EJGO European Journal of Gynaecological Oncology

Stromal cell-derived factor 1α induce epithelial mesenchymal transition of Hela cells

J.P. Zhang[1], L.L. Feng[2], H.F. Zhan[1], Y. Liu[1], X.C. Wang[3]

[1] Department of Gynecology, Jing'an District Centre Hospital of Shanghai; Huashan Hospital Fudan University Jing'An Branch, Shanghai
[2] Department of Obstetric and Gynecology, Taizhou Second Hospital, Taizhou
[3] Department of Anatomy, Medical College, Soochow University, Suzhou (China)

Summary

Epithelial mesenchymal transition endows cells with migratory and invasive properties.Two groups of Hela cells (invasive group and non-invasive group) were retrieved utilizing Transwell Permeable Supports by Stromal cell-derived factor 1α (SDF-1α). In both the invasive and non-invasive groups, the morphological changes were observed under an inverted microscope, the expressions of mRNA and protein of E-cadherin and Vimentin were analyzed by RT-PCR assay and Western blot, and the levels of c were detected using RT-PCR. The authors found that compared with the non-invasive group, Hela cells in invasive group showed the mesenchymal morphologic changes, decreased expression of E-cadherin mRNA, and protein ($p < 0.05$), increased expression of Vimentin mRNA, and protein ($p < 0.05$), up-regulated expression of Snail, Slug, and Twist mRNA ($p < 0.05$). SDF-1α was able to induce epithelial mesenchymal transition of Hela cells, and obtain the invasive features through up-regulation of Snail, Slug, and Twist.

Key words: Hela cell; Epithelial mesenchymal transition; E-cadherin; Vimentin; Snail; Twist.

Introduction

An estimated 14.1 million new cancer cases and 8.2 million cancer deaths occurred in 2012 worldwide [1]. There were an estimated 527,600 new cervical cancer cases and 265,700 deaths worldwide in 2012. It is the second most commonly diagnosed cancer and third leading cause of cancer death among females in less developed countries [1]. In spite of various existing screening programs and novel therapeutic strategies that have the mortality rates, the molecular mechanisms underlying the pathogenesis of uterine cervical cancer are only partially understood.

In the present authors' previous study [2], it was found that CXCR4 was expressed on the cytomembrane and in the cytoplasm of Hela cells. CXCR4 ligand, stromal cell-derived factor 1α (SDF-1α) induced directional invasion of Hela cell concentration-depended, which can be blocked by using CXCR4 mAb in vitro.

Epithelial mesenchymal transition (EMT) endows cells with migratory and invasive properties, a prerequisite for the establishment of tumour invasion and metastasis. However, the role EMT might play in the pathophysiology of Hela cell invasion is still unknown. Therefore, the authors examined five recognized markers for EMT in Hela cells before/after base membrane: E-cadherin, Vimentin, Snail, Slug, and Twist.

Materials and Methods

The human uterine cervical cancer cell line (Hela cells) were purchased and maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 100μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Medium changes and cell subculture were conducted every one or two days.

Transwell Permeable Supports, a 24-well cell-culture chambers with eight-mm pore, have inserts with 6.5-mm-diameter polycarbonate membranes. 0.1 ml Hela cells in log phase growth, in which the cells were adjusted to a concentration of 5×10^5 cells/ml in RPMI-1640 containing 10% FCS, were added on the upper chambers. Simultaneously, 0.6 ml completed culture medium containing 100 ng/ml synthetic human SDF-1α was placed in the lower chambers. The micropore polycarbonate membranes coated with extracellular matrix (ECM)/matrix proteins were isolated two chambers. The ECM layer occludes the membrane pores, blocking non-invasive cells from migrating through, but allowing the chemokine SDF-1α to penetrate through the membrane to activate the cells. Invasive cells migrate into and pass through the coated membrane of the lower side of the filter. After 24 hours of incubation at 37°C in 5% CO_2, the invasive cells on the underside of the membrane and the non-invasive cells on the top of the membrane were harvested aseptically. The cells collected from the upper chambers were non-migrated as non-invasive group, those collected from the lower chambers as invasive group. The cell invasion assay was performed in triplicate. Cells from the two groups were evaluated for mRNA and/or protein levels of E-Cadherin, Vimentin, Snail, Slug, and Twist.

Under an inverted microscope, the authors investigated and obtained images of the morphological changes between the two group cells.

Total proteins were extracted using RIPA extraction reagents

Revised manuscript accepted for publication May 4, 2016

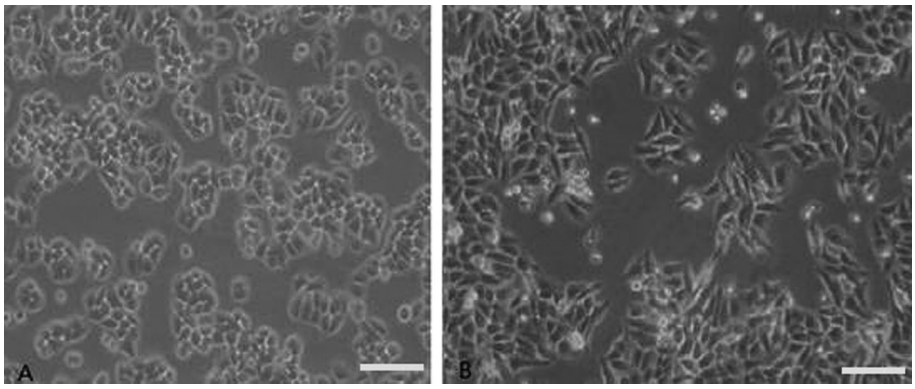

Figure 1. — A) Hela cells in the non-invasive group showing the classical epithelial morphology-polygon or cube, slabstone-like, tight ligation. B) Hela cells in invasive group showing the mesenchymal morphologic changes: shuttle or spindle and loose ligation.

kit. A total of 60 μg of protein was separated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking the membrane with 5% non-fat milk for two hours at room temperature, the membrane was incubated with rabbit anti-human E-cadherin antibody (dilution 1:1000, rabbit anti-human Vimentin antibody (dilution 1:1000, and mouse anti-human GAPDH antibody (dilution 1:8000) overnight at 4°C. The membrane was then incubated for one hour at 37°C with Horseradish peroxidase-conjugated (HRP) antibodies against mouse (dilution 1:1000) or against rabbit (dilution 1:1000) and washed extensively with TBS. The relative optical density (OD) value ratio was calculated by comparing it with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the National Institutes of Health software Image. Western blots were repeated three times for each sample.

RT-PCR was performed to determine the messenger RNA (mRNA) levels of E-cadherin, Vimentin, Snail, Slug, Twist, and GAPDH. Total RNA was extracted using Trizol reagent according to the manufacturer's protocol, and reverse transcribed to complementary DNA using the ReverTra Ace qPCR RT Kit. The real-time experiments were conducted on an iQ5 multicolor RT-PCR detection system using a SYBR Green Real-time PCR Master Mix. The PCR reactions consisted of 30 seconds at 95°C followed by 40 cycles of at 95°C for ten seconds, at 57°C for 30 seconds, and at 72°C for 30 seconds. The PCR primer sequences for E-cadherin, Vimentin, Snail, Slug, Twist, and GAPDH were designed and synthesized (Table 1). The comparative C (T) method was used to quantitate the expression of each target gene using GAPDH as an internal standard. Each measurement was performed in triplicate. Expression levels of E-cadherin, Vimentin, Snail, Slug, and Twist mRNA were evaluated using a relative quantification approach ($2^{-\Delta\Delta Ct}$ method) against GAPDH levels.

The results were presented as the mean ± standard error. The analysis of difference was performed with SPSS version 19.0 software. $P < 0.05$ was considered as significant difference.

Table 1. — *Primer sequences used for RT-PCR.*

Gene	Primer sequences (5'-3')
E-cadherin	5'-aagcctcaggtcataaacatc-3'
	5'-cgcctccttcttcatcatag-3'
Vimentin	5'-cgcttcgccaactacat-3'
	5'-agggcatccacttcacag-3'
Snail	5'-acctccctgtcagatgagga-3'
	5'-ggactcttggtgcttgtgga-3'
Slug	5'-catctttggggcgagtgagt-3'
	5'-acacagcagccagattcctc-3'
Twist1	5'-tgagcaacagcgaggaagag-3'
	5'-ctccatcctccagaccgaga-3'
Twist2	5'-cgaggaggagctcgagagg-3'
	5'-ctagtgggaggcggacatg-3'
GAPDH	5'-gaacgggaagctcactgg-3'
	5'-gcctgcttcaccaccttct-3'

Results

Under an inverted microscope, the non-invasive Hela cells showed the classical epithelial morphology-polygon or cube, slabstone-like, tight ligation (Figure 1A). Compared with the non-invasive group, Hela cells in invasive group showed the mesenchymal morphologic changes-shuttle or spindle, and loose ligation (Figure 1B).

Western blotting analysis showed that compared with the non-invasive group, Hela cells in invasive group showed decreased expression of epithelial marker protein E-cadherin, increased expression of mesenchymal marker protein Vimentin (Table 2).

Compared with the non-invasive group, Hela cells in invasive group showed decreased expression of E-cadherin mRNA, increased expression of Vimentin, Snail, Slug, and Twist mRNA ($p < 0.05$) (Figure 2).

Discussion

Invasiveness is a feature of malignant tumor cells, which reflects their dissemination ability from the primary site to distant organs to form metastases. EMT is a cell process by which epithelial cells converse into mesenchymal cells and obtain the capacity of cell motility. EMT process is required for tumor cell invasion and metastasis in many solid tumor types [3-5]. EMT can be induced by some of the cellular secreting factors, such as transforming growth factor beta1 (TGF-β1), bone morphogenetic protein 4 (BMP4) [6], vascular endothelial cell growth factor (VEGF) [7], inflammatory factors (e.g. IL-8) [8], hypoxia associated factors (e.g. HIF-1) [9], and cell junction proteins (e.g. Claudin-1) [10]. Stromal cell-derived factor 1 (SDF-1) and its receptor,

Table 2. — *E-cadherin and Vimentin protein expression between non-invasive group and invasive group using Western blot analysis ($\bar{x} \pm s$)*

Groups	OD	
	E-cadherin	Vimentin
non-invasive	105.06±0.185	42.14±0.92
invasive group	85.68±0.286*	65.46±1.71*

Note: *Comparison between non-invasive group and invasive group, $P<0.05$.

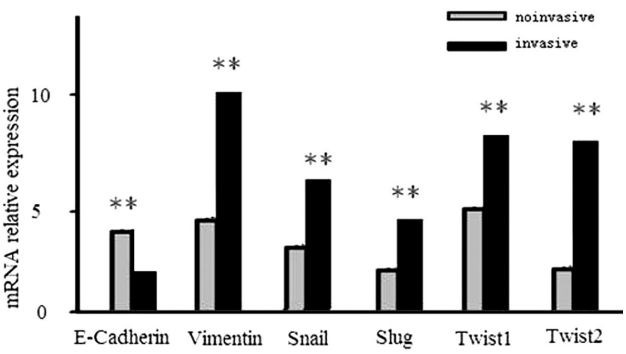

Figure 2. — Hela cells in invasive group showed decreased expression of E-cadherin mRNA, increased expression of Vimentin, Snail, Slug, and Twist mRNA (p <0.05).

CXCR4, play an important role in angiogenesis and are associated with tumor progression. In the authors' previous study [2] it was found that CXCR4 is expressed in Hela cells. By a Matrigel invasion model [2], the authors demonstrated a directional migration of Hela cells stimulated by chemokine SDF-1α with concentration-dependent mode and a maximum response at 100 ng/ml concentration. Moreover, the chemotactic response to SDF-1α was inhibited by co-incubating the cells with CXCR4 mAb [2]. In the present study, the authors used 100 ng/ml SDF-1± to induce Hela cells' migration by a Matrigel invasion model again, to investigate whether EMT occurred.

EMT is a complex process, which involves cytoskeletal remodeling and cell–cell and cell–matrix adhesion, leading to the transition from a polarized, epithelial phenotype to a highly motile mesenchymal phenotype [11] and become more migratory and invasive [12], losing their apical-basal polarity and extensive adhesions to neighboring cells and basement membranes. In the present study, the authors found the similar representation of Hela cells under an inverted microscope using the Matrigel invasion assay.

Epithelial cells undergoing EMT lose the expression of epithelial marker (E-cadherin and ZO-1) and acquire the expression of mesenchymal markers (N-cadherin, α-SMA, and vimentin) and EMT biomarker β-catenin [13]. Vimentin is an intermediate-sized filament that is highly expressed in mesenchymal cells and is commonly used to identify cancer cells undergoing EMT based on a positive correlation of Vimentin expression with increased invasiveness and metastasis [14]. In this study, the authors found compared with the non-invasive group, that Vimentin was upregulated and E-cadherin was downregulated in invasive group.

EMT can be triggered or mediated by several transcription factors including Snail, Twist1/2, Slug, ZEB1, ZEB2, and CtBP2 [15], which commonly function as oncogenes in a variety of cancers. In particular, it has been well documented that Snail and Slug (a closely related member of the Snail family) regulate several genes involved in cell adhesion and cell junctions [16]. Snail (SNAI1) and Slug (SNAI2) are master regulatory transcription factors for organogenesis and wound healing, and they are involved in EMT of cancer cells [17]. Snail is a zinc-finger transcription factor that belongs to a larger superfamily known as SNAI and participates in cell differentiation and survival [18]. Snail's main action mode is by inducing EMT by suppression of E-cadherin transcription, which is responsible for cell adhesion and migratory capabilities [19]. Slug is also a zinc-finger transcription factor that has activities similar to Snail, including E-cadherin transcriptional repression and anti-apoptotic activity, and it plays a crucial role in organogenesis and neuralization. Slug expression is increased in patients with melanoma, lung, colon, and ovarian cancers [20]. Snail and Slug are linked to tumor progression and invasiveness by their ability to alter E-cadherin and Vimentin gene expression [19, 21, 22]. Both Snail and Slug are direct repressors of E-cadherin and act by binding to the specific E-boxes of E-cadherin's proximal promoter [23]. When Snail was overexpressed in epidermoid cancer cells, E-cadherin expression was lost with a concomitant change in cell morphology to a fibroblastic phenotype and Vimentin gene expression was upregulated, which indicated that Snail induced an EMT [24, 25]. In the present study, the authors found the expression of Snail and Slug was up-regulated in invasive group.

Twist (also called Twist1), a highly conserved basic Helix-Loop-Helix transcription factor, is a critical transcriptional factor essential for neural tube formation, cell migration, and differentiation in embryonic development [26]. Twist has been found to induce EMT [27] and tumor metastasis in different cells and tumor tissues, including murine isogenic breast cancer [28], prostate cancer [29], hepatocellular carcinoma (HCC) [30], gastric cancer [31], oesophageal squamous cell carcinoma [32], and bladder cancer [33]. Twist exerts its effects on EMT through modulation of E-cadherin, Vimentin, miR10b, AKT2, and IL-8 [34]. Oncogenes such as RAS, ErbB2, and c-Myc can elicit EMT in mammary tumors by up-regulating Twist [35]. Twist plays an essential role in cancer metastasis through different signaling pathways, such as AKT signaling in breast cancer [36], WNT in cervical cancer [37], VEGF in

HCC [38], and p53 in gastric cancer cells [31]. Thus, induction and regulation of EMT by Twist may involve multiple molecular mechanisms in different cells and tumor tissues. Interestingly, the present results show that the expression of Twist1/2 are up-regulated in Hela cells in the presence of exogenous SDF-1α.

However, the mechanism governing the downstream regulation of SDF-1/CXCR4-mediated invasion remains unclear. Here the authors report the role of SDF-1/CXCR4 in Hela cells and the possible mechanism of SDF-1/CXCR4-mediated Hela cell invasion. The present authors show that there is a cross-talk between SDF-1/CXCR4 axis and EMT in Hela cell invasion. Taken together, SDF-1/CXCR4 axis may represent a promising therapeutic target to prevent uterine cervical cancer progression. SDF-1 could induce Hela cell EMT.

References

[1] Torre L.A., Bray F., Siegel R.L., Ferlay J., Lortet-Tieulent J., Jemal A.: "Global cancer statistics, 2012". *CA Cancer J. Clin.*, 2015, *65*, 87.

[2] Zhang J.P., Lu W.G., Ye F., Chen H.Z., Zhou C.Y., Xie X.: "Study on CXCR4/SDF-1alpha axis in lymph node metastasis of cervical squamous cell carcinoma". *Int. J. Gynecol. Cancer*, 2007, *17*, 478.

[3] Bronsert P., Enderle-Ammour K., Bader M., Timme S., Kuehs M., Csanadi A., *et al.*: "Cancer cell invasion and EMT marker expression: a three-dimensional study of the human cancer-host interface". *J. Pathol.*, 2014, *234*, 410.

[4] Kouso H., Yano T., Maruyama R., Shikada Y., Okamoto T., Haro A., *et al.*: "Differences in the expression of epithelial-mesenchymal transition related molecules between primary tumors and pulmonary metastatic tumors in colorectal cancer". *Surg. Today*, 2013, *43*, 73.

[5] Liu L., Dai Y., Chen J., Zeng T., Li Y., Chen L., *et al.*: "Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial-mesenchymal transition by way of Akt/GSK-3beta/Snail signaling". *Hepatology*, 2014, *59*, 531.

[6] Xu T., Yu C.Y., Sun J.J., Liu Y., Wang X.W., Pi L.M., *et al.*: "Bone morphogenetic protein-4-induced epithelial-mesenchymal transition and invasiveness through Smad1-mediated signal pathway in squamous cell carcinoma of the head and neck". *Arch. Med. Res.*, 2011, *42*, 128.

[7] Desai S., Laskar S., Pandey B.N.: "Autocrine IL-8 and VEGF mediate epithelial-mesenchymal transition and invasiveness via p38/JNK-ATF-2 signalling in A549 lung cancer cells". *Cell Signal.*, 2013, *25*, 1780.

[8] Bates R.C., DeLeo M.J. 3rd, Mercurio A.M.: "The epithelial-mesenchymal transition of colon carcinoma involves expression of IL-8 and CXCR-1-mediated chemotaxis". Exp. Cell. Res., 2004, *299*, 315.

[9] Yang M.H., Wu M.Z., Chiou S.H., Chen P.M., Chang S.Y., Liu C.J., *et al.*: "Direct regulation of TWIST by HIF-1alpha promotes metastasis". *Nat. Cell. Biol.*, 2008, *10*, 295.

[10] Stebbing J., Filipovic A., Giamas G.: "Claudin-1 as a promoter of EMT in hepatocellular carcinoma". *Oncogene*, 2013, *32*, 4871.

[11] Huber M.A., Kraut N., Beug H.: "Molecular requirements for epithelial-mesenchymal transition during tumor progression". *Curr. Opin. Cell. Biol.*, 2005, *17*, 548.

[12] Choi Y., Lee H.J., Jang M.H., Gwak J.M., Lee K.S., Kim E.J., *et al.*: "Epithelial-mesenchymal transition increases during the progression of in situ to invasive basal-like breast cancer". *Hum. Pathol.*, 2013, *44*, 2581.

[13] Zeisberg M., Neilson E.G.: "Biomarkers for epithelial-mesenchymal transitions". *J. Clin. Invest.*, 2009, *119*, 1429.

[14] Cao H., Xu E., Liu H., Wan L., Lai M.: "Epithelial-mesenchymal transition in colorectal cancer metastasis: a system review". *Pathol. Res. Pract.*, 2015, *211*, 557.

[15] Martin T.A., Goyal A., Watkins G., Jiang W.G.: "Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer". *Ann. Surg. Oncol.*, 2005, *12*, 488.

[16] Hajra K.M., Chen D.Y., Fearon E.R.: "The SLUG zinc-finger protein represses E-cadherin in breast cancer". *Cancer Res.*, 2002, *62*, 1613.

[17] Ganesan R., Mallets E., Gomez-Cambronero J.: "The transcription factors Slug (SNAI2) and Snail (SNAI1) regulate phospholipase D (PLD) promoter in opposite ways towards cancer cell invasion". *Mol. Oncol.*, 2016, *10*, 663

[18] Nieto M.A.: "The snail superfamily of zinc-finger transcription factors". *Nat. Rev. Mol. Cell. Biol.*, 2002, *3*, 155.

[19] Bolos V., Peinado H., Perez-Moreno M.A., Fraga M.F., Esteller M., Cano A.: "The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors". *J. Cell. Sci.*, 2003, *116*, 499.

[20] Elloul S., Elstrand M.B., Nesland J.M., Trope C.G., Kvalheim G., Goldberg I., *et al.*: "Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma". *Cancer*, 2005, *103*, 1631.

[21] Come C., Arnoux V., Bibeau F., Savagner P.: "Roles of the transcription factors snail and slug during mammary morphogenesis and breast carcinoma progression". *J. Mammary Gland Biol. Neoplasia*, 2004, *9*, 183.

[22] Lee M.Y., Chou C.Y., Tang M.J., Shen M.R.: "Epithelial-mesenchymal transition in cervical cancer: correlation with tumor progression, epidermal growth factor receptor overexpression, and snail up-regulation". *Clin. Cancer Res.*, 2008, *14*, 4743.

[23] Seki K., Fujimori T., Savagner P., Hata A., Aikawa T., Ogata N., *et al.*: "Mouse Snail family transcription repressors regulate chondrocyte, extracellular matrix, type II collagen, and aggrecan". *J. Biol. Chem.*, 2003, *278*, 41862.

[24] Kaufhold S., Bonavida B.: "Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention". *J. Exp. Clin. Cancer Res.*, 2014, *33*, 62.

[25] Yokoyama K., Kamata N., Fujimoto R., Tsutsumi S., Tomonari M., Taki M., *et al.*: "Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas". *Int. J. Oncol.*, 2003, *22*, 891.

[26] Perez-Pomares J.M., Munoz-Chapuli R.: "Epithelial-mesenchymal transitions: a mesodermal cell strategy for evolutive innovation in Metazoans". *Anat. Rec.*, 2002, *268*, 343.

[27] Yang J., Hou Y., Zhou M., Wen S., Zhou J., Xu L., *et al.*: "Twist induces epithelial-mesenchymal transition and cell motility in breast cancer via ITGB1-FAK/ILK signaling axis and its associated downstream network". *Int. J. Biochem. Cell. Biol.*, 2016, *71*, 62.

[28] Yang J., Mani S.A., Donaher J.L., Ramaswamy S., Itzykson R.A., Come C., et al.: "Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis". *Cell*, 2004, *117*, 927.

[29] Yuen H.F., Chua C.W., Chan Y.P., Wong Y.C., Wang X., Chan K.W.: "Significance of TWIST and E-cadherin expression in the metastatic progression of prostatic cancer". *Histopathology*, 2007, *50*, 648.

[30] Yang M.H., Chen C.L., Chau G.Y., Chiou S.H., Su C.W., Chou T.Y., *et al.*: "Comprehensive analysis of the independent effect of twist and snail in promoting metastasis of hepatocellular carcinoma". *Hepatology*, 2009, *50*, 1464.

[31] Feng M.Y., Wang K., Song H.T., Yu H.W., Qin Y., Shi Q.T., *et al.*: "Metastasis-induction and apoptosis-protection by TWIST in gastric cancer cells". *Clin. Exp. Metastasis*, 2009, *26*, 1013.

[32] Sasaki K., Natsugoe S., Ishigami S., Matsumoto M., Okumura H., Setoyama T., *et al.*: "Significance of Twist expression and its association with E-cadherin in esophageal squamous cell carcinoma". *J. Exp. Clin. Cancer Res.*, 2009, *28*, 158.

[33] Wallerand H., Robert G., Pasticier G., Ravaud A., Ballanger P., Reiter R.E., *et al.*: "The epithelial-mesenchymal transition-inducing

factor TWIST is an attractive target in advanced and/or metastatic bladder and prostate cancers". *Urol. Oncol.*, 2010, *28*, 473.

[34] Ma L., Teruya-Feldstein J., Weinberg R.A.: "Tumour invasion and metastasis initiated by microRNA-10b in breast cancer". *Nature*, 2007, *449*, 682.

[35] Liu M., Casimiro M.C., Wang C., Shirley L.A., Jiao X., Katiyar S., *et al.*: "p21CIP1 attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo". *Proc. Natl. Acad. Sci. U S A*, 2009, *106*, 19035.

[36] Zhang Y.Q., Wei X.L., Liang Y.K., Chen W.L., Zhang F., Bai J.W., *et al.*: "Over-Expressed Twist Associates with Markers of Epithelial Mesenchymal Transition and Predicts Poor Prognosis in Breast Cancers via ERK and Akt Activation. *PLoS One*, 2015, *10*, e0135851.

[37] Li J., Zhou B.P.: "Activation of beta-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters". *BMC Cancer*, 2011, *11*, 49.

[38] Niu R.F., Zhang L., Xi G.M., Wei X.Y., Yang Y., Shi Y.R., *et al.*: "Up-regulation of Twist induces angiogenesis and correlates with metastasis in hepatocellular carcinoma". *J. Exp. Clin. Cancer Res.*, 2007, *26*, 385.

Corresponding Author:
J.P. ZHANG, M.D.
Huashan Hospital Fudan University
Jing'An Branch
No 259 Xikang Road
Shanghai 200040 (China)
e-mail: g5615335@126.com