Effect of Over-Expression of Zinc-Finger Protein (ZFX) on Self-Renewal and Drug-Resistance of Hepatocellular Carcinoma

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Background: X-chromosome-coupled zinc finger protein (ZFX) in the Zfy protein family is abundantly expressed in both embryonic and hematopoietic stem cells (HSCs). ZFX exist in various tumor cells and is correlated with proliferation and survival of tumor cells. As a malignant tumor with high invasiveness, hepatocellular carcinoma (HCC) may present resistance against chemotherapy and features of stem cells. This study aimed to explore the expression of ZFX in HCC cells, in an attempt to illustrate the role of ZFX in tumorigenesis.

Material/Methods: The expression of ZFX in tumor tissues was quantified by RT-PCR. The ZFX expression was then silenced to evaluate the stem cell-like features of HCC cells, including self-renewal, colony formation, and cell cycle, along with the sensitivity to cisplatin. Xenograft of ZFX-overexpressed HCC on nude mice was performed to evaluate the in vivo effect of ZFX on tumor growth.

Results: Quantitative RT-PCR showed over-expression of ZFX in 51.8% of HCC tumors. The silencing of ZFX gene inhibited the self-renewal, colony formation, and proliferation ability of HCC cells (p<0.05 in all cases) via the cell cycle arrest at G0/G1 phase, in addition to the elevated sensitivity of tumor cells to cisplatin (p<0.001). Further studies showed that binding between ZFX and promoter regions of Nanog or SOX-2 regulatory factor initiate their expression in HCC cells. The xenograft experiment indicated the potentiation of tumor growth by ZFX over-expression.

Conclusions: ZFX is over-expressed in HCC cells, and correlates with stem cell-like features and pleiotropic characteristics.

MeSH Keywords: Carcinoma, Hepatocellular • Self Administration • Self-Injurious Behavior • Tristetraprolin

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Background

Hepatocellular carcinoma (HCC) accounts for 80–90% of all liver cancers and is the third leading mortality factor among all cancers worldwide [1]. Due to its insidious onset and course, HCC is often at late stage at the time of primary diagnosis, causing its high invasiveness, unsatisfactory treatment efficacy, and high mortality [2,3]. Most HCC patients presented recurrence even after surgical resection and/or chemotherapy, and rapid progression into terminal stage cancer. It is now recognized that the stem cell-like feature of tumor cells during the progression is closely related with the chemo-resistance and recurrence [4–9]. Recently, there has been an increasing number of potential prognosis biomarkers associated with tumor progression of hepatocellular carcinoma [10–13]. Elevated ZFX expression level has been detected in both pleiotropic embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs) to keep the self-renewal ability of cells. ZFX over-expression has also been found in various cancer cells, including esophageal, gastric and prostate cancer cells, whose stem cell-like feature, colony formation ability, drug resistance, cell survival, and metastasis have all been confirmed to be regulated by ZFX [14–17]. The function of ZFX in HCC, however, remains unknown. This study thus aimed to investigate the expressionional and functional characteristics of ZFX in HCC.

Material and Methods

Tissue sample collection

A total of 83 liver cancer patients were recruited in this study, including 48 cases of HCC, 22 cases of intrahepatic cholangiocarcinoma (ICC), and 13 cases of mixed hepatocarcinoma (MHC). All patients received confirmed diagnosis by pre-operative biopsy. Both tumor tissue and tumor-adjacent tissue (2 cm from the tumor) samples were collected during surgical resection. This study was pre-approved by the ethics committee of our hospital and we obtained written consent from all patients. Another 6 samples of normal liver tissues were recruited as the control group.

Cell culture

Human HCC cell lines HKCl-10, HKCl-C2, and HKCl-8 were cultured in RPMI 1640 medium (Invitrogen, US) containing 10% fetal bovine serum (FBS). Normal hepatocyte cell line LO2 was cultured in DMEM medium (Invitrogen, USA) containing 10% FBS. Normal hepatocyte cell line L02 was cultured in RPMI 1640 medium (Invitrogen, US) containing 10% fetal bovine serum (FBS).

Human HCC cell lines HKCI-10, HKCI-C2, and HKCI-8 were cultured in DMEM medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS). Normal hepatocyte cell line LO2 was cultured in DMEM medium (Invitrogen, USA) containing 10% FBS as previously reported [18].

RNA extraction and RT-PCR

Total RNA was extracted from cultured cells by Trizol reagents (Invitrogen, USA). Residual DNA was degraded by RNase-free DNase I (Promega, USA). First-strand cDNA was synthesized using TaqMan Master Mix (ABI, US) followed by RT-PCR. The fluorescent intensity was detected by FAM490 system and replicated in triplicate. The gene expression level was normalized against 18s rRNA using 2(-ΔΔCt) method. When compared to the mean value of normal liver tissues, genes with more than 2-fold increase of expression were defined as having elevated expression.

Western blot

Total protein from lysis buffer was separated in 8% SDS-PAGE and transferred onto PVDF membrane. After blocking non-specific binding sites, the membrane was incubated in anti-ZFX, anti-SOX-2, anti-Nanog antibody (1:1 000, Cell Signaling, USA) or anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:25 000, Chemicon). Horseradish peroxidase (HRP) was again used to incubate the membrane. The signal was detected by ECL detector (GE Healthcare, USA).

Packaging and transfection of lentivirus

A FUGENE transfection kit (Roche, Switzerland) was used to transfact HEK293FT cells with pCMV-HSV1-G, pRS-Rev, pMDLg/pRRE, pLKO.1-NS Ctrl, or pLKO.1-hZFX shRNA (Sigma, USA) vectors. At 48 h after transfection, virus was precipitated from supernatants by buffer (1:4). At 24 h before transfection, HKCI-10 and HKCI-C2 cells were seeded into 6-well plates. At 48 h after transfection, cells were incubated in medium containing 2 mg/mL puromycin for 10-day selection as previously reported [19].

Expression of ZFX in HKCI-8 cell line

We used pCMV6-ZFX (Origene, USA) vector containing neomycin-resistant gene or blank pCMV6 vector to transfact HKCI-8 cells with the help of Lipofectamine 2000 reagent (Invitrogen, USA). At 48 h after transfection, neomycin (500 mg/mL) was added into the culture medium for cell selection. Quantitative PCR was used to describe the over-expression of ZFX in cells.

Cell cycle analysis

Stable transfected cells (3×10^6) were digested by trypsin and rinsed in PBS. Cells were fixed using 3 mL 70% ethanol at –20°C overnight, followed by precipitation at 1000 g centrifugation for 5 min. PBS was used to gently wash cells twice. Cells were then stained by 50 mg/mL propidium iodide (PI) and 0.5 mg/mL nuclease A for 30 min, followed by flow cytometry analysis.

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The mean value of G0/G1, S, and G2/M stage was calculated from independent experiments performed 3 times each.

**Cell proliferation assay**

Stable transfected cells (1×10^5) were seeded into a 96-well plate, in which 8 replicates were performed for a certain time-point (D0–D7). Using MTT assay, the optical density value at 570 nm was measured on 7 consecutive days. The Click-iT EdU flow cytometry approach was used to detect the level of DNA replication, using the integration of uridine analogs 5-ethynyl-2-0-deoxyuridine (EdU) of newly synthesized DNA strands. In brief, stable transfected cells (3×10^5) were first incubated in complete medium containing 10 mM EdU for 2 h, followed by PBS washing and Click-iT fixation. After 15-min incubation at room temperature, cells were re-suspended in 500 μL 1× Click-iT buffer and 0.5-mL reaction cocktails were added. After rinsing and re-suspension, cells were analyzed in the FACS Calibur system (BD, US). The percentage of various fluorescent cells were compared by use of WinMDI 2.9 software.

**Colony formation assay**

Stable transfected cells were inoculated into 6-well plates (200 cells per well). After 7-day incubation, medium was removed and cells were washed twice with PBS. After staining by 1% crystal violet (in absolute methanol) for 15 min, the number of colonies (defined as more than 20 cells per colony) was counted and recorded.

**Single cell clonal analysis**

To evaluate the in vitro self-renewal ability of transfected cells, they were seeded into 96-well plates (1 cell per well) and cultured in DMEM medium containing 10% FBS. Twenty-four hours later, those wells with no cells or more than 1 single cell were excluded, leaving only wells with a single cell. Seven days later, the number of clones with more than 20 cells was counted.

**In vitro cytotoxicity assay**

Stable transfected cells (5×10^5) were inoculated into 96-well plates. Cisplatin (20 mM) was added for 48-h incubation, followed by MTT assay for cell viability at an absorbance value of 570 nm.

**ChiP-PCR assay**

Forty-eight hours after ZFX-flag transient transfection, L02 cells were fixed in 1% formaldehyde and lysed in RIPA lysis buffer containing protease inhibitor. Chromatin was fragmented into ~600 bp lengths by ultrasonic processing. After centrifugation, chromatin precipitation was diluted 10-fold by ChiP diluents. With 1-h pre-incubation in protein G-agarose, anti-flag antibody or IgG-controlled serum was used for 4°C overnight incubation. After immunoprecipitation, protein G-agarose beads were used to collect immunoprecipitation complex, which was rinsed by low- and high-saline buffer, LiCl rinsing buffer, and TE buffer, and was eluted by elution buffer. The eluted compounds were decoupled and processed in proteinase K, and purified using a PCR DNA purification kit (Qiagen, USA). Real-time ChiP-PCR was used to quantify ZFX level based on SYBR Green reaction mixtures, along with pre-designed primers flanking possible ZFX binding sites [20].

**Xenograft of tumor cells**

Stable transfected HKCI-8 cells (1×10^5) were re-suspended in 0.1 mL PBS, which was subcutaneously injected into the backs of nude mice (7 weeks old). After 5 days, the tumor size (equals to width^2 × length/2) was measured and calculated. Three weeks later, mice were sacrificed to extract the tumor tissue for measurement.

**Statistical analysis**

All collected data were analyzed by SPSS16.0 software. The paired t test or Mann-Whitney test, as appropriate, was used to compare the ZFX expression level between tumor and tumor-adjacent tissues. The non-paired t test was used to analyze the correlation between ZFX expression and clinical pathological parameters. The paired t test was used to compare the function of ZFX. Statistical significance was defined as p<0.05.

**Results**

**ZFX is over-expressed in human HCC**

Quantitative RT-PCR revealed the significantly elevated ZFX expression in HCC tumor samples (T) when compared to normal liver tissues (NL). Among all 83 cases of HCC, 43 (51%) tumor samples had elevated ZFX expression compared to tumor adjacent (TN) tissues (Figure 1A). Both mRNA and protein levels of ZFX were elevated in tumor tissues (Figure 1B). No correlation existed between ZFX expression level and major clinical parameters, including age, sex, HBV level, histological type, number of lesions, and microvascular invasion. Stage III HCC tumors, however, had higher ZFX expression levels compared to stage I or II tumors (Figure 1C, p<0.05 by non-paired t test), suggesting the correlation between ZFX expression and tumor stage. Further analysis revealed elevated ZFX expression level in liver cirrhosis-induced HCC compared to those tumors not caused by liver cirrhosis (Figure 1D, p<0.05). Because tumor-adjacent liver cirrhosis lesions are often recognized as precancerous lesions, ZFX expression level may serve as an index for evaluating the risk of HCC.
ZFX induced the G0/G1 phase arrest of HCC cells

To explore the mechanism underlying up-regulation of ZFX in HCC tissues, we transfected 2 HCC cell lines, HKCI-10 and HKCI-C2, which have increased ZFX expression (Figure 2A, p<0.01), with lentivirus vector for stable ZFX-deficient clones. Both qRT-PCR and Western blotting revealed the efficiency of ZFX gene silencing (Figure 2B, p<0.001). PI staining showed significantly elevated numbers of cells at G0/G1 phase with fewer cells at S or G2/M phase (Figure 2C, p<0.01), suggesting the cell cycle arrest at G0/G1 phase of HCC cells by the silencing of ZFX gene.

ZFX facilitated HCC cell growth and proliferation

MTT assay showed decreased in vitro proliferation of HCC cells with ZFX gene silencing (Figure 3A, p<0.001), consistent with results from EdU staining (Figure 3B, p<0.001 in both cell lines). Colony formation assay revealed a decreased number and size of cell colonies after ZFX silencing (Figure 3C, p<0.001). The over-expression of ZFX facilitated HCC cell proliferation and colony formation ability (p<0.001 in both cases). These results collectively suggest the important role of ZFX on in vitro proliferation and growth of HCC cells.
ZFX induced stem cell-like features of HCC

Due to its critical role in preserving the pluripotency of cells, ZFX’s function in mediating stem cell-like characteristics of HCC cells was further studied. We mainly studied 2 major features, self-renewal and drug resistance, of stem cell-like features. The silencing of ZFX in HKCI-10 and HKCI-C2 cell lines inhibited self-renewal ability, with formation of fewer and smaller colonies (Figure 4A, p<0.001). Higher sensitivity to chemotherapeutic agents occurred in HCC cells after ZFX silencing, as IC_{50} value decreased from 16.86 μg/mL ±1.07 μg/mL (control) to 6.73 μg/mL ±1.10 μg/mL (shZFX-C1) or 7.03 μg/mL ±1.12 μg/mL (shZFX-C2) (Figure 4B, p<0.001). Therefore, ZFX modulates the stem cell-like features of HCC cells.

ZFX induced stem cell-like features of HCC

ESC pluripotent-related transcriptional factors include SOX-2, Nanog, OCT-4, and Notch1 [21,22]. In this study, we focused on the potential effect of ZFX on the expression of those transcriptional factors. Our study found significantly decreased mRNA and protein expressions of SOX-2 and Nanog after silencing with ZFX.
ZFX (Figure 5A, 5B, p<0.001), leaving the expression of OCT-4 and Notch1 largely unchanged. The over-expression of ZFX in HCC cells significantly altered the expression level of SOX-2 and Nanog mRNA (Figure 5C, p<0.001). In total, there were 6 potential binding sites for ZFX within the 2k promoter region of SOX-2 gene, along with 3 binding sites in Nanog gene

Figure 3. Inhibition of in vitro HCC cell proliferation by ZFX gene silencing. (A) MTT analysis showed cell viability. (B) EdU staining for cell proliferation. Left panels, non-proliferated (P1) and proliferated (P2) cells; right panels, percentage change of proliferated cells. (C) Decreased colony formation ability after ZFX silencing. ** p<0.01; *** p<0.001.
promoter (Figure 5D). To check if ZFX can actually bind onto the promoter region of those 2 genes, we used ChIP-PCR assay and showed the decreased DNA enriching in SOX-2 promoter binding sites by ZFX (Figure 5E, p<0.05). Although no DNA enriching phenomena occurred in the promoter region of Nanog gene, a positive relationship did exist between ZFX expression and Nanog (Figure 5F, p<0.05). These results collectively indicate the role of SOX-2 and Nanog as the downstream target genes of ZFX.

ZFX induced stem cell-like features of HCC

In a further in vivo xenograft study on nude mice, we found the over-expression of ZFX in HCC cells significantly facilitated the growth of xenograft tumors (Figure 6), suggesting the potentiation of ZFX on in vivo growth of HCC lesions.

Discussion

Zinc finger protein coupled with X chromosome (ZFX) is a highly reserved protein coded by a gene located in the X chromosome...
of mammals. As 1 family of zinc finger protein, ZFX contains acidic transcriptional activation domain, nuclear targeting sequence, and DNA structural domain [23,24]. Recent studies have supported the role of ZFX in tumorigenesis. In this study, we, for the first time, confirmed the up-regulation of ZFX in HCC tissues and the important role of ZFX on the stem cell-like properties of HCC. In liver cirrhosis-derived HCC cells, the elevation of ZFX expression was even more significant, which has not been reported previously. About 70–90% of HCC were derived from liver cirrhosis, which may be caused by viral hepatitis, alcohol abuse, and metabolic disorders. Our discovery that liver cirrhosis-derived HCC cells had elevated ZFX expression indicates the value of ZFX as a predictive index for evaluating cancer risk in patients with precancerous lesions. The silencing of ZFX significantly inhibited the self-renewal of HCC cells, in addition to the potentiation of its sensitivity to chemotherapy, thereby suggesting the novel role of ZFX as a target for gene therapy of HCC.

Figure 5. ZFX regulated SOX-2 and Nanog expression. (A) qRT-PCR shows decreased SOX-2 and Nanog mRNA expressions after ZFX silencing. (B) SOX-2 and Nanog protein levels were down-regulated in Western blotting. (C) The over-expression of ZFX induced SOX-2 and Nanog expression. (D) Possible ZFX binding sites within promoter regions of SOX-2 and Nanog genes. (E) SOX-2 and Nanog promoter can bind with ZFX by ChIP-PCR assay. (F) A positive relationship existed between ZFX and Nanog mRNA levels from 83 cases of primary HCC tissues and adjacent tissues. * p<0.05; ** p<0.01; *** p<0.001.

Figure 6. ZFX over-expression facilitated in vivo tumor growth. (A) Nude mouse model showed over-growth of tumors derived from ZFX-overexpression HCC cells (far right panels). (B) Tumor volume of ZFX-overexpressed xenograft was significantly larger than control ones. * p<0.05.
The cancer stem cell theory has been postulated in recent years and has been strengthened by an increasing body of evidence. Some scholars recognize the primary tumor as a complex of heterogeneous cell populations, in which certain cancer stem cells keep various features, including low differentiation, unlimited cell division, resistance to chemotherapy, and self-renewal [13,25–27]. The existence of such tumor-initiated stem cells (TISCs), however, remains controversial as some researchers believe that the stem cell-like features existed not just in a certain sub-population of cancer cells, but in all solid tumor cells. Our findings generally agree with the latter opinion, as most HCC cells had potentiated stem cell features with the help of ZFX. The inhibition of ZFX expression led to broad suppression of cell growth and self-renewal ability. The over-expression of ZFX in HCC cells facilitated both in vitro proliferation of HCC cells and in vivo growth of xenograft.

We also investigated the downstream effector of ZFX in HCC cells, focusing on the effects on expression of stem cell-related genes, including Nanog, Oct-4, Notch1, and SOX-2. No significant change of Oct-4 or Notch1 occurred. The expression of Nanog and SOX-2, however, was significantly altered by ZFX, which possibly binds onto the promoter region of SOX-2 gene as shown by ChiP-PCR analysis. Recent studies showed that the synergistic expression of Nanog and SOX-2 can re-program somatic cells for induction into pluripotent ESCs [28,29], suggesting the potency of co-expression of stem cell factors in keeping cells at the undifferentiated status. Moreover, both Nanog and SOX-2 have been suggested to be related with cancer progression because they may potentiate the expression of ZFX, further causing the oncogenesis of HCC [30–32]. Nanog has been shown to significantly potentiate to the chemoresistance of both oral squamous cell carcinoma and prostate cancer [33,34]. In this study, we further demonstrated a positive relationship between ZFX expression and Nanog, indicating that the elevated sensitivity of HCC cells with ZFX silencing may be related with Nanog down-regulation.

Conclusions

The elevated expression of ZFX in HCC, plus its relationship with cancer stem cells and chemoresistance, indicate the importance of further studies on the signal pathway of ZFX in tumor cells. Our results and further studies should provide a new potential target for novel therapy against HCC.

References:

1. Li Y, Tang ZY, Hou JX: Hepatocellular carcinoma: insight from animal models. Nat Rev Gastroenterol Hepatol, 2012; 9: 32–43
2. Motola-Kuba D, Zamora-Valdes O, Unhe M, Mendez-Sanchez N: Hepatocellular carcinoma. An overview. Ann Hepatol, 2006; 5: 16–24
3. Wang Q, Xue Y. Characterization of solid tumors induced by polycyclic aromatic hydrocarbons in mice. Med Sci Monit Basic Res, 2015; 21: 81–85
4. Chiba T, Kamiya A, Yokosuka O, Iwama A: Cancer stem cells in hepatocellular carcinoma: Recent progress and perspective. Cancer Lett, 2009; 286: 145–53
5. Tong CM, Ma S, Guan XY: Biology of hepatocellular cancer stem cells. J Gastroenterol Hepatol, 2011; 26: 1229–37
6. Ma S, Tang KH, Chan YP et al: miR-130b Promotes CD133+(e) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell, 2010; 7: 694–707
7. Woo HG, Wang XW, Budhu A et al: Association of TP53 mutations with stem cell-like gene expression and survival of patients with hepatocellular carcinoma. Gastroenterology, 2011; 140: 1963–70
8. Oishi N, Wang XW: Novel therapeutic strategies for targeting liver cancer stem cells. Int J Biol Sci, 2011; 7: 517–35
9. Tang G, Shen X, Lv K et al: Different normalization strategies might cause inconsistent variation in circulating microRNAs in patients with hepatocellular carcinoma. Med Sci Monit, 2015; 21: 617–24
10. Zhang JW, Li Y, Zeng XC et al: miR-630 overexpression in hepatocellular carcinoma tissues is positively correlated with alpha-fetoprotein. Med Sci Monit, 2015; 21: 667–73
11. Zhou H, Wang L, Li X et al: IFN-α/β potentiates the expression of SOX-2 in primary HCC tissues. Cancer Res, 2008; 68: 848–85
12. Huang D, Gao Q, Guo L et al: Isolation and identification of cancer stem-like cells in esophageal carcinoma cell lines. Stem Cells Dev, 2009; 18: 465–73
13. Akisawa S, Fukagawa T, Ueh O et al: Clinical significance of mir-144-ZFX axis in disseminated tumor cells in bone marrow in gastric cancer cases. Br J Cancer, 2012; 107: 1345–53
14. Jiang H, Zhang L, Liu J et al: Knockdown of zinc finger protein X-linked inhibits prostate cancer cell proliferation and induces apoptosis by activating caspase-3 and caspase-9. Cancer Gene Ther, 2012; 19: 684–89
15. Zhou Y, Su Z, Huang Y et al: The ZFX gene is expressed in human gliomas and is important in the proliferation and apoptosis of the human malignant glioma cell line U251. J Exp Clin Cancer Res, 2011; 30: 114
16. Gho JW, Ip WK, Chan KY et al: Re-expression of transcription factor ATF5 is a potential target for novel therapy against HCC. Cancer Lett, 2008; 68: 673–81
17. Wang XQ, Ongkeko WM, Chen L et al: Octamer 4 (Oct4) mediates chemotherapy drug resistance in liver cancer through a potential Oct4-AKT-ATP-binding cassette G2 pathway. Hepatology, 2010; 52: 528–39
18. Liu H, Xu Y, Li K et al: Photocrosslinked CR2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. Science, 2008; 322: 1353–39
19. Masui S, Nakatake Y, Toyooka Y et al: Pluripotency governed by Sox2 via regulation of Oct4/silencing in expression in mouse embryonic stem cells. Nat Cell Biol, 2007; 9: 625–35
20. Chen X, Hu Y, Yuan P et al: Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell, 2008; 133: 1106–17
21. Galan-Caridad JM, Harel S, Arenzana TL et al: Zfx controls the self-renewal of embryonic and hematopoietic stem cells. Cell, 2007; 129: 345–57
22. Cellot S, Sauvageau G: Zfx: At the crossroads of survival and self-renewal. Genes Cells, 2007; 12: 239–41
23. Heidel FH, Mar BG, Armstrong SA: Self-renewal related signaling in myeloid leukemia stem cells. Int J Hematol, 2011; 94: 109–17
24. Al-Hajj M, Wicha MS, Benito-Hernandez A et al: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA, 2003; 100: 3983–88

Zhang S. et al.: Zinc-finger protein in liver cancer cells
© Med Sci Monit, 2016; 22: 3025-3034
27. O’Brien CA, Pollett A, Gallinger S, Dick JE: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature, 2007; 445: 106–10
28. Aoi T, Yae K, Nakagawa M et al: Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science, 2008; 321: 699–702
29. Yu J, Hu K, Smuga-Otto K et al: Human induced pluripotent stem cells free of vector and transgene sequences. Science, 2009; 324: 797–801
30. Jeter CR, Badeaux M, Choy G et al: Functional evidence that the self-renewal gene NANOG regulates human tumor development. Stem Cells, 2009; 27: 993–1005
31. Lu Y, Futtner C, Rock JR et al: Evidence that SOX2 overexpression is oncogenic in the lung. PLoS One, 2010; 5: e11022
32. Sholl LM, Barletta JA, Yeap BY et al: Sox2 protein expression is an independent poor prognostic indicator in stage I lung adenocarcinoma. Am J Surg Pathol, 2010; 34: 1193–98
33. Tsai LL, Yu CC, Chang YC et al: Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. J Oral Pathol Med, 2011; 40: 621–28
34. Lu Y, Zhu H, Shan H et al: Knockdown of Oct4 and Nanog expression inhibits the stemness of pancreatic cancer cells. Cancer Lett, 2013; 340: 113–23