Pathol. Oncol. Res. (2009) 15:91–96
DOI 10.1007/s12253-008-9096-y

ORIGINAL PAPER

Inhibitor of DNA Binding-1 Overexpression in Prostate Cancer: Relevance to Tumor Differentiation

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Received: 19 April 2008 / Accepted: 1 August 2008 / Published online: 28 August 2008

Abstract Inhibitor of DNA binding-1 (Id1) is a dominant-negative regulator of basic helix–loop–helix transcription factor, which control malignant cell behaviors in several types of carcinomas. This study aimed to find the relationship between Id1 expression and some clinical parameters. Paraffin-embedded tissue specimens from two normal human prostates, 12 benign prostatic hyperplasia (BPH), 43 prostate cancers(PCa) were detected by immunofluorescence assay. Prostatectomy samples from 11 BPH and 28 PCa were used for real time RT-PCR. The relationship between Id1 staining and several patient's clinical parameters, including Gleason grade, PSA, clinical stage, and size of tumor, was further analyzed. Significant up-regulated Id1 protein was shown in prostate cancer specimens, while only weak expression in some BPH samples (5/12). Analyzed by image software, the mean proportion of Id1 positive staining remarkably increased with the increasing of Gleason grade in prostate cancer specimens (r=0.9967, P<0.01). Id1 expression was not significantly associated with PSA, TNM stage or tumor size. Furthermore, the average mRNA of prostate cancer was 3.09 times of BPH. This study confirms that Id1 protein and mRNA are over expressed in prostate cancer tissues. Overexpression of Id1 protein correlates with tumor tissue differentiation. We propose that Id1 over expression can be used in the analysis of the progression of prostate cancer.

Keywords Prostate cancer · Id1 · Protein expression · mRNA quantitation · Differentiation

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Introduction

Prostate cancer is the most common malignancy in men and the second leading cause of male cancer deaths in the United States [1]. In oriental population, the incidence is much lower [2, 3]. However, with the changing of life-style in recent years, the incidence has increased [4, 5]. The early detection of prostate cancer using clinical sensitive procedures and/or tumor biomarkers is of prime importance. To our knowledge, an ideal biomarker-Id1 (inhibitor of DNA binding 1 or inhibitor of differentiation 1) for determining the malignancy of some human cancers is actively investigated in recent years [6–10].

Id proteins belong to a helix–loop–helix family, which play fundamental roles in the control of developmental processes. As a transcriptional regulator, Id1 prevents basic helix–loop–helix transcription factors from binding to DNA [11], thus, inhibiting the transcription of differentiation associated genes. Id1 expression not only restores the ability of DNA synthesis in senescent human fibroblast cells, but also results in the immortalization of human keratinocytes [12]. This finding suggests its role in the regulation of cell differentiation and proliferation. Further studies have revealed much wider biological roles for Id family proteins that impinge on the fields of developmental biology, cell cycle research, and tumor biology, including important roles in oncogenesis [13–16]. Chetcuti A et al (2001) first identified differentially expressed Id1 gene in

human organ-confined prostate cancer by gene expression array [17]. Then Id1 overexpression was further detected in human prostate cancer specimens by immunohistochemical study and in situ hybridization [18]. It was hypothesized that Id1 may serve as a useful prognostic marker for prostate cancer [19]. However, only little clinical data on the expression of Id proteins in human prostate cancer exists thus far. The present study aimed to detect the expression of Id1 mRNA and protein in prostate cancer tissues by two sensitive methods: Real time quantitative PCR for mRNA expression and immunofluorescence assay for protein expression. The relationship between Id1 expression and some clinical parameters was further analyzed.

Materials and Methods

Immunofluorescence Assay for Id1 Protein

Tissue Specimens Formalin-fixed, paraffin-embedded prostate specimens were obtained from the archival tissue bank of the Department of Pathology, Affiliated Hospital of Qingdao University between 2003 and 2005. Prostate cancer specimens were selected from patients 59 to 79 years old diagnosed with prostate cancer. A total of 43 prostate cancer specimens were chosen by Haematoxylin and Eosin Staining (HE) to represent the range of Gleason grade: eight cases were graded as Gleason 2 (well-differentiated), eleven cases were graded as Gleason 3 (moderately-differentiated), fourteen cases and ten cases were graded as Gleason 4 and Gleason 5 (poorly-differentiated). None of these specimens were obtained from patients who underwent any preoperative hormonal ablative therapy. Twelve cases of BPH were used as controls. In addition, two cases of normal prostate tissues were also detected.

Immunofluorescence Microscopy Formalin-fixed, paraffin-embedded prostate tissues were sliced and placed on microscope glass slides. The sections were deparaffinization, hydration, and boiled in antigen retrieval buffer. After incubation with 10% normal goat serum for 20 min to block non-specific binding, the sections were incubated for 16 h at 4°C with Id1 primary antibody (Specific rabbit anti-human Id1, sc-488, Santa Cruz, CA, 1:250). Sections were washed several times with phosphate-buffered saline, then were counterstained with second antibody (FITC-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) Kit, USA™ Jackson, 1:50), dehydrated, cleared and mounted. For negative control, PBS was used as a substitute for the primary antibody to verify the possibility of false positive responses from the secondary antibody. No positive staining was observed. For positive control, sample of breast cancer overexpressing Id1 protein was used in the respective staining procedures. The positive control displayed high cytoplasmic staining. Mounted slides were observed with a LSM510 confocal laser scanning microscope (Olympus). Images were captured on a high-resolution Olympus camera. All slides were interpreted in a blinded manner without knowledge of the clinical and pathological parameters by one of the investigators (BJ Han) and the final evaluation was cooperatively determined by two investigators (XH Xu and XL Yu).

Evaluation of Immunostaining For quantitative assay of Id1 immunostaining, the immunofluorescent images were scanned and calculated by Jieda Image software (Jieda, China). By choosing the typical positive staining cell, the mean fluorescence intensity in the prostate epithelium and the proportion of positive staining cells were calculated. Combining the proportion of staining cells and the mean staining intensity together, the immunofluorescent images of the prostate samples were qualitatively scored (− negative, + faint, ++ moderate, +++ intense).

Clinical Parameters Some clinical parameters were presented, such as TNM stage, serum prostate specific antigen (PSA) by ELISA, prostate size by B ultrasonic waves.

Statistical Analysis Statistical analysis was performed by SPSS statistical software. The association of Id-1 expression levels with some clinical parameters was performed by the chi-square test for categorical variables and by the Spearman correlation test for continuous variables. The *P*-value <0.05 was considered statistically significant.

Real Time Quantitative RT-PCR

Tissue Specimens Radical prostatectomy samples including 28 prostate cancer and 11 BPH samples were obtained from Affiliated Hospital of Qingdao University during 2004 to 2005. Immediately following surgery, the samples were snap frozen in liquid nitrogen, and stored at −80°C. The mean age of the prostate cancer patients was 66.2 years (range 59～79) and that of BPH was 64 years (range 58～70). None of the patients underwent any preoperative hormonal ablative therapy. All samples were confirmed by pathologic diagnosis. The procedure of collection of the samples was in accordance with the Human Ethics standards of Qingdao University.

Total RNA Extraction The frozen tissue samples were homogenized in TRIzol reagent (Invitrogen, USA) using 1ml TRIzol per 50 mg tissue. Total RNA was extracted from the tissue according to the manufacturer's suggested

protocol. RNA pellets were dissolved in 100 μl DEPC (diethylpyrocarbonate) treated water. Total RNA concentration was determined by spectrophotometer analysis at 260 nm. RNA quality was assessed by electrophoresis on a 1% formalin–agarose gel. All extracted RNA had well-defined 28S and 18S rRNA bands.

Real Time RT-PCR Immediately after RNA extraction, 1 μg total RNA was reverse transcribed into cDNA by BcaBEST™ RNA PCR Kit (TaKaRa bio, Japan), following the manufacturer's instructions using random primer. To determine if contaminating genomic DNA after DNAse treatment in RNA extraction, controls lacking reverse transcriptase were run at the same time. Primers specificity for Id1 target sequence, as well as for endogenous β-actin control were designed and provided by TaKaRa biotechnology Co., Ltd (Dalian, China). Id1 forward: 5′-acgacat gaacggctgttactcac-3′; Id1 reverse: 5′-ctccaactgaaggtccct gatgtag-3′. The product of PCR is 125 bp. β-actin as an endogenous RNA control was used to normalize each sample. β-actin forward: 5′-attgccgacaggatggaga-3′; β-actin reverse: 5'-gagtacttgcgctcaggagga-3'. The product of PCR is 89 bp. All PCR reactions were carried out in Line-Gene (Bio flux, Japan) using SYBR Premix Ex Tag™ (perfect Real Time,TaKaRa bio, Japan) and specific primers. The thermal cycling conditions comprised an initial denaturation step of 30 s at 95°C, followed by 40 thermal cycles of 5s at 95°C and 30s at 60°C. Standard curves were generated from decreasing amounts of one prostate cancer cDNA diluted at 10-times intervals to evaluate the efficiency of real-time PCR. Experiments were performed in triplicate for each data point. β-actin was used as a housekeeping gene to normalize the gene expression in each sample.

Calculation the Relative Times of mRNA Relative times of gene expression at each sampling time point were calculated according to the following equation:

Relative times=$A \times B/C$, A=fluorescence quantity of Id1 in each sample; B=β-actin relative times of each sample; C=quantity of background. Data were expressed as mean± S.D. Statistical significance of mRNA quantitation between BPH and prostate cancer was evaluated by t-test. Differences were considered to be significant if P-value <0.05.

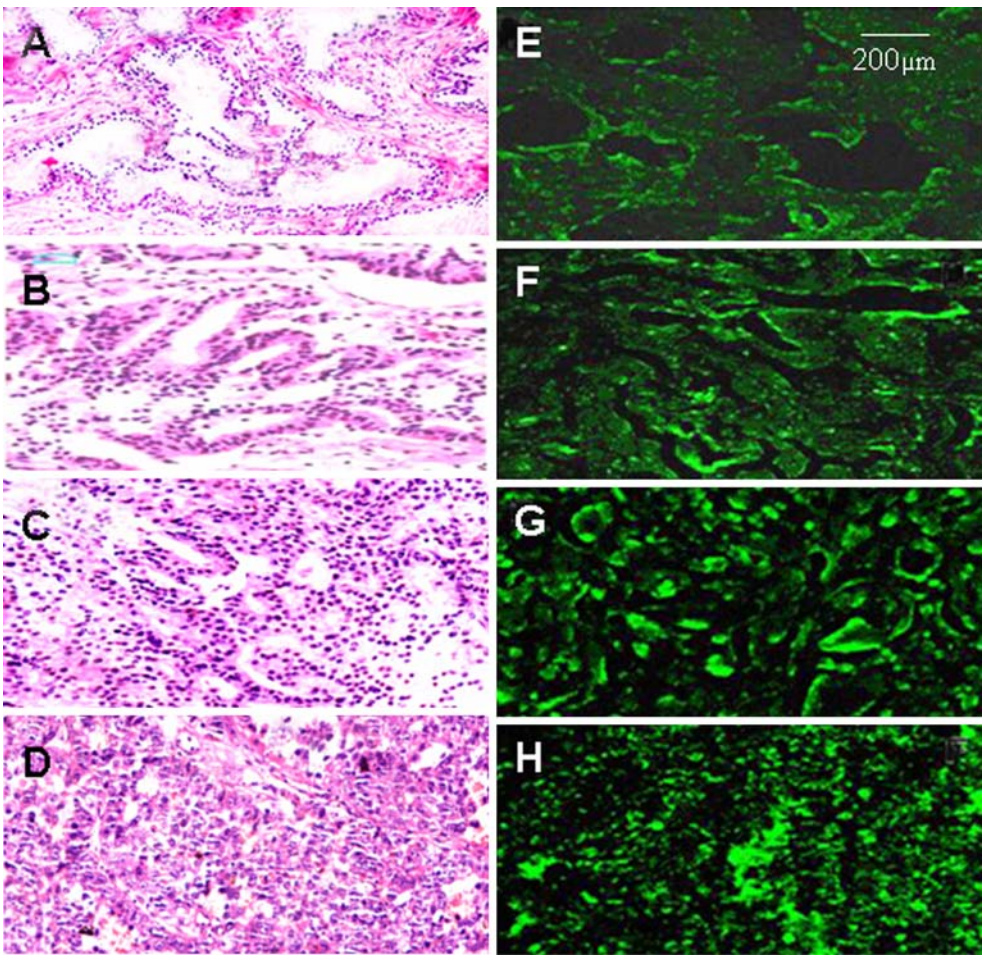

Fig. 1 Immunostaining of Id1 protein in prostate cancer sections with different Gleason grades. Panels **a**, **b**, **c**, **d** stained by HE represent Gleason grade 2~5, respectively. Panels **e**, **f**, **g**, **h** represent Id1 protein expression in tumors with different Gleason grades. *Magnification bar* located in panel **e** represents 200 μm and is equal for all panels

Results

Id1 Protein Expression by Immunofluorescence Assay

No positive fluorescent staining was detected among the two normal prostate samples. The BPH tissues commonly displayed no (7/12) or weak (5/12) immunostaining of Id1 protein in the epithelial cells. All 43 prostate cancer specimens displayed clear but variable levels of Id1 immunoreactivity in cancerous glandular epithelia. Positive staining for Id1 was found mainly in the cytoplasm of prostate cancer cells. Figure 1 illustrates typical staining for Id1 protein in tumors with different Gleason grades. Figure 2 shows the proportion of Id1 positive staining and the mean staining intensity in different Gleason grades. The proportion of Id1 positive staining were increased significantly with the increasing of Gleason grade (r=0.9967, P< 0.01). The proportion of cases with strong expression of Id1 (+++), was mostly in poor-differentiated cancer tissues (Gleason grade 4~5), whereas those of well-differentiated cancer specimens (Gleason grade 2) mostly exhibited weak fluorescent staining (+).The Spearman correlation between Id1 and Gleason grade was statistically significant (r_s= 0.88088, P<0.01). Table 1 shows the relationship between Id1 expression and some clinical parameters in the representative cancers. There is no significant association between Id1 and PSA, TNM stage or tumor size.

Id1 mRNA Expression by Real Time Quantitative RT-PCR

After PCR amplification with specific real-time PCR primers, a single band of expecting size in agarose gel was observed. A single dissociation peak was obtained after dissociation analysis. These mean that real-time PCR assays were gene-specific. The results showed that in all of prostate cancer tissues, Id1 mRNA expression was significantly up-regulated (Fig. 3). It was 3.09 times in prostate cancer (5.34±1.73) as much as in BPH (1.73± 0.59; P<0.01).

Table 1 Id1 protein expression and some clinical parameters in prostate cancer

Clinicopathological parameters	Case	Id1 protein expression			
		−	+	++	+++
Gleason grade[a]					
2	8	0	6	2	0
3	11	0	3	5	3
4	14	0	1	6	7
5	10	0	0	3	7
PSA(ng/ml)					
4–10	18	0	3	6	9
≥10	25	0	7	10	8
TNM stage					
T1~T2	15	0	4	6	5
T3~T4	28	0	6	10	12
Prostate size (cm^3)					
≤25	6	0	4	2	0
25~50	12	0	3	6	3
51~75	18	0	2	4	12
≥75	7	0	1	4	2

[a] The Spearman correlation between Id1 and Gleason grade was statistically significant (r_s=0.88088, P<0.01).

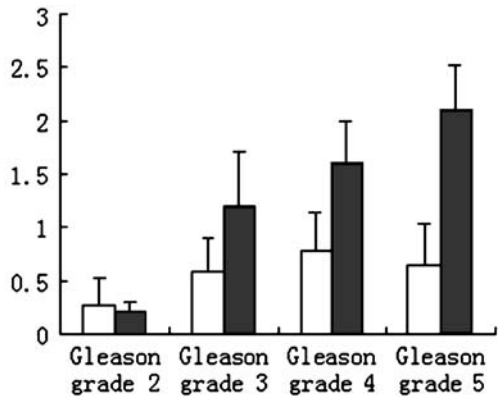

Fig. 2 Quantitative measurement of Id1 protein expression in prostate cancer. *Empty bars* represent mean fluorescence intensity of immunostaining images. *Solid bars* represent average proportion of staining cells. All data are expressed as average±SD. *Vertical scale* represents arbitrary units. The average proportion of Id1 staining closely correlated with increased Gleason grade(r=0.9967, P<0.01)

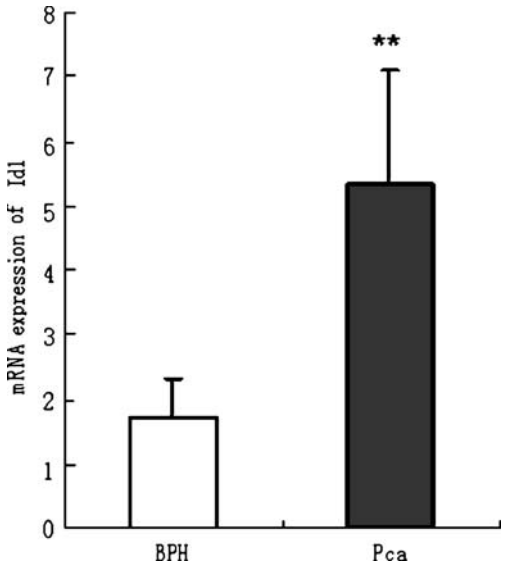

Fig. 3 Relative quantitation of Id1 mRNA expression by Real time PCR in BPH and prostate cancer (PCa) samples. Each lane shows increased times of Id1 mRNA. Statistically significant up-regulation of Id1 mRNA expression in prostate cancer compared to BPH control is P<0.001

Discussion

The genes of Id family have four members, Id1 to Id4. Several studies have shown that Id1 protein upregulated in prostate cancer cell lines, and functioned as a key mediator of cell proliferation, differentiation and drug resistance [20–23]. In this study, we used immunofluorescence assay to detect the Id1 protein in some Chinese prostate cancer tissues. We found that Id1 exhibited overexpression in all of the prostate carcinomas. Consistent with Perk J et al (2006) findings by a specific monoclonal antibody [24], we also confirmed that over expressed Id1 mostly localized in cytoplasm. This probably represents the region of its protein production. Moreover, high levels of Id1 protein expression in prostate cancers were closely correlated with the increased levels of Gleason grade, suggesting that the Id1 overexpression is associated with the increasing malignant potential of prostate cancer. This association of Id1 with poor differentiation of tumor cells is in good concordance with the role of Id1 as an inhibitor of cellular differentiation. The up-regulation of Id1 protein in prostate cancer that we detected is in agreement with a previous immunohistochemistry analysis [18], as also shown previously for Id1 in other types of carcinomas [8, 25, 26]. We propose that Id1 over expression may have strong potential to be used in the analysis of progression of prostate cancer.

Besides Gleason grade, we did not find Id1 protein expression had significant association with PSA, TNM stage or tumor size. We are presently investigating the relationship between Id1 expression and patient outcome.

To our knowledge, the functional studies in prostate cancer cells found that transfected Id1 stimulated prostate cancer cell proliferation through activation of EGFR [27], inactivation of p16/Rb [22], or inducing activation of the MAPK pathways [28]. Id1 expression also protected cell from TNFα-induced apoptosis [29]. Loss of Id1 by siRNA further confirmed that Id1 target prostate cancer cellular proliferation [23]. Considering the Id1 protein expression and its functional study in prostate cancer, we think that Id1 is a predominant gene in proliferation and aggravation of prostate cancer. It has been speculated that interfering with Id1 may as a promising target in some cancer therapy [30–32]. We also support the speculation that inhibition of Id1 might also be beneficial for prostate cancer patients.

In this study, real time RT-PCR method was used to detect Id1 mRNA expression in prostate cancer. The high expression of Id1 mRNA in prostate cancer specimens suggested that upregulation of Id1 occurred at the transcriptional level in human prostate cancer. The mRNA expression of Id1 is also an important biomarker for prostate cancer.

In conclusion, our data suggests that Id1 protein and mRNA represent potential predictive markers in the progression of prostate cancer. Targeting Id1 gene or protein might be a promising way of diagnosis or therapy for prostate cancer.

Acknowledgment This study was supported by the Medical Science Fund (05-1-NS-76) of Qingdao Municipal Science and Technology Commission.

References

1. Jemal A, Siegel R, Ward E et al (2007) Cancer statistics, 2007. CA Cancer J Clin 57:43–66
2. Hsing AW, Tsao L, Devesa SS (2000) International trends and patterns of prostate cancer incidence and mortality. Int Cancer 85:60–67, (review)
3. Sim HG, Cheng CW (2005) Changing demography of prostate cancer in Asia. Eur J Cancer 41:834–845
4. Gu F (2000) Epidemiological survey of benign prostatic hyperplasia and prostate cancer in China. Chin Med J (Engl) 113:299–302
5. Hsing AW, Devesa SS, Jin F et al (1998) Rising incidence of prostate cancer in Shanghai. Cancer Epidemiol Biomarkers Prev 7:83–84 (letter)
6. Hasskarl J, Munger K (2002) Id proteins—tumor marker or oncogenes? Cancer Biology and Therapy 1:91–96 (review)
7. Perk J, Iavarone A, Benezra R (2005) Id family of helix–loop–helix proteins in cancer. Nat Rev Cancer 5:603–604
8. Han S, Guo C, Hong L et al (2004) Expression and significance of Id1 helix–loop–helix protein overexpression in gastric cancer. Cancer Lett 216:63–71
9. Matsuda Y, Yamagiwa S, Takamura M et al (2005) Overexpressed Id-1 is associated with a high risk of hepatocellular carcinoma development in patients with cirrhosis without transcriptional repression of p16. Cancer 104:1037–1044
10. Straume O, Akslen LA (2005) Strong expression of ID1 protein is associated with decreased survival, increased expression of ephrin-A1/EPHA2, and reduced thrombospondin-1 in malignant melanoma. Br J Cancer 93:933–938
11. Benezra R, Davis RL, Lockshon D et al (1990) The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. Cell 61:49–59
12. Alani RM, Hasskarl J, Grace M et al (1999) Immortalization of primary human keratinocytes by the helix–loop–helix protein,Id-1. Proc Natl Acad Sci USA 96:9637–9641
13. Lyden D, Young AZ, Zagzag D et al (1999) Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature 401:670–677
14. Ruzinova MB, Benezra R (2003) Id proteins in development, cell cycle and cancer. Trends Cell Biol 13:410–418
15. Wong YC, Wang X, Ling MT (2004) Id-1 expression and cell survival. Apoptosis 9:279–289, (review)
16. Jang KS, Han HX, Paik SS et al (2006) Id-1 overexpression in invasive ductal carcinoma cells is significantly associated with intratumoral microvessel density in ER-negative/node-positive breast cancer. Cancer Lett 244:203–210
17. Chetcuti A, Marqan S, Mann S et al (2001) Identification of differentially expressed genes in organ-confined prostate cancer by gene expression array. Prostate 47:132–140
18. Ouyang XS, Wang X, Lee DT et al (2002) Overexpression of ID-1 in prostate cancer. J Urol 167:2598–2602
19. Coppe JP, Itahana Y, Moore DH et al (2004) Id-1 and Id-2 proteins as molecular markers for human prostate cancer progression. Clin Cancer Res 10:2044–2051

20. Yu DS, Hsieh DS, Chang SY (2006) Increasing expression of GST-pi MIF, and ID1 genes in chemoresistant prostate cancer cells. Arch Androl 52:275–281
21. Lin JC, Chang SY, Hsieh DS et al (2005) Modulation of mitogen-activated protein kinase cascades by differentiation-1 protein: acquired drug resistance of hormone independent prostate cancer cells. J Urol 174:2022–2026
22. Ouyang XS, Wang X, Ling MT et al (2002) Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of p16(INK4a)/pRB pathway. Carcinogenesis 23: 721–725
23. Asirvatham AJ, Schmidt MA, Chaudhary J (2006) Non-redundant inhibitor of differentiation (Id) gene expression and function in human prostate epithelial cells. Prostate 66:921–935
24. Perk J, Gil-Bazo I, Chin Y et al (2006) Reassessment of id1 protein expression in human mammary, prostate, and bladder cancers using a monospecific rabbit monoclonal anti-id1 antibody. Cancer Res 66:10870–10877
25. Schindl M, Schoppmann SF, Strobel T et al (2003) 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
26. Lin CQ, Singh J, Murata K et al (2000) A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res 60:1332–1340
27. Ling MT, Wang X, Lee DT et al (2004) Id1 expression induces androgen-independent prostate cancer cell growth through activation of epidermal growth factor receptor (EGF-R). Carcinogenesis 25:517–525
28. Ling MT, Wanq X, Ouyanq XS et al (2002) Activation of MAPK signaling pathway is essential for Id1 induced serum independent prostate cancer cell growth. Oncogene 21:8498–8505
29. Ling MT, Wang X, Ouyang XS et al (2003) Id1 expression promotes cell survival through activation of NF-kB signaling pathway in prostate cancer cells. Oncogene 22:4498–4508
30. Fong S, Itahana Y, Sumida T et al (2003) Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. Proc Natl Acad Sci USA 100:13543–13548
31. Fong S, Debs RJ, Desprez PY (2004) Id genes and proteins as promising targets in cancer therapy. Trends Mol Med 10:387–392 (review)
32. Tsuchiya T, Okaji Y, Tsuno NH et al (2005) Targeting Id1 and Id3 inhibits peritoneal metastasis of gastric cancer. Cancer Sci 96:784–790