

# Ulinastatin Inhibits Nasopharyngeal Carcinoma Metastasis by Suppressing uPA/uPAR Signaling

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**Research**

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# Abstract

**Background:** Distant metastasis is the main reason for treatment failure in patients with nasopharyngeal carcinoma (NPC). In this study, we evaluated the effect of ulinastatin (UTI) on NPC metastasis and investigated its underlying mechanism.

**Methods:** Highly-metastatic NPC cell lines S18 and 5-8F were treated with UTI and the effect on cellular growth, migration, and invasion of NPC cells was determined using MTS and transwell assays. The luciferase-expressing S18 cells (S18-1C3) were injected into the left hind footpad of nude mice to establish a spontaneous footpad to popliteal lymph node (LN) metastasis model. The luciferase mRNA was measured by qPCR to calculate the metastatic inhibition rate. The UTI-related uPA, uPAR and the main molecular members of the JAT/STAT3 pathway were detected by qPCR and immunoblotting.

**Results:** UTI inhibited the migration and invasion of S18 and 5-8F cells with no effect on cellular growth *in vitro*, and suppressed metastasis of S18 cells *in vivo*. uPAR expression was reduced from 24 to 48 hours after UTI treatment. The anti-metastasis effect of UTI partially relies on the suppression of uPA and uPAR.

**Conclusion:** UTI partially inhibits NPC metastasis via downregulating the expression of uPA and uPAR.

## 1. Background

Nasopharyngeal carcinoma (NPC) is a rare malignancy worldwide; however, it is highly prevalent in southern China and Southeast Asia [1, 2]. Although a large number of NPC patients benefit from radiotherapy and chemoradiotherapy [3], the 5-year survival rate of locoregionally advanced NPC patients is unsatisfying [4]. Current therapies have limited efficacy in inhibiting NPC metastasis [5]. Thus, identifying potential drug agents capable of preventing and/or inhibiting NPC metastasis is critical if we are to improve the treatment outcomes.

Plasminogen activators (PAs) are serine proteases that catalyze the conversion of plasminogen into plasmin. Plasmin is a kind of serine protease that degrades a variety of proteins, including fibrin, fibronectin, laminin, and other components of the extracellular matrix (ECM) and basement membrane [6, 7]. Tissue-type tPA and urokinase-type uPA are two kinds of mammalian PAs. tPA is mainly found in the circulatory system, while uPA is present in cells and involved in tissue remodeling events which are closely related to tumor cell invasion and metastasis [8, 9]. uPA expression is elevated in many malignant tumors, such as breast cancer [10], melanoma [11], prostate cancer [12], colon cancer [13], stomach cancer [14] and lung cancer [15], and its mediated-plasminogen activation is said to be dependent on the uPAR receptor in cells [7, 16]. In a previous study we demonstrated that uPAR plays a crucial role in regulating NPC cell growth, motility, and metastasis [17].

Ulinastatin (UTI), a urinary trypsin inhibitor, is a physiological serine protease inhibitor found in human urine and serum, which can inhibit a series of proteases [18]. UTI has been widely used in the treatment of

severe inflammatory diseases, including pancreatitis, shock, and disseminated intravascular coagulation [19]. In mice, it has been reported that UTI can inhibit metastasis in 3LL cells and the invasion of ovarian cancer by inhibiting uPA and uPAR [20, 21]. However, the role of UTI in blocking NPC metastasis is unknown. We therefore conducted this study to evaluate the effect of UTI on NPC cells in order to unveil its underlying molecular mechanism.

## **2. Materials And Methods**

### **2.1 Major reagents and apparatus**

UTI and placebo (phosphoric acid buffer solution containing sodium chloride and mannitol) were generous gifts from Techpool Bio-Pharma (Guangzhou, China). The RT-PCR kit (AQ201-01) was purchased from TransGen Biotech (China). P38 (14064-1-AP), ERK1/2 (16443-1-AP), JAK1 (66466-1-Ig), JAK2 (17670-1-AP), and Stat3 (10253-2-AP) were purchased from Proteintech. Phospho-p38 MAPK (CST,4511) and Phospho-p44/42 MAPK (Erk1/2) (CST, 4370) were purchased from CST. JAK1 (Phospho-Tyr1022) (AB11149-4), JAK2 (Phospho-Tyr1007) (AB11151-4), and STAT3 (Phospho-Tyr705) (AB11045-4) were purchased from Abcitech. UPA (AP30202b) and UPAR (AP8156c) were purchased from ABGENT. The Jak-Stat pathway inhibitor Ruxolitinib (INCB018424) (S1378) and S3I-201 (S1155) were purchased from Selleck Chemicals. ChemiDoc Gel Imaging System was purchased from Bio-Rad Company (CA, USA), and the inverted CX41 fluorescent microscope was purchased from Olympus (Tokyo, Japan).

### **2.2 Cell cultures**

Human NPC cell lines S18, S26, 5-8F, and SUNE-1 were from our laboratory [22, 23]. S18-1C3, a subclone of S18 was generated by permanent transfection with a GFP-luciferase reporter, which was provided by Exploring Health LLC. S26 and S18 were isolated from their parental line CNE-2 [24], and 5-8F from its parental line SUNE-1 [25]. The human NPC cell lines S18, S26, 5-8F, SUNE-1, and the S18-1C3 with luciferase were cultured in DMEM (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco) with 0.1 mg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### **2.3 Grouping and drug administration**

#### **2.3.1 Cellular experiment**

This experiment comprised of three groups: 1) a control group treated with physiological saline only; 2) a UTI medium dose group treated with UTI at a concentration of 800 U/mL (S18) or 3200 U/ml (5-8F); and 3) a UTI high dose group treated with UTI at a concentration of 1600 U/mL (S18) or 6400 U/ml (5-8F). All drugs were freshly prepared 4h before administration.

#### **2.3.2 Animal experiment**

A total of 50 female BALB/c nu/nu mice aged 4–5 weeks old were purchased from Guangdong Medical Animal Center (Production License No. SCXK [Yue] 2013-0002). FVB/N-Tg (MMTV-PyMT) 634Mul/J mice

were purchased from the Jackson Laboratory (Stock No: 002374, <https://www.jax.org/strain/002374>). Nude mice and MMTV-PyMT mice were kept in a specific pathogen free environment at 22–25°C with 50–65% humidity. Drinking water, food, and experimental materials were sterilized, and the rule of aseptic operation strictly followed. This study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University Cancer Center.

The spontaneous footpad to popliteal lymph node (LN) metastasis experiments were conducted as previously reported [24]. Briefly,  $1 \times 10^5$  cells in 20  $\mu$ l DMEM were subcutaneously injected into the footpad of the left hind limb of each mouse to generate a primary tumor. After 11 days, the mice were randomly divided into two groups for subsequent UTI treatment through intraperitoneal injections as follows: 1) The UTI group (n = 17) was injected with UTI at 1600 U/day/mouse for 38 consecutive days; 2) the control group (n = 16) was injected with an equal volume of placebo for 38 consecutive days. The animals were euthanized for sample collection 38 days after administration. At the termination of the experiment, the mice were injected with 150 mg/kg of D-Luciferin Potassium Salt (Mkbio, MX4603-100MG) before isoflurane anesthesia. The radiance was measured after substrate injection using the ChemiDoc Gel Imaging System (Bio-Rad, USA) and the popliteal LNs of the left hind feet were isolated. LNs were homogenized in TRIzol for total RNA extraction. Reverse transcription and real-time qPCR were performed to assess metastasis using specific primers for luciferase, which was only expressed in human nasopharyngeal carcinoma cells.

For the xenograft tumor experiments, the S18 cell lines were harvested and washed with PBS, then resuspended in serum-free DMEM medium. The cell concentration was adjusted to  $1 \times 10^7$  cells/mL. Cells were inoculated subcutaneously into the right armpits of 16 nude mice at 0.2 mL/mouse. Seven days after inoculation, the animals were randomly divided into two groups for subsequent intraperitoneal injections as follows: 1) The UTI group (n = 9) was injected with UTI at 1600 U/day/mouse for 17 consecutive days; and 2) the control group (n = 7) was injected with an equal volume of placebo for 17 consecutive days.

The development of breast cancer was spontaneous in the MMTV-PyMT mice. The nine week old MMTV-PyMT mice with breast cancer were randomly divided into two groups for subsequent intraperitoneal injections as follows: 1) The UTI group (n = 6) was injected with UTI at 1600 U/day/mouse for 5 weeks; 2) the control group (n = 16) was injected with an equal volume of placebo for 5 weeks. The animals were euthanized for sample collection 5 weeks after administration. Lung tissues were collected and fixed in Bouin's solution (PHYGENE, PH0976) for 24 to 48 hours, and metastatic lung nodules were examined and counted.

## 2.4 Quantifying cell proliferation using MTS assay

Cells were seeded into 96-well plates at a density of  $1 \times 10^5$  cells/well in 100  $\mu$ l normal culture medium. UTI and PBS were added 24h later. Cell growth was determined using MTS (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay solution; sigma), 10  $\mu$ l MTS reagents were added to 100ul culture

medium per well and incubated for 2–4 h at 37°C. The OD490 value was measured with a microplate reader.

## 2.5 Wound healing assays

Cells were seeded into a 6-well plate and cultured until 90% confluence. A sterile 200- $\mu$ L tip was used to create artificial wounds in the cell monolayer, and the floating cells removed by a single wash with 1 $\times$ PBS. UTI and PBS were added separately. Respective images were captured at 0 h and 24 h using an inverted microscope. Wound healings were monitored under a microscope and quantified at 0 h and 24 h.

## 2.6 Migration and invasion assays

Migration assays were conducted with Biocoat without Matrigel (Corning. Life sciences), and invasion assays were performed with Biocoat with Matrigel (Corning. Life sciences) following the manufacturer's instructions. The harvested Biocoats were then stained with crystal violet, and invaded cells were counted under a microscope. Both experiments were repeated independently three times.

## 2.7 RNA isolation and real-time quantitative reverse-transcription PCR (qPCR)

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, USA). Complementary DNA (cDNA) synthesis was completed using the reverse transcription kit (Transgene, China) following the manufacturer's instructions. qRT-PCR analysis was performed using the SYBR Green PCR Kit (Transgene, China). The relative mRNA levels are shown as the value  $2^{-\Delta Ct}$ .

The following PCR primers were used:

Luciferase F: 5'-AGAGATACGCCCTGGTTCCT-3";

Luciferase R: 5'-ATCCCCCTCGGGTGTAATCA-3";

GAPDH-F: 5'- CTCATGACCACAGTCCATGC-3";

GAPDH-R: 5'- CAGTGAGCTTCCCGTTCAG-3";

uPA forward: 5'-GCCACACACT GCTTCATTGA-3';

uPA reverse: 5'-TATACATCGA GGGCAGGCAG-3';

uPAR forward: 5'-GCCTTACCGA GGTTGTGTGT-3';

uPAR reverse: 5'-CATCCAGGCA CTGTTCTTCA-3';

GAPDH was used as the internal control for measuring the relative level of luciferase.

## 2.8 Immunoblotting analyses

NPC cells treated with UTI or Placebo were lysed with a 25  $\mu$ L lysis buffer and mixed with a 2  $\times$  sample buffer. Cell lysates/proteins were subjected to SDS-PAGE and then transferred onto a PVDF membrane. The membrane was incubated overnight at 4°C and subsequently with secondary antibodies for 1 h. After being washed with PBST, signals were visualized by incubation with ECL luminescence substrate and detected with the ChemiDoc Gel Imaging System (Bio-Rad, USA).

## 2.9 Statistical analysis

One-Way ANOVA was used to compare different independent groups of data. A p-value < 0.05 was considered statistically significant in all cases.

## 3. Results

### 3.1 UTI inhibits the migration and invasion of NPC cells

We previously reported that among the cellular clones isolated from the NPC cell line CNE2, and S18 had the highest metastatic ability, while S22, and S26, and their parent line CNE2 had low metastatic ability [22]. Another highly-metastatic clone, 5-8F, was isolated from a lowly-metastatic parental NPC cell line SUNE-1 [25].

After treatment with different concentrations of UTI, the migration of S18 was suppressed in a concentration-dependent manner (Fig. 1a-b, 1d). A consistent concentration-dependent inhibitory effect of UTI was also observed in 5-8F cells (Fig. 2a-b, 2d). Using invasion assay, we further confirmed that UTI significantly suppressed the invasion of both S18 cells and 5-8F cells (Figs. 1c, 2c).

### 3.2 UTI reduces metastasis of NPC cells

To evaluate the effects of UTI on NPC metastasis *in vivo*, we applied a footpad to popliteal lymph node (LN) metastasis model (Fig. 3a). Live popliteal lymph nodes were imaged and the luciferase signal of lymph node metastasis was detected in the placebo treatment group (Fig. 3c). The popliteal LN metastasis rate was significantly reduced from 69% (11/16) to 29% (5/17) following UTI treatment in the spontaneous metastasis model (Fig. 3d). Notably, the body weight of the mice was not affected by UTI (Fig. 3e). Another orthotopic mammary tumor metastasis model of MMTV-PyMT mice was used to validate the anti-metastasis effect of UTI [26]. The lung metastatic foci were significantly reduced by UTI treatment in this model (Fig. 5e-f) whereas, the body weight alteration was insignificant compared with the placebo treatment group (Fig. 5g).

### 3.3 UTI has no effect on NPC cellular proliferation, or on NPC xenograft tumor growth

In contrast to the inhibitory effect on breast cancer cells reported by another laboratory [27], the proliferation of S18 and 5-8F cells was not inhibited by UTI treatment in our study, even with prolonged

treatment time or increased UTI concentrations (Figs. 1e and 2e.).

To evaluate whether UTI affects tumor growth *in vivo*, we subcutaneously injected S18 cells into the armpits of nude mice. After 17 consecutive days of UTI administration, we found that UTI treatment had no effect on the growth of S18 xenograft tumors (Fig. 4a-c) or the body weight of the animals (Fig. 4d), suggesting that UTI does not act as a cytotoxic drug.

## **3.4 The expression of uPA and uPAR was downregulated upon UTI treatment**

Although transient upregulation of the uPA mRNA level was seen in both S18 and 5-8F cell lines, suppression of uPA mRNA at 24 h was consistent with the suppression of uPA protein at 48 h in S18 cells, and this phenomenon was also observed in 5-8F cells. (Fig. 5a-5c). Both S18 and 5-8F cells showed downregulation of uPAR in both mRNA and protein levels upon UTI treatment (Fig. 5a-5c), suggesting that the impact on uPAR was more profound in comparison with uPA.

## **3.1 Impact of UTI on epithelial to mesenchymal transition**

Epithelial to mesenchymal transition (EMT) is regarded as an important step in the metastatic cascade of tumor cells. To explore whether UTI suppresses NPC cellular motility through inhibition of EMT, we evaluated the expression of EMT markers by qPCR and immunoblotting in UTI-treated NPC cells. The results revealed that UTI treatment significantly upregulated the expression epithelial markers E-cadherin and DSP but downregulated the expression of mesenchymal markers N-cadherin, Vimentin, and Slug at the RNA level after 48h of UTI incubation (Fig. 5d, e). Although the expression of the markers at both the mRNA and protein level fluctuated along with treatment time, the epithelial markers consistently increased while the mesenchymal marks uniformly decreased at 48h of treatment, which is consistent with the results of functional studies. Collectively, these results suggest that UTI treatment restrains NPC cells from metastasis while inhibiting EMT.

## **3.6 UTI treatment does not affect the AKT, ERK, or STAT3 signaling pathways**

We evaluated the activation of AKT, ERK and STAT3 signaling upon UTI treatment. No significant alteration in any of these signaling pathways was observed after 24 h of treatment (Fig. 6). In contrast to our assumption, the levels of phosphorylated JAK2 and phosphorylated STAT3 increased in some NPC cell lines after 48 h of UTI treatment. Interestingly, the JAK1/2 inhibitor, Ruxolitinib ( $IC_{50} = 18.88 \mu M$ , Fig S1a), partially reversed UTI's inhibitory effect on the invasion of S18 (Fig. 6d). The inhibitor of STAT3, S3I-201 ( $IC_{50} = 139.6 \mu M$ , Fig S1c), reversed UTI's induced-inhibition of S18 cellular migration at a concentration of 50  $\mu M$ .

## **4. Discussion**

Distant metastases are the main cause of treatment failure in NPC patients. In this study, we demonstrated that UTI may inhibit the migration, invasion and metastasis of NPC cells without affecting cancer cell proliferation.

Urokinase-type plasminogen activator (uPA), a type of multifunctional serine protease, is a key player of the plasminogen activator system. uPA promotes the degradation of the extracellular matrix, and activates the matrix metalloproteinase [28], thereby facilitating the migration and invasion of cancer cells. In addition, uPA is involved in angiogenesis and lymphangiogenesis [29]. The binding of uPA to uPAR can lead to the subsequent conversion of plasminogen into plasmin [30]. Our previous study demonstrated that uPAR promotes NPC cellular growth, colony formation, migration, and invasion and facilitates EMT of NPC cells [17]. In our present study, real time PCR results showed that UTI treatment may decrease the RNA levels of uPA and uPAR 24 to 48 hours after treatment. The protein expression of uPA was also reduced correspondingly. UTI induced-reduction of uPA and uPAR was consistent with the time of UTI inhibition on NPC cellular migration and invasion. uPAR is overexpressed in numerous human cancers. It has been reported that uPAR can be activated independently to uPA [17, 31–33], therefore, suppression of uPAR by UTI is of the most importance. It has been reported that UTI binds to cells through its domain I, and exerts its anti-fibrinolytic activity through domain II [34], in which the anti-metastatic effect is said to be dependent on the COOH-terminal domain II of UTI [UTI-(78–136)-peptide] [35]. The synthesized conjugate between amino-terminal fragment of human uPA and a native UTI molecule or domain II of UTI can also inhibit cancer cell invasion [36]. And a positive feedback loop between uPA and uPAR has been reported [37, 38]. These suggest that uPAR plays a vital role in UTI's inhibition of metastasis.

Interestingly, UTI treatment showed no effect on the key proteins in the ERK/MAPK and PI3K-AKT pathways in NPC cells, which is different from the effects on breast cancer [34]. Our results on the inhibitory effects on both uPA and uPAR suggest therapeutic value of UTI treatment on NPC metastasis, which has favorable clinical potential.

## 5. Conclusions

We observed that UTI can inhibit NPC metastasis, while having little effect on NPC cellular proliferation. UTI suppressed the expression of uPA and uPAR between 24 and 48 hours after treatment. The inhibition on metastasis of NPC cells may be partly due to the downregulation of the uPA and uPAR signaling pathway as well as the activation of JAK/STAT3 signaling. UTI may serve as a potential treatment agent for inhibiting NPC metastasis.

## Abbreviations

UTI Ulinastatin

FBS Fetal bovine serum

NPC Nasopharyngeal carcinoma

PCR Polymerase chain reaction

EMT Epithelial-mesenchymal transition

GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase

E-cad E-cadherin

N-cad N-cadherin

DSP Desmoplakin

## **Declarations**

### **Ethics approval and consent to participate**

All animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University Cancer Center.

### **Consent for publication**

Not applicable

### **Availability of data and materials**

All data have been uploaded onto the Research Data Deposit ([www.researchdata.org.cn](http://www.researchdata.org.cn)) with the approval number RDD2021000XXX.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

CZ and YY carried out most of the experimental work. CZ drafted the manuscript. ZJ and LX carried out the RT-qPCR. LS and YM participated in the design of the study and performed the statistical analysis. CN and JD conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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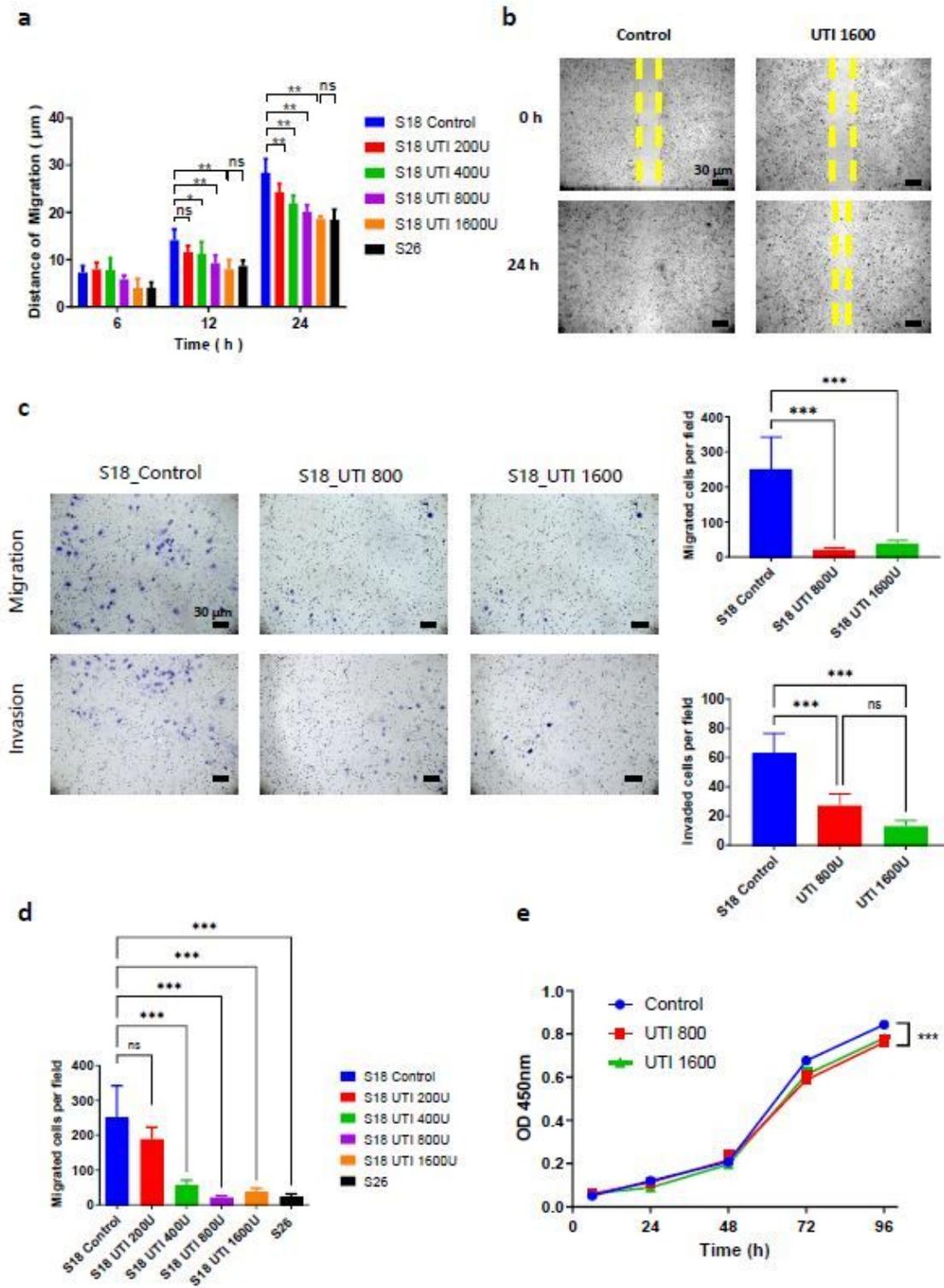
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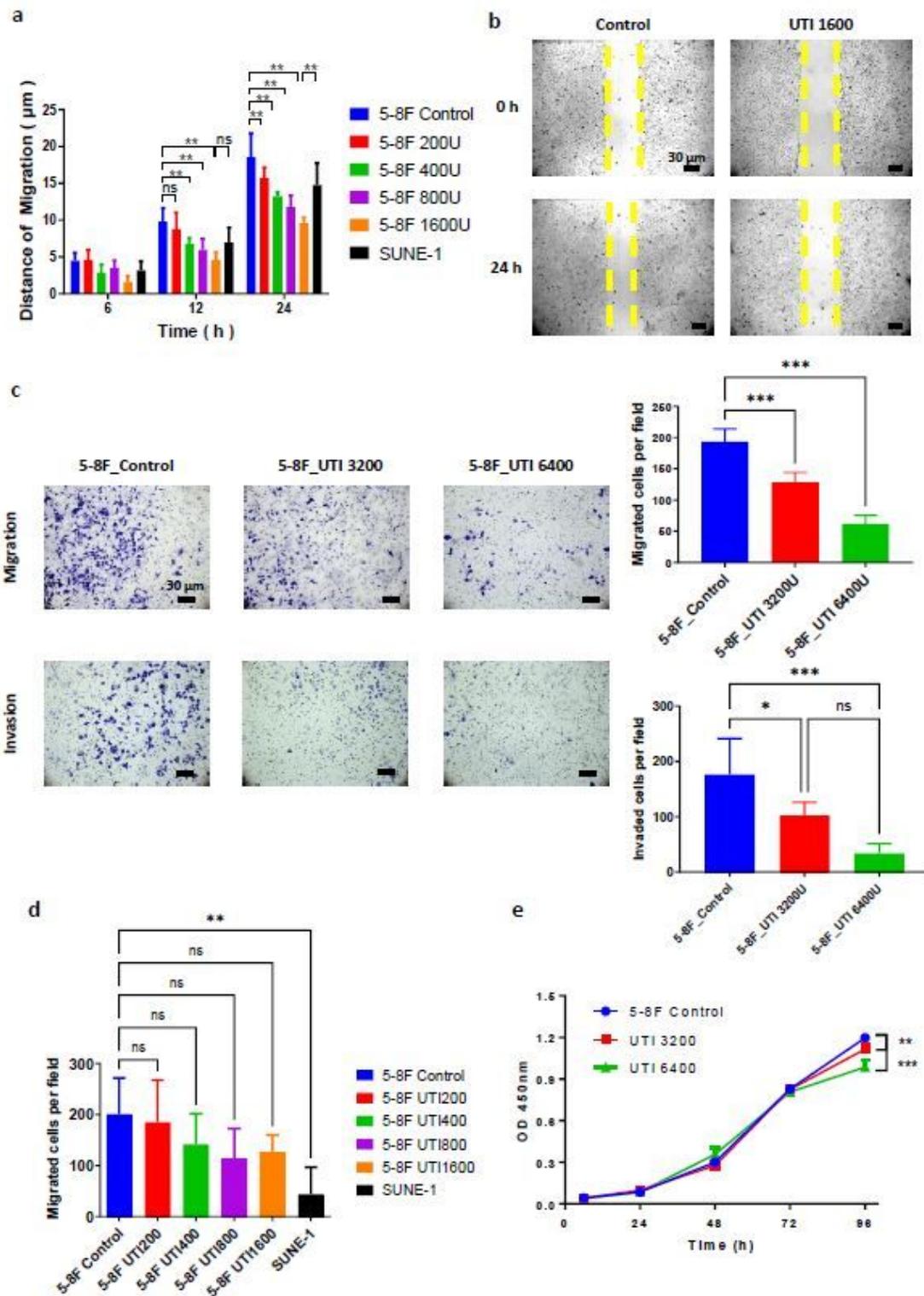
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## Figures



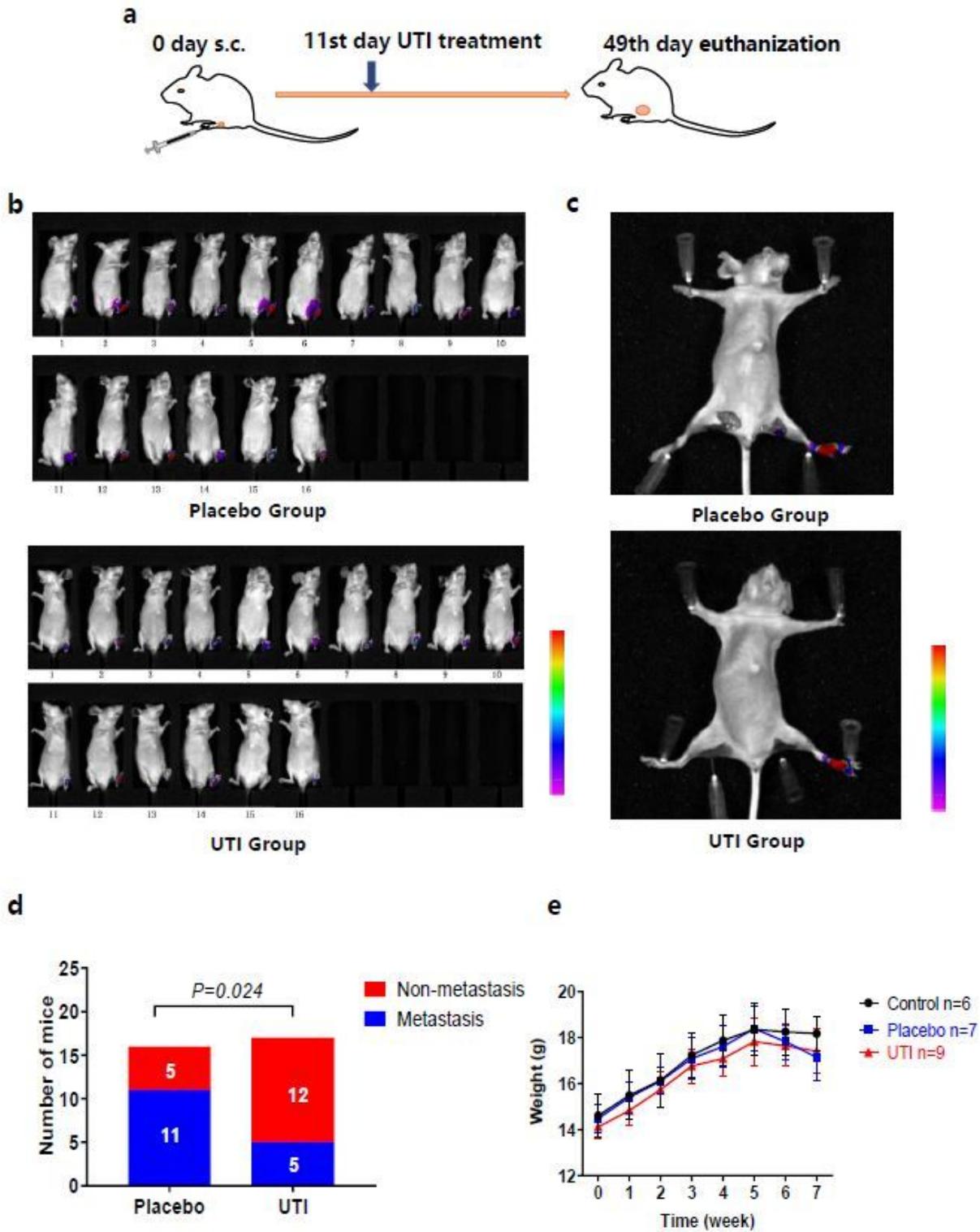
**Figure 1**

UTI inhibits the migration and invasion of S18 without influencing cellular growth. a-b. Wound scratch assay revealed that UTI inhibited S18 motility dose-dependently. c. UTI can inhibit the migration and invasion of S18 in vitro. d. A dose-dependent inhibition of S18 cell migration by UTI in transwell assay. e. UTI 800 U/ml and 1600 U/ml had little effect on the proliferation of S18 cells assessed by MTS assay. \* $P < 0.05$ , \*\* $P < 0.01$ , results of One-Way ANOVA.



**Figure 2**

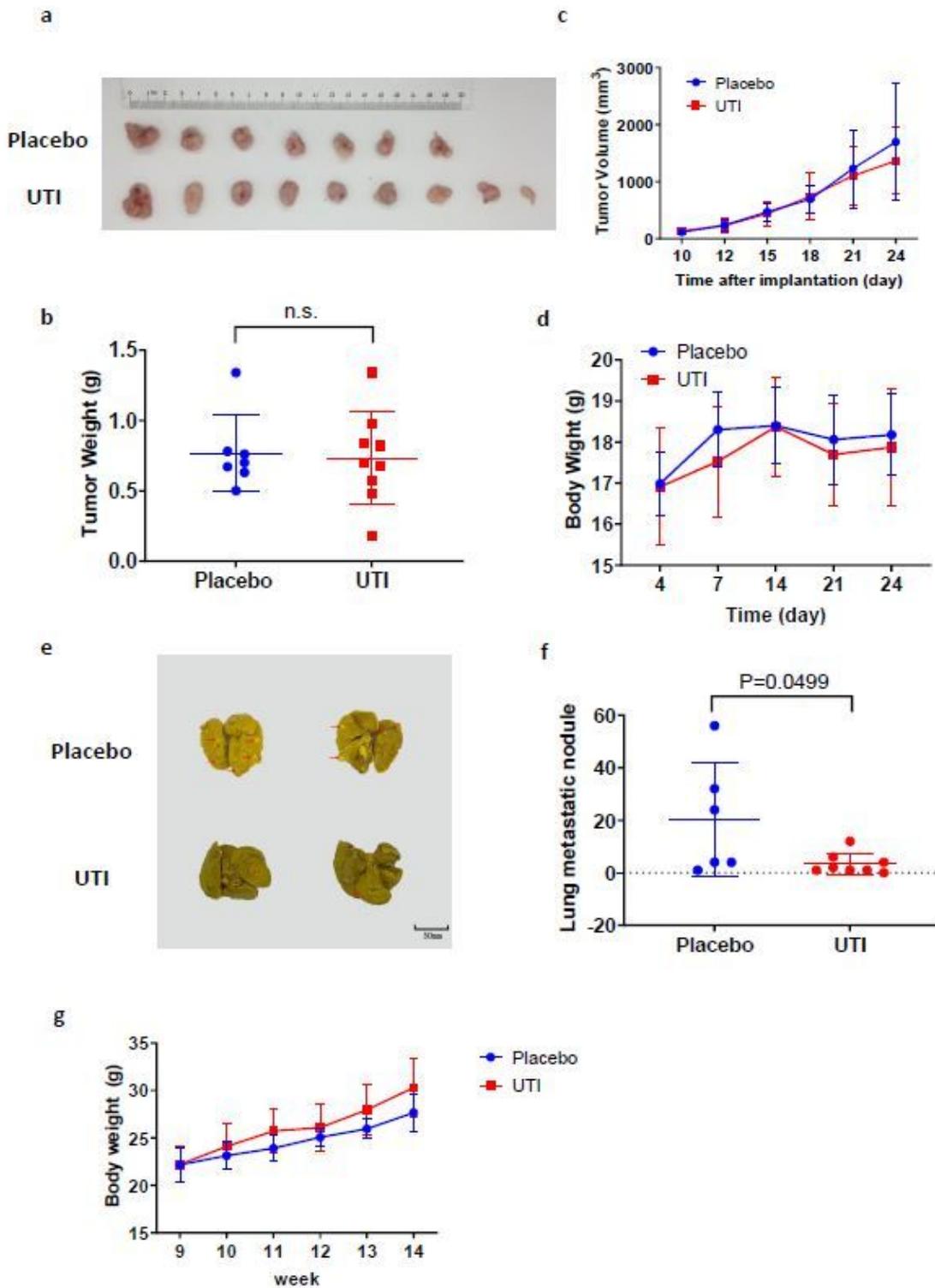
UTI inhibits the migration and invasion of 5-8F without influencing cell growth. a-b. Wound scratch assay revealed that UTI treatment inhibited 5-8F motility dose-dependently. c. UTI inhibited the migration and invasion of 5-8F in vitro. d. A dose-dependent inhibition of 5-8F cell migration by UTI in transwell assay. e. UTI 3200 U/ml and 6400 U/ml had little effect on the growth of 5-8F cells assessed by MTS assay. \* $P < 0.05$ , \*\* $P < 0.01$ , results of One-Way ANOVA.



**Figure 3**

UTI inhibits lymph node metastasis of NPC cells in vivo. a. Ideogram of the mice lymph node metastasis treated with UTI. The left footpad of each mouse was subcutaneously injected with S18-1C3 cells and intraperitoneally injected with UTI 20,000 U per day for 38 days starting on the 11th day after cell implantation. b-c. The in vivo imaging of mice treated with placebo (up) and UTI (down) showed that tumors in situ of the UTI group were smaller than the placebo group and the lymph node of the placebo

group had obvious NPC metastases. d. Chi-square test between the placebo and UTI group showed that UTI could significantly reduce the S18 metastasis rate. e. UTI treatment had little effect on the body weight of the mice.



**Figure 4**

UTI had little effect on mammary tumor growth while it significantly reduced pulmonary metastasis in MMTV-PyMT mice. a. A photograph of a subcutaneous tumor treated with placebo and UTI. There was

no significant difference between the mice treated with placebo and UTI in regards to tumor weight (b.), tumor volume (c.) and (d.) body weight. e-f. The number of pulmonary metastatic nodules was significantly reduced upon UTI treatment. g. The body weight of MMTV-PyMT mice was not affected by UTI treatment.

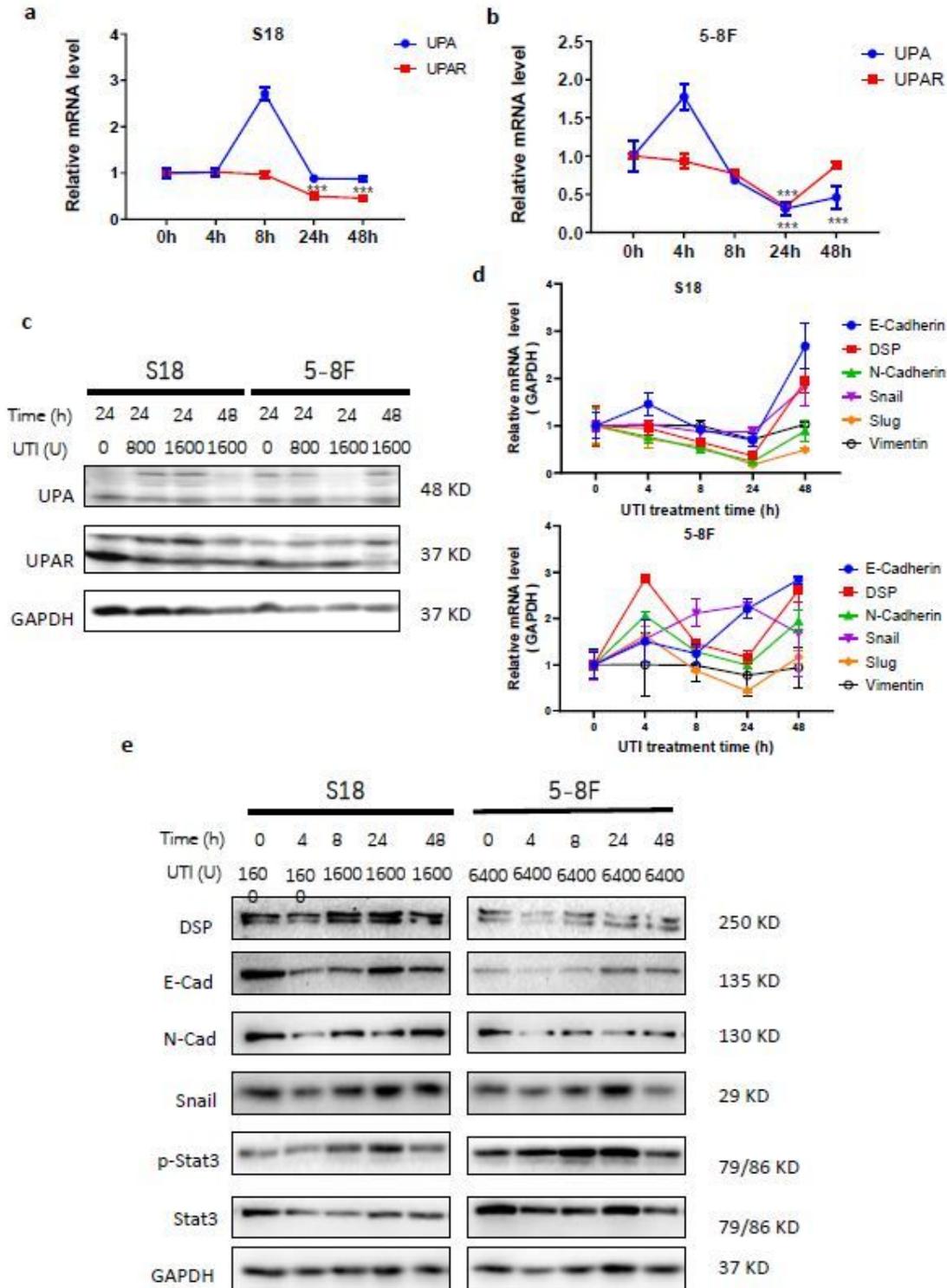


Figure 5

UTI downregulates the levels of uPA and uPAR. a-b. uPAR mRNA was suppressed by UTI at 24 h of treatment in both S18 and 5-8F cell lines. While a reduction of uPA mRNA was only seen in 5-8F cell lines at 24-48 h. c. The uPA and uPAR protein levels were reduced after the treatment of UTI between 24 and 48 h. d-e. UTI downregulated the mesenchymal marker and increased the epithelial marker expression after the treatment of UTI between 24 and 48 h. Interestingly, phosphorylated STAT3 levels were upregulated by UTI treatment.

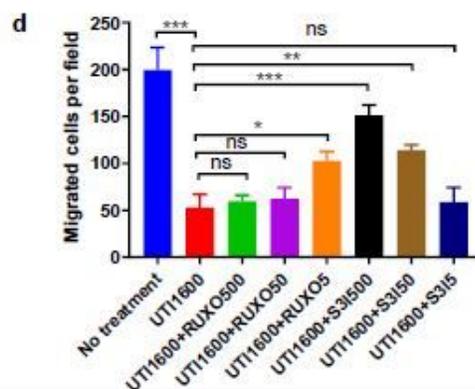
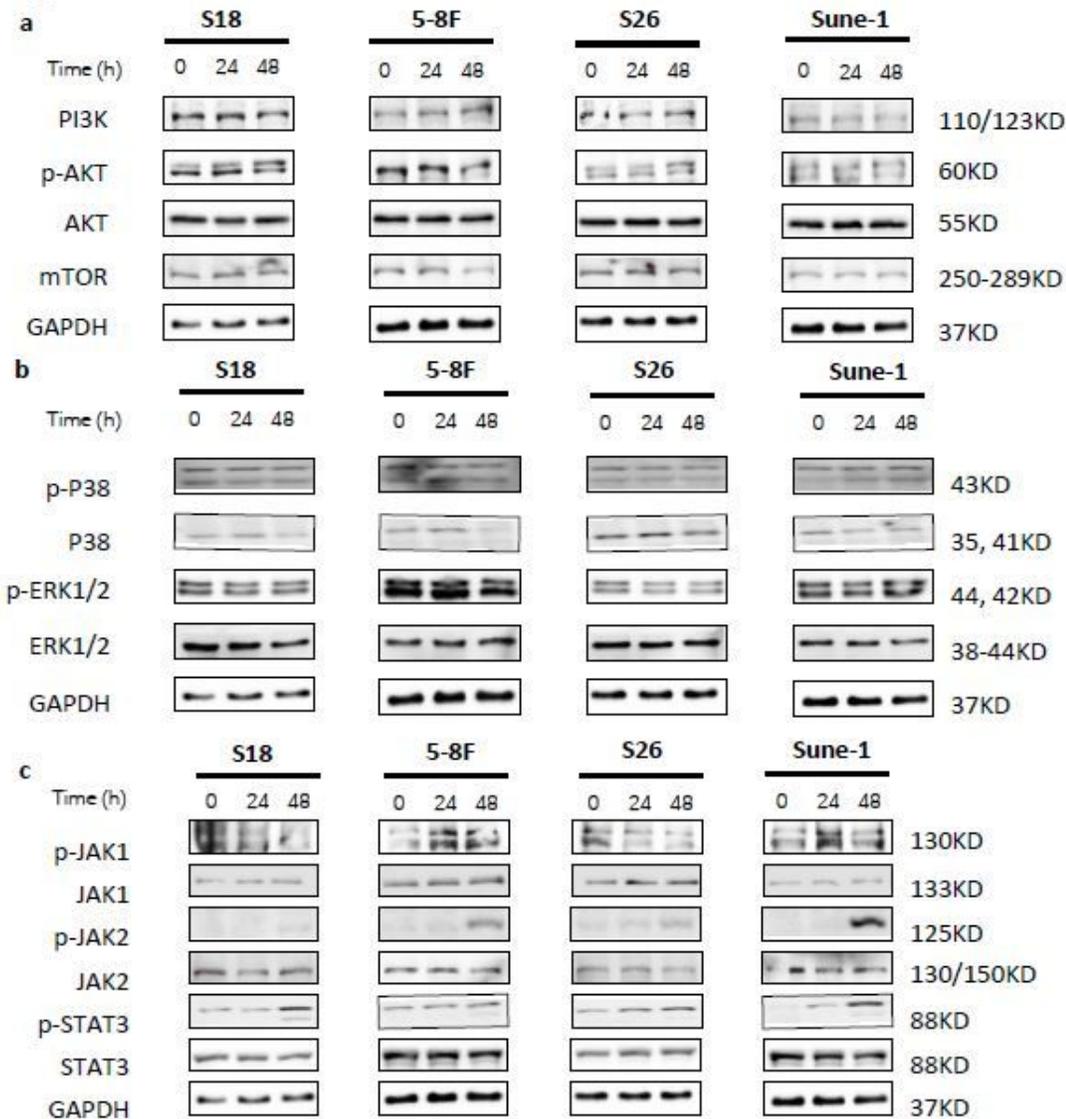


Figure 6

UTI treatment does not affect AKT and ERK signaling pathways in NPC cells. a-b. UTI treatment did not affect the key proteins in the PI3K-AKT and ERK pathways. c. UTI treatment activated STAT3 in S18. d. The inhibitor of JAK1/2, Ruxolitinib, and inhibitor of STAT3, S3I-201, may reverse UTI-induced inhibition of S18 cellular migration. \*P<0.05, \*\*P <0.01, result of One-Way ANOVA.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [figureS1.pdf](#)