Over-expression of Id-1 induces cell proliferation in hepatocellular carcinoma through inactivation of p16

Introduction

Inhibitors of differentiation and DNA binding (Id) proteins are transcription factors that belong to a group of helix-loop-helix proteins lacking the DNA binding domain. Therefore, these proteins act as dominant inhibitors of basic helix-loop-helix transcription factors by forming transcriptionally inactive heterodimers. Four Id genes (Id-1 through Id-4) are important for cell fate decisions of growth and differentiation. Their expression is typically high in actively proliferating cells and is down-regulated as a prerequisite for exit from cell cycle and differentiation (1–3). The Id family member Id-1 has been implicated in regulating cellular life span, immortalization and delayed senescence in mammalian cells (4–6). Over-expression of Id-1 has been reported in several types of primary tumors including breast (7), pancreatic (8), prostate (9), cervical (10) and colorectal adenocarcinoma (11). Previous findings showed that ectopic expression of Id-1 induced aggressiveness and metastasis in breast cancer cells (7), and up-regulation of Id-1 has been correlated with tumor stage in squamous cell carcinoma (12). Most recently, over-expression of Id-1 protein has been correlated with patients’ poor clinical outcome and mitotic index in several human cancers (10,11). This evidence strongly supports that Id-1 plays an important role not only in tumorigenesis, but also in tumor progression. However, Id-1 expression in hepatocellular carcinoma (HCC) has not been studied and its role remains unknown.

Recently, Id-1 has been demonstrated to oppose Ets-mediated activation of p16INK4a via Ras-Raf-MEK signaling (13). The p16INK4a/retinoblastoma (RB) pathway has been shown to be down-regulated in various human tumors including HCC, either through loss of p16INK4a or RB function, or through down-regulated expression of cyclin D or cdk4 (14–16). Several mechanisms of inactivation of p16INK4a/RB pathway have been proposed including promoter methylation, protein sequestration and post-translational modification (17). However, little is known about the direct transcriptional control of genes within the p16INK4a/RB family and their role in HCC tumorigenesis. Therefore, we first examined the Id-1 expression in messenger RNA (mRNA) and protein levels in HCC and then investigated whether Id-1 may play a role in regulating p16INK4a expression during the development of HCC.

Materials and methods

Patient samples

Samples were obtained with informed consent from 10 healthy liver transplant donors and 62 patients undergoing hepatectomy for HCC from 1997 to 1999 in the Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong.

Abbreviations: FBS, fetal bovine serum; HCC, hepatocellular carcinoma; Id, inhibitors of differentiation and DNA binding; mRNA, messenger RNA; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, flow cytometry, immunostaining and western blot. Our results showed that Id-1 was over-expressed in HCC specimens both at mRNA and protein levels. Over-expression of Id-1 protein was correlated with PCNA (r = 0.334, P = 0.033). HCC samples showing low Id-1 protein expression had a lower Id-1 mRNA level (340.2 versus 1467%, P = 0.039) and higher p16INK4a expression (195 versus −78.6%, P = 0.039) than samples with high Id-1 protein expression. In the PLC/PRF/5 HCC cell line study, ectopic Id-1 expression resulted in proliferation of HCC cells and an increased percentage of S phase cells and PCNA expression. The results showed that over-expression of Id-1 induces cell proliferation in HCC through inactivation of p16INK4a/retinoblastoma pathway. In conclusion, the results provided an insight for the understanding of the role of Id-1 in functional inactivation of p16INK4a in HCC.
Biotecnology) (Figure 1G). A standard avidin–biotin peroxidase technique (DAKO, Carpinteria, CA) was applied. Briefly, biotinylated goat anti-mouse Ig or goat anti-rabbit Ig and avidin–biotin peroxidase complex were applied for 30 min each, with 15-min washes in phosphate-buffered saline. The reaction was finally developed by Dako Liquid DAB+ Substrate-chromogen System (DAKO).

Cytoplasmic expression of Id-1 and nuclear staining of PCNA were determined by two independent observers who assessed semi-quantitatively the percentage of stained tumor cells as well as staining intensity. The percentage of positive cells was rated as follows: 2 points, 11–50% positive tumor cells; 3 points, 51–80% positive cells; and 4 points, >81% positive cells. Staining intensity was rated as follows (18): 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for expression and percentage of positive cells were added, and specimens were attributed to four groups according to their overall scores: negative, ≤10% of cells stained positive, regardless of intensity; weak expression, 3 points; moderate expression, 4–5 points; and strong expression, 6–7 points. Negative to weak Id-1 expression was graded as group 1, which represented low Id-1 expression; whereas moderate to strong Id-1 expression was graded as group 2, which represented high Id-1 expression. Expression of PCNA was also graded as above.

mRNA levels of Id-1 and p16INK4a in HCC by quantitative reverse transcription–polymerase chain reaction (RT–PCR)

The liver specimen was stored at –80°C until total RNA extraction. The total RNA was extracted using the Rneasy Midi Kit (Qiagen Company, GmbH, Germany) and the quality of the total RNA was detected by the spectrophotometer (DU-65, BECKAM, Germany). About 0.5 µg total RNA from each sample was used to perform reverse transcription reaction. Taqman Reverse Transcription Reagents (Applied Biosystem, Foster City, CA) were used according to the manufacturer’s instruction (25°C × 10 min, 48°C × 30 min, 95°C × 5 min). Reverse transcription product (1 µl) was used to perform real-time quantitative polymerase chain reaction (PCR) with a reaction volume of 50 µl (TaqMan PCR Core Reagent Kit, Applied Biosystem) by the ABI PRISM 7700 Sequence Detection System (Applied Biosystem). Probes and primers of Id-1 and p16INK4a were designed under the Primer Express software (Applied Biosystem) according to the criteria for real-time PCR. The sequences are listed in Table I. The Taqman Ribosomal RNA Control Reagent (18S RNA probe (VIC) and primers; PE Applied Biosystem) was used for internal control in the same PCR plate well to normalize the target genes amplification copies. The PCR protocol was according to the manufacturer’s recommendation [50°C × 2 min, 95°C × 10 min (95°C × 15 s, 60°C × 1 min) ×50 cycles]. All the samples were detected in triplicate and the readings from each sample and its internal control were used to calculate the gene expression level. After normalization with the internal control, the gene expression levels in HCC were calculated as the percentage of the levels in normal liver tissue and non-tumor tissue.

Cell line transfaction

PLC/PRF/5 was obtained from the Japanese Cancer Research Bank (Tokyo, Japan). The cells were transfected with 2 µg of plasmid DNA of either Id-1 or pCDNA3.1(−) (kindly provided by Prof. Y.C.Wong of the University of Hong Kong) containing the entire coding of Id-1 or expression vector pCDNA3.1(−) alone using FuGENE 6 according to the manufacturer’s protocol (Boehringer, Mannheim, GmbH, Germany). After 48 h, the medium was replaced with fresh Dulbecco’s modified Eagle minimal essential medium (Sigma, St Louis, MO) was added every day and the plates were incubated for 12 h in a moist chamber at 37°C. Optical density was determined by eluting the dye with dimethyl sulfoxide (Sigma), and the absorbance was measured at 570 nm. Three independent experiments were performed.

Cell cycle analysis

Cells (5 × 10⁵) were trypsinized and washed once in PBS. They were then fixed in cold 70% ethanol and stored at 4°C. Before testing, the ethanol was removed and the cells were resuspended in PBS. The fixed cells were then washed with PBS and treated with RNase (1 µg/ml) and stained with propidium iodide (50 µg/ml) for 30 min at 37°C. Cell cycle analysis was performed on an EPICS profile analyzer and analyzed using the ModFit LT2.0 software (Coulter Electronics, Hialeah, FL).

Western blotting

The cells were lysed and protein extraction was performed. The samples were separated in 10% sodium dodecyl-sulfate acrylamide gel and electrophoretically transferred to PVDF membrane (Amersham, Buckinghamshire, UK). The membrane was blotted with 10% non-fat milk, washed and then probed with Id-1 (1:500, Santa Cruz Biotechnology), CDK4 (1:500, Calbiochem, La Jolla, CA), p16INK4a (1:500, Santa Cruz Biotechnology) and pRB (1:200, Santa Cruz Biotechnology). After washing, the membrane was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (Amersham) and then visualized by enhanced chemiluminescence plus according to the manufacturer’s protocol.

Statistical analysis

Continuous variables were expressed as median and range. The Mann–Whitney U test was used for statistical comparison. The Pearson test was used for bivariate correlation comparison. Significance was defined as P < 0.05. Calculations were made with the help of SPSS computer software (SPSS, Chicago, IL).

Results

Expression of Id-1 protein in normal, non-tumor and tumor tissue from human HCC by immunostaining

In order to determine the significance of Id-1 expression in HCC, we evaluated 62 samples of HCC and their corresponding non-tumor tissues by immunostaining. In normal liver, no cytoplasmatic expression of Id-1 in hepatocytes was observed (Figure 1A). However, in non-tumor liver (cirrhotic liver or chronic hepatitis), absent to weak cytoplasmatic expression of Id-1 was observed (Figure 1B). In HCC samples, cytoplasmatic expression of Id-1 was not found in eight cases (12.9%) (Figure 1C), weak in 16 cases (25.8%) (Figure 1D), moderate in 25 cases (40.3%), and strong in 13 cases (20%) (Figure 1E). The margin between non-tumor liver and tumor was shown in Figure 1F. Twenty-four cases were graded as group 1, which represented low Id-1 expression, whereas 38 cases were graded as group 2, which represented high Id-1 expression.

mRNA levels of Id-1 and p16INK4a in normal, non-tumor and tumor tissues by quantitative RT–PCR

The mRNA level of Id-1 and its correlation to p16INK4a were examined by quantitative RT–PCR. The Id-1mRNA expression in non-tumor and tumor tissues showed higher levels when compared with the normal liver from healthy liver transplant donors. For non-tumor liver, the median level was 192% (range 12.8–562%) of normal liver level (100%). There was no

| Id-1 | CACGCTCAGAGGACTGGTGC | Sense: CTCCTACGAGATGAGCGTTT | Anti-sense: TGGCCTACGAGCACTGCAT |
| P16INK4a | CCCCCATGGAAGAAGACGAGAGGCT | Anti-sense: TCTAAGTTCGGATTCTCAGAC |
Fig. 1. Expression of Id-1 in normal liver, non-tumor liver and HCC. Immunostaining analysis showing no Id-1 expression in (A) normal liver, weak Id-1 expression in (B) non-tumorous liver. Differential expression of Id-1 is observed in HCC with (C) absent, (D) weak expression and (E) high expression. Cytoplasmic Id-1 expression at the margin between non-tumor liver and HCC is shown in (F). Negative control of immunohistochemistry for Id-1 on HCC specimen with known strong Id-1 expression. The antigen-binding site of the antibody has been blocked by a specific blocking peptide (G). HCC specimens with absent expression of Id-1 with strong Id-1 expression in tumor vascular endothelia (arrows) (H) (×200 magnification).
significant difference in Id-1 at mRNA level in non-tumor liver between groups 1 and 2 [182.3 (12.8±473%) versus 198.7% (74.2±562%); $P = 0.78$]. There was significant difference in Id-1 at mRNA level in tumors between group 1 and group 2 [260 (82±817) versus 1467% (141±7313%); $P = 0.022$] (Figure 2A). As for the mRNA levels of p16INK4a, there was significantly less expression in patients of group 2 than patients of group 1 [194 (99.94–6480%) versus 78.6 (98.9–64.28%); $P = 0.039$] (Figure 2B).

Correlation of Id-1 and PCNA in HCC tissues
Id-1 was suggested to play an important role in proliferation. To determine whether over-expression of Id-1 will be correlated with increased proliferation in HCC, we evaluated the expression of Id-1 and a proliferative marker PCNA by immunostaining. All cases showed PCNA immunoreactivity in which it was strong in nine cases (15%), moderate in 31 cases (50%) and weak in 22 cases (35%). Id-1 protein was found to significantly and positively correlate with PCNA expression ($r = 0.334, P = 0.033$) (Figure 3A and B).

Correlation of Id-1 and PCNA in HCC cell line
The effect of Id-1 expression in proliferation was also studied by directly transfecting Id-1 or pCDNA3.1 into PLC/PRF/5. After clonal selection, five clones were isolated. From the in vitro result, clone 3 showed high Id-1 expression when compared with PLC/PRF/5 harboring empty vector (Figure 3C and D). Clone 3 showed increased PCNA expression when compared with PLC/PRF/5 harboring empty vector (Figure 3E and F).

The effect of ectopic Id-1 introduction on HCC cell growth
The effect of FBS on Id-1 expression in PLC/PRF/5 was shown in western blot. In the absence of FBS in the culture medium, the level of Id-1 protein was barely detectable when compared with the presence of FBS in the culture medium (Figure 4A). As shown in Figure 4A, in the absence of FBS, all 5 clones showed different levels of Id-1 expression.

After transfection of Id-1, PLC/PRF/5 exhibited a relatively different morphology with flatter structure when compared with the parental cell. We evaluated the effect of ectopic Id-1 introduction by MTT assay.

Introduction of Id-1 resulted in increased cell growth when compared with the parental cell and PLC/PRF/5 harboring empty vector (Figure 5). The increase in cell growth was correlated with the level of Id-1 expression.

Effect of Id-1 introduction in cell cycle distribution
Next, we studied if Id-1 induced cell growth was a result of its ability to initiate DNA synthesis in HCC cell line in FBS free medium. Cell cycle analysis showed that there was 18% of S phase in the PLC/PRF/5 harboring pCDNA3.1 (control), but the percentage of S phase cells significantly increased (25.1–37%) in Id-1 transfectants. There was no significant change in the percentage of G2 phase (Figure 6).

Effect of Id-1 expression in p16INK4a/RB pathway
We have shown that expression of Id-1 was negatively correlated with p16INK4a in HCC samples and positively correlated with increased growth in PLC/PRF/5 in cell culture. To examine whether the increased proliferation was through inactivation of p16INK4a/RB pathway, we evaluated the expression levels of p16INK4a, CDK4 and RB in Id-1 expressing clones and PLC/PRF/5 harboring pCDNA3.1. As shown in Figure 4B, the level of p16INK4a was weak and barely detectable in 5 Id-1 transfectants and was inversely proportional to ectopic Id-1 expression. This in vitro result confirmed our data from the clinical samples that over-expression of Id-1 correlated with decreased p16INK4a expression. The phosphorylated form of CDK4 (upper band in Figure 4C) was also found in all five Id-1 transfectants and parental cells in the presence of FBS in the culture medium, but not in parental cells in the absence of FBS in the culture medium. Moreover, phosphorylated RB protein expression was also found in all five Id-1 transfectants (Figure 4D).

Discussion
In the present study, we first demonstrated that deregulation of Id-1 expression both at the mRNA and protein levels in human HCC, and level of immunohistologically detected protein correlated with levels of Id-1 mRNA. Some reports have found the difference in Id-1 expression level between the mRNA level by RT–PCR and protein level by western blot in ovarian cancer tissues and the discrepancy is due to strong expression of Id-1 in tumor vascular endothelia (19). From our immunostaining result, we observed nearly all cases showing positive...
Fig. 3. Expression of Id-1 and PCNA in samples of HCC patients and PLC/PRF/5 harboring empty vector and Id-1 transfectants. Some HCC specimens show strong cytoplasmic expression of Id-1 (A), and also strongly positive for PCNA (B) in the same specimens. Id-1 expression is positively correlated with PCNA ($r = 0.334, P = 0.033$) ($\times 200$ magnification). In in vitro cell culture model, strong Id-1 expression is observed in (C) Id-1 transfectant (clone 3) when compared with (D) PLC/PRF/5 harboring empty vector. Increased PCNA expression is also observed in (E) Id-1 transfectant (clone 3) when compared with (F) PLC/PRF/5 harboring empty vector.

Fig. 4. Assessment of Id-1, p16$^{INK4a}$, CDK4, RB and $\beta$-actin (for protein level reference) by western blot in Id-1 transfectants and PLC/PRF/5 harboring empty vector. (A) Id-1 expression in control and Id-1 transfectants after 48 h FBS starving. (B) Decreased p16$^{INK4a}$ expression is found in Id-1 transfectants. (C) Presence of CDK4 phosphorylation in Id-1 transfectants. (D) Presence of RB phosphorylation in Id-1 transfectants but not in PLC/PRF/5-harboring empty vector.
vascular endothelia staining in both non-tumor and tumor tissue. Some cases with absent Id-1 expression in tumor cells also show strong staining of Id-1 in tumor vascular endothelia. This result is consistent with the previous finding that Id-1 is also strongly expressed in vascular smooth muscle cells (20). However, there is no significant increase in Id-1 expression in tumor vascular endothelia. Evaluation of the whole patients’ series revealed several significant associations with histopathological and other features of tumors. Of these, the correlation with mitotic index and p16INK4a were highly significant. We found a positive and significant correlation between the protein expression of Id-1 and PCNA, which indirectly suggested the role of Id-1 in the proliferation of HCC cells. This was further confirmed by our in vitro cell culture model. Increased PCNA expression in clone 3 with the highest ectopic Id-1 expression was also observed when compared with the control in the absence of FBS. Our data further confirmed the function of Id-1 as a promoter of proliferation in cancers (21–24). From the RT–PCR result, it was also noted that Id-1 mRNA level in the non-tumor liver is relatively high when compared with the normal liver, but it was low when compared with the tumor tissue. Since most of our non-tumor cases are cirrhotic, which has a higher proliferative rate when compared with normal liver (25), it accounts for the enhanced Id-1 expression.
p16INK4a was found to be frequently inactivated in HCC through promoter methylation (16,26–28). However, few reports have demonstrated the direct transcriptional inactivation of p16INK4a in HCC. Since the transcriptional regulator Id-1 has recently been identified as a repressor of p16INK4a transcription (13,29,30), we sought to determine whether transcriptional inactivation of p16INK4a by Id-1 might play a role in the initiation and progression of HCC. Recent reports showed that high level of Id-1 expression is correlated with the loss of p16INK4a in early stage melanoma (31), but is positively correlated with the expression of p16INK4a in breast cancer samples (32). In our study, we found that high Id-1 expression was significantly correlated with decreased p16INK4a expression and the result is similar to melanoma. Hypermethylation of p16INK4a within the promoter was suggested to be one of the late events in hepatocarcinogenesis for tumor progression and metastases (33). With reference to the hypothesis by Polsky et al. (31), HCC hepatocarcinogenesis might also occur via...
multi-step that entails reversible Id-1 transcriptional inactivation of p16INK4a in the early growth phase that allows bypass of cellular senescence, and subsequent acquired epigenetic changes in cells such as promoter methylation for vertical growth phase for later stage. Further study on this subject is required. Conclusively, these results suggested that inactivation of p16INK4a in HCC might be, in part, through transcription control of Id-1.

In order to determine whether Id-1 plays a role in proliferation of HCC through inactivation of p16INK4a, we transfected PLC/PRF/5 by Id-1. Five Id-1 transfectants were isolated and showed differential Id-1 expression. All these five clones showed FBS-independent proliferation accompanied by increased PCNA expression and increased percentage of cell cycle S phase from G1 phase. Our in vitro results were consistent with the previous findings that ectopic Id-1 expression stimulated DNA synthesis from G1 to S phase (7,21,24,28) and resulted in down-regulation of p16INK4a in the Id-1 transfectants. This result further supported that p16INK4a inactivation was due to transcription control of Id-1. Other than p16INK4a, ectopic Id-1 expression induced RB phosphorylation in human keratinocytes (28). One of the functions of p16INK4a is to prevent cyclin-dependent kinases such as CDK4 and results in prevention of RB phosphorylation. In our study, we found that increased expression of phosphorylated CDK4 and RB was only observed in the five Id-1 transfectants but not in the control. This showed that down-regulation of p16INK4a was associated with increased expression of phosphorylated CDK4 and RB but not with CDK and RB levels. RB phosphorylation is proposed to regulate cell cycle regulation from G1 to S phase through cyclin D and CDK4/6 complex. Without the inhibition of p16INK4a, CDK4 becomes phosphorylated and can prevent the binding of E2F with RB, resulting in G1 to S phase conversion by RB phosphorylation (34). In our study, all Id-1 transfectants showed a variable degree of G1 to S phase conversion. Therefore, these results suggested that the effect of Id-1 on proliferation on HCC cells might be caused by the decreased p16INK4a, which in turn inactivated RB.

In summary, our in vitro and in vivo data provided evidence for the first time on over-expression of Id-1 and its role in HCC. Over-expression of Id-1 played a role in HCC cell proliferation. Id-1 induced HCC proliferation through inactivation of p16INK4a/pRB pathway as shown by the evidence of decreased p16INK4a expression and activation of CDK and RB in the five Id-1 transfectants. Our results provided an insight for the understanding of the role of Id-1 in functional inactivation of p16INK4a in HCC.

References
1. Norton, J.D., Deed, R.W., Craggs, G. and Sablitzky, F. (1998) Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol., 8, 58–65.
2. Norton, J.D. (2000) Id helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J. Cell Sci., 113, 3897–3905.
3. Israel, M.A., Hernandez, M.C., Florio, M., Andres-Barquin, P.J., Mantani, A., Carter, J.H. and Julin, C.M. (1999) Id gene expression as a key mediator of tumor cell biology. Cancer Res., 59 (7 suppl.), 1726–1730.
4. Lundberg, A.S., Hahn, W.C., Gupta, P. and Weinberg, R.A. (2000) Genes involved in senescence and immortalization. Curr. Opin. Cell Biol., 12, 705–709.
5. Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W. and Weinberg, R.A. (1999) Creation of human tumour cells with defined genetic elements. Nature, 400, 464–468.
6. Kiyono, T., Foster, S.A., Koop, J.L., McDougall, J.K., Galloway, D.A. and Klingelfuss, A.J. (1998) Both Rb/p16INK4a inactivation and telomerization activity are required to immortalize human epithelial cells. Nature, 396, 84–88.
7. Lin, C.Q., Singh, J., Murak, T., Itahana, Y., Parrinello, L., Liang, S.H., Ohi, J. and Campisi, J. (2000) A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res., 60, 1332–1340.
8. Kleeff, J., Ishiwata, T., Friss, H., Buchler, M.W., Israel, M.A. and Korc, M. (1998) The helix-loop-helix protein Id2 is overexpressed in human pancreatic cancer. Cancer Res., 58, 3769–3772.
9. Ouyang, X.S., Wang, X., Lee, D.T., Tsao, S.W. and Wong, Y.C. (2002) Overexpression of Id-1 in prostate cancer. J. Urol., 167, 2598–2602.
10. Schindl, M., Oberhuber, G., Obermair, A., Schopppmann, S.F., Karner, B. and Birner, P. (2001) Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. Cancer Res., 61, 5703–5706.
11. Wilson, J.W., Deed, R.W., Inoue, T., Balzio, M., Becciolini, A., Faraoni, P., Potten, C.S. and Norton, J.D. (2001) Expression of Id helix-loop-helix proteins in colorectal adenocarcinoma correlates with p53 expression and mitotic index. Cancer Res., 61, 8803–8810.
12. Langlards, K., Down, G.A. and Kealey, T. (2000) Id proteins are dynamically expressed in normal epithelium and dysregulated in squamous cell carcinoma. Oncogene, 20, 7104–7109.
13. Ohnani, N., Zedbecz, Z., Hout, T.J., Stinson, J.A., Sugimoto, M., Ohashi, S., Sharrocks, A.D., Peters, G. and Hara, E. (2001) Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. Nature, 409, 1067–1070.
14. Sherr, C.J. (1996) Cancer cell cycles. Science, 274, 1672–1677.
15. Jin, M., Piao, Z., Kim, N.G., Park, C., Shin, E.C., Park, J.H., Jung, H.J., Kim, C.G. and Kim, M. (2000) p16 is a major inactivation target in hepatocellular carcinoma. Cancer, 89, 60–68.
16. Tannapfel, A., Busse, C., Weimans, L., Benicke, M., Katalinic, A., Geissler, F., Hauss, J. and Wittekind, C. (2001) IdINK4A-ARF alterations and p53 mutations in hepatocellular carcinomas. Oncogene, 20, 7104–7109.
17. Dynlacht, B.D. (1997) Regulation of transcription by proteins that control the cell cycle. Nature, 389, 149–152.
18. Birner, P., Schindl, M., Obermair, A., Plank, C., Breitecnecker, G. and Oberhuber, G. (2000) Overexpression of hypoxia-inducible factor alpha1 is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res., 60, 4693–4696.
19. Schindl, M., Schroppmann, S.F., Strobel, T., Heinzl, H., Leisser, C., Horvat, R. and Birner, P. (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.
20. Uehara, N., Chou, Y.C., Galvez, J.J., de-Candia, P., Cardill, R.D., Benerezza, R. and Shyamala, G. (2003) Id-1 is not expressed in the luminal epithelial cells of mammary glands. Breast Cancer Res., 5, R25–R30.
21. Ouyang, X.S., Wang, X., Ling, M.T., Wong, H.L., Tsao, S.W. and Wong, Y.C. (2002) Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of p16 (INK4a)/pRB pathway. Carcinogenesis, 23, 721–725.
22. Wicke, E.M. and Gordon, J.I. (1998) Forced expression of Id-1 in the adult mouse small intestinal epithelium is associated with development of adenomas. J. Biol. Chem., 273, 25310–25319.
23. Alani, R.M., Hasskarl, J., Grace, M., Hernandez, M.C., Israel, M.A. and Munger, K. (1999) Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1. Proc. Natl Acad. Sci. USA, 96, 9637–9641.
24. Hara, E., Yamaguchi, T., Nojima, H., Ide, T., Campisi, J., Okayama, H. and Oda, K. (1994) Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. J. Biol. Chem., 269, 2139–2145.
25. Donato, M.F., Arosio, E., Del ninno, E., Ronchi, G., Lampertico, P., Morabito, A., Balestrieri, M.R. and Colombo, M. (2001) High rates of hepatocellular carcinoma in cirrhotic patients with high liver cell proliferative activity are required to immortalize human hepatic preneoplastic cells. Oncology, 60, 346–354.
26. Morabito, A., Balestrieri, M.R. and Colombo, M. (2001) Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. Hepatology, 33, 561–568.
28. Alani, R.M., Young, A.Z. and Shifflett, C.B. (2001) Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. Proc. Natl Acad. Sci. USA, 98, 7812–7816.

29. Pagliuca, A., Gallo, P., De, Luca, P. and Lania, L. (2000) Class A helix-loop-helix proteins are positive regulators of several cyclin-dependent kinase inhibitors’ promoter activity and negatively affect cell growth. Cancer Res., 60, 1376–1382.

30. Peverali, F.A., Ramqvist, T., Saffrich, R., Pepperkok, R., Barone, M.V. and Philipson, L. (1994) Regulation of G1 progression by E2A and Id helix-loop-helix proteins. EMBO J., 13, 4291–4301.

31. Polsky, D., Young, A.Z., Busam, K.J. and Alani, R.M. (2001) The transcriptional repressor of p16/Ink4a, Id1, is up-regulated in early melanomas. Cancer Res., 61, 6008–6011.

32. Schoppmann, S.F., Schindl, M., Bayer, G., Aumayr, K., Dienes, J., Horvat, R., Rudas, M., Granit, M., Jakesz, R. and Birner, P. (2003) Overexpression of Id-1 is associated with poor clinical outcome in node negative breast cancer. Int. J. Cancer, 104, 677–682.

33. Feitelson, M.A., Sun, B., Satiroglu Tufan, N.L., Liu, J., Pan, J. and Lian, Z. (2002) Genetic mechanisms of hepatocarcinogenesis. Oncogene, 21, 2593–2604.

34. Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E. and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev., 7, 331–342.

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