Expression and prognostic value of Id protein family in human breast carcinoma

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Abstract. Inhibitors of DNA binding/inhibitors of differentiation (Id) protein family (Id-1, -2, -3 and -4) of helix-loop-helix proteins have been shown to be involved in carcinogenesis and are regarded as prognostic markers in several types of human cancers. However, the roles of Id proteins during breast carcinoma progression remain unclear. The objective was to study the effects of Id proteins in breast cancer. The expression of Id-1, Id-2, Id-3 and Id-4 proteins was examined in 122 dissected female human breast carcinoma tissues and 22 normal female breast specimens by immunohistochemical assay and the relationship between Id staining and clinical pathological characteristics of breast cancer was also analyzed. The over-expressed Id-1 and down-regulated Id-4 proteins were found both correlated with poorer differentiation and more aggressive behavior of the tumor. Id-1 protein could be termed as a negative prognostic marker while Id-4 protein as a positive marker for patients with breast carcinoma. Although the differentially expressed Id-2 and -3 may be correlated with some clinical parameters, they could not be used as independent prognostic factors in human breast cancer.

Introduction

Breast cancer remains one of the main cancers for women in Western countries, currently with no permanent cure. Despite considerable diagnostic and therapeutic advances for breast carcinoma in recent years, it is still crucial to provide clinicians with further molecular markers concerning useful information about possible therapeutic options and prognosis (1). Many genetic alterations, which occur during transformation of normal breast epithelial cells, have been explored. Identifying these genes involved in the alterations affecting cell growth control, maintenance of differentiated phenotype and functions, is not only essential for understanding how breast carcinoma develops and progresses, but also for deriving better methods for treatment and prognosis.

Inhibitors of DNA binding/inhibitors of differentiation (Id) protein family, a group of basic helix-loop-helix transcription (bHLH) factors, have been shown to interfere with the activities of the other bHLH transcription factors by heterodimerization in many cellular processes including carcinogenesis (2). Their participation in cell cycle control, cancer development, angiogenesis and apoptosis has been suggested in a previous study (3). Therefore, it can be considered as a potential target for cancer therapy.

There are four members of the Id family, namely Id-1, Id-2, Id-3 and Id-4. Although they belong to the same family, their localization to chromosomes and their pattern of expression and function have marked differences (2). Expressions of Id-1, Id-2 and Id-3 are elevated in colorectal adenocarcinoma specimens when compared to normal tissues (4), while Id-4 is hypermethylated and shows reduced expression correlating with a poor prognosis in colorectal adenocarcinoma (5). Id-1 is a prognostic marker of cervical carcinoma, in which highly expressed Id-1 is associated with poor prognosis (6). Id-1, Id-2 and Id-3 also participate in early stages of hepatocarcinogenesis but not in progression, and reduced expression of Id-1 is associated with a better prognosis in hepatocellular carcinoma (7). Id-1 is over-expressed in gastric cancer, in which Id-1 expression is positively correlated with tumor progression (8), while Id-4, is hypermethylated, and reduced (9). Id-1 and -3 are indispensable to the metastasis of gastric cancer, since their metastatic potentials are significantly decreased in Id-1 and -3 double-knockdown cells (10). Over-expressions of Id-1 and -2 have also been described in pancreatic cancer and could be early markers of pancreatic malignant transformation. These results suggest that expression of different Id proteins in certain of cancers could be used as prognostic markers.

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As to human breast cancer, deregulations of Id expression have been reported. Id-1 protein was highly expressed in infiltrating human breast cancer specimens with a significant correlation with tumor angiogenesis (11), while high cytoplasmic level of Id-2 reflects a favorable prognosis in patients with primary breast cancer and reduces invasiveness of breast cancer cells (12). Id-4 is highly expressed in normal human mammary epithelium and ER-negative carcinomas, but is suppressed in ER-positive breast carcinomas and preneoplastic lesions (13). Hypermethylation of Id-4, which leads to reduced gene expression, is significantly associated with the risk of regional lymph node metastasis of human breast cancer (14). The aims of our study were to investigate the expression of Id proteins in breast carcinoma, their influence on the survival of patients, and the association of Id proteins expression.

Materials and methods

Tissue samples. One hundred and twenty-two consecutive formalin-fixed, paraffin-embedded cases of human breast cancer and 20 adjacent normal mammary tissues (all females, ranging in age from 28 to 80 years; median, 50 years), dating from 1995 to 2002, were retrieved from the Department of Pathology at the First Affiliated Hospital of Harbin Medical University. All the patients lived in Harbin or nearby. They were initially evaluated by clinical and ultrasound examination or molybdenum target X-ray of the breast. Treatment of cancer patients consisted of radical mastectomy and modified radical mastectomy in breast cancer. All of the patients, except for those stage I, were treated adjuvant chemotherapy with 5-FU during the procession of operation. All of the patients had received chemotherapy or radiation therapy after surgical therapy. All the deceased patients in this study died of breast cancer, not from other diseases or accidents. The adjacent tissues were chosen >2 cm away from the tumor margin and proved to be normal mammary tissues without cancer infiltrating or hyperplasia of mammary glands by H&E staining.

Immunohistochemistry. Sections (4 μm), cut from the original paraffin blocks, were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. After inhibition of endogenous peroxidase activity for 10 min with 0.3% H_2O_2, the sections were blocked with 10% normal goat serum (Zymed Co., Ltd.) for 20 min and incubated overnight with rabbit anti-human Id-1 (sc-488, diluted 1:100), Id-2 (sc-489, diluted 1:150), Id-3 (sc-490, diluted 1:100), or Id-4 (sc-491, diluted 1:150) polyclonal antibody (Santa Cruz Biotechnology, CA) at 4˚C. The sections were then incubated with biotinylated anti-rabbit IgG (diluted 1:200) for 30 min at 37˚C followed by incubation with peroxidase-conjugated avidin/biotin complexes and stained with 3,3-diaminobenzidine (DAB) (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). Finally, the sections were counterstained with hematoxylin. Normal goat serum and blocking with antibody specific peptides were used as a negative control for the staining reactions.

Evaluation of immunostaining. All sections were examined by immunohistochemical assessment system, Motic Images
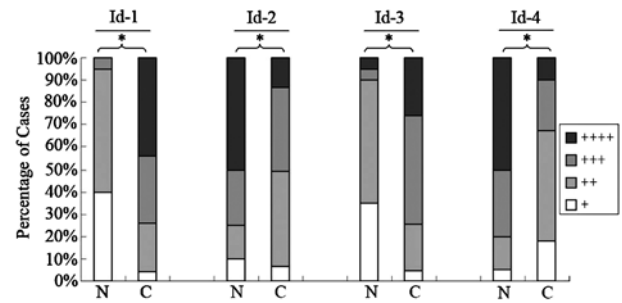

Figure 1. Summary of Id protein immunohistochemical staining results in breast carcinoma and adjacent benign tissues. Distribution of the Id-1, Id-2, Id-3 and Id-4 expression in each histopathological group of breast carcinoma and non-cancer breast tissues are shown as follows: grey (intensity ++++), hatched (intensity +++), dotted (intensity ++), and white, (intensity +). The cytoplasmic staining of Id-1 and Id-3 were significantly increased in breast cancer (P<0.01) when compared to the adjacent benign tissues, while Id-2 and Id-4 were down-regulated (P<0.01). These results suggest that Id-1 and Id-3 proteins play a positive role in neoplastic transformation of the breast (N, normal breast tissues; C, breast carcinoma tissues).

Advanced 3.0. The staining was semi-quantitatively evaluated by assigning a score for the proportion of area positively stained, which was classified into four groups (6,7): +, 0-10%; ++, 10-20%; +++, 20-30%; ++++, 30-40%. The intensity of '+, ++' were labeled as 'low expression', and '+++, ++++' were labeled as 'high expression'.

Statistical analysis. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL). Differences in Id expressions between breast cancer tissues and adjacent tissues were analyzed by non-parametric Mann-Whitney U test. Kruskal-Wallis and non-parametric Mann-Whitney U test were employed for analysis of the association between Id expression and clinicopathological parameters. The 3-year survival rate was performed by non-parametric Mann-Whitney U test. Significance was defined as P<0.05. The value for prognosis of Id expression was assessed by the method of Cox regression, and significance was affirmed at P=0.1.

Results

Differential expression of Id proteins in breast cancer tissues and adjacent normal mammary tissues. To investigate whether Id proteins are dysregulated in breast cancer, the expressions of Id proteins in breast cancer (n=122) and adjacent breast tissue (n=20) specimens were analyzed. The results of Id-1, 2, 3, 4 are summarized in Fig. 1. The expression of Id-1, 2, 3, 4 proteins were located in cytoplasmic staining (Fig. 2). The expression of Id-1 and Id-3 was significantly increased in breast cancer tissues when compared to the adjacent normal tissues (P<0.01) (Fig. 1). However, marked down-regulation of Id-2 and Id-4 were also observed in breast cancer tissues (P<0.001) (Fig. 1). These results suggest that Id-1 and Id-3, not Id-2 and Id-4, may have positive influence in neoplastic transformation of human mammary tissue.

Correlation between Id proteins expression and clinicopathological characteristics. The relationship between Id protein expression and clinicopathological features was analyzed in this study. The findings are summarized in Table I.
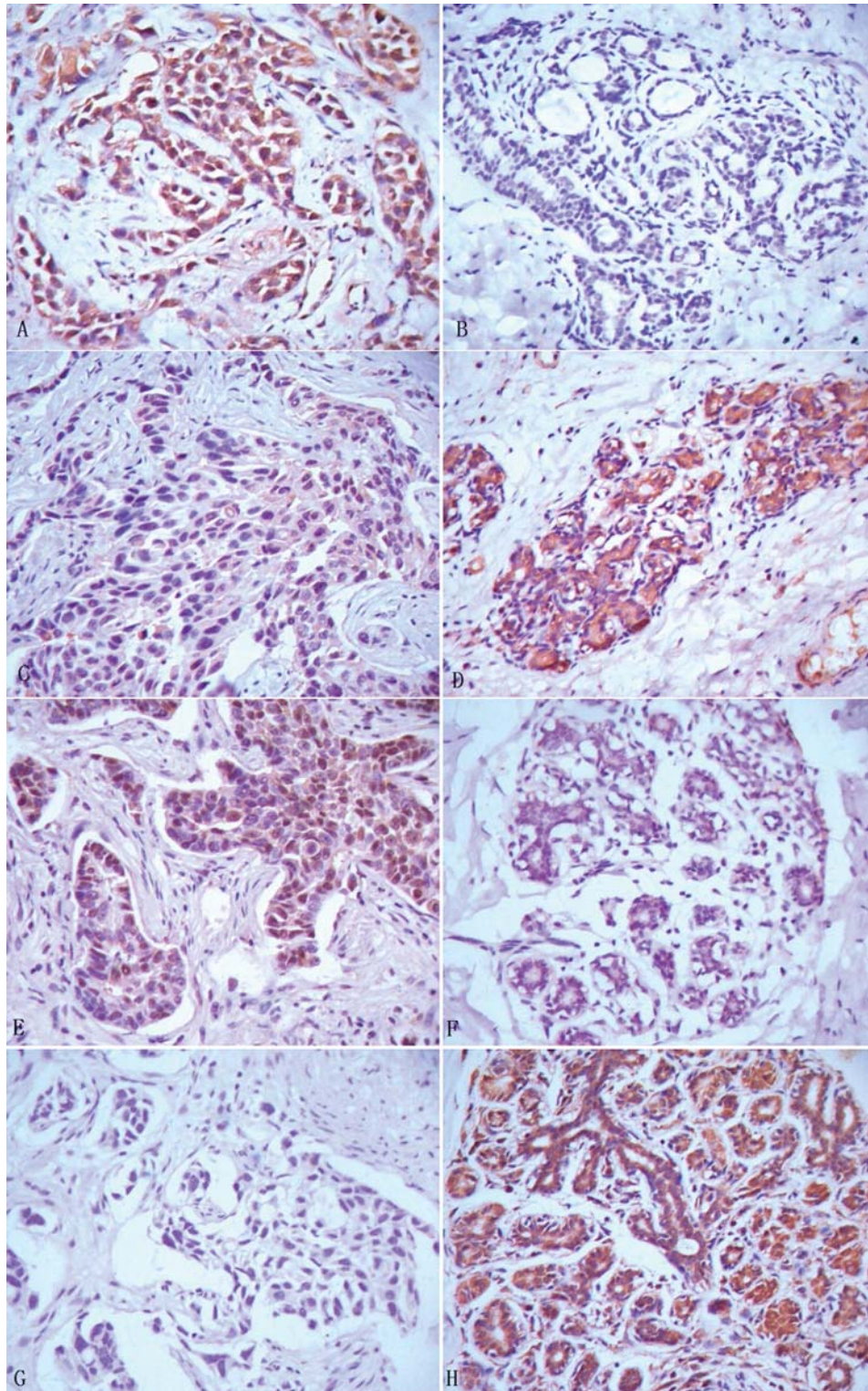
Figure 2. The expression of Id proteins in normal breast tissue, well and poorly differentiated breast carcinomas using immunohistochemistry. Expression of Id-1 in breast carcinomas (A) was much higher than that of normal breast tissues (B). The cytoplasmic staining of Id-2 (C) in the normal breast tissues was increased when compared to breast carcinoma (D). Expression of Id-3 in breast carcinoma (E) was much higher than that of normal breast tissues (F) while cytoplasmic staining of Id-4 (G) in the normal breast tissues was increased in comparison with breast carcinoma (H) (magnifications: A-H, x400).

Id-2 and Id-4 protein expression showed an inverse correlation with tumor size (P=0.003 and P=0.047), while Id-3 protein expression increased in larger tumors (P=0.011). Only Id-1 expression showed a positive relationship between Id proteins and metastatic potential (P=0.015). Expression of Id-1 protein was also shown to be positively correlated with TNM stage while Id-4 expression was negatively related to TNM stage.

To examine the roles of Id proteins during neoplastic transformation and progression of breast cancer, the differential expressions of Id protein staining among normal human

Table I. Expression of Id proteins was analyzed with pathological features.

Characteristics	Staining intensity															
	Id-1				Id-2				Id-3				Id-4			
	+	++	+++	++++	+	++	+++	++++	+	++	+++	++++	+	++	+++	++++
Age																
<50	2	13	20	28	5	24	25	9	3	12	32	16	10	34	16	3
≥50	3	14	16	26	3	28	21	7	3	13	27	16	12	26	12	9
Cell types																
Infiltrate duct cancer	2	21	23	36	7	30	34	11	4	20	37	21 ⌉	15	42	21	4 ⌉
Infiltrate lobular carcer	1	1	4	12	1	13	3	1	0	2	9	7	6	8	4	0
Adenocarcinoma	2	3	4	2	0	5	4	2	2	3	6	0 ⎮a	0	4	0	7 ⎮b
Carcinoma molle	0	0	1	1	0	1	1	0	0	0	1	1	0	2	0	0
Intraductal carcinoma	0	1	1	0	0	2	0	0	0	2	0	0	1	1	0	0
Mucinous adenocarcinoma	0	1	3	3	0	1	4	2	0	0	4	3 ⌋	0	3	3	1 ⌋
Post-menopause																
Yes	3	11	10	24	2	24	15	7	3	9	21	15	11	21	10	6
No	2	16	26	30	6	28	31	9	3	16	38	17	11	39	18	6
Family history																
Yes	2	7	5	13	2	8	10	7	1	4	13	9	5	11	6	5
No	3	20	31	41	6	44	36	9	5	21	46	23	17	49	22	7
Differentiation																
Poor	3	23	30	53 ⌉b	8	44	42	14	4	22	50	32 ⌉a	21	54	28	5 ⌉b
Well	2	4	6	2 ⌋	0	8	4	2	2	3	9	0 ⌋	1	6	0	7 ⌋
Tumor size																
≤2cm	2	14	11	13	1	9	23	7 ⌉	3	11	18	8 ⌉	5	15	15	5 ⌉
2-5 cm	2	9	22	32	5	35	19	6 ⎮b	3	13	34	15 ⎮b	13	38	8	6 ⎮a
≥5cm	1	4	3	9	2	7	4	3 ⌋	0	1	7	9 ⌋	4	7	5	1 ⌋
Lymph node metastasis																
Without	2	13	10	11 ⌉a	2	13	18	3	1	10	20	5	8	15	5	8
With	3	14	26	43 ⌋	6	39	28	13	5	15	39	27	14	45	23	4
TNM stage																
I, II	4	22	31	35 ⌉a	4	37	38	13	5	20	47	20	12	46	23	11 ⌉a
III, IV	1	5	5	19 ⌋	4	15	8	3	1	5	12	12	10	14	5	1 ⌋

[a]P<0.05; [b]P<0.01. Id-3 expression was much higher with the increase of tumor volume (P=0.011), while Id-2, and Id-4 expressions decreased in large tunors (P=0.003, and 0.047). Id-1 expression was higher in patients with lymph node metastasis when compared to the negative ones (P=0.015). Id-1 and Id-4 immunoreactivity decreased in patients with higher TNM stage.

breast tissues were investigated in moderate and poorly differentiated breast cancer tissues (Fig. 2). Cytoplasmic Id-1 and Id-3 staining significantly increased in moderate or poorly differentiated cancer specimens when compared to the normal breast tissues (P<0.05 or P<0.01). An apparently lower expressed of Id-4 was observed in poorly differentiated

Table II. OS and DFS in 100 patients with breast cancer (Cox regression).

	DFS			OS		
Prognostic characteristics	B	P-value	Relative risk	B	P-value	Relative risk
Clinical stage	-1.670	0.000	0.188	-1.500	0.000	0.223
Id-1	-1.127	0.128	0.324	-1.713	0.095	0.180
Id-4	0.952	0.082	2.592	1.103	0.077	3.014

The independent factor affected the patient's prognosis based on Cox regression analysis. Of all the four Id proteins, Id-1 is a negative independent factor similar to clinical stage while Id-4 is a positive independent factor.
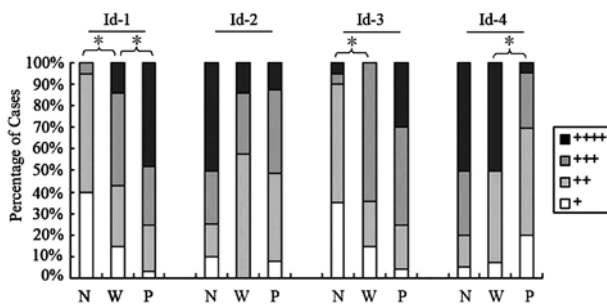

Figure 3. Cytoplasmic staining intensity for the four Id proteins in various differentiated tissues. *A significant difference ($P<0.025$, $0.05/2$). Cytoplasmic Id-1 and Id-3 staining significantly increased in well differentiated cancer specimens when compared to normal breast tissues ($P=0.005$ and $P=0.012$), and also elevated in poorly differentiated cancer specimens over well differentiated ones ($P=0.014$ and $P=0.032$), while apparently lower expression of Id-4 ($P=0.015$) was observed in poorly differentiated breast cancer tissues over well differentiated breast cancer specimens (N, normal breast tissues; W, well differentiated breast cancer tissues; P, poorly differentiated breast cancer tissues).

breast cancer tissues, compared to well and moderate breast cancer specimens ($P<0.05$). These results suggest that Id-1 and Id-3 proteins played a positive role while Id-4 protein played a negative role in neoplastic transformation and cancer progression of human breast cancer.

Prognostic significance of Id proteins. We investigated whether expressions of Id proteins in breast cancer could serve as

prognostic biomarkers in breast cancer by a follow-up of 100 patients who underwent curative surgical operations, and analyzed the disease-free survival (DFS) and overall survival (OS) using a Cox regression analysis (Table II). The patients were followed for a period of 1120-3913 days (mean, 1892 days). During this observation period, 26 patients (26%) developed recurrent or distal metastasis and 23 of the 26 patients died. Kaplan-Meier survival curves showed that expression of Id-1, and Id-3 was inversely correlated with DFS and OS and both expression of Id-2 and Id-4 presented positive association (Fig. 3). Although patient's age, adjuvant therapy, lymph node metastasis, tumor size, and cancer cell differentiation had no impact on OS or DFS by COX regression analysis ($P>0.05$), significant prognostic influences of tumor stage, Id-1 and Id-4 expressions were found. We examined the 3-year survival rate of these patients using non-parametric Mann-Whitney U test (Table III), and found that patients with higher expressed Id-1 and Id-3 had poorer 3-year survival than those with lower expressed Id-1 and Id-3. Id-4 showed the opposite correlation.

Discussion

The expressions of the four members of Id protein family in breast cancer (n=120) and normal mammary tissues (n=20) were investigated by immunohistochemistry. There was a dysregulation of the four Id proteins in breast cancer, including up-regulation of Id-1 and Id-3 proteins and down-regulation of Id-2 and Id-4 proteins in cancer tissues when

Table III. The effects of Id protein expression on 3-year survival of patients.

	Breast tumor tissues								P-value			
	Id-1		Id-2		Id-3		Id-4					
Groups	High	Low	High	Low	High	Low	High	Low	Id-1	Id-2	Id-3	Id-4
<3 years	15	0	5	10	13	2	2	13	0.000	0.329	0.008	0.003
≥3 years	62	23	46	39	61	21	32	53				

The 3-year survival rate was examined using non-parametric Mann-Whitney U test. Patients with higher expressed Id-1 and -3 had a poor 3-year survival compared with lower expressed ones, while Id-4 expression showed the opposite indication.
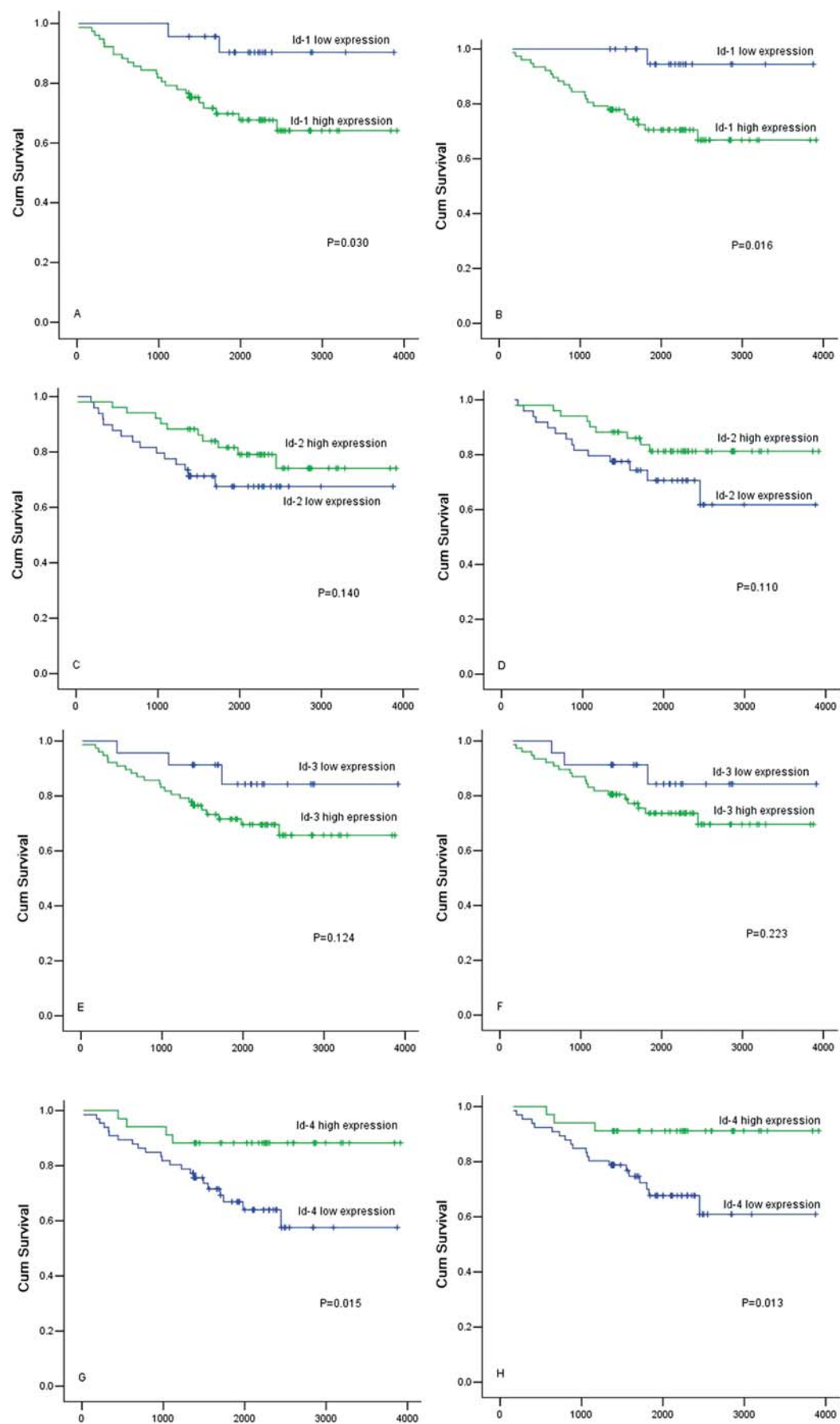
Figure 4. Kaplan-Meier survival curves for patients with high and low stained Id proteins. Patients were followed-up for a period of 1120-3913 days (mean, 1892 days). Kaplan-Meier survival curves showed that expression of Id-1 and Id-3 were inversely correlated with DFS and OS and the positive association was presented both in Id-2 and Id-4 expression. A, C, E and G, disease-free survival curve of Id-1, 2, 3, 4; B, D, F and H, overall survival curve of Id-1, 2, 3, 4.

compared to the adjacent breast tissue specimens. Id proteins play a role in the cytoplasm, where Id proteins bind with the transcription factors to block them from passing through the nuclear membrane into the nuclear. Thus, the transcription factors can not bind with DNA and Id proteins and this may explain why the Id proteins express only in the cytoplasm. The results were analyzed with clinicopathological characteristics. Expression of Id-1 and Id-3 proteins was positively correlated with tumor differentiation, and associated with a higher risk of lymph node metastasis. Higher Id-1 protein expression in breast cancer suggested metastatic potential of breast cancer. Id-3 protein expression was up-regulated in larger tumors, while Id-2 and Id-4 protein expressions tended to be lower in larger tumors. Kaplan-Meier survival curves showed an inverse correlation between expression of Id-1 and Id-3 proteins, and DFS and OS. Expression of Id-2 and Id-4 proteins showed a negative relationship with DFS and OS. Taken together, these data showed that the differentially expressed Id proteins were involved in the neoplastic transformation and progression of breast cancer. It suggests that Id protein may serve in the long-term survival expectation in this study. Our findings warrant further studies to investigate the exact mechanism by which Id proteins are involved in breast cancer and pathogenesis.

Id-1 protein has been previously reported to be up-regulated in human breast cancer and to show its cancer promotion effects. Constitutively expressed Id-1 could transform a non-aggressive breast cancer cell line (T47D cells) into a more aggressive one (11), while down-regulating Id-1 protein expression in the highly metastatic breast cancer cells by antisense therapy can significantly suppress its invasive and metastatic potential (15). In this study, over-expression of Id-1 protein was found in the breast cancer tissues accompanying lymph node metastasis, increased TNM stage and associated with poor long-term survival of breast cancer patients, consistent with the study by Kim *et al* (16), which provided the molecular mechanism of the cross-talk between Id-1 and HIF-1α that plays a critical role in tumor angiogenesis. A significant increase of Id-1 protein expression was observed in low differentiated breast cancer over high differentiation, and the latter over normal mammary tissue also indicated its possible involvement in breast cancer neoplastic transformation and progression, which is also in accord with the results of Fong *et al* (15).

Previous studies have suggested that Id-2 protein may promote differentiation in breast cancer (12). Id-2 protein expresses at a high level in well differentiated breast cancer cells (T47D and MCF-7 cells), at a low level in more aggressive, and at metastatic breast cancer cells (MDA-MB-231 and MDA-MB-435 cells). Little Id-2 protein expression is detectable in human biopsies from aggressive and invasive carcinomas compared with *in situ* carcinomas (12). In our study, we also found evident decrease of Id-2 protein expression in breast cancer tissues, and Kaplan-Meier survival curve suggested its influence on prognosis of breast cancer. Forced expression of Id-2 protein in aggressive breast cancer cells reduced the proliferative and invasive phenotypes of the cells, and decreased its level of matrix metalloproteinase 9 secretions while increasing syndecan-1 expression possibly explaining its beneficial role in preventing breast tumors (12).

Dysregulation of Id-3 protein has been found in many tumor types including its up-regulation in colorectal adeno-carcinoma (4) and pancreatic cancer (17), and down-regulation in ovarian cancer (18). In this study, Id-3 protein was apparently over-expressed in breast cancer specimens. Although Id-3 protein has been shown to participate in the angiogenesis process of human breast cancer (19), no noticeable association between Id-3 protein expression and TNM stage or lymph node metastasis was found in this study. However, highly expressed Id-3 protein can still be viewed as a negative indicator for poor long-term survival of breast cancer patients. Such a discrepancy may be due to the differences among the distribution of tumor stage, and ethnicity of the population.

Hypermethylation and consequential reduced expression of Id-4 protein has been described in T1 breast cancer, and Id-4 protein may play an important suppressive role in tumor progression. In the present study, the expression of Id-4 protein increased in breast cancer in comparison with the adjacent breast tissues, suggesting that Id-4 protein may play a positive role in carcinogenesis of human breast cancer, consistent with the study by Shan *et al* (20). Moreover, expression of Id-4 protein was negatively correlated with TNM stage and survival time, suggesting that decreased Id-4 expression may promote human breast cancer progression, which is different from the results in rats that Id-4 protein positively correlated with invasive ability of a mouse breast cancer cell line (21), and this result is also different from the results that Id-4 protein was a crucial gene regulating BRCA1 expression and might therefore be important for BRCA1 regulatory pathway involved in the pathogenesis of sporadic breast cancer (13,22). This illustrated that future work is required to elucidate the function of Id-4 protein in breast cancer. The difference of *in vivo* and *in vitro* may also lead to this contradictory conclusion.

Lymph node metastasis, tumor size and differentiation should be the factors that affect the prognosis of the patients. We also confirmed this through the single-factor regression analysis; however, these factors were not independent affecting the prognosis of patients based on the multifactor Cox regression analysis. Expression of Id-1 and Id-4 proteins, and TNM staging could be independent factors affecting prognosis of patients. At follow-up, the causes of death for patients were recurrence and distant metastasis. Recurrence and distant metastasis of patient's tumor are affected by the proliferation speed of tumor cells except for tumor size, lymph node metastasis and differentiation. Proteins of Id-1 and Id-4 may control the speed of breast cancer cell proliferation. Intraoperative smaller tumors may metastasize earlier hematogenously rather than via lymphatics, which may eventually worsen the prognosis of patients. It needs to be studied further why tumor differentiation is not an independent prognostic factor in breast cancer.

We hyposized that Id-1 regulated by estrogen can worsen the prognosis of the patients through promoting the tumor proliferation and enhance the speed of the tumor growth, so we analyzed the relationship between expression of Id proteins and menopause, but no correlation was found. This

can not result in the conclusion that Id-1 is not regulated by estrogen, because the estrogen level is normal in pre-menopausal and post-menopausal women, however, because of lower progesterone level, the estrogen level is relatively high in the peri-menopausal women. To confirm the relationship between Id-1 and estrogen, we could not neglect the factors affecting the estrogen level including obesity, infertility and menoxenia. This experiment can not affirm the relationship between Id-1 and estrogen because many parameters were not obtained during consulting the case history and follow-up. Further study is needed to confirm the hypothesis.

In conclusion, our study showed differential expressions of Id proteins in benign and malignant mammary tissues. Id-1, Id-3 and Id-4 proteins might be involved in neoplastic transformation, cancer progression and development of metastases. We found up-regulation of Id-1 and Id-3 proteins and down-regulation of Id-2 and Id-4 proteins in breast cancer, cytoplasmic staining of Id-1 and Id-3 were positively correlated with tumor differentiation. Id-1 protein had a higher risk of lymph node metastasis, and Id-3 protein was up-regulated in larger tumors while Id-2 and 4 proteins tended to be lowered in smaller ones. Kaplan-Meier survival curves showed that expression of Id-1, and -3 proteins may serve as negative indicators, and Id-2, and -4 proteins may be the positive indicators of long-term survival in breast carcinoma patients. Further study is needed to clarify the mechanisms involved and to understand the development of targeted therapy against breast cancer.

Acknowledgements

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