhibits STAT3 activity involved in gastric cancer cell proliferation via MEK-ERK-dependent pathway. *Oncogene* 22: 548-554, 2003.
- 26 Kadowaki Y, Ishihara S, Miyaoka Y, Rumi MA, Sato H, Kazumori H, Adachi K, Takasawa S, Okamoto H, Chiba T and Kinoshita Y: Reg protein is overexpressed in gastric cancer cells, where it activates a signal transduction pathway that converges on ERK1/2 to stimulate growth. *FEBS Lett* 530: 59-64, 2002.
- 27 Sasaki N, Morisaki T, Hashizume K, Yao T, Tsuneyoshi M, Noshiro H, Nakamura K, Yamanaka T, Uchiyama A, Tanaka M and Katano M: Nuclear factor-kappaB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. *Clin Cancer Res* 7: 4136-4142, 2001.
- 28 Schindl M, Schoppmann SF, Strobel T, Heinzl H, Leisser C, Horvat R and Birner P: Level of Id-1 protein expression correlates with poor differentiation, enhanced malignant potential, and more aggressive clinical behavior of epithelial ovarian tumors. *Clin Cancer Res* 9: 779-785, 2003.
- 29 Schoppmann SF, Schindl M, Bayer G, Aumayr K, Dienes J, Horvat R, Rudas M, Gnant M, Jakesz R and Birner P: Overexpression of Id-1 is associated with poor clinical outcome in node negative breast cancer. *Int J Cancer* 104: 677-682, 2003.

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