Diagnostic relevance of overexpressed Nanog gene in early lung cancers

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Abstract. Currently, no target molecules have been identified that enable the diagnosis of lung cancer with high sensitivity and specificity, especially in the early clinical stages of cancer. Recently, Nanog has been reported to play an important role in the self-renewal and regeneration of ES cells by maintaining these cells in the undifferentiated state and by accelerating cell proliferation. Here, we compared the degree of Nanog mRNA expression in lung cancer tissues with that in non-cancerous tissues. Nanog mRNA was detected in 84.8% (39/46) of lung cancer tissues. The sensitivity and specificity of this diagnostic technique was 80.4 and 93.3%, respectively, as estimated using the cut-off obtained from the analysis of the receiver operating characteristic curve. Further, comparison of paired cancerous and non-cancerous tissues from the same patient revealed elevated Nanog mRNA levels in all patients. No obvious correlations were detected between the clinicopathological factors and Nanog mRNA expression; however, Nanog mRNA was expressed at high levels even in the early clinical stages of the cancer. In addition, the transduction of Nanog siRNA in lung carcinoma cells resulted in growth inhibition. These results suggest that Nanog mRNA might be a new tool to support the diagnosis of lung cancers, irrespective of the clinical stage.

Introduction

Although there has been improvement in the prognosis of lung cancer, the prognosis is still very poor (1). To increase the efficacy of lung cancer therapy, it is important to urgently identify candidate genes that would facilitate tumour detection at high sensitivity and specificity rates, even in the early stages of lung cancer. Measuring highly amplified gene expression of such molecules would be useful to support the diagnosis, especially when very few cells can be obtained and malignancy cannot be definitive by histological or cytological diagnosis.

Cancer cells exhibit the characteristics of continuous proliferation and uncontrollable differentiation, similar to embryonic stem (ES) cells. Previous studies have demonstrated that Nanog (Tir Na Nog) is expressed at high levels in ES cells and that the expression levels decrease after differentiation of ES cells (2,3). Nanog is first expressed in the morula stage during embryonic development; its maximal expression is observed in the inner cell mass of the blastocyst. Nanog cannot be detected in normal mouse tissues (2).

Recent studies have clarified that the inhibition of *Nanog* results in the accelerated differentiation of ES cells (2,4,5). Okita *et al* reported that induced-pluripotent stem (iPS) cells, which were generated by the transduction of *c-myc*, *Oct3/4*, *Sox2* and *Klf4* in the embryonic fibroblasts derived from *Nanog*-transgenic mice, can be selected by using the Nanog gene as a marker because it is expressed in high amounts in the cells (6). Moreover, Yu *et al* demonstrated that the transduction of 4 genes, including *Nanog*, into human somatic cells results in the formation of iPS cells (7). These findings suggest that Nanog plays an important role in the self-renewal of ES cells by maintaining these cells in an undifferentiated state and by accelerating their proliferation, thereby leading to the regeneration of the cells. The features exhibited by cells that express Nanog are characteristic of cancer cells. Therefore, it is speculated that the level of Nanog expression in cancerous cells is higher than in non-cancerous cells and that its expression may play a key role in the acceleration of cell proliferation. Regarding this comparison, only one study has demonstrated that the level of Nanog protein expression was higher in cancerous tissues than in normal cervical epithelia and dysplastic cells of the patients with squamous cervical carcinomas (SCCs) (8). In the above-mentioned study, data were obtained using immunohistochemistry and quantitative analysis was not performed because the aim of the study was not to demonstrate the diagnostic sensitivity of Nanog. Owing to the regenerative ability of Nanog, its expression in non-regenerative organs is speculated to be very low. Hence, we

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hypothesized that lung cancers might be good targets to measure Nanog as diagnostic marker and that gene expression should be measured because of high detection sensitivity of RT-PCR. For this purpose, we quantitatively analyzed the degree of Nanog mRNA expression in non-cancerous tissues and in cancerous tissues that were obtained from patients with lung cancer. In addition, we examined the importance of Nanog expression in the proliferation of lung cancer cells.

Materials and methods

Patients and frozen tissue samples. Cancerous and non-cancerous tissue specimens were obtained from patients who underwent surgery for the treatment of lung, gastric, colon, or breast cancers at Sapporo Medical University Hospital and Minami-ichijo Hospital (Sapporo, Japan). After informed consents were obtained, the samples were dissected from these tissue specimens and were immediately frozen. The tissue samples were stained with haematoxylin/eosin and reviewed by experienced pathologists. Clinicopathological factors and clinical stages were evaluated according to the criteria of the Japanese Society of Lung Cancer, which are based on the tumour-node-metastasis (TNM) system.

Cell culture. The human lung cancer cell lines A549 and SBC-1 (LC817) were cultured in minimum essential medium (Eagle) (MEM; Sigma-Aldrich, St. Louis, MO) and Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and grown at 37˚C in a humidified atmosphere of 5% CO_2.

Immunocytochemistry. The level of Nanog protein expression in the culture cells was examined by conventional methods. The cells were plated and cultured in 2-chamber culture slides (BD Biosciences, Bedford, MA) and subjected to immunocytochemistry. The cells were fixed with 4% para-formaldehyde for 15 min and washed with phosphate-buffered saline (PBS). Next, the cells were treated with 0.2% Triton X-100 in PBS for 5 min, washed and treated with 0.2% Tween-PBS to block non-specific staining. The cells were then stained with rabbit anti-human-Nanog polyclonal antibody (1:100 dilution; ReproCELL, Tokyo, Japan) at 4˚C for 18 h. Subsequently, the cells were washed thrice with PBS and reacted with peroxidase-labeled goat anti-rabbit polyclonal antibody at 25˚C for 2 h. Thereafter, the stained cells were washed thrice using PBS and visualized using the DAB substrate chromogen system (DakoCytomation, Dako Japan, Kyoto, Japan). Non-specific staining was examined by repeating this procedure, except that rabbit anti-human Nanog polyclonal antibody was not used. The stained cells were photographed using the BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and images were captured using the DP-70 digital camera (Olympus) the software DP controller ver. 1.2 (Olympus).

Quantitative reverse transcription-polymerase chain reaction for Nanog mRNA. RNA from the culture cells or tissues was extracted using the Isogen reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's protocol. The RNA concentration of the extract was assayed using the GeneQuant DNA/RNA Calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The expression levels of Nanog mRNA and 18S ribosomal RNA (rRNA) were determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) by using an ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). The gene-specific primers and fluorescent hybridization probes for *Nanog* used in quantitative RT-PCR were as follows: forward primer, 5'-CCG ACT GTA AAG AAT CTT CAC CTA TG-3'; reverse primer, 5'-TCA GGG CTG TCC TGA ATA AGC-3'; and probe, 5'-(FAM) CCT TGC AAA TGT CTT CTG CTG AGA TGC C (TAMRA)-3'. In order to compare the concentrations of Nanog mRNA in the different samples, the quantity of specific mRNA was normalized as a ratio to the concentration of 18S rRNA, which was determined using the TaqMan Ribosomal RNA Control Reagents (Perkin-Elmer Applied Biosystems), according to the manufacturer's protocol. Quantitative RT-PCR was performed using the TaqMan Core Reagent kit (Perkin-Elmer Applied Biosystems). Standard curves were generated on the basis of the linear relationship between the cycle number at which the fluorescence signal significantly increased for the first time (Ct value) and the logarithm of the initial mRNA concentration. The concentration of target mRNA in the test samples was determined on the basis of the Ct value by using the standard curve. A control without a template was included in each experiment.

Transduction of small inhibitory RNA for Nanog. A short inhibitory RNA (siRNA), Hs_NANOG_4_HP, designed by targeting the coding region of the *Nanog* gene (GenBank accession no: NM-024865), was purchased from Qiagen (Tokyo, Japan). Single-strand RNAs were annealed by incubating each strand in siRNA suspension buffer for 1 min at 90˚C followed by incubation of 1 h at 37˚C. SiRNA transduction was performed using the Nucleofector® II Device and Nucleofector® kit T for Cell Line (Amaxa Inc., Gaithersburg, MD), according to the manufacturer's protocol. In brief, SBC-1 cells at a concentration of $1x10^6$ were transfected with siRNA or siRNA-suspension buffer and transfected cells at a concentration of $1x10^5$ were cultured in 6-well plates (Costar, Tokyo, Japan) containing 3 ml DMEM supplemented with 10% FBS. After 48 h, the expression of silenced mRNA was estimated and the number of cells was counted on a hemacytometer.

Statistical analysis. Statistical analysis of the differences in the expression levels of Nanog mRNA were analysed using the Mann-Whitney rank-sum test. Statistical significance was defined as $p<0.05$. The receiver operating characteristics (ROC) curve was generated, and the sensitivity/specificity at various cut-off values was calculated using the StatFlex software version 5.0 (Artech, Osaka, Japan). First, the software automatically arranges the data that are manually inputted for both groups (non-cancerous and cancerous tissues) according to the magnitude of the values. Next, it calculates the sensitivity and specificity at each cut-off value that is determined for each data set and automatically generates the ROC curve.

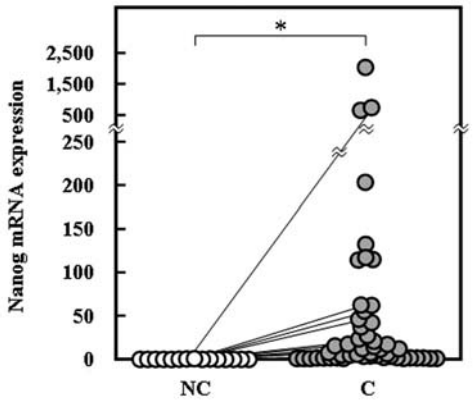

Figure 1. Expression of Nanog mRNA in cancerous and adjacent non-cancerous tissues obtained from lung cancer patients. The level of Nanog mRNA expression was measured using quantitative RT-PCR and is reported relative to the amount of 18S rRNA. The solid line indicates a paired sample. NC, non-cancerous tissues; C, cancerous tissues. Statistical analysis was performed using the Mann-Whitney rank-sum test. *P<0.00001.

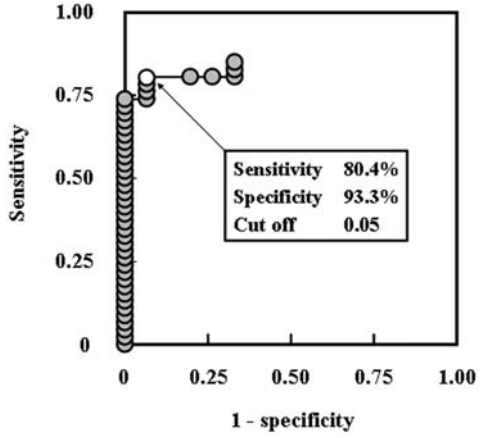

Figure 2. ROC curve for the detection of lung cancer. The curve was automatically generated from 44 points of cut-off values set using StatFlex software.

Results

Nanog mRNA expression in lung cancers. RT-PCR was performed on 15 samples of non-cancerous tissues and 46 samples of cancerous tissues obtained from patients with lung cancer (Fig. 1). In the cancerous tissues, Nanog mRNA was detected in 39 of the 46 samples (84.8%). The mean Nanog mRNA expression in cancerous tissues (94.71±310.39) was extremely high as compared to that in non-cancerous tissues (0.02±0.04) ($p<0.00001$). When the cut-off value for the level of Nanog mRNA expression was set as the mean + 2 SD (0.096) of its expression in non-cancerous lung tissues, 76.1% of the cancerous tissues (35 of 46; Fig. 1) were found to be positive for Nanog mRNA. Further, in the case of all patients from whom samples were obtained from cancerous tissue and adjacent non-cancerous tissues, Nanog mRNA expression was elevated in the cancerous samples. On the basis of the ROC curve, the sensitivity of Nanog mRNA was 80.4% and its specificity was 93.3% at the appropriate cut-off value (0.05, Fig. 2).

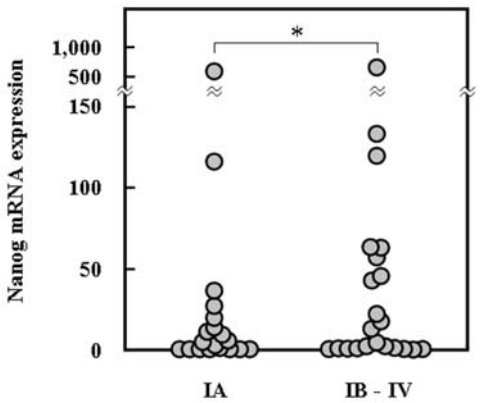

Figure 3. Nanog mRNA expression in stage IA and other stages of lung cancer. The level of Nanog mRNA expression was measured using quantitative RT-PCR and is reported relative to the amount of 18S rRNA. Statistical analysis was performed using the Mann-Whitney rank-sum test. *P>0.05.

Further, we analysed the relationship between clinico-pathological factors and Nanog mRNA expression in cancerous tissues. We did not find any correlation of between age, gender and tissue type (adenocarcinoma vs. SCC) with Nanog mRNA expression. Nanog mRNA expression was examined at different clinical stages that were determined on the basis of the tumour size and invasion and metastasis to lymph nodes and distant organs. Nanog mRNA expression was high even in stage IA (Fig. 3). Further, the mean value and the range of SD at stage IA (43.22±132.45, n=19) were almost equal to those in the other clinical stages [53.13±141.17 (stage IB-IV, n=22) $p>0.05$].

Next, we examined the levels of Nanog mRNA expression in cancers derived from other organs. Mean expression in cancerous tissues and non-cancerous tissues was 5.35±6.34 vs. 3.41±2.28 ($p>0.05$, gastric cancer), 854.79±1.273.16 vs. 326.43±738.47 ($p>0.05$, colon cancer) and 5.47±4.61 vs. 3.55±6.80 ($p>0.05$, breast cancer). In these cancers, in contrast to the expression profile in the lung, no significant difference in the expression level of Nanog mRNA was observed in non-cancerous and cancerous tissues (Fig. 4).

Effect of siRNA transduction on cell proliferation. To determine the importance of *Nanog* expression in the proliferation of cancer cells, we transduced Nanog siRNA into lung cancer cells. We selected small cell lung cancer SBC-1 cells (9,10) that displayed rapid cell growth. The expression of Nanog mRNA and protein was more evident in SBC-1 cells than in the A549 cells that exhibited low expression levels (Fig. 5A and B). Transduction of siRNA decreased the Nanog mRNA expression to 58% from the baseline after 24 h (Fig. 5C). Thereafter, we examined how the siRNA transduction affects the number of cells. The growth inhibition was observed in 82.5 and 66.3% of siRNA-transduced cells and control cells, respectively, after electroporation with siRNA suspension buffer (Fig. 5C).

Discussion

This study was performed to determine whether *Nanog* is a useful gene marker for the diagnosis of lung cancer. We have

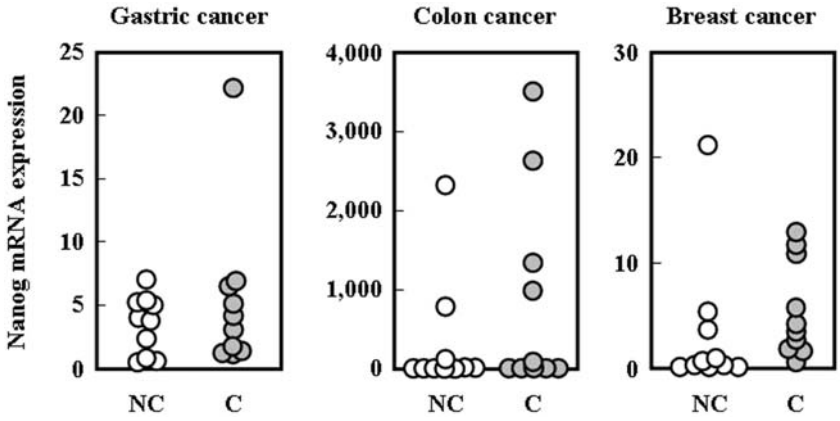

Figure 4. Nanog mRNA expression in non-cancerous and cancerous part from various cancer patients. The level of Nanog mRNA expression was measured using quantitative RT-PCR and is reported relative to the amount of 18S rRNA. NC, non-cancerous tissues; C, cancerous tissues.

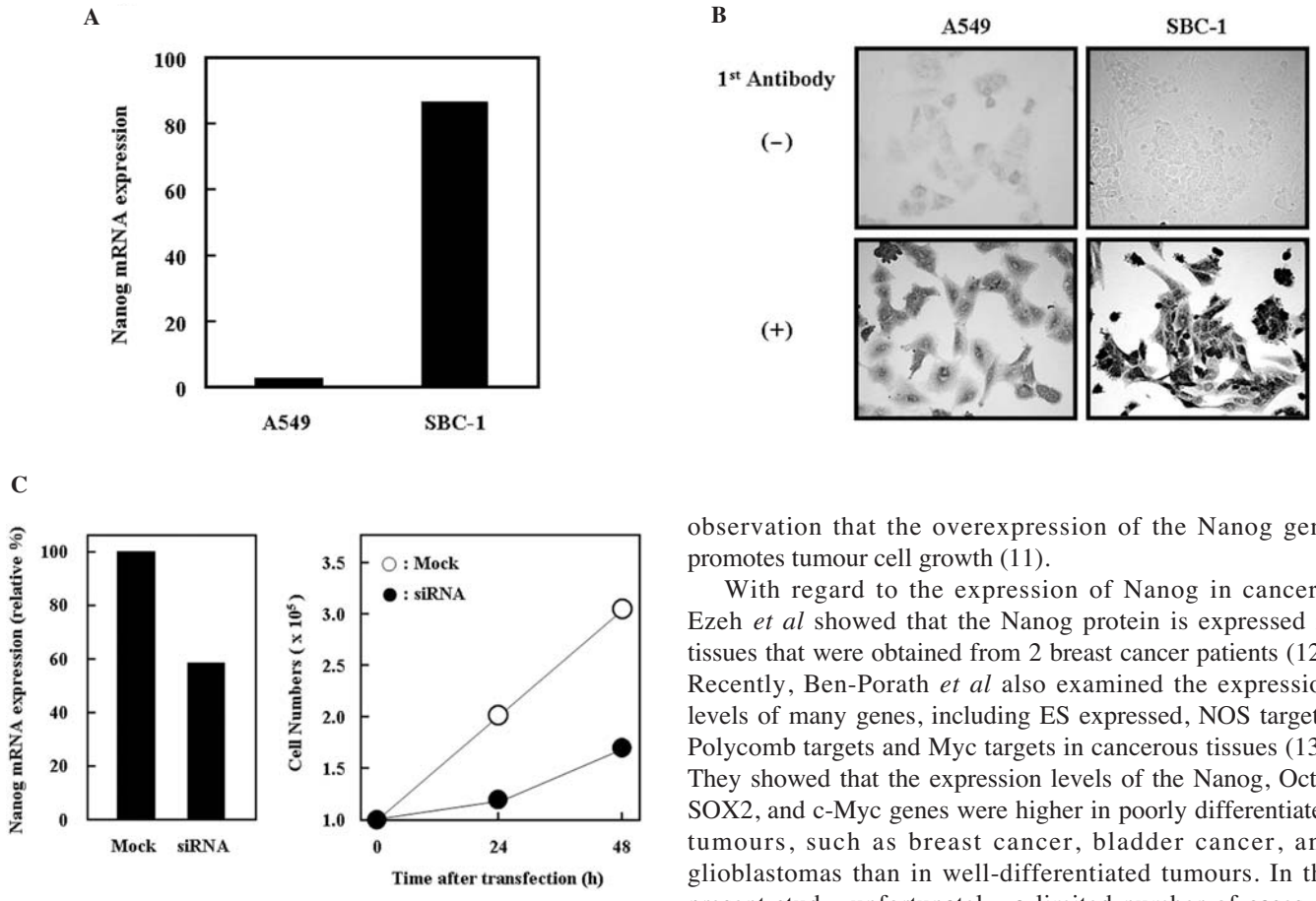

Figure 5. (A) Nanog mRNA expression in A549 and SBC-1 cells. The level of Nanog mRNA expression was measured using quantitative RT-PCR and is reported relative to the amount of 18S rRNA. (B) The Nanog protein was detected by immunocytochemistry in A549 and SBC-1 cells. (C) Left, decrease in the expression levels of Nanog mRNA due to transduction of siRNA against the *Nanog* in SBC-1 cells. Right, effect of transduction of siRNA against *Nanog* on the cell proliferation of SBC-1 cells.

demonstrated for the first time that Nanog mRNA expression can be detected with high sensitivity and specificity in lung cancer tissues. In addition, experiments on siRNA revealed that the constitutive expression of Nanog in cancer cells maintains proliferation ability; this is consistent with the observation that the overexpression of the Nanog gene promotes tumour cell growth (11).

With regard to the expression of Nanog in cancers, Ezeh *et al* showed that the Nanog protein is expressed in tissues that were obtained from 2 breast cancer patients (12). Recently, Ben-Porath *et al* also examined the expression levels of many genes, including ES expressed, NOS targets, Polycomb targets and Myc targets in cancerous tissues (13). They showed that the expression levels of the Nanog, Oct4, SOX2, and c-Myc genes were higher in poorly differentiated tumours, such as breast cancer, bladder cancer, and glioblastomas than in well-differentiated tumours. In the present study, unfortunately, a limited number of cases of poorly-differentiated lung cancers were included and we could not confirm whether or not the expression of *Nanog* was higher in these cancers. It remains to be investigated whether the expression of the Nanog gene is higher in small-cell lung cancers than in differentiated lung cancers.

With regard to the analysis of other organs, extremely high Nanog mRNA expression was observed in the colon, even in non-cancerous specimens. Further, no significant difference was noted in the levels of Nanog gene expression between the cancerous and non-cancerous tissues. Considering the fact that the rate of physiological proliferation of the epithelial cells in the colon is remarkably high, the finding might reflect on the regenerative ability of Nanog. The contrasting results obtained

with lung tissue sample may be explained by the fact that among the 4 organs tested in the study, the level of cellular regeneration is the lowest in the lungs. However, we could not determine the exact reason behind the varying expression profiles of *Nanog*.

In most previous studies the mean + 2 SD of the expression levels of target mRNA in non-cancerous tissues has been used as the cut-off value to determine sensitivity. The ideal cut-off value from taking into consideration both sensitivity and specificity was nearly identical to the cut-off value used in this study; thus, indicating that the cut-off value of the mean + 2 SD is statistically appropriate.

However, it remains unclear whether the high expression of Nanog mRNA causes carcinogenesis or *vice versa*. Thus far, Nanog expression in cancerous and non-cancerous tissues has been compared in only one study (8). Ye *et al* examined Nanog expression among in normal cervical epithelia, dysplastic cells and SCC cells (8). They demonstrated that the level of the Nanog protein expression was higher in SCC cells than in dysplastic cells and concluded that Nanog may be involved in cervix carcinogenesis. However, this study did not include data regarding Nanog expression and clinical stages of cancer. We found that Nanog mRNA was expressed at high levels even in the early clinical stages of the cancer. On the basis of these findings, we speculate that Nanog plays a role in the development of lung cancer. In particular, in the patients with small lesions that are suspected lung cancers, Nanog mRNA, which can be detected with high sensitivity, may be an ideal diagnostic marker to support diagnosis even when very few cells can be obtained.

References

1. Betticher DC: Adjuvant and neoadjuvant chemotherapy in NSCLC: a paradigm shift. Lung Cancer 50: S9-S16, 2005.
2. Mitsui K, Tokuzawa Y, Itoh H, *et al*: The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113: 631-642, 2003.
3. Chambers I, Colby D, Robertson M, *et al*: Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643-655, 2003.
4. Yasuda SY, Tsuneyoshi N, Sumi T, *et al*: NANOG maintains self-renewal of primate ES cells in the absence of a feeder layer. Genes Cells 11: 1115-1123, 2006.
5. Liu N, Feng X, Fang Z, Ma F, Lu S, Lu M and Han Z: Identification of genes regulated by nanog which is involved in ES cells pluripotency and early differentiation. J Cell Biochem 104: 2348-2362, 2008.
6. Okita K, Ichisaka T and Yamanaka S: Generation of germline-competent induced pluripotent stem cells. Nature 448: 313-317, 2007.
7. Yu J, Vodyanik MA, Smuga-Otto K, *et al*: Induced pluripotent stem cell lines derived from human somatic cells. Science 318: 1917-1920, 2007.
8. Ye F, Zhou C, Cheng Q, Shen J and Chen H: Stem-cell-abundant proteins Nanog, Nucleostemin and Musashi1 are highly expressed in malignant cervical epithelial cells. BMC Cancer 8: 108-112, 2008.
9. Tanabe H, Yagihashi A, Tsuji N, Shijubo Y, Abe S and Watanabe N: Expression of survivin mRNA and livin mRNA in non-small-cell lung cancer. Lung Cancer 46: 299-304, 2004.
10. Amachika T, Kobayashi D, Moriai R, Tsuji N and Watanabe N: Diagnostic relevance of overexpressed mRNA of novel oncogene with kinase-domain (NOK) in lung cancers. Lung Cancer 56: 337-340, 2007.
11. Bourguignon LY, Peyrollier K, Xia W and Gilad E: Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. J Biol Chem 283: 17635-17651, 2008.
12. Ezeh UI, Turek PJ, Reijo RA and Clark AT: Human embryonic stem cell genes OCT4, NANOG, STELLAR, and GDF3 are expressed in both seminoma and breast carcinoma. Cancer 104: 2255-2265, 2005.
13. Ben-Porath I, Thomson MW, Carey VJ, *et al*: An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40: 499-507, 2008.