Original Paper

Increased Nanog Expression Promotes Tumor Development and Cisplatin Resistance in Human Esophageal Cancer Cells

Li Yang^a Xudong Zhang^b Mingzhi Zhang^b Junhui Zhang^c Yuqiao Sheng^d
Xiangdong Sun^e Qingjiang Chen^b Le-Xin Wang^f

Departments of ^aReproductive Medicine Center, ^bOncology and ^dInstitute of Clinical Medicine, the First Affiliated Hospital, Zhengzhou University, Zhengzhou; ^cDepartments of Otolaryngology, the Third Affiliated Hospital, Zhengzhou University, Zhengzhou; ^eDepartments of Biology, Henan Normal University, Xinxing; ^fSchool of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW

Key Words

Esophageal cancer • Nanog • RNAi • Cisplatin • Drug sensitivity

Abstract

Background/Aims: Nanog plays a key role in stem cell self-renewal and pluripotency differentiation in embryonic stem cells (ESCs). Recently, some studies reported that abnormal expression of Nanog could be detected in several tumors, indicating that Nanog might be related to tumor development. However, studies on the correlation between Nanog expression and esophageal cancer are sparse. **Methods:** In this study, we established two esophageal cancer cell lines 9706-Nanog and 9706-shNanog which stably expressed Nanog and Nanog-short-hairpin RNA (shRNA) genes. **Results:** We found that Nanog expression could promote the proliferation and invasiveness of the cancer cells, and inhibit the apoptosis. We also treated 9706-Nanog, EC9706 and 9706-shNanog cell lines with cisplatin and evaluated the drug sensitivity of the three cell lines. We found that the sensitivity of cisplatin was decreased with increased expression of Nanog. The expression of MDR-1 was also increased in 9706-Nanog cells. **Conclusions:** Nanog may play an important role in human esophageal cancer development, and could be used as a therapeutic target in esophageal cancer treatment.

Copyright © 2012 S. Karger AG, Basel

Dr Qing-Jiang Chen
or Prof Le-Xin Wang

Oncology Department, the First Affiliated Hospital, Zhengzhou University, Zhengzhou 450000, (P.R. China), E-Mail 13633825183@163.com
or School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2678 (Australia); Tel. +61 2 69332905, E-Mail lwang@csu.edu.au

Introduction

Esophageal cancer is one of the most common malignant tumors with high mortality rates in Hebei, Henan and east coast of China [1]. The treatments for esophageal cancer include surgery, chemotherapy and radiotherapy but the efficacy of these therapies is unsatisfactory [2, 3]. Thus it's important to develop new therapeutic strategies for esophageal cancer. Previous studies found that a core regulatory network including three stemness factors Sox2, Oct3/4, and Nanog coordinately determines embryonic stem cells (ESCs) self-renewal and differentiation, and further studies have demonstrated that Nanog is expressed at high levels in ESCs and that the expression levels decrease after differentiation of ESCs [4-7]. There were many similar biological properties between cancer cells and embryonic stem (ES) cells, such as the characteristics of continuous proliferation and uncontrollable differentiation, which indicating these ESC self-renewal molecules may conceptually also contribute to tumorigenesis and development. Some studies have suggested that these factors could regulate self-renewal and pluripotency differentiation of cancer stem cell [8] and may play a role in human malignancy [9]. Previous studies have found that Sox2 might promote cell proliferation and tumorigenesis of breast cancer [10], and Oct3/4 might associated with the early stage of pancreatic cancer carcinogenesis [11, 12], and even correlated with lymph node metastasis in colorectal cancer cells [13]. High expression of OCT-4 has been observed in esophageal cancer [14], suggesting association between OCT-4 and proliferation of esophageal cancer.

Nanog is a member of ANTP class NK family genes and plays a key role in stem cell self-renewal and pluripotency differentiation [4, 5]. In addition to self-renewal regulation of embryonic development, the abnormal expression of Nanog gene is found in malignant germ cell tumors, such as embryonic carcinoma and seminoma [8]. The abnormal expression of Nanog is also detected in solid tumors, such as pulmonary [15], breast [16], cervix [17], oral cavity [18], ovary [19], gastrointestinal [20] and kidney [21] cancer. Jeter CR et al. [9] evaluated the expression, origin, and functions of NANOG in different tumor cells, and found that multiple tumor cells *in vitro* and *in vivo* express NANOG (NANOG mRNA is derived from a Transcribed pseudogene, NANOGP8), and down regulation of NANOG inhibits tumor cells development associated with an inhibition of cell proliferation, clonal expansion, and clonogenic growth of tumor cells. This systematical investigation demonstrated that NANOG expression in human cancer cells is biologically functional in regulating tumor development. In addition, researchers also found that Nanog overexpression may induce chemo-resistance in oral squamous cell carcinoma [22] and prostate cancer [23], promote the tumor recurrence to resist cisplatin. Comprehensive and systematic studies of NANOG expression in human tumor cells have been proceeded, but research of the correlation between Nanog expression and esophageal cancer cells development is lacking.

In our pilot study, the suppression effect of shRNA target Nanog gene was demonstrated *in vitro*. The suppression effect was compared with the off-target effect of control shRNA, and eukaryotic expression vectors pcDNA3.1-Nanog and pSUPER-EGFP-shNanog, which could respectively express and knock-down Nanog gene in human esophageal cancer cell line EC9706, were also constructed. In this study, we detected the expression of Nanog in EC9706 cells, and used pcDNA3.1-Nanog and pSUPER-EGFP-shNanog to transfect into EC9706 cells, and established two esophageal cancer cell lines 9706-Nanog and 9706-shNanog, which could express Nanog and Nanog-shRNA gene stably. Using these cell lines, the impact of Nanog expression on the tumor development including proliferation, apoptosis and invasion behavior of esophageal cancer was evaluated. In addition, drug resistance of cisplatin, a widely used chemotherapeutic agent for esophageal cancer treatment, was also investigated in these cell lines.

Materials and Methods

Cells and plasmids

Human esophageal cancer cell line EC9706 was provided from Henan Key Laboratory for Tumor Pathology and grown in RPMI 1640 with 10% fetal bovine serum. Eukaryotic expression vectors pcDNA3.1-Nanog and pSUPER-EGFP-shNanog were obtained from Institute of Clinical Medicine in the First Affiliated Hospital of Zhengzhou University. pcDNA3.1-Nanog was inserted cDNA of Nanog, reverse transcribed from NANOG mRNA (NM_024865.2), length 920bp. The sequences of the primers to amplify Nanog cDNA and Nanog short-hairpin RNA (shRNA) with restriction enzyme sites were as below: Nanog-F: 5'-CCGCTCGAGATGAGTGTGGATCCAGCTT-3'; Nanog-R: 5'-CGGGATCCTCACACGTCCTCAGGTTGCAT-3'. shNanogF: GATCT GAAGATGAGTGAAGCTGATATTATTCAAGAGATAATATCAGTTTCACTCATCTTCTTTTAA; shNanogR: AGCTTAAAAAAGAAGATGAGTGAAGCTGA TATTATCTCTTGAATAATATCAGTTTCACTCATCTTCA.

Establishment of esophageal cancer cell lines

EC9706 cells were transfected with pcDNA3.1-Nanog and pSUPER-EGFP-shNanog respectively using liposome 2000, and screened by G418 reagent, and the cells that have stably incorporated pcDNA3.1-Nanog and pSUPER-EGFP-Nanog-shRNA could not be killed by G418. Initially these cells were treated with 400 µg/mL of G418 then the dose was increased to 800 µg/mL after 2 weeks. EC9706 cells transfected with normal medium were regarded as control. Cells were cultured until cell clusters derived from replication of cell clones were observed. Then, one of the cell clusters was picked by trypsin filter paper and expanded for further experiments. Finally, Real-time PCR and western blot was carried out for identify the Nanog expression of cell lines.

Real-time PCR and Western blot

Real-time PCR and Western blot analysis were used to evaluate mRNA and protein expression of target gene. Total RNA and protein was isolated from esophageal cancer cells. cDNA was synthesized by random priming and real-time PCR was performed used SYBR green mixture. The sequences of the primers used for real-time PCR are as follows: Nanog-F(5'- ACCTGGTGCACCCAATCCTGG -3'); Nanog-R(5'- CCCAGCAGCTTCCAAGGCAG -3'). MDR1-F(5'- CCGAGCACACCTGGGCATCG -3'); MDR1-R(5'- GGCCTCCTTTGCTGCCCTCAC -3'); β-actin-R (5'- GTCGTCGACAACGGCTCCG -3'); β-actin-F(5'- TGGGCCTCGTCGCCACATA -3'). Following amplification, compare CT values of samples (normalized to β-actin) in order to assess fold differences in mRNA levels of the target genes. 2-Delta Delta CT Method was used in relative gene expression data analysis. Total protein was subjected to SDS-PAGE, and transferred to PVDF membrane, probed with the antibodies as indicated. Primary antibodies were applied and incubated overnight at 4°C as follows: anti-Nanog and anti-MDR-1 (Santa Cruz Biotechnology) at 1:200; β-actin (Santa Cruz Biotechnology) at 1:500. Immunoreactive bands were visualized by ECL Chemiluminescence method and quantified with Gelpro32 image processing and analyzing program.

Colony formation assay

Colony formation was analyzed by plating clone assay. First, 1000 cells/well in logarithmic growth were seeded onto 6-well, and incubated for 10 days. After incubation cells were fixed with 4% paraformaldehyde for 20 min and stained with haematoxylin for 5 min. Finally, staining solution was washed away and colony formation was recorded the microphotographs. The numbers of colony were counted by viewing multiple fields under a microscope.

FACS analysis

EC9706 cells were collected and washed twice using cold PBS, centrifuged at 1000 rpm for 5 min and re-suspend in 500 µL PBS. The re-suspension was added with 5 µL Annexin V-FITC and 2.5 µL PI and cultured for 15 min in darkroom before studied by flow cytometry. The test was described as below: intact cells (FITC-/PI-), apoptotic cells (FITC+/PI-) and necrotic cells (FITC+/PI+). Rate of apoptotic cells was calculated.

Fig. 1. Establishment of esophageal cancer cell lines 9706-Nanog and 9706-shNanog. After 30 days of culture by G418 screening (Initially these cells were treated with 400 $\mu\text{g/mL}$ of G418 then the dose was increased to 800 $\mu\text{g/mL}$ after 2 weeks), stable clones of these cells were selected and expanded, and two esophageal cancer cells lines were established and named as 9706-Nanog(A) and 9706-shNanog(B). Observation of morphology showed that 40 days were required from the cell cluster of 9706-Nanog to cell line establishment, whereas with 9706-shNanog cell line, u2 months were required.

Transwell filter invasion assay

Transwell filter invasion assay is one of the most frequently used methods to analyze cell migration *in vitro* assays. This assay involved a two-compartment system where cells may be induced to migrate from an upper compartment through a porous membrane into a lower compartment. Invasion assays were performed in a 24-well Transwell chamber (Corning, Lowell, MA, USA). Six Transwell chambers were set up for each group, and each chamber was coated with 100 μg Matrigel, and 1×10^5 cells were seeded to each chamber. The lower compartments were filled with 500 μL of RPMI 1640 medium containing 10% FBS, and were incubated in a 5% CO_2 humidified incubator at 37°C for 48 h. To determine the extent to which cells have migrated, the filter inserts are removed from culture wells, the cells that have not migrated are cleared from the top surface of the filter, and the remaining cells that have migrated to the underside of the filter were fixed with 95% alcohol for 10 min and stained with hematoxylin-eosin for 10 min. Finally, the invasion behavior of the cells were quantitated by viewing multiple fields under a microscope, an area corresponding to 20% of the filter are counted.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT assay and trypan blue/trypan blue staining were used to determine the cisplatin sensitivity of EC9706 cell lines. Briefly, cells were harvested and seeded in a 96-well. After 24 h, fresh RPMI 1640 medium containing cisplatin (3 $\mu\text{g/mL}$) was added. After incubation for different time intervals (12, 24, 36, 48, 60, 72 h), 10 μL of MTT (5 mg/mL) was added to each well and the cells were further incubated at 37°C for 4 h. The supernatant was then removed and 100 μL DMSO was added into each well. The absorbance at 450 nm was measured using a microplate reader. Six replicate wells were used for each group.

Statistical analysis

Statistical analysis was performed with one-way ANOVA and student *t* test. Statistical significance was set at $P < 0.05$.

Results

Esophageal cancer cell lines expressing Nanog and shNanog

After 30 days of culture by G418 screening, stable clones of these cells were selected and expanded, and two esophageal cancer cells lines were established and named as 9706-Nanog and 9706-shNanog (Fig. 1). Observation of morphology showed that the growth of 9706-Nanog was faster than 9706-shNanog. Real-time PCR and Western blot were performed to identify the expression of Nanog in these cell lines. The results showed that expression of

Fig. 2. Expression of Nanog in 9706-Nanog and 9706-shNanog cell lines. Cells were collected and total RNA and protein were isolated. The results showed Nanog expressions were up- and down-regulated respectively in 9706-Nanog and 9706-shNanog cell lines at both mRNA and protein levels (A) Real-time PCR analysis; (B) Quantification of Nanog protein expression by Gelpro32 image processing and analyzing program; (C) Western blot analysis. Values are mean $\pm$ standard deviation representative of three independent experiments (n=3).

Fig. 3. Colony formation assay of 9706-Nanog, EC9706 and 9706-shNanog cell lines. (A) Colonies of 9706-Nanog were bigger than EC9706 and 9706-shNanog, and the number colonies was also greater than other groups. Cell colonies of 9706-shNanog were smaller and less in numbers; (B) Colony formation rate. Values are mean $\pm$ standard deviation representative of three independent experiments.

Nanog in 9706-Nanog was up-regulated and higher than normal EC9706 cells at both mRNA and protein levels ($P < 0.05$). The expression of Nanog in 9706-shNanog was lower than normal EC9706 cells because of the down-regulation of Nanog by shRNA ($P < 0.05$, Fig. 2).

Nanog promote clonogenicity and proliferation of esophageal cancer cells

To determine the proliferation and replication abilities of 9706-Nanog and 9706-shNanog cell lines, colony formation assay was performed. The results showed that clonogenicity of 9706-Nanog cell was enhanced according to the size and number of cell colonies. Obviously, colonies of 9706-Nanog were bigger than EC9706 and 9706-shNanog, meanwhile the number was also greater than other groups. To the contrary, cell colonies of 9706-shNanog were smaller and less. In addition, colony formation rate of 9706-Nanog, EC9706 and 9706-shNanog was $34.55 \pm 4.66\%$, $21.11 \pm 3.56\%$ and $12.8 \pm 3.33\%$ respectively. The clonogenicity and proliferation abilities of 9706-Nanog cells were stronger than in the EC9706 and 9706-shNanog cells ($P < 0.05$, Fig. 3).

Apoptosis of esophageal cancer cells

Flow cytometry was applied to assess the apoptosis of 9706-Nanog, EC9706 and 9706-shNanog cell lines. There was little apoptosis being observed in 9706-Nanog and EC9706 cells, and the apoptotic rate of 9706-Nanog and EC9706 cells was $1.63 \pm 1.08\%$ and $1.54 \pm 0.98\%$, respectively ($P > 0.05$, Fig. 4). The apoptotic rate of 9706-shNanog cells was $19.97 \pm 2.08\%$, which was higher than in the 9706-Nanog and EC9706 cells ($P < 0.05$, Fig. 4).

Fig. 4. Apoptosis assay of 9706-Nanog, EC9706 and 9706-shNanog cell lines. Cells were collected and washed twice using cold PBS, centrifuged at 1000 rpm for 5 min and re-suspend in 500 μ L PBS. The re-suspension was added with 5 μ L Annexin V-FITC and 2.5 μ L PI and cultured for 15 min in darkroom before studied by flow cytometry. The test was described as below: intact cells (FITC-/PI-), apoptotic cells (FITC+/PI-) and necrotic cells (FITC+/PI+). (A) FACS showed that apoptotic cells (FITC+/PI-) of 9706-shNanog increased obviously compared to 9706-Nanog and EC9706. (B) Apoptotic rate. Values are mean $\pm$ SD representative of three independent experiments.

Fig. 5. Invasion assay of 9706-Nanog, EC9706 and 9706-shNanog cell lines. Six Transwell chambers were set up for each group, and each chamber was coated with 100 μ g Matrigel, and 1×10^5 cells were seeded to each chamber. The lower compartments were filled with 500 μ L of RPMI 1640 medium containing 10% FBS, and were incubated in a 5% CO_2 humidified incubator at 37°C for 48 h. (A) Transwell assay showed that invasion cells decreased in 9706-Nanog, EC9706 and 9706-shNanog cell lines; (B) Invasion cell number. Values are mean $\pm$ standard deviation representative of three independent experiments.

Nanog promote invasion of esophageal cancer cells

The results of Transwell cell invasion are presented in Figure 5. Overexpression of Nanog resulted in significant promotion of invasion of 9706-Nanog cells with 109.6 ± 6.65 cells of invasion. Only 80.08 ± 4.56 and 49.81 ± 5.09 invasion cells were observed in EC9706 and 9706-shNanog cell lines respectively ($P < 0.05$).

Effect of Nanog on cisplatin sensitivity of EC9706

To evaluate the effect of Nanog on cisplatin sensitivity of EC9706, we test the cell viability of 9706-Nanog, EC9706 and 9706-shNanog cell lines treated with cisplatin by using MTT

Fig. 6. Effect of Nanog on cisplatin sensitivity of 9706-Nanog, EC9706 and 9706-shNanog cell lines. (A), (B) and (C) respectively represent 9706-Nanog, EC9706 and 9706-shNanog cell lines 48 h after treatment with cisplatin. Compared to EC9706, cells of 9706-Nanog exhibited a weaker proliferation inhibition, most of 9706-Nanog cells remained in a good growth condition even 48 h after treatment with cisplatin, but most of 9706-shNanog cells were dead; (D) Compared to EC9706, the growth inhibition rate of cells by cisplatin was increased in 9706-shNanog and decreased in 9706-Nanog. Values are mean $\pm$ standard deviation representative of three independent experiments.

Fig. 7. Apoptosis assay after treatment with cisplatin. (A) There was an apoptosis of EC9706 cells after treatment with cisplatin, and the apoptotic rate of 9706-Nanog was lower than EC9706 cells, while in 9706-shNanog cells it was higher than EC9706 and 9706-Nanog; (B) Apoptotic rate. Values are mean $\pm$ standard deviation representative of three independent experiments.

method. The results showed that cisplatin inhibited proliferation of EC9706 cells, and the inhibition rate increased with time dependence. Compared to EC9706, cells of 9706-Nanog exhibited a weaker proliferation inhibition, most of 9706-Nanog cells could remain a good growth condition even 48 h after treatment with cisplatin (Fig. 6). But the growth of 9706-shNanog cells was extremely inhibited and most of cells were dead 48 h after treatment with cisplatin. Compared to EC9706, the growth inhibition rate of cells by cisplatin was increased in 9706-shNanog and decreased in 9706-Nanog ($P < 0.05$), and these data indicated that Nanog could influence the cisplatin sensitivity of EC9706.

The changes in apoptosis upon treatment with cisplatin were also evaluated by Flow cytometry. There was an obvious apoptosis of EC9706 cells after treatment with cisplatin ($16.44 \pm 2.48\%$), and the apoptotic cells of 9706-Nanog was less than EC9706 ($7.9 \pm 1.52\%$), while more apoptotic cells in 9706-shNanog cells ($23.59 \pm 1.78\%$) was detected ($P < 0.05$, Fig. 7).

Fig. 8. Expression of MDR-1 in 9706-Nanog, EC9706 and 9706-shNanog cell lines. Compared to EC9706, the expression of MDR-1 was increased in 9706-Nanog cells and decreased in 9706-shNanog cells at both mRNA and protein levels. (A) Real-time PCR analysis; (B) Quantification of Nanog protein expression by Gelpro32 image processing and analyzing program; (C) Western blot analysis. Values are mean $\pm$ standard deviation representative of three independent experiments.

To more precisely determine the effect of Nanog on cisplatin sensitivity of EC9706, we also detected the expression of MDR-1 which was regarded as an important factor on drug resistance and sensitivity of chemotherapy to esophageal cancer. The real-time and Western blot assay showed that MDR-1 expression was related closely with Nanog expression. Compared to EC9706, the Expression of MDR-1 was increased in 9706-Nanog cells and decreased in 9706-shNanog cells at both mRNA and protein levels with significant difference ($P < 0.05$) (Fig. 8).

Discussion

Nanog serves as a novel transcription factor which maintains self-renewal and pluripotency of stem cells [4, 5]. As a key self-renewal molecule, Nanog is detected not only in embryonic stem cells (ESCs), but also in germ cell tumors [8]. Ezech et al. [16] showed that the Nanog protein was expressed in tissues of breast cancer. Zhang et al. [24] reported that several tumor cell lines express NANOGP8, a processed pseudogene of Nanog. Jeter CR et al. [9] previously finished a systematical investigation on the expression, origin, and functions of NANOG in different tumor cells, and found that multiple tumor cells *in vitro* and *in vivo* express NANOG and demonstrated that NANOG expression in human cancer cells might be related to tumor development. The results suggest that Nanog may play an important role in tumor development.

In the present study, we used two Eukaryotic expression vectors, pcDNA3.1-Nanog and pSUPER-EGFP-shNanog, which were transfected into human esophageal cancer cell line EC9706, and established two cell lines 9706-Nanog and 9706-shNanog by screening cells with G418 reagent. In addition, Real-time PCR and Western blot assay were carried out to identify the expression of Nanog in 9706-Nanog and 9706-shNanog cell lines. Based on these cell lines, we evaluated the relations between Nanog and the biological characteristics of human esophageal cancer cells, including clonogenicity and proliferation, invasion and apoptosis of cell lines with Nanog expression and loss-of-function. We showed that the colony formation rate of 9706-Nanog cells was higher than EC9706 and 9706-shNanog cell lines, and there were more invasion cells in 9706-Nanog than in other cell lines. These results indicate that expression of Nanog could promote clonogenicity, proliferation and invasion abilities of human esophageal cancer cell line EC9706. Our data also indicate that Nanog inhibits apoptosis of esophageal cancer cells, as increased apoptotic cells were observed by FACS in 9706-shNanog cells. Taken together, not only Nanog is expressed in human esophageal cancer cell line EC9706, it is also closely related to the malignant characteristics which usually

gave rise to tumorigenesis and progression of esophageal cancer. Furthermore, inhibition of Nanog expression may be an effective treatment for patients with esophageal cancer.

Multidrug resistance (MDR) enables cancer cells to resist anticancer drugs of a wide variety of structure and function [25, 26], and inducing reduced drug sensitivity in cancer cells [27, 28]. Cisplatin is a commonly used chemotherapeutic drug for human esophageal cancer, but drug resistance has become a major issue. Expression of Nanog was increased when cancer cells turned into MDR condition [22], suggesting a possible relationship between Nanog and MDR in cancer cells [18, 29]. In our study, we treated 9706-Nanog, EC9706 and 9706-shNanog cell lines with cisplatin and evaluated the drug sensitivity of these three cell lines. We found that the expression of MDR-1 was increased in 9706-Nanog cells but it was inhibited in 9706-shNanog cells. The sensitivity to cisplatin was decreased as Nanog expression levels were increased. These data indicate Nanog was related to the expression of MDR-1 gene and further changed the drug sensitivity of human esophageal cancer to cisplatin. Therefore, Nanog may be used as a novel target to study drug resistance of esophageal cancer.

In this study, we were unable to discriminate between Nanog and NanogP8 gene, so we could not demonstrate the expression level of these two genes and biological differences among Nanog and its pseudogenes in esophageal cancer. Future studies are required to assay for Nanog and NanogP8 mRNA, and to perform the sequence analysis of 3'UTR region to discriminate among the Nanog alleles and NanogP8.

In conclusion, this study showed that Nanog was expressed in human esophageal cancer cell line EC9706 and promotes tumor cells development. Overexpression or loss of function of Nanog was associated with ability of clonogenicity, proliferation and invasion, and apoptosis in EC9706 cells. In addition, expression of Nanog was related to cisplatin resistance of EC9706. However, the biological function of NANOG expressed in human esophageal cancer cells remains unclear, and more importantly, the function of multiple pseudogenes of Nanog genes in regulating tumorigenesis and tumor development is still controversial.

References

- 1 Yeung PS, Boor KJ: Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. *Foodborne Pathog Dis* 2004;1:74-88.
- 2 Lin SH, Chang JY: Esophageal cancer: diagnosis and management. *Chin J Cancer* 2010;29:843-854.
- 3 Rice TW, Rusch VW, Apperson-Hansen C, Allen MS, Chen LQ, Hunter JG, Kesler KA, Law S, Lerut TE, Reed CE, Salo JA, Scott WJ, Swisher SG, Watson TJ, Blackstone EH: Worldwide esophageal cancer collaboration. *Dis Esophagus* 2009;22:1-8.
- 4 Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A: Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 2003;113:643-655.
- 5 Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S: The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 2003;113:631-642.
- 6 Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA: Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 2005;122:947-956.
- 7 Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH: A protein interaction network for pluripotency of embryonic stem cells. *Nature* 2006;444:364-368.
- 8 Hoei-Hansen CE: Application of stem cell markers in search for neoplastic germ cells in dysgenetic gonads, extragonadal tumours, and in semen of infertile men. *Cancer Treat Rev* 2008;34:348-367.
- 9 Jeter CR, Badeaux M, Choy G, Chandra D, Patrawala L, Liu C, Calhoun-Davis T, Zaehres H, Daley GQ, Tang DG: Functional evidence that the self-renewal gene NANOG regulates human tumor development. *Stem Cells* 2009;27:993-1005.

- 10 Chen Y, Shi L, Zhang L, Li R, Liang J, Yu W, Sun L, Yang X, Wang Y, Zhang Y, Shang Y: The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. *J Biol Chem* 2008;283:17969-17978.
- 11 Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG: Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005;65:5506-5511.
- 12 Wen J, Park JY, Park KH, Chung HW, Bang S, Park SW, Song SY: Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. *Pancreas* 2010;39:622-626.
- 13 Saiki Y, Ishimaru S, Mimori K, Takatsuno Y, Nagahara M, Ishii H, Yamada K, Mori M: Comprehensive analysis of the clinical significance of inducing pluripotent stemness-related gene expression in colorectal cancer cells. *Ann Surg Oncol* 2009;16:2638-2644.
- 14 Zhou X, Huang GR, Hu P: Over-expression of Oct4 in human esophageal squamous cell carcinoma. *Mol Cells* 2011;32:39-45.
- 15 Nirasawa S, Kobayashi D, Tsuji N, Kuribayashi K, Watanabe N: Diagnostic relevance of overexpressed Nanog gene in early lung cancers. *Oncol Rep* 2009;22:587-591.
- 16 Ezech UI, Turek PJ, Reijo RA, Clark AT: Human embryonic stem cell genes OCT4, NANOG, STELLAR, and GDF3 are expressed in both seminoma and breast carcinoma. *Cancer* 2005;104:2255-2265.
- 17 Ye F, Zhou C, Cheng Q, Shen J, Chen H: Stem-cell-abundant proteins Nanog, Nucleostemin and Musashi1 are highly expressed in malignant cervical epithelial cells. *BMC Cancer* 2008;8:108.
- 18 Chiou SH, Yu CC, Huang CY, Lin SC, Liu CJ, Tsai TH, Chou SH, Chien CS, Ku HH, Lo JF: Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin Cancer Res* 2008;14:4085-4095.
- 19 Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP: Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008;68:4311-4320.
- 20 Miyoshi N, Ishii H, Nagai K, Hoshino H, Mimori K, Tanaka F, Nagano H, Sekimoto M, Doki Y, Mori M: Defined factors induce reprogramming of gastrointestinal cancer cells. *Proc Natl Acad Sci U S A* 2010;107:40-45.
- 21 Bussolati B, Bruno S, Grange C, Ferrando U, Camussi G: Identification of a tumor-initiating stem cell population in human renal carcinomas. *FASEB J* 2008;22:3696-3705.
- 22 Tsai LL, Yu CC, Chang YC, Yu CH, Chou MY: Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. *J Oral Pathol Med* 2011;40:621-628.
- 23 Jeter CR, Liu B, Liu X, Chen X, Liu C, Calhoun-Davis T, Repass J, Zaehres H, Shen JJ, Tang DG: NANOG promotes cancer stem cell characteristics and prostate cancer resistance to androgen deprivation. *Oncogene* 2011;30:3833-3845.
- 24 Zhang J, Wang X, Li M, Han J, Chen B, Wang B, Dai J: NANOGP8 is a retrogene expressed in cancers. *FEBS J* 2006;273:1723-1730.
- 25 Baguley BC: Multiple drug resistance mechanisms in cancer. *Mol Biotechnol* 2010;46:308-316.
- 26 Chen T: Overcoming drug resistance by regulating nuclear receptors. *Adv Drug Deliv Rev* 2010;62:1257-1264.
- 27 Gan SY, Zhong XY, Xie SM, Li SM, Peng H, Luo F: Expression and significance of tumor drug resistance related proteins and beta-catenin in esophageal squamous cell carcinoma. *Chin J Cancer* 2010;29:300-305.
- 28 Seruga B, Hertz PC, Le LW, Tannock IF: Global drug development in cancer: a cross-sectional study of clinical trial registries. *Ann Oncol* 2010;21:895-900.
- 29 Yin BB, Wu SJ, Zong HJ, Ma BJ, Cai D: Preliminary screening and identification of stem cell-like sphere clones in a gallbladder cancer cell line GBC-SD. *J Zhejiang Univ Sci B* 2011;12:256-263.