Malaysian J Pathol 2017; 39(3) : 227 – 234

ORIGINAL ARTICLE

Immunohistochemical expression of NANOG in urothelial carcinoma of the bladder

Mohd Khairul Anuar MD AKHIR *BSc*, Huzlinda HUSSIN *MD, MPath*, Abhimanyu VEERAKUMARASIVAM *PhD*, Chan Soon CHOY* *PhD*, Maizaton Atmadini ABDULLAH *MPath, PhD* and Fauzah ABDUL GHANI *MBBS, MPath*

*Universiti Putra Malaysia, Department of Pathology, Serdang, Selangor, Malaysia, *Perdana University, Serdang, Selangor, Malaysia*

Abstract

Urothelial carcinoma is a common malignant neoplasm that has a poor prognosis and a high frequency of recurrence and metastasis. Constant disease surveillance with periodic and long term cystoscopy examination is necessary for management of the disease. However, the monitoring and therapy regimen is expensive, incurring a massive burden to patients and the government. Therefore, the development of specific biomarkers for urothelial carcinoma at an early stage and recurrence detection becomes a priority. Homeobox genes are a family of genes that are involved in tumourigenesis. They might be potential prognostic markers for urothelial carcinoma. The study investigated the expression pattern of NANOG which is one of a homeobox gene in different stages and grades of urothelial carcinoma. NANOG expressions were also correlated with patient demographic factors and clinicopathological parameters. The expression of NANOG in 100 formalin-fixed paraffin-embedded urothelial carcinoma tissues was determined by immunohistochemistry. Immunohistochemistry showed positive expression of NANOG in all specimens with detection in the cytoplasm, nuclei and the nuclear membrane of the cancer cells. The immunohistochemical expression of NANOG increased across stages and grades of the tumour. The expression of NANOG was not significantly associated with demographic factors; gender ($p = 0.376$), race ($p = 0.718$) and age ($p = 0.058$) as well as with most of the clinicopathological parameters; pathological stage ($p = 0.144$), grade ($p = 0.625$), lymph node involvement ($p = 0.174$) and distant metastasis ($p = 0.228$). However, NANOG expression showed significant correlation with tumour invasion ($p = 0.019$). We concluded that NANOG might be a potential biomarker for early diagnosis of urothelial carcinoma of the bladder.

Keywords: homeobox genes, NANOG, urothelial carcinoma, immunohistochemistry, histopathology

INTRODUCTION

Bladder cancer is the tenth leading cause of death worldwide. However, the incidence of this cancer varies worldwide and it was reported to be high in North America and Europe.[1] In Peninsular Malaysia, bladder cancer is the sixth most common cancer in males but is less common in females.[2] The most common type of bladder cancer is urothelial carcinoma which is a heterogeneous neoplasm that either presents as non-invasive or invasive urothelial carcinoma.[3,4] Several studies have showed that advanced pathological stage, grade, nodal involvement and urinary obstruction are prognostic factors for recurrence and survival of the disease.[3] About 70% of superficial urothelial carcinoma (Ta and T1) recurred after the first treatment and 10-20% progressed to invasive urothelial carcinoma.[5,6] Thus, frequent and long-term surveillance is needed for the management and treatment of the disease. This poses a massive economic burden to the patient as well as to the government, making it an expensive cancer to manage.

Therapeutic resistance and failure has often been reported in urothelial cancer. Studies showed that the presence of cancer stem-like cells (CSCs) are involved with the failure to halt the tumour proliferation in many cancer patients. This involved the lack of response towards conventional treatment including radiation,

Address for correspondence: Dr. Huzlinda Hussin, MD, MPath, Department of Pathology, Faculty of Medicine and Health Sciences, 43400 University Putra Malaysia Serdang, Selangor Darul Ehsan, Malaysia. Tel: +60389472781. Email: huzlinda@upm.edu.my

chemotherapy and hormonal therapies.[7-9] The failure leads to recurrence of the tumour and chemo-resistance which may pose a problem in management and treatment of the patient. A study by Wang and Guda[10] in 2013 suggested that embryonic stem cells (ESCs) and cancer cells shared essential regulatory networks.

NANOG is one of the master regulators for embryonic stem cells transcription regulatory network reported to be involved with many tumours. NANOG is one of the homeobox genes that act as a transcription factor involved in maintaining pluripotency and self-renewal of embryonic stem cells. Recent studies reported that NANOG was also involved in self-renewal and the tumourigenicity of cancer stem cells in a variety of human cancers.[11-14] The upregulation of NANOG was found to be associated with tumour metastasis and poor prognosis in various human malignancies including prostate cancer, lung adenocarcinoma, gliomas, rectal cancer, gastric cancer and oral squamous cell carcinoma.[15] In bladder cancer cases, it was reported that increased expression of NANOG was associated with an increase in pathological grade.[9]

In this study, we investigated the expression pattern of NANOG across the stages and grades of urothelial carcinoma to gain insight into the correlation of NANOG expression with clinicopathological parameters of urothelial carcinoma.

MATERIALS AND METHODS

Patients and tissue samples

Histological blocks of paraffin-embedded tissue of urothelial carcinoma cases between 2003 to March 2013 were selected from the Department of Pathology, Hospital Kuala Lumpur. The following inclusion and exclusion criteria were used to select the cases; only histologically proven urothelial carcinoma of the bladder with FFPE blocks having adequate tissues material were selected. Tis stage cases and samples with large areas of necrosis were excluded. Urothelial bladder carcinoma samples which fulfilled the inclusion and exclusion criteria were from biopsies and surgical resections (cystectomies, cystoprostatectomies and cystectomy plus TAHBSO). The cancers were staged using the TNM system. Haematoxylin and eosin (H&E) stained slides of all the cancers were reviewed independently by two pathologists to confirm the diagnosis and tumour grades and also to select the best blocks for immunohistochemical stains. In case of any discrepancy in grading, the pathologists reviewed the slides together to reach a consensus agreement. Patients' demographic data (age, gender, ethnic group) and clinicopathological parameters (tumour stage, lymph node involvement and metastasis) were obtained from patient records (Table 1).

Haematoxylin and eosin (H&E) staining

The original H&E slides were reviewed for all selected cases to choose the appropriate blocks that contained sufficient amount and satisfactory tumour tissue. H&E staining was performed on all selected blocks for further confirmation.

Immunohistochemical staining with NANOG antibody

Immunohistochemistry was performed to examine the expression of NANOG in the urothelial carcinoma tissues. Four-micrometre thick sections were cut from the selected paraffin blocks and fixed on poly-L-lysine glass slides (Thermo Scientific, U.S.A). The slides were deparaffinized by incubating at 60°C for 45 minutes in an oven VENTICELL (MMM Group, Germany) followed by soaking in two xylene solutions for 5 minutes. The slides were dehydrated by soaking into a sequence of 100%, 100%, 95%, 80% and 70% ethanol for 5 minutes. Then the slides were washed with running tap water for 5 minutes. Antigen retrieval was performed with citrate buffer (10 mM, pH 6) in high mode for 5 minutes until boiling, followed by defrosting mode for 10 minutes in the oven (ELBA, Republic of Korea). Slides were cooled at room temperature for about 35 minutes. A circle was drawn onto the glass slides surrounding the tissue sample using Pap pen (Daiko Sangyo, Japan) before washing the slides with TBS plus Tween 20 solutions (TBST-20) for five times, 2 minutes each. The slides were then blocked with 150 µl of 3% hydrogen peroxide for 30 minutes.

The slides were washed with TBS-T 20 solutions five times, 2 minutes each time. Hundred microlitres of primary antibody were added onto the slides. The following monoclonal antibodies were applied as primary antibodies; mouse monoclonal anti-Nanog antibody with 1:200 dilutions (Abcam [2C4], ab129045). The primary antibodies were incubated onto the slides for 4 hours at room temperature. The slides were then washed with TBST-20 solutions 5 times for 2 minutes before adding 4 drops of polymer (DAKO REAL™ EnVision™/HRP) onto the slides. The incubation of polymer was done for 30 minutes.

TABLE 1: Demographic and clinicopathological parameters of study cases

Description		No. of cases (%) n = 100
Gender		
	Male	92 (92)
	Female	8 (8)
Age (years)		
	< 50	15 (15)
	≥ 50	85 (85)
Ethnic group		
	Malay	59 (59)
	Chinese	31 (31)
	Indian	6 (6)
	Others	4 (4)
Depth of tumour extension		
	Ta	21 (21)
	T1	21 (21)
	T2	19 (19)
	T3	21 (21)
	T4	18 (18)
Stage		
	Stage 0	21 (21)
	Stage 1	21 (21)
	Stage 2	14 (14)
	Stage 3	13 (13)
	Stage 4	31 (31)
Invasiveness		
	NMIBC	42 (42)
	MIBC	58 (58)
Grade		
	G1	25 (25)
	G2	33 (33)
	G3	42 (42)
Lymph node metastasis		
	Yes	25 (25)
	No	75 (75)
Distant metastasis		
	Yes	23 (23)
	No	77 (77)

NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer

The slides were washed again with TBST-20 solutions 5 times for 2 minutes. Next, 100 µl of chromogen diaminobenzidine (DAB) [1 of Dako REAL™DAB : 50 chromogen in of Dako REAl™ Substrate Buffer] was applied onto the slides for 5 minutes to develop a brown colour. Then, the slides were rinsed with running tap water for 10 minutes. The slides were then counterstained with haematoxylin-Z (CellPath, UK) for 2 minutes and then rinsed again under running tap water for 10 minutes. Slides were dehydrated by soaking in a sequence of 70%, 80%, 95% and 100% ethanol solution for 3 minutes respectively. The slides were then soaked in xylene solution for 5 minutes twice and then were mounted with DPX.

Sections of normal testis tissue were used as positive control. Matched negative controls were sections stained without the primary antibody.

Scoring for immunohistochemical expression of NANOG

After the staining process was completed, the slides were examined under a light microscope (Olympus, UK) for scoring. Immunostaining was semi-quantitatively scored by two pathologists independently. Based on Luo *et al* 2013[15], the slides were scored according to percentage of tumour cells expressing positive staining and the intensity of staining (Table 2). The final score was obtained by multiplying the percentage positivity score and the staining intensity score. Final scores which were less than or equal to 4 (≤ 4) and more than or equal to (≥ 6) were interpreted as low and high NANOG protein expression respectively. Where there was a discrepancy in scoring by the two pathologists, the slides were reviewed together to reach a consensus agreement.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software package for Windows. The association between NANOG expression (final score) with demographic and clinicopathological parameters was analysed using chi-square test. A p-value <0.05 was considered statistically significant.

Ethics review

Ethics approval for this study was obtained from the Medical Research and Ethics Committee, Ministry of Health Malaysia on 7th of January 2013 (NMRR-12-970-13096).

RESULTS

Immunohistochemical expression and localisation of NANOG in urothelial carcinoma

All samples showed positive expression for NANOG with 15 (15%) showing low expression and 85 (85%) showing high expression. In 53 (53%) samples, NANOG expression was detected in both nucleus and cytoplasm of the cancer cells. 36 (36%) samples showed cytoplasmic localization (Fig.1), 6 (6%) samples showed expression in the nuclear membrane and 5 (5%) samples showed only nuclear expression.

Immunohistochemical expression of NANOG across different stages and grades of urothelial carcinoma of the bladder

There was an incremental increase in the percentage of cancer cells with high expression of NANOG as the cancer progressed from stage 0 to stage 3; thereafter, there was a slight decrease in percentage of high expression tumours in stage 4. Similarly, NANOG showed an increase in high expression pattern as the grades increased. The percentage distribution of NANOG expression across the different stages and grades of urothelial carcinoma of the bladder is summarised in Fig. 2.

TABLE 2: Semi-quantitative scoring system for immunohistochemical expression of NANOG

Percentage of positivity	Score	Staining intensity	Score
No positive tumour cells	0	No staining	0
Less than 10% positive tumour cells	1	Weak staining	1
10% to 50% positive tumour cells	2	Moderate staining	2
More than 50% positive tumour cells	3	Strong staining	3

Final score = Percent positivity score X Staining intensity score
Score: 0 = negative
≤ 4 = low expression
≥ 6 = high expression

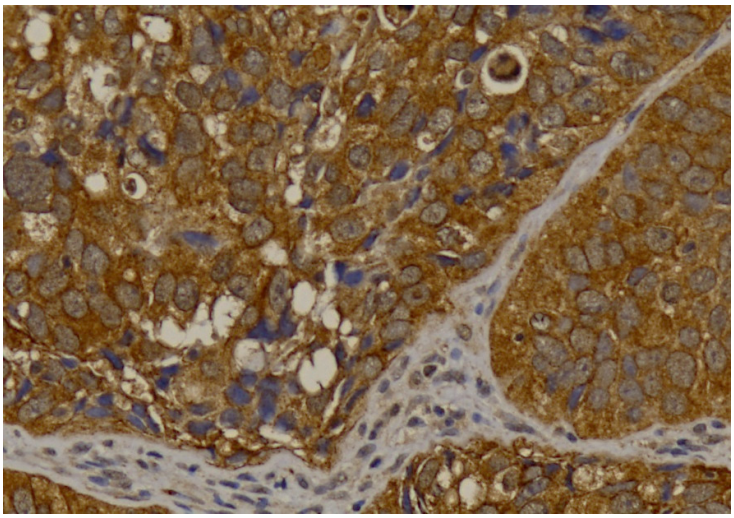

FIG. 1: Immunohistochemical staining of NANOG in urothelial carcinoma showing 2+ staining intensity and intracytoplasmic localisation (400X)

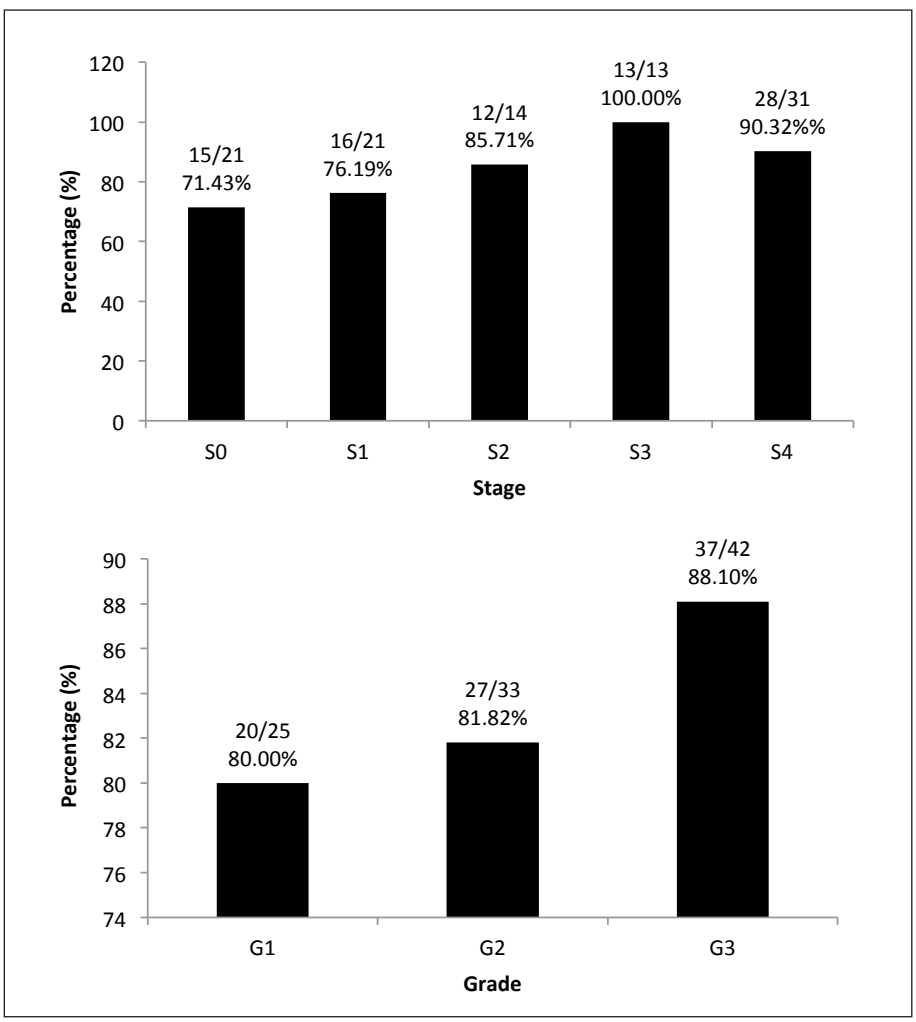

FIG. 2: Percentage of tumours with high expression of NANOG across the stages and grades of urothelial carcinoma

The association of NANOG immunohistochemical expression with demographic and clinicopathological parameters of urothelial carcinoma of the bladder

Statistical analysis did not show any significant association of NANOG expression with all the demographic parameters. A few clinicopathological parameters were analysed. One of them was the expression of NANOG in non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). The study showed a higher percentage of cancer cells with high expression of NANOG in MIBC (91.38%) compared to NMIBC (73.81%).

There was a higher percentage of cancer cells with high NANOG expression in the urothelial bladder carcinoma with lymph node metastasis (92%) compared with those without lymph node metastasis (81.3%). Additionally, a higher percentage of cancer cells with high NANOG expression was observed in urothelial cancers with distant metastasis (91.3%) compared with those without distant metastasis (81.8%).

Interestingly, when assessing the relationship between NANOG expression and clinicopathological parameters, there was a significant association between NANOG expression and tumour invasion ($p = 0.019$) (Table 3). However, there was no significant association between NANOG expression with other clinicopathological parameters.

DISCUSSION

Our study showed a predominance of males compared to females (11.5:1 male to female ratio), and a lower percentage of NMIBC cases compared to MIBC cases. These findings differed from the literature which showed 3:1 male to female ratio and a higher percentage of NMIBC (75%) than MIBC.[16] The reasons for our differing findings might be because our samples came from only a single institution (Hospital Kuala Lumpur) which might not be representative of the whole population. Furthermore, Hospital Kuala Lumpur is a referral government centre for further surgical interventions and further management of higher stage bladder cancers and that may explain why there were more MIBC cases compared to NMIBC. When compared to the studies done in two other institutions (Universiti Kebangsaan Malaysia Medical Centre) in 2010 and (King Abdulah University Hospital) 2008, the male to female patients ratio was 9.4:1 and 10:1 respectively,[17,18] which were quite similar to our study finding. Kong *et al* 2010[17] also reported a study done in Universiti Kebangsaan Malaysia Medical Centre, where 58.6% of bladder cancer cases were NMIBC which is slightly higher than MIBC.

The study also found that some of the superficial tumours were higher in tumour grade. The finding was concordant with a study by Kong *et al* 2012[17] which showed 32.5% of the superficial tumours with a higher grade.

The subcellular localisation of the proteins of genes in specific tissues determines their function in the cells. Our study found positive expression of NANOG in both nucleus and cytoplasm. Different findings were found in studies that were conducted in germline stem cells as well as in oral squamous cell carcinoma where positive immunostaining for NANOG was only found in the nucleus.[19] However, in nasopharyngeal carcinoma and non-small cell lung cancer, high cytoplasmic expression of NANOG was detected.[15,20] A study done by Gu *et al* 2012[21] showed that NANOG was expressed in the nucleus of human embryonic carcinoma cell lines while in human cervical cancer cell lines it was expressed in the cytoplasm. The authors suggested that localisation of NANOG depended on cell type and tumour stage. The cellular translocation of NANOG from the nucleus to cytoplasm could also relate to its molecular characteristics for example its molecular size.[19] Moreover, protein modification and its spatial structural changes also contribute to the translocation of NANOG in a cell.[21,22]

NANOG is one of the transcription regulators that are involved in inner cell mass and embryonic stem (ES) cell proliferation and self-renewal.[23] Overexpression of NANOG promotes cells to enter the S phase and proliferate. This suggests that the function and regulation of NANOG are essential for cancer stem cell renewal and tumourigenesis.[9] Zhang *et al*[9] reported that NANOG was expressed in both low and high-grade bladder cancers and higher grade tumours showed intense expression as compared to low grade and PUNLMP tissue. The previous studies supported the finding that NANOG expression was increased across the stages and grades of urothelial carcinoma.

Our study found a significant correlation between NANOG expression with tumour invasion in urothelial carcinoma of the bladder. A previous study also showed that NANOG played a role in the regulation of tumour metastasis in lung adenocarcinoma by enhancing the epithelial-mesenchymal transition (EMT)

TABLE 3: The association between NANOG immunohistochemical expression with demographic and clinicopathological parameters

Parameters	Expression			Total (%) n = 100	P value
	Negative n (%)	Low n (%)	High n (%)		
Gender					
Male	-	14 (15.2)	78 (84.8)	92 (100)	0.376
Female	-	2 (25.0)	6 (75.0)	8 (100)	
Age					
<50	-	0 (0)	15 (100)	15 (100)	0.058
≥50	-	16 (18.8)	69 (81.2)	85 (100)	
Race					
Malay	-	11 (18.6)	48 (81.4)	59 (100)	0.718
Chinese	-	3 (9.7)	28 (90.3)	31 (100)	
Indian	-	1 (20.0)	4 (80.0)	5 (100)	
Others	-	1 (20.0)	4 (80.0)	5 (100)	
Stage					
Stage 0	-	6 (28.6)	15 (71.4)	21 (100)	0.144
Stage 1	-	5 (23.8)	16 (76.2)	21 (100)	
Stage 2	-	2 (14.3)	12 (85.7)	14 (100)	
Stage 3	-	0 (0)	13 (100)	13 (100)	
Stage 4	-	3 (9.7)	28 (90.3)	31 (100)	
Tumor invasion					
NMIBC	-	11 (26.2)	31 (73.8)	42 (100)	0.019*
MIBC	-	5 (8.6)	53 (91.4)	58 (100)	
Grade					
G1	-	5 (20.0)	20 (80.0)	25 (100)	0.625
G2	-	6 (18.2)	27 (81.8)	33 (100)	
G3	-	5 (11.9)	37 (88.1)	42 (100)	
Lymph node metastasis					
Yes	-	2 (8.0)	23 (92.0)	25 (100)	0.174
No	-	14 (18.7)	61 (81.3)	75 (100)	
Distant metastasis					
Yes	-	2 (8.7)	21 (91.3)	23 (100)	
No	-	14 (18.2)	63 (81.8)	77 (100)	0.228

*Statistically significant ($p < 0.05$); NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer

process.[20] EMT is the process whereby epithelial cells undergo changes in cell morphology and motility into mesenchymal characteristics. The process is known to play an important role in tumour invasion or migration as well as metastasis.[24] However there was no significant association between NANOG expression with other clinicopathological parameters and the demographic data. NANOG expression might not be dependent on gender, age and ethnicity of the patients. There was no other previous study that correlated the demographic factors with immunohistochemical expression of NANOG. It also reflects the heterogeneity of the tumours, suggesting there is a significant variation in the type of genes an individual tumour expresses as compared to other tumours, even within the same subtype. This supports the model

of clonal evolution of tumours where a single tumour cell continuous to acquire mutations and dysregulation of expression throughout tumourigenesis.

Conclusion

A significant correlation between NANOG with the invasive potential of urothelial carcinoma suggests that it may play a role in the development of urothelial carcinoma. An increase in its expression throughout the stages and grades of urothelial carcinoma raises its potential as a biomarker for early diagnosis of urothelial carcinoma of the bladder and possibility as a target for therapy in the future. However, further studies such as addressing functional expression modulation need to be done to confirm the functional significance of the expression and prognostic role of NANOG in urothelial carcinoma.

ACKNOWLEDGEMENT

We would like to thank the Ministry of Health Malaysia and Hospital Kuala Lumpur for allowing collecting data and samples from the hospital. This project was funded by Research and University Graduate School (RUGS) grant. The authors declare no conflict of interest.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61: 69-90.
2. Omar ZA, Ali ZM, Ibrahim Tamin NS. Malaysian Cancer Statistics: Data and Figure Peninsular Malaysia 2006. Putrajaya: National Cancer Registry, Malaysia Ministry of Health Malaysia; 2006.
3. Eble JN, Sauter G, Esptein JI, Sesterhenn IA, editors. Pathology and genetics tumours of the urinary system and male genital organs. 2nd ed. 2004. Lyon: IARC Press; 2004. p. 92-123.
4. Lingren D, Liedberg F, Andersson A, *et al*. Molecular characterization of early-stage bladder carcinomas by expression profiles, *FGFR3* mutation status, and loss of 9q. Oncogene. 2006; 25: 2685.
5. de Braud F, Maffezzini M, Vitale V, *et al*. Bladder cancer. Crit Rev Oncol Hematol. 2002; 41: 89-106.
6. Zhao M, He XL, Teng XD. Understanding the molecular pathogenesis and prognostics of bladder cancer: an overview. Chinese J Cancer Res. 2016; 28: 92-8.
7. Kitamura H, Okudela K, Yazawa T, Sato H, Shimoyamada H. Cancer stem cells: implications in cancer biology and theraphy with special reference to lung cancer. Lung Cancer. 2009; 66: 275-81.
8. Yu Z, Pestell TG, Lisanti MP, Pestell RG. Cancer stem cells. Int J Biochem Cell Biol. 2012; 44: 2144-51.
9. Zhang Y, Wang Z, Yu J, *et al*. Cancer stem-like cells contribute to cisplatin resistance and progression in bladder cancer. Cancer Lett. 2012; 322: 70-7.
10. Wang X, Guda C. Computational analysis of transcriptional circuitries in human embryonic stem cells reveals multiple and independent networks. Biomed Res Int. 2014; 2014: 725780.
11. Boyer LA, Lee TI, Cole MF, *et al*. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122: 947-56.
12. Wang J, Rao S, Chu J, *et al*. A protein interaction network for pluripotency of embryonic stem cells. Nature. 2006; 444: 364-8.
13. Hu L, McArthur C, Jafte RB. Ovarian cancer stem-like side population cells are tumourigenic and chemoresistant. Br J Cancer. 2010; 102: 1276-83.
14. Wen J, Park JY, Chung HW, *et al*. Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. Pancreas. 2010; 39: 622-6.
15. Luo W, Li S, Peng B, Ye Y, Deng X, Yao K. Embryonic stem cell markers SOX2, OCT4 and Nanog expression and their correlations with epithelial-mesenchymal transition in nasopharyngeal carcinoma. PLoS ONE. 2013; 8: e56324.
16. Messing EM, Young TB, Hunt VB, *et al*. Comparison of bladder cancer outcome in men undergoing hematuria home screening versus those with standard clinical presentations. Urology. 1995; 45: 387-96.
17. Kong CH, Singam P, Hong GE, *et al*. Clinicopathological features of bladder tumours in a single institution in Malaysia. Asian Pac J Cancer Prev. 2010; 11: 149-52.
18. Matalka I, Bani-Hani K, Shotar A, Bani Hani O, Bani-Hani I. Transitional cell carcinoma of the urinary bladder: a clinicopathological study. Singapore Med J. 2008; 49: 790-4.
19. Chiou SH, Yu CC, Huang CY, *et al*. Positive correlation of Oct-4 and Nanog in oral cancer stem-like cells and high grade oral squamous cell carcinoma. Clin Cancer Res. 2008; 14: 4085-95.
20. Li XQ, Yang XL, Zhang G, *et al*. Nuclear β-catenin accumulation is associated with increased expression of Nanog protein and predicts poor prognosis of non-small cell lung cancer. J Transl Med. 2013; 11: 114.
21. Gu TT, Liu SY, Zheng PS. Cytoplasmic NANOG-positive stromal cells promote human cervical cancer progression. Am J Pathol. 2012; 181: 652-61.
22. Chang DF, Tsai, SC, Wang XC, Xia P, Senadheera, D, Lutzko C. Molecular characterization of the human NANOG protein. Stem Cells. 2009; 27: 812-82.
23. Pan G, Thompson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res. 2007; 17: 42-9.
24. Adam L, Zhong M, Choi W, *et al*. miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reserves resistance to epidermal growth factor receptor theraphy. Clin Cancer Res. 2009; 15: 5060-72.