JBUON 2021; 26(6): 2475-2479
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

NTF4 promoted the malignant progression of NSCLC by negatively regulating FOXL1

Lin Mu[1], Hong Zhao[1], Yifeng Zhang[1], Chun Xiao[2]

[1]Department of Pulmonary and Critical Care Medicine, Heping Hospital Affiliated to Changzhi Medical College, Changzhi, China.
[2]Department of General Surgery, PLA Rocket Force Characteristic Medical Center, Beijing, China.

Summary

Purpose: *To uncover the biological function of NTF4 in affecting the progression of non-small-cell lung cancer (NSCLC).*

Methods: *NTF4 levels in NSCLC and paracancer tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Knockdown of NTF4 in A549 and H1299 cells was achieved by transfection of sh-NTF4. Subsequently, proliferative and migratory changes in NSCLC cells with NTF4 knockdown were determined by cell counting kit-8 (CCK-8), Transwell and wound healing assay. The target gene binding NTF4 was predicted by bioinformatic software and verified by dual-luciferase reporter assay. The role of NTF4/FOXL1 axis in mediating NSCLC cell behaviors was finally explored through rescue experiments.*

Results: *NTF4 was highly expressed in NSCLC tissues than in normal ones. Knockdown of NTF4 remarkably reduced proliferative and migratory rates in A549 and H1299 cells. FOXL1 was confirmed as a target gene of NTF4 by bioinformatic software and verified by dual-luciferase reporter assay. Knockdown of FOXL1 was able to reverse the reduced proliferative and migratory rates in A549 and H1299 cells transfected with sh-NTF4.*

Conclusions: *NTF4 triggers NSCLC to proliferate and migrate via negatively regulating FOXL1.*

Key words: *NTF4, FOXL1, NSCLC*

Introduction

Lung carcinoma is the malignant tumor with the highest incidence in the world. In 2018, lung carcinoma patients accounted for 20.27% and 12.59% of all male and female cancer cases, respectively [1,2]. NSCLC is the main subtype of lung carcinoma, including squamous cell carcinoma and adenocarcinoma [3,4]. The 5-year survival of non-small-cell lung cancer (NSCLC) is less than 15% even in patients who have active treatment [5,6]. At present, diagnostic and therapeutic targets for NSCLC are lacking, and great efforts should be made on seeking for effective biomarkers [7,8].

NTF4 is a newly discovered pathogenic gene [9,10]. NTF4 protein can activate the tyrosine kinase-B receptor (TrkB) and prevents ocular hypertension, ischemia and release of cytotoxins [11]. In addition, NTF4 variation impairs the TrkB signaling, thus affecting the growth of tumor cells [11,12]. Therefore, it is believed that NTF4 variation is of significance in tumor process. Literature shows NTF4 is able to aggravate the malignant progression of colorectal carcinoma [9,13].

With the rapid development of molecular biology, the complicated network involving NTF4 and its downstream target is conductive to provide novel strategies for clinical treatment of NSCLC. This study first detected differential level of NTF4 in NSCLC samples, and later explored its biological functions in mediating malignant phenotypes of NSCLC and the underlying molecular mechanism.

Corresponding author: Chun Xiao, MM. Department of General Surgery, PLA Rocket Force Characteristic Medical Center, No. 16 Xinwai Street, Xicheng District, Beijing 100088, China.
Tel: +86 018513533662; Email: helloxiaochun@live.cn
Received: 12/05/2021; Accepted: 29/06/2021

Methods

NSCLC samples

19 pairs of NSCLC and adjacent normal tissues were collected from surgery, puncture biopsy or bronchoscopic biopsy. Adjacent tissues were at least 5 cm away from tumor lesions. None of recruited patients had preoperative chemotherapy or radiotherapy. This study was approved by the research Ethics Committee of our hospital and complied with the Helsinki Declaration. Signed informed consent was obtained from all patients.

Cell lines and reagents

NSCLC cell lines A549, H1299, PC-9, H358 and SPC-A1 and the human bronchial epithelial cell line BEAS-2B were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 (Gibco, Rockville, MD, USA).

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-60% density in 6-well plates, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48-h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in 96-well plates with 2×10^3 cells/well. At 24, 48, 72 and 96 h, optical density (OD) at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell assay

Cell suspension was prepared at 5×10^5 cells/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS were respectively added on the top and bottom of a Transwell insert, and cultured for 48 h. Cells were migrated from the top to the bottom, which was induced with methanol for 15 min, 0.2% crystal violet for 20 min and captured using a microscope. Five random fields per sample were selected for capturing and counting cells.

Wound healing assay

Cell suspension in serum-free medium was prepared at 5×10^5/mL, and implanted in 6-well plates. Cells were cultured to 90% confluence, followed by creating an artificial scratch using a sterilized pipette tip. Cells were washed in phosphate buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 h later, wound closure percentage was calculated.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary DNAs (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Japan), followed by qRT-PCR using StepOne Plus Real-time PCR (Applied Biosystems, CA, USA). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. NTF4: Forward: 5'-AGATGTCAGGAAGGAGGGGG-3', Reverse: 5'-CATCTCTCGGAGCACCTGTC-3'; FOXL1: Forward: 5'-TTCAACGCTTCCCTGATGCT-3', Reverse: 5'-GAACCGTGCCATTGTTTGCT-3'; GAPDH: Forward: 5'-GGAGCGAGATCCCTCCAAAAT -3', Reverse: 5'- GGCTGTTGTCATACTTCTCATGG -3'.

Western blot

Cells were lysed with radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) on ice for 15 min, and the mixture was centrifuged at 14000×g, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses using alpha SP.

Dual-luciferase reporter assay

Cells were seeded in 24-well plates, which were co-transfected with pcDNA-FOXL1/pcDNA-NC and

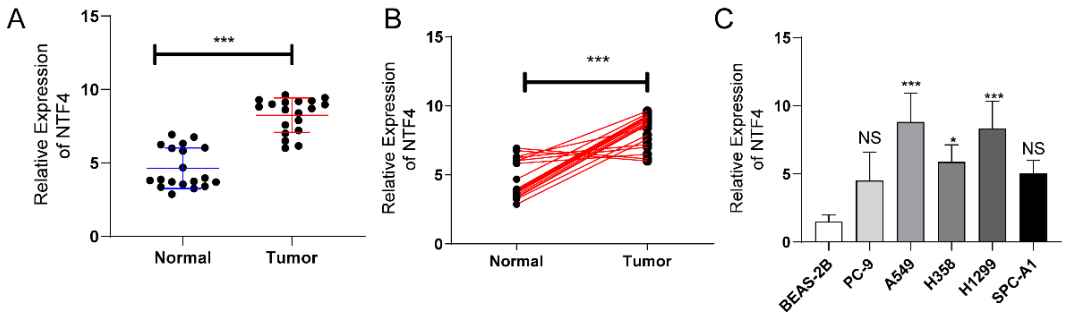

Figure 1. NTF4 was highly expressed in NSCLC. **A** and **B:** Differential levels of NTF4 in NSCLC and adjacent normal tissues. **C:** NTF4 levels in NSCLC cell lines; *p<0.05, ***p<0.001, NS: no significance.

pmirGLO-NTF4-WT/pmirGLO-NTF4-MUT, respectively. Luciferase activity (Promega, Madison, WI, USA) was measured at 48 h in a standard method.

Statistics

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the t-test. Potential influences of NTF4 and FOXL1 on NSCLC pathology were analyzed by Chi-square test. Kaplan-Meier survival curves were created, followed by log-rank test for comparing differences between curves. Pearson correlation test was conducted to assess the relationship between expressions of NTF4 and FOXL1 in NSCLC tissues. P<0.05 was considered as statistically significant.

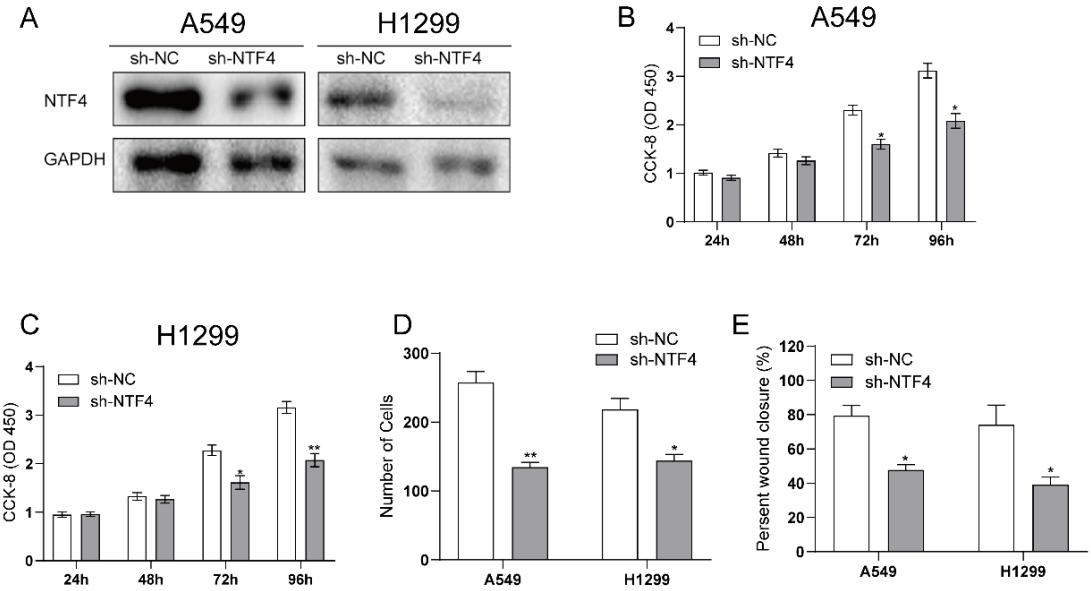

Figure 2. Knockdown of NTF4 reduced proliferative and migratory rates in NSCLC. **A:** Transfection efficacy of sh-NTF4 in A549 and H1299 cells. **B** and **C:** Viability in A549 and H1299 cells regulated by NTF4. **D:** Migration in A549 and H1299 cells regulated by NTF4. **E:** Wound closure in A549 and H1299 cells regulated by NTF4. *p<0.05, **p<0.01.

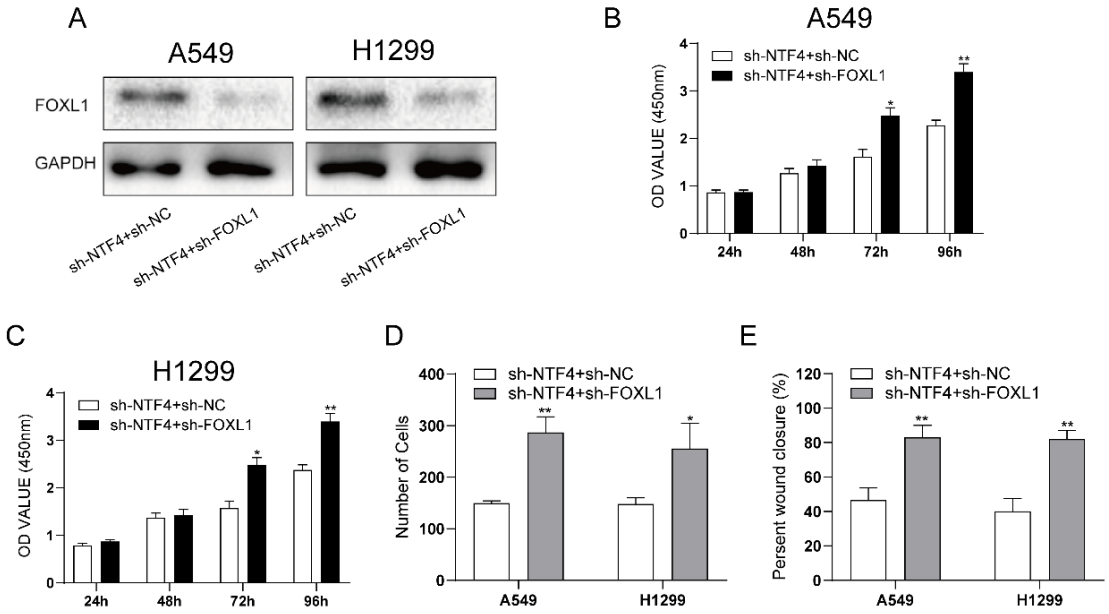

Figure 3. NTF4 regulated NSCLC cell phenotypes by negatively regulating FOXL1. **A:** Transfection efficacy of sh-FOXL1 in A549 and H1299 cells. **B:** Viability in A549 and H1299 cells co-regulated by NTF4 and FOXL1. **C:** Migration in A549 and H1299 cells co-regulated by NTF4 and FOXL1. **D:** Wound closure in A549 and H1299 cells co-regulated by NTF4 and FOXL1. *p<0.05, **p<0.01.

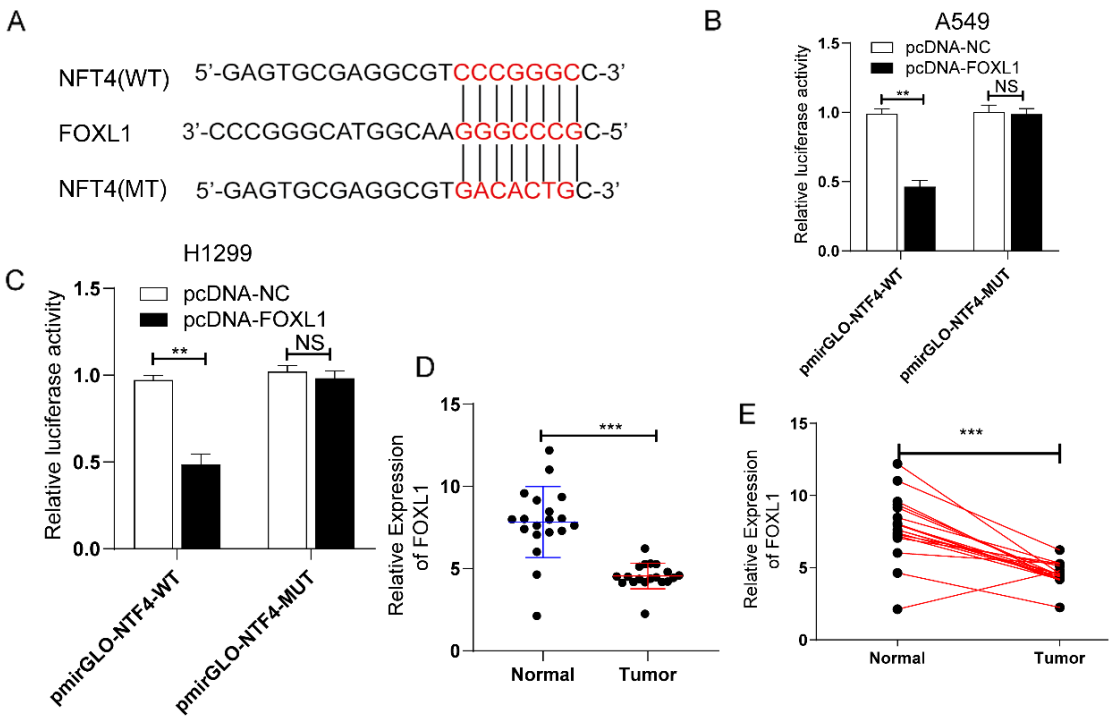

Figure 3. NTF4 targeted the regulation of FOXL1. **A:** FOXL1 was predicted to be a target gene of NTF4 by bioinformatic. **B** and **C:** FOXL1 was confirmed to be a target gene of NTF4 by dual-luciferase reporter assay. **D** and **E:** Differential levels of FOXL1 in NSCLC and adjacent normal tissues; **p<0.01, ***p<0.001.

Results

NTF4 was highly expressed in NSCLC

Differential levels of NTF4 were detected in 19 pairs of NSCLC and adjacent normal tissues. As qRT-PCR data revealed, NTF4 was upregulated in NSCLC tissues (Figure 1A and 1B). Besides, NTF4 was highly expressed in NSCLC cell lines (Figure 1C).

Knockdown of NTF4 reduced proliferative and migratory rates in NSCLC

Transfection efficacy of sh-NTF4 was examined in A549 and H1299 cells by Western blot (Figure 2A). CCK-8 assay revealed a reduction in cell viability after transfection of sh-NTF4 in A549 and H1299 cells, suggesting the inhibited proliferative potential (Figure 2B and 2C). In addition, declined migratory cell number and wound closure percentage owing to knockdown of NTF4 in NSCLC cells indicated that NTF4 stimulated migratory ability (Figure 2D, 2E).

NTF4 targeted the regulation of FOXL1

A potential interaction between NTF4 and FOXL1 was predicted using bioinformatic tools (Figure 3A). Subsequently, overexpression of FOXL1 was detected to reduce luciferase activity in pmirGLO-NTF4-WT, while it had no impact on luciferase activity in pmirGLO-NTF4-MUT (Figure 3B and 3C). It is suggested that NTF4 could be targeted by FOXL1 through the predicted binding site. Compared with normal tissues, FOXL1 was lowly expressed in NSCLC tissues (Figure 3D and 3E).

NTF4 regulated NSCLC cell phenotypes by negatively regulating FOXL1

A series of rescue experiments were conducted to analyze the involvement of FOXL1 in NTF4-regulated NSCLC cell phenotypes. Transfection efficacy of sh-FOXL1 was examined in A549 and H1299 cells with NTF4 knockdown (Figure 4A). Compared with NSCLC cells with NTF4 knockdown, those with co-knockdown of NTF4 and FOXL1 had higher viability, migratory cell number and wound closure percentage (Figure 4B-4E). It is concluded that the inhibited proliferative and migratory rates in NSCLC cells with NTF4 knockdown were reversed by co-knockdown of FOXL1.

Discussion

The incidence and mortality of NSCLC are both in the first place of malignant tumors [1-3]. Lung squamous cell carcinoma is the major histological subtype of NSCLC. In recent years, the incidence of lung adenocarcinoma has annually increased [4,5]. A series of novel medical technologies have improved the therapeutic efficiency of NSCLC to a

certain degree. Nevertheless, the 5-year survival of NSCLC remains far away from satisfying [6]. Invasiveness and metastasis are the leading risk factors for the poor prognosis of NSCLC [7,8]. It is of significance to deeply clarify the molecular mechanisms underlying NSCLC progression.

Effective biomarkers for diagnosis of NSCLC and prediction of cancer metastasis are lacking [14,15]. Here, differential level of NTF4 was identified, which was upregulated in NSCLC tissues than in adjacent normal ones. We believe that NTF4 could be an oncogene involved in NSCLC.

Tumorigenesis and tumor progression involve biological characteristic changes, including persistent proliferation, apoptosis blockage, strengthened invasiveness, etc [16,17]. Differentially expressed NTF4 was previously reported in many types of tumors [9,11-13]. Our results demonstrated that knockdown of NTF4 markedly attenuated the proliferative and migratory abilities in A549 and H1299 cells.

Bioinformatic prediction suggested an interaction between FOXL1 and NTF4. Later, we confirmed their binding relationship through dual-luciferase reporter assay. FOXL1 is a transcription factor that is expressed in gastrointestinal mesenchymal cells. It is responsible for regulating tumor cell phenotypes *via* activating the Wnt signaling through upregulating LRP5 [18,19]. In the present study, FOXL1 was lowly expressed in NSCLC tissues. Notably, knockdown of FOXL1 could abolish the regulatory effects of NTF4 knockdown on malignant phenotypes of NSCLC. To sum up, NTF4 is an oncogene that triggered NSCLC to proliferate and migrate through negatively regulating FOXL1.

Conclusions

NTF4 triggers NSCLC to proliferate and migrate *via* negatively regulating FOXL1.

Conflict of interests

The authors declare no conflict of interests.

References

1. Mao Y, Yang D, He J, Krasna MJ. Epidemiology of Lung Cancer. Surg Oncol Clin N Am 2016;25:439-45.
2. Usman AM, Miller J, Peirson L et al. Screening for lung cancer: A systematic review and meta-analysis. Prev Med 2016;89:301-14.
3. Fitzgerald K, Simone CN. Combining Immunotherapy with Radiation Therapy in Non-Small Cell Lung Cancer. Thorac Surg Clin 2020;30:221-39.
4. Broderick SR. Adjuvant and Neoadjuvant Immunotherapy in Non-small Cell Lung Cancer. Thorac Surg Clin 2020;30:215-20.
5. Bagcchi S. Lung cancer survival only increases by a small amount despite recent treatment advances. Lancet Respir Med 2017;5:169.
6. Deng HY, Zha P, Hou L, Huang KL. Does sarcopenia have any impact on survival of patients with surgically treated non-small-cell lung cancer? Interact Cardiovasc Thorac Surg 2019;29:144-7.
7. Balkan E, Bilici M, Gundogdu B et al. ERCC2 Lys-751Gln rs13181 and XRCC2 Arg188His rs3218536 Gene Polymorphisms Contribute to Subsceptibility of Colon, Gastric, HCC, Lung And Prostate Cancer. JBUON 2020;25:574-81.
8. Puri S, Saltos A, Perez B, Le X, Gray JE. Locally Advanced, Unresectable Non-Small Cell Lung Cancer. Curr Oncol Rep 2020;22:31.
9. Yang Z, Chen Y, Wei X, Wu D, Min Z, Quan Y. Upregulated NTF4 in colorectal cancer promotes tumor development via regulating autophagy. Int J Oncol 2020;56:1442-54.
10. Huang C, Xie L, Wu Z et al. Detection of mutations in MYOC, OPTN, NTF4, WDR36 and CYP1B1 in Chinese juvenile onset open-angle glaucoma using exome sequencing. Sci Rep 2018;8:4498.
11. Huang T, Krimm RF. Developmental expression of Bdnf, Ntf4/5, and TrkB in the mouse peripheral taste system. Dev Dyn 2010;239:2637-46.
12. Vithana EN, Nongpiur ME, Venkataraman D, Chan SH, Mavinahalli J, Aung T. Identification of a novel mutation in the NTF4 gene that causes primary open-angle glaucoma in a Chinese population. Mol Vis 2010;16:1640-5.
13. Patel AV, Krimm RF. Neurotrophin-4 regulates the survival of gustatory neurons earlier in development using a different mechanism than brain-derived neurotrophic factor. Dev Biol 2012;365:50-60.
14. Ye Y, Luo Z, Shi D. Use of cell free DNA as a prognostic biomarker in non-small cell lung cancer patients with bone metastasis. Int J Biol Markers 2019;34:381-8.
15. Ren S, Ren XD, Guo LF et al. Urine cell-free DNA as a promising biomarker for early detection of non-small cell lung cancer. J Clin Lab Anal 2020;34:e23321.
16. Zeeshan R, Mutahir Z. Cancer metastasis - tricks of the trade. Bosn J Basic Med Sci 2017;17:172-82.
17. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med 2013;19:1423-37.
18. Kaestner KH. The Intestinal Stem Cell Niche: A Central Role for Foxl1-Expressing Subepithelial Telocytes. Cell Mol Gastroenterol Hepatol 2019;8:111-7.
19. Katoh M, Katoh M. Human FOX gene family (Review). Int J Oncol 2004;25:1495-500.