The Stat3 signaling pathway mediates sunitinib resistance in the renal cell carcinoma cell line

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Directed by Professor Seung Choul Yang
and Professor Kyung-Sup Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Kyung Hwa Choi

December 2013

This certifies that the Doctoral Dissertation of
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December 2013

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my esteemed Prof. Seung Choul Yang and Kyung-Sup Kim, my supervisors, for their expert guidance and valuable suggestions throughout my doctoral degree course. I especially would like to thank Prof. Woong Kyu Han for his excellent guidance and valuable advice on this doctoral dissertation and on other research works in many ways. With their willingness, advices and guidance, I have been able to complete my research successfully. I would like to also acknowledge Prof. Beom Seok Kim and Sae-Chul Kim for their critical comments and thoughtful suggestions. With their guidance, this thesis could be produced with completeness.

My special thanks go to my colleagues Eunjin Koh, Hee Eun Kim, Eunju Bae, Deok-yoon Jeong, Soobin Jung and Seok-Jun Kim. It was a great pleasure to work with them and many inspiring discussions with them were encouraged and helpful to my research. I would also like to recognize Prof. Yong-Ho Ahn, Kun-Hong Kim, Man-Wook Hur, Sahng-Wook Park, Jae-Woo Kim, Ho-Geun Yoon, Kyung-Hee Chun and all colleagues of the department of Biochemistry and Molecular Biology for their healthy criticism, suggestion and cooperation during the period of this study.

Especially, I would like to express my sincere thanks to my parents, husband and other family members who constantly provided emotional support and took care of me in every aspect

December, 2013

Kyung Hwa Choi

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ABSTRACT

The Stat3 signaling pathway mediates sunitinib resistance in the renal cell carcinoma cell line

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Sunitinib is a first line treatment modality for patients with metastatic clear cell renal cell carcinoma. However, the development of resistance to sunitinib has emerged as an important clinical problem for molecular-targeted therapy in metastatic renal cell carcinoma. In this study, we have established the sunitinib-resistant renal cell carcinoma sub-lines (designated as OR) by culturing 786-O cells (O) with increasing concentrations of sunitinib for 6 months. Interestingly, exposure of OR cells to sunitinib failed to show the decline of Stat3 phosphorylation and upstream JAK2 phosphorylation. Subsequent experiments showed activated Stat3 promotes cell proliferation and prevents apoptosis in sunitinib resistant cell line by regulating cell cycle associated proteins (skp2, p21, p27, Cyclin E and Cdk2) and apoptosis associated proteins (p53, Bax and Bcl-2) in protein levels. Parental cells acquired sunitinib resistance and reduced apoptosis after forced expression of Stat3, and knock-down of Stat3 in OR cells markedly increased the sensitivity

to sunitinib to similar level of parental cells. These findings indicate that activation of JAK2/Stat3 signaling plays critical role in sunitinib resistance. OR cells exhibited increased expression of IL-6, by which JAK2/Stat3 is stimulated. And this IL-6/JAK2/Stat3 pathway is less sensitive to sunitinib and is thought to be relevant for the escape pathway of inhibitory effect of sunitinib.

In addition, we showed a series of processes of JAK2 activation, PKM2 phosphorylation, nuclear translocation of PKM2 and concomitant Stat3 phosphorylation by IL-6 stimulation and JAK2/Stat3 signaling were severely attenuated by PKM2 knock-down, leading to increased sunitinib-sensitivity. These results indicate that nuclear PKM2 has an important role in JAK2/Stat3 signaling activation by sustaining Stat3 phosphorylation.

In summary, our findings suggest that Stat3 activation by IL-6/JAK2 activation is a key modulation in sunitinib resistant mechanism against direct anti-cancer cell effect in renal cell carcinoma cell lines. Increased nuclear level of PKM2 which enhances transcriptional activity of Stat3 also does an important role in sunitinib resistance. With further investigations of in vivo experiment and linkage of the clinical data, our results may be improve the information we have for overcoming sunitinib resistance in metastatic renal cell carcinoma patients.

Key words : renal cell carcinoma, sunitinib, resistance, Stat3

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I. INTRODUCTION

Renal cell carcinoma is the most common malignant tumor in the kidney. The life expectancy of patients with metastatic kidney cancer is less than two years. In the United States, 57,000 people are diagnosed with renal cell carcinoma every year, and approximately 13,000 people die from kidney cancer. Thirty percent of renal cell carcinomas accompany the metastatic lesion at the time of diagnosis, and 30% of non-metastatic cancers that are treated with curative surgical resection eventually progress to the metastatic stage[1]. In localized renal cell carcinoma, surgical resection is the treatment of choice, and over 90% report a 5-year survival rate. Metastatic renal cell carcinoma usually does not respond to classical chemotherapy or radiotherapy. Recently, therapy that targets the Von Hippel-Lindau (VHL) gene defect, which causes 70% of total incidences of renal cell carcinoma, has been used as the primary treatment for metastatic renal cell carcinoma[2,3].

Sunitinib is a first-line medication for metastatic clear cell renal cell carcinoma. It is a multi-targeted tyrosine kinase inhibitor (TKI), which has been mainly

explored for its anti-angiogenic effect of targeting the vascular endothelial growth factor receptor (VEGFR) and the platelet-derived growth factor receptor (PDGFR)[2,4]. Most of clear renal cell carcinomas are characterized by defects in *VHL*, followed by activation of hypoxia-inducible factor (HIF) and the excessive protein secretion of HIF-related factors, such as VEGF and PDGF. Sunitinib acts as an adenosine triphosphate-competitive TKI to block the binding of VEGF and PDGF to their respective receptors in the surrounding endothelial cells, thus inhibiting angiogenesis and cancer progression[5,6].

Recently, the effect of sunitinib on directly inhibiting cancer cells has been reported in numerous cancers, such as thyroid cancer and gastrointestinal stromal tumor (GIST)[7,89]. In the cancer cell, TKIs including sunitinib block tyrosine kinases (e.g., c-Kit), receptor tyrosine kinases (RTKs), VEGFR, and PDGFR, thus inhibiting downstream signaling pathways such as the janus kinase 2 (JAK2)/Stat3, PI3K/Akt, and RAS/RAF signaling pathways[7-10]. These signaling pathways are well-known cancer survival pathways, which are important for the proliferation, apoptosis prevention, and metastasis of cancer cells. As such, renal cell carcinoma cells that express cytokine receptors and tyrosine kinase receptors (e.g., VEGFR, PDGFR, and RTKs) are the direct targets of sunitinib[11]. Although sunitinib primarily has an anti-angiogenic effect, recent reports demonstrate dramatic tumor cell responses without the destabilization of surrounding vessels in GIST biopsies[8]. This suggests that the anti-cancer cell effect of sunitinib is dominant over its anti-angiogenic effect in certain cases.

Three main theories have been described to explain the resistance mechanisms of TKIs. The first theory is that advanced metastatic cancer exhibits different expression levels of growth factor receptors, in comparison to those in early metastatic stage cancer. Unlike early metastatic cancer, the advanced stages of cancer demonstrate resistance to TKIs. Early metastatic cancers, which mainly express VEGFR in surrounding endothelial cells, are sensitive to TKIs. In

advanced cases, receptors that are not targets of TKIs, such as fibroblast growth factor receptors, are mainly expressed and angiogenesis can be maintained. Therefore, cancers may be resistant to TKIs according to their progression[12]. The second theory hypothesizes that during treatment with TKIs the surrounding tumor environment is modified to promote angiogenesis that is not as dependent on VEGF or other TKI targets[13]. The third theory is that cancer cell itself directly develops mechanisms of resistance against TKIs. There is evidence showing that cancer cells under TKI treatment escape growth inhibition by using a variety of TK-independent growth-signaling mechanisms to promote proliferation. In thyroid cancer, when VEGFR and PDGFR are blocked by sunitinib treatment, the epidermal growth factor receptor (EGFR) is compensatively activated. In addition, extracellular signal-regulated kinase (ERK) signaling, which promotes cancer survival, is also activated in sunitinib-resistant thyroid cancer[7]. The epigenetic silencing of phosphatase and tensin homolog (PTEN), which results in the activation of Akt signaling, has also been reported in sunitinib-resistant GIST cells[14]. Similarly, Makhov et al. discovered mutations in PTEN in some renal cell carcinoma and prostate carcinoma cells, which contributes to cell survival and sunitinib resistance[15]. Therefore, newly developed intracellular oncogenic events can occur in TKI-resistant cancer cells. The three theories described above can be deemed equally important. However, in renal cell clear cell carcinoma, the resistant mechanism against the direct anti-cancer cell effect (third theory) has not been well elucidated.

Signal transducer and activator of transcription 3 (Stat3) is activated in renal cell carcinoma[16]. Stat3 signaling is activated by diverse growth factors and cytokines, including EGF, hepatocyte growth factor, and interleukin-6 (IL-6), via various tyrosine kinase receptors, such as EGFR, c-MET, JAK2, and Src[17,18]. Stat3 activation has an oncogenic effect in numerous cancers[11,18-20]. Upon receiving upstream signals, Stat3 is activated by phosphorylation at tyrosine 705,

thus up-regulating the expression of oncogenes (e.g., Skp2) and anti-apoptotic genes (e.g., Bcl-2, Mcl-1), and down-regulating the expression of apoptotic genes (e.g., p53, Bax) and cell cycle-associated genes (e.g., p27, p21)[18,21]. Ara et al. showed that environment-mediated drug resistance is acquired under IL-6-mediated Stat3 activation in neuroblastoma cell lines, in which Stat3 is not constitutively active[22]. Xin et al. reported that sunitinib inhibits Stat3 in renal cell carcinoma and induces tumor cell apoptosis[11]. These reports suggest that the activation of Stat3 may contribute to sunitinib resistance in renal cell carcinoma.

PKM2 converts phosphoenolpyruvate (PEP) to pyruvate in the glycolytic pathway. However, in cancer cells, the stimulation of growth induces PKM2 phosphorylation, and this change induces the conversion of pyruvate kinase activity to protein kinase activity[23]. PKM2 phosphorylation is decreased due to the inhibition of JAK2, which is regulated by growth signals such as IL-6 in the leukemia cell line[24]. Also, the phosphorylated form of PKM2 can translocate to the nucleus and act as a multifunctional signaling molecule, such as a transcriptional co-activator[25].

Little is known about the roles of Stat3 and PKM2 in sunitinib resistance in clear cell renal cell carcinoma. In this study, we assessed the mechanisms associated with the Stat3 and PKM2 signaling pathways that are involved in sunitinib resistance against the direct anti-cancer cell effects in clear cell renal cell carcinoma.

II. MATERIALS AND METHODS

1. Cell culture

786-O human renal cell carcinoma cell lines (O) were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in a 5% CO_2 atmosphere at 37°C with 25 mM or 5 mM of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS; Gibco), 100 μg/ml streptomycin (Gibco), and 100 U/ml penicillin (Gibco). Six-well, 96-well (Corning, New York, NY), 60-mm, and 100-mm culture dishes (TPP, St. Louis, MO) were used for cell culture.

Sunitinib (Sutent® ; LC laboratories, Woburn, MA) and WP1066 (Selleckchem, Houston, TX) were prepared at concentrations of 10 mM in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Recombinant human IL-6 (Peprotech, Rocky Hill, NJ) was prepared at a concentration of 200 μg/ml in phosphate-buffered saline (PBS; Gibco). Prior to IL-6 treatment, cells that were 70% confluent were washed with PBS, and then incubated in FBS-free DMEM overnight.

2. Generation of a sunitinib-resistant cell line

Sunitinib-resistant 786-O (OR) cells were generated from O cells. To generate the OR line, O cell lines were continuously exposed to sunitinib from concentrations of 1 μM to 13 μM for more than 6 months, as previously reported[14]. In each dose-escalation step, the sunitinib concentration was elevated by 1-2 μM per 2 or 3 cell passages. Cells were maintained in a humidified incubator containing 5% CO_2 at 37℃.

3. Plasmids

Amplification of *Stat3* cDNA from O cell cDNA was performed, and a pSG5-Stat3 overexpression vector was generated by inserting the *Stat3* cDNA

into the pSG5 vector. For stable knockdown of PKM2, a shPKM2-lentivirus plasmid was purchased from Sigma-Aldrich.

4. Virus production and infection

To generate stable cells with PKM2 knockdown, a lentiviral vector was produced by inserting the shPKM2-lentivirus plasmid into the pLL-CMV-puro vector. PKM2 shRNA vectors were mixed with viral packing vectors (pMD2.G, pMDLG/PRRE, pRSV-Rev), and the transient transfection was conducted in HEK293T cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). After 36 h, the culture medium was obtained and purified, after which 7 mg/ml polybrene was added (Sigma-Aldrich), and the O cells were infected. Puromycin (2.5 μg/ml; Sigma-Aldrich) was used for selection.

5. Transient transfection

The Neon™ Transfection System set (Invitrogen) was used as an electroporator. Cells were suspended gently in Resuspension Buffer R, which has an optimal density of 1.5×10^6 cells per sample. The cell suspensions (0.2 ml) were transferred to each tube (i.e., 1.5×10^6/0.2 ml), and the pEGFP, pSG5-empty plasmid, and pSG5-wildtype Stat3 plasmid were added (total of 5.0 μg/tube). A Neon™ Tube with 3 mL Electrolytic E2 Buffer was set up into the Neon™ Pipette Station containing the cell-DNA mixture. Electroporation was performed following settings that were previously optimized for O cells: pulse voltage = 1400, pulse width = 20, and pulse number = 2. After adding 3.8 ml DMEM, the electroporated cells were pipetted gently and transferred immediately to a 60-mm culture dish. After 4 h, transfection efficiency was over 70%, and cell viability was over 90%. The expressions of GFP and Stat3 were confirmed after 24 h of incubation.

6. RNA interference (silencing RNA [siRNA])

The following double-stranded stealth siRNA oligonucleotides were kindly provided by Dr. Kyung-Hee Chun: human Stat3 (sense 5'-UGA AAG UGG UAG AGA AUC U [dTdT]-3', antisense 5'-AGA UUC UCU ACC ACU UUC A [dTdT]-3'), along with control oligonucleotides with comparable GC contents. Cells were prepared at an optimal density of 2×10^6 cells per 10 ml for each sample. For Stat3 knockdown, cells were transfected with control or Stat3 siRNA in 2 ml OPTI-MEM medium, using Lipofectamine RNAiMax (Invitrogen), according to the manufacturer's protocol. The cells were incubated for 72 h before protein preparation.

7. MTT assay for measurement of IC_{50}

Cells were transferred to 96-well culture dishes ($3x10^3$ cells/100.0 μg/well) and grown for 1 day, after which they were treated with different concentrations of sunitinib for 3 days. The cells were incubated for 4 h at 37℃ with 20.0 μg/well MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5.0 mg/ml, Promega, Madison, WI). The supernatant was aspirated, and the precipitates were dissolved in 100.0 μg DMSO. Cellular proliferation was determined from the conversion of MTT to formazan, using a SpectraMax 250 at 560 nm (Molecular Device Co, Sunnyvale, CA). The IC_{50} (50% inhibitory concentration of a drug) was measured as the drug resistance index.

8. Soft agar colony formation

Briefly, cells (5×10^3 cells/well) were seeded in 6-well plates containing 2 ml 0.3% low-melting agarose (Sigma-Aldrich) over 1.5 ml-layers of 0.5% agarose, in the presence of sunitinib (10 μM) or DMSO in 0.5 ml DMEM for 2 weeks. The plates were maintained at 37℃ in a humidified atmosphere of 5% CO_2. The colonies were stained with iodonitrotetrazolium chloride (1 mg/ml; Sigma-Aldrich), fixed with 3% acetic acid (Sigma-Aldrich), and then counted

and photographed under a microscope at a magnification of 40×. All assays were performed in triplicate.

9. RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was purified from cultured cells using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. First-strand cDNA was synthesized using oligodT-primer with SuperScriptTM II reverse transcriptionase (Invitrogen), following the supplier's protocol.

The following primers were used:

human IL-6 (S) 5'- TCG GTC CAG TTG CCT TCT CCC T - 3'

human IL-6 (AS) 5'- TGC AGC CAC TGG TTC TGT GCC T - 3'

human LIF (S) 5'- TGT CAA CGC CAC CTG TGC CAT A - 3'

human LIF (AS) 5'- TGG CGT TGA GCT TGC TGT GGA - 3'

human CNTF (S) 5'- TTC TCC AAG TCG CTG CCT TTG CA - 3'

human CNTF (AS) 5'- TTG TTG TTA GCA ATA TAA TGG CTC CCA - 3'

human OSM (S) 5'- TCC TGT TTC CAA GCA TGG CGA - 3'

human OSM(AS) 5'- AGT GTG GCA TTG AGG GTC TGC A - 3'

human IL-11(S) 5'- ACT GTG TTT GCC GCC TGG TCC T - 3'

human IL-11(AS) 5'- AGG GTC TTC AGG GAA GAG CCA CCT - 3'

human CTF1(S) 5'- ACC TCC TCA CCA AAT ACG CTG AGC A - 3'

human CTF1(AS) 5'- ACT CGC GGT AGA GGC CGC AAA - 3'

Equal amounts of cDNA were loaded onto the lanes of a 1% agarose gel and separated by electrophoresis, and the proteins were then transferred to a nylon membrane. A probe labeled with radioactive isotopes was manufactured for each target gene, and the bands were identified via autoradiography.

10. Western blot analysis

NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific, Rockford, IL) were used to separate the nuclear and cytoplasmic fractions, according to the manufacturer's protocol. Cell lysate preparation and western blot analysis were performed as previously reported[26]. The following antibodies were used: JAK2, phospho-JAK2 (Tyr1007/1008), Stat3, phospho-Stat3 (Tyr705), Akt, phospho-Akt (Thr308), p44/42 ERK1/2, phospho-p44/42 ERK1/2 (Thr202/Tyr204), phospho-PKM2 (Tyr105), GAPDH (Cell Signaling Technology, Danvers, MA), VEGFR-1, PKM2 (Abcam, Cambridge, UK), phospho-VEGFR-1 (RD systems, Minneapolis, MN), cyclin E, Cdk2, Skp2, p53, Bcl-2, HDAC-1 (Santa Cruz Biotechnology, Dallas TX), p27 (BD Transduction Laboratories, San Jose, CA), p21, α-tubulin (Millipore, Tumecula, CA), and Bax (Delta Biolabs, Muraoka Drive Gilroy, CA). The membranes were then incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Thermo Scientific) for 1 h at room temperature. The protein bands were developed using the Supersignal West Pico Chemiluminescent Substrate System (Thermo Scientific, Rockford, IL).

11. Cell cycle analysis

O cells at 80% confluency were prepared and treated with DMSO or 10 mM sunitinib to construct O (control) and O-Sunitinib groups, respectively. Sunitinib was deprived for 24 h in OR cells, after which sunitinib was added to all groups. Confluent Stat3-overexpressed cells and control cells in 60-mm culture dishes were also prepared. The cells were trypsinized, washed with PBS, and fixed with ice-cold 70% ethanol (Merck, Darmstadt, Germany) for 2 h. After washing with PBS, the cells were resuspended with 300 μl PBS and stained with 15 μl propidium iodide (1.0 mg/ml, Sigma-Aldrich) in 3 μl ribonuclease A (10 mg/ml, Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature in the dark. DNA contents, cell cycle profiles, and forward scatter

were analyzed using a BD LSR II FACS caliber (BD Biosciences) with emission detection at 488 nm (excitation) and 561 nm (peak emission). Data were analyzed using BD FACS Diva software (BD Biosciences)

12. Apoptosis analysis

Cells were grown in 100-mm culture dishes, and then treated with sunitinib and DMSO for 24 h. The annexin V-FITC apoptosis kit (BD Pharmingen, San Diego, CA) was used, according to the manufacturer's protocol. Cells were harvested 24 h after treatment and suspended in binding buffer at a concentration of 1×10^6 cells/ml. Cells in 100 μl biding buffer were then stained with 5 μl FITC-annexin V and 5 μl propidium iodide (50.0 μl/ml, Sigma-Aldrich) in the dark at room temperature, and then analyzed on a BD LSR II FACS caliber (BD Biosciences) using BD FACS Diva software (BD Biosciences), with a laser excitation wavelength at 488 nm (excitation) and 530 nm (peak emission).

13. Statistical analysis

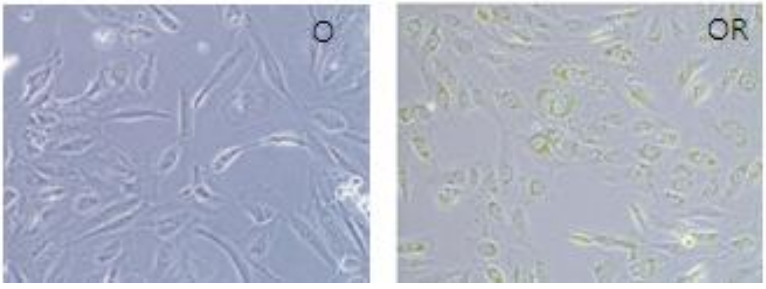
All results are expressed as mean ± SD.

III. RESULTS

1. Generation of the sunitinib-resistant 786-O (OR) cell line

OR cells were generated from O cells by exposing the cells to gradually increasing concentrations of sunitinib (up to 13 μM) for 6 months. In each dose-escalation step, the concentration of sunitinib was elevated by 1-2 μM for every 2 or 3 cell passages. OR cells were more flattened, pleomorphic, and had enlarged nuclei, compared with the O cells (Figure 1A). To evaluate the resistance to sunitinib in OR cells, the IC_{50} was measured using the MTT assay. The IC_{50} of OR cells was over 15 μM, which was much higher than that of O cells (<5 μM), thus showing that OR cells were resistant to sunitinib (Figure 1B).

A



B

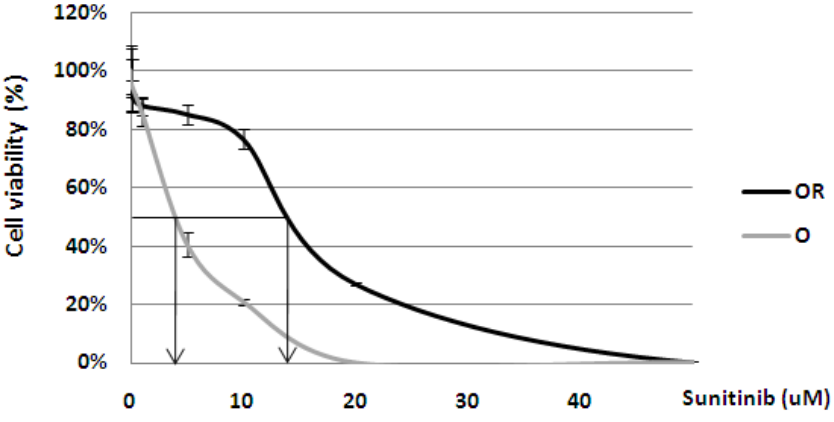


Figure 1. Generation of the sunitinib-resistant (OR) cell line. OR cells were generated from 786-O (O) cells. O cells were continuously exposed to gradually increasing concentrations of sunitinib (up to 13 μM) for more than 6 months. (A) The microscopic appearance of O cells and OR cells. (B) The MTT assay was performed to determine IC_{50}. Cells were plated in 96-well culture dishes and treated with different concentrations of sunitinib (0-50 μM) for 3 days, after which the IC_{50} was measured. The cell viability was represented with the relative absorbance. Experiments were performed in triplicate.

2. Decreased sunitinib-induced cell cycle arrest and apoptosis in OR cells

We examined whether cell proliferation was altered in OR cells, using FACS cell cycle analysis (Figure 2A and B). The proportion of G0/G1 phase cells was relatively higher 12 h after sunitinib (10 μM) treatment in O cells (②; 76.9%), compared with non-treated O cells (①; 62.2%). However, after 72 h of sunitinib treatment (10 μM), the G0/G1 proportion (③) was decreased (52.2%), whereas the proportion of apoptotic cells was markedly increased (8.02%). On the contrary, sunitinib-maintained OR cells (④) showed much lower proportions of apoptotic cells (2.4%) than O cells that were treated for 72 h (③). In addition, treatment of OR cells with sunitinib resulted in G0/G1 proportions (69.7%) that were similar to those of O cells that were treated with sunitinib for 12 h (②). Based on these findings, in the presence of sunitinib, OR cells showed increased cell proliferation and decreased apoptosis compared to that of O cells. This indicates that OR cells acquire sunitinib resistance to a great extent.

The apoptosis was further confirmed by FACS using annexin V staining (Figure 3A and B). The proportion of apoptotic cells in non-treated O cells was 0.4%. Sunitinib treatment (10 μM) for 24 h induced apoptosis up to 5.1%, whereas OR cells that were maintained under sunitinib (10 μM) exhibited only 2.3%. These results suggest that OR cells may acquire anti-apoptotic potential against sunitinib-induced apoptosis.

The effects of sunitinib on various regulators of the cell cycle and apoptosis were analyzed and evaluated by western blot analysis (Figure 3C). The levels of negative regulators of the cell cycle, such as p21, p27, and p53, were decreased in the sunitinib maintained OR cells, compared with that of 24 h treated O cells.

Conversely, positive regulators of the G1/S cell cycle, such as cyclin E and cdk2, and Skp2 which is involved in the downregulation of p27 were increased in OR cells. Decreases in the levels of apoptotic proteins (p53 and Bax) were

observed in treated OR cells, whereas levels of Bcl-2, an anti-apoptotic factor, were increased in OR cells, compared with that of sunitinib treated O cells. These results demonstrate that cell cycle arrest, followed by apoptosis, in sunitinib-treated O cells were rescued in OR cells.

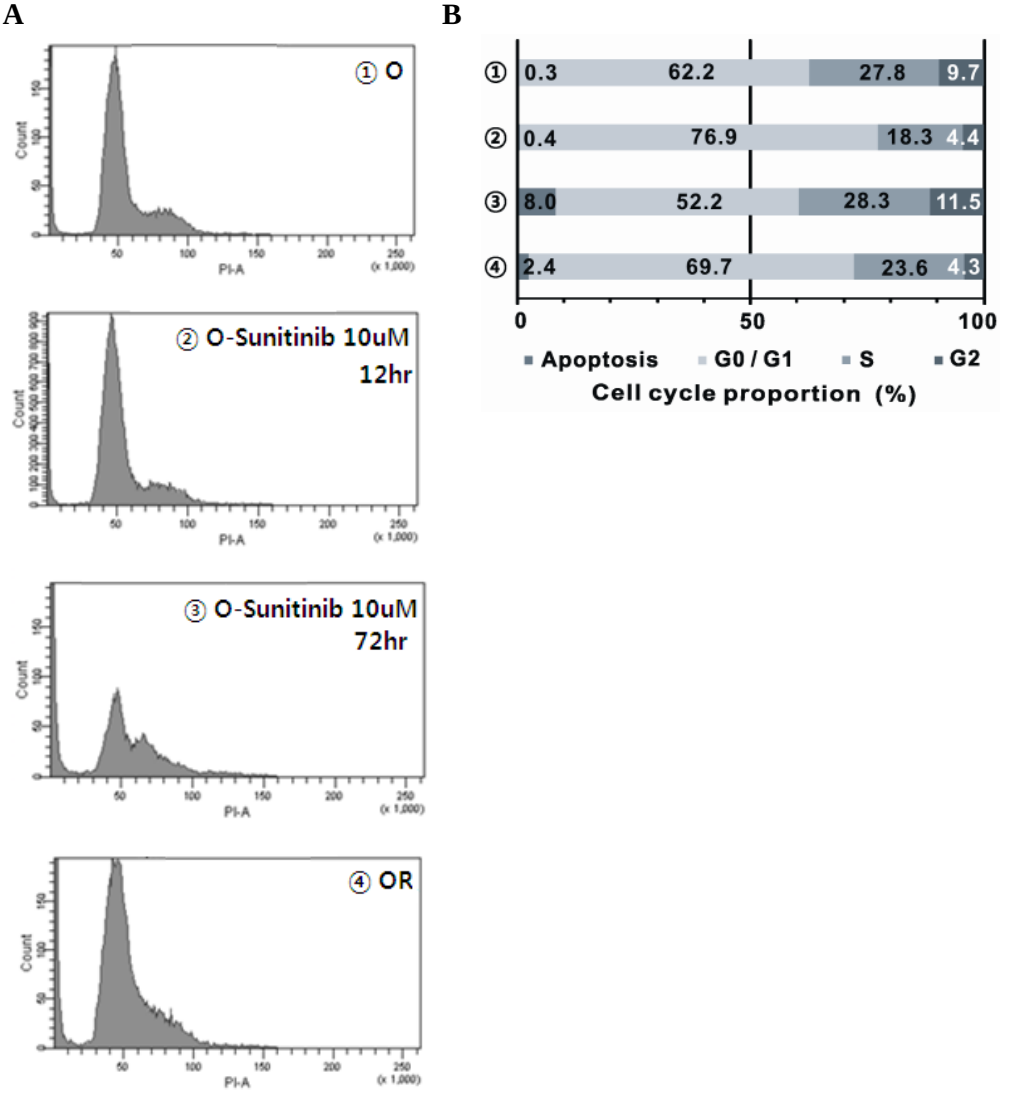
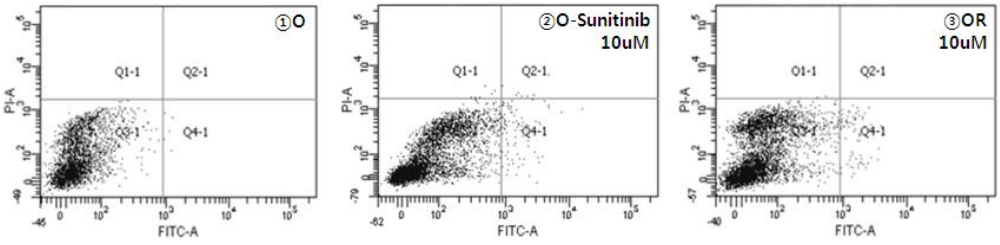
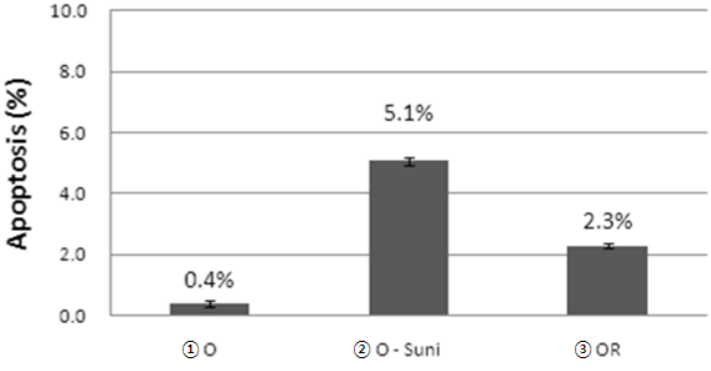


Figure. 2. Decreased cell cycle arrest in OR cells. (A) To evaluate the effect of sunitinib on cell cycle regulation, FACS analysis was performed in O and OR cells. O cells were treated with 10 μM of sunitinib for 12 h and 72 h and compared with OR cells. (B) The proportion of each cell cycle phase was quantified. Data are expressed as percent total cells. Means ± SD of three independent experiments are shown.

A


①O
②O-Sunitinib 10uM
③OR 10uM
Q1-1
Q2-1
Q3-1
Q4-1
PI-A
FITC-A

B


Apoptosis (%)
10.0
8.0
6.0
4.0
2.0
0.0
0.4%
5.1%
2.3%
① O
② O - Suni
③ OR

C

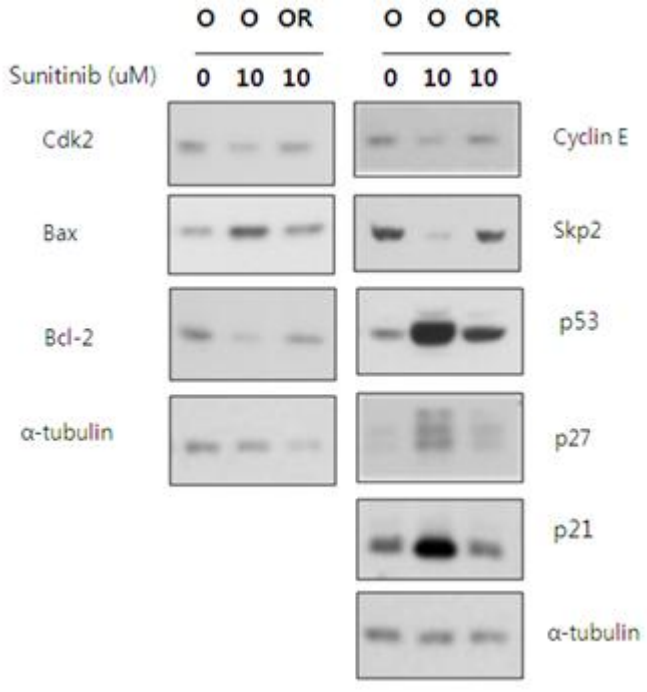
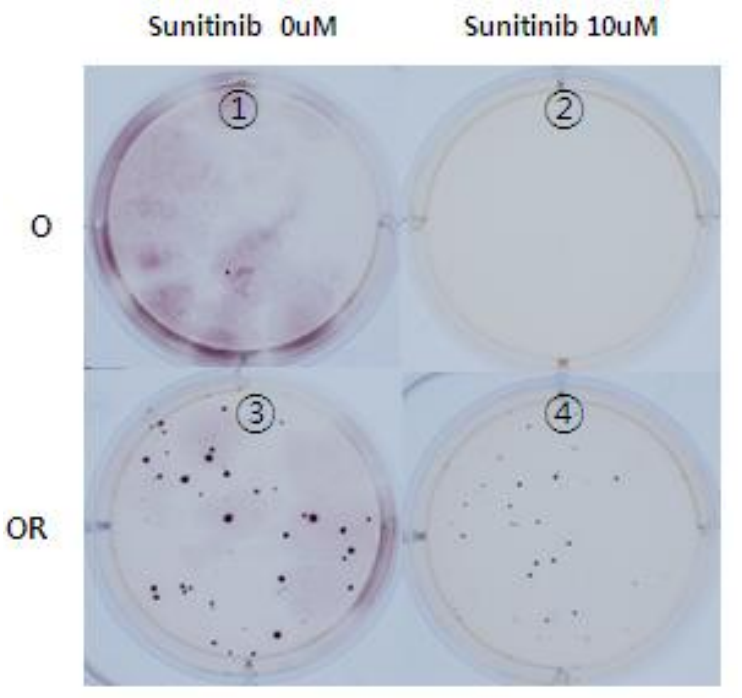

O O OR
O O OR
Sunitinib (uM)
0 10 10
0 10 10
Cdk2
Bax
Bcl-2
α-tubulin
Cyclin E
Skp2
p53
p27
p21
α-tubulin

Figure 3. Decreased apoptosis rate in OR cells. (A) To evaluate the effect of sunitinib on apoptosis, FACS analysis with annexin V/PI staining was performed in O and OR cells. O cells were treated with 10 μM of sunitinib for 24 h and compared with OR cells. (B) Data are expressed as % apoptotic cells. Means ± SD of three independent experiments are shown. (C) Western blot analysis was performed for proteins that are associated with the cell cycle and apoptosis. O cells were treated with 10 μM of sunitinib for 24 h and compared with OR cells. The levels of GAPDH or α-tubulin were used as loading controls.

3. The anchorage-independent growth ability of OR cells

To evaluate the effect of sunitinib on the anchorage-independent growth of O and OR cells, the soft agar colony formation assay was performed. In the presence of sunitinib (10 μM), the colony-forming ability of O cells was almost abolished. OR cells formed much higher numbers of colonies than O cells in the absence of sunitinib, and they showed high colony-forming ability even in the presence of sunitinib (Figure 4A and B). These findings show that OR cells acquired higher anchorage-independent growth ability, which is an important cancer-specific characteristic. Thus OR cells exhibited more aggressive cancer characteristics.

A


Sunitinib 0uM
Sunitinib 10uM
O
OR
①
②
③
④

B

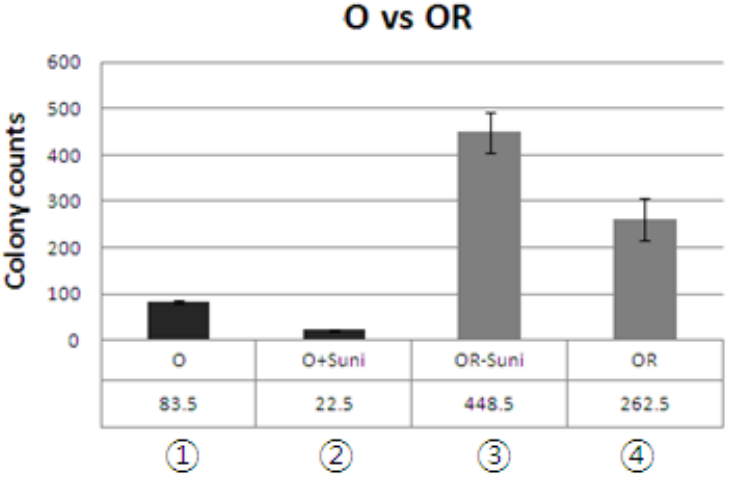

O vs OR
Colony counts
600
500
400
300
200
100
0
O
O+Suni
OR-Suni
OR
83.5
22.5
448.5
262.5
①
②
③
④

Figure 4. Soft agar colony formation assay. O and OR cells were seeded in 6-well plates containing 0.5% low-melting agarose over a 0.3% agarose layer, and they were incubated for 14 days in the absence or presence of sunitinib. (A), Colonies were observed after iodonitrotetrazolium staining and (B) counted under a microscope at a magnification of 40×. Data are the means ± SD of three independent experiments.

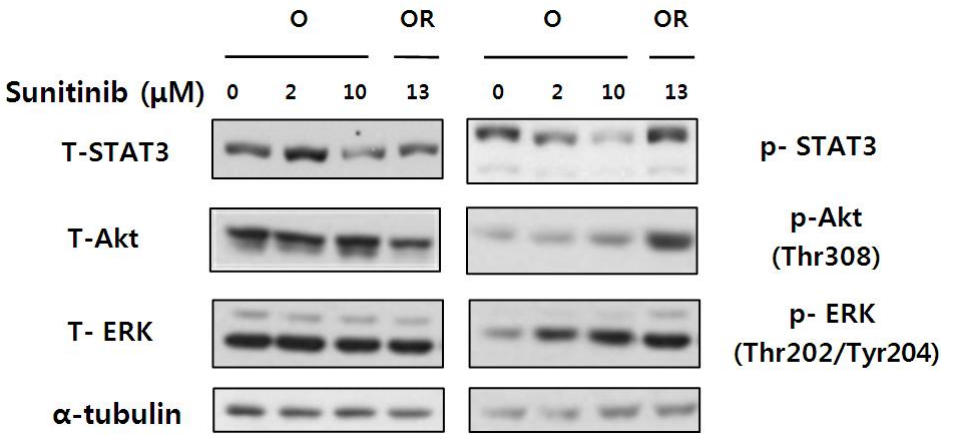
4. Stat3 is important for the survival of O cells against sunitinib treatment

To determine which signaling pathway plays a key role in developing sunitinib resistance, major candidate signals were assessed in O cells that were treated with various doses of sunitinib, as well as in OR cells that were maintained in sunitinib (13 μM). In O cells, 10 μM sunitinib inhibited the phosphorylation of Stat3 to very low levels, while phospho-Stat3 levels remained high even with 13 μM sunitinib in OR cells. However, phosphorylation of Akt and ERK was increased both in sunitinib-treated O and OR cells (Figure 5A).

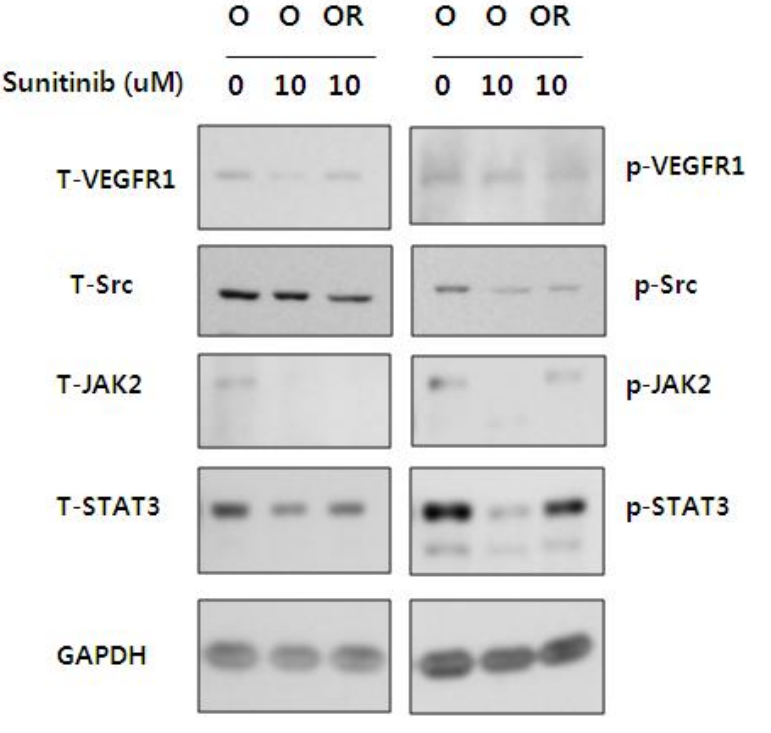
To evaluate changes in the upstream signaling factors of Stat3, western blot analysis was performed. OR cells exhibited increased phosphorylation of JAK2. However, the phosphorylation of neither Src, which is another upstream signal of Stat3, nor VEGFR1 was increased, even though both are known targets of sunitinib (Figure 5B).

To assess the importance of Stat3 signaling in the survival of O cells, cell viability was evaluated in cells that were treated with a Stat3 inhibitor, WP1066. Treatment with WP1066 almost completely inhibited the phosphorylation of Stat3 without changing its total level (Figure 5C), and it severely reduced cell viability (Figure 5D). Inhibition of Stat3 decreased levels of cyclin E, a cell cycle-associated protein, and increased the levels of p53 and other cell cycle inhibitors, such as p27 and p21. In other words, Stat3 inhibition induced cell cycle arrest in O cells (Figure 5C). These results demonstrate that Stat3 signaling played a critical role in the survival of O cells, and provide evidence showing that maintenance of Stat3 signaling may serve as a key modulator for OR cells to survive against sunitinib.

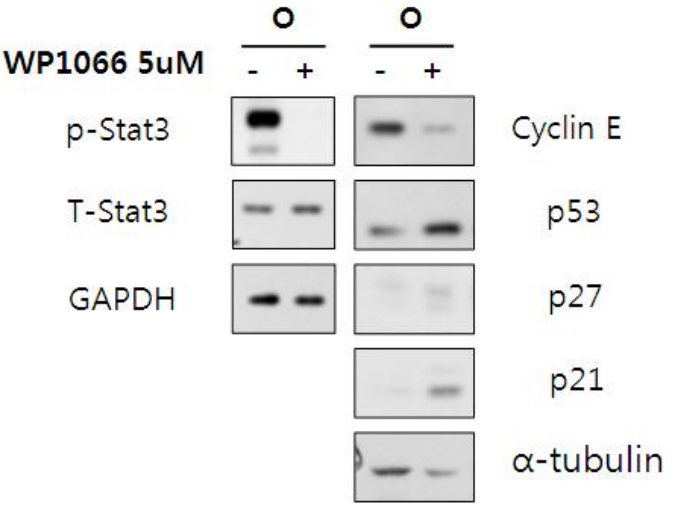
A


O
OR
O
OR
Sunitinib (μM)
0
2
10
13
0
2
10
13
T-STAT3
p- STAT3
T-Akt
p-Akt
(Thr308)
T- ERK
p- ERK
(Thr202/Tyr204)
α-tubulin

B


O
O
OR
O
O
OR
Sunitinib (uM)
0
10
10
0
10
10
T-VEGFR1
p-VEGFR1
T-Src
p-Src
T-JAK2
p-JAK2
T-STAT3
p-STAT3
GAPDH

C


WP1066 5uM
- + - +
p-Stat3
T-Stat3
GAPDH
Cyclin E
p53
p27
p21
α-tubulin

D.

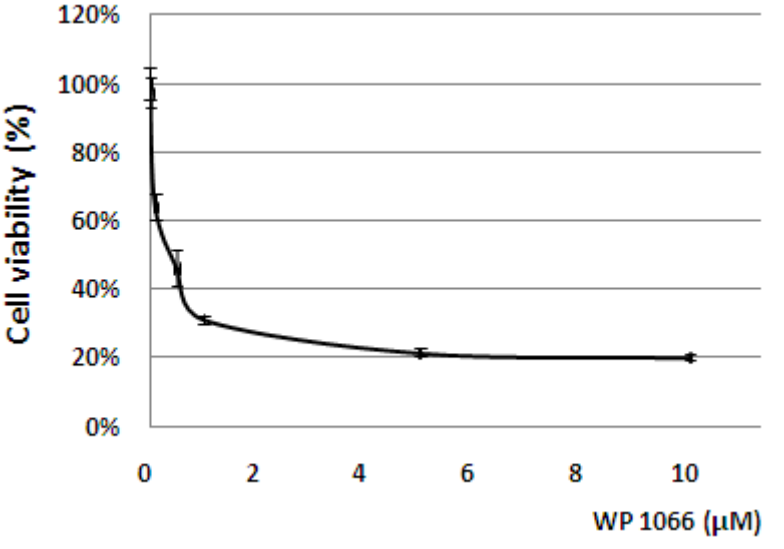

120%
100%
80%
60%
40%
20%
0%
Cell viability (%)
0
2
4
6
8
10
WP 1066 (μM)

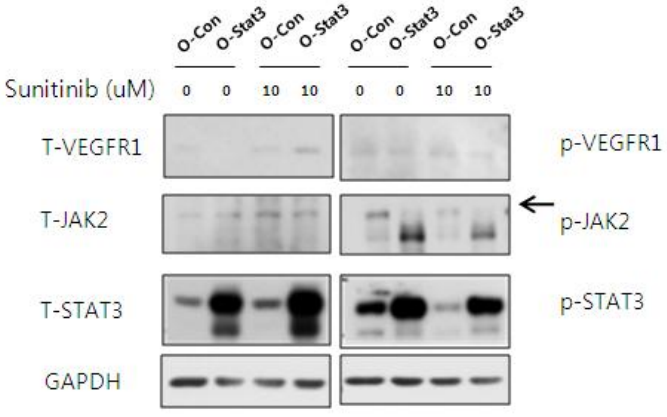
Figure 5. Stat3 signaling is important for O cells to have sunitinib resistance. (A) The major candidate target signals of TKIs (Stat3, Akt and ERK), the target cytosolic signaling proteins of Src/Stat3 or JAK2/Stat3, and the components of the PI3K/Akt/mTORC and RAS/RAF/ERK signaling pathways were evaluated by western blot analysis. (B) Stat3, Src, JAK2, and VEGFR1 (target protein of sunitinib) were also evaluated. O cells were treated with different concentrations of sunitinib for 24 h and compared with OR cells. (C) Western blot analysis for cell cycle proteins in Stat3-inhibited O cells using 5 μM WP1066 (Stat3 inhibitor) for 24 h. The levels of α-tubulin and GAPDH were used as a loading control. (D) Cell viability was measured by the MTT assay after treatment of WP1066. Cells were plated in 96-well culture plates and treated with different concentrations of WP1066 (0-10 μM) for 3 days, after which the assay was performed.

5. Stat3 overexpression induces sunitinib resistance in O cells

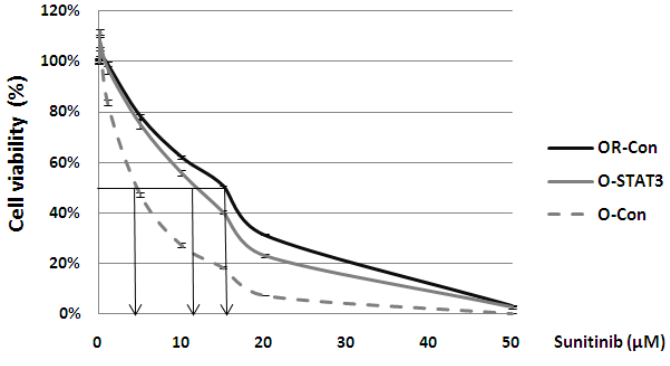
Because the inhibition of Stat3 severely affected cell viability, and sunitinib inhibited Stat3 phosphorylation, we examined whether forced Stat3 activation may contribute to the rescue from sunitinib-induced cell cycle arrest and apoptosis. When Stat3 was exogenously overexpressed, phospho-Stat3 was markedly increased even in the presence of sunitinib, and upstream phospho-JAK2 was decreased, thus indicating that overexpression of Stat3 is sufficient to induce Stat3 activation (Figure 6A). The activation of Stat3 in O cells drastically increased the IC_{50} to a level that was similar to that in OR cells. This suggests that the activation of Stat3 is sufficient to induce resistance to sunitinib in O cells (Figure 6B).

Next, the levels of proteins associated with cell cycle progression and apoptosis were evaluated. The forced expression of Stat3 (O-Stat3) prevented the sunitinib-induced down-regulation and up-regulation of positive (cyclin E, cdk2, and Skp2) and negative (p21, p27, and p53) cell cycle regulators, respectively. Likewise, the level of Bcl-2 was up-regulated, and the level of Bax was down-regulated (Figure 6C). To monitor the effect of Stat3 overexpression on apoptosis, FACS analysis using annexin V staining was performed. In the absence of sunitinib, the proportions of apoptotic cells were very low (0.3%), and no effect was observed with Stat3 overexpression both in control and Stat3-overexpressed cells. However, in the presence of sunitinib, apoptosis was induced up to 5.0%, and Stat3 overexpression significantly suppressed the percentage of apoptotic cells (2.6%) (Figure 6D and E). These results show that Stat3 overexpression prevented sunitinib-induced cell cycle arrest and apoptosis, thus leading to sunitinib resistance in the renal cell carcinoma cell line. Furthermore, the findings suggest that sunitinib resistance may be acquired via modulations in intracellular Stat3 signaling.

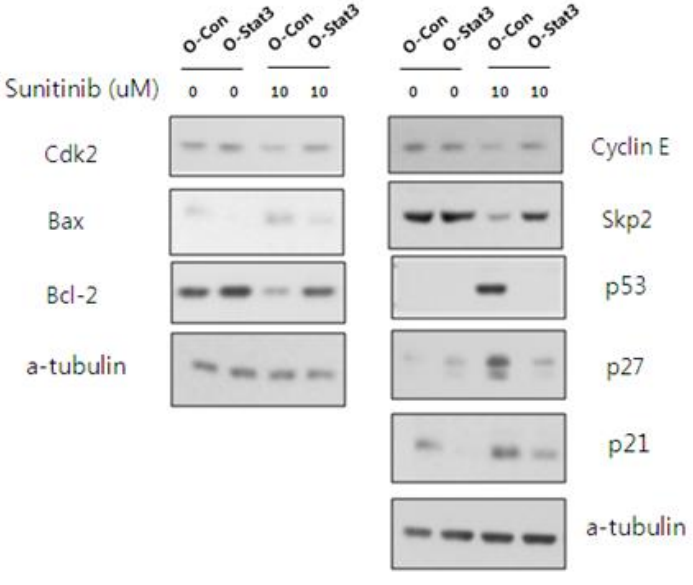
A


O-Con
O-Stat3
O-Con
O-Stat3
O-Con
O-Stat3
O-Con
O-Stat3
Sunitinib (uM)
0 0 10 10 0 0 10 10
T-VEGFR1
p-VEGFR1
T-JAK2
p-JAK2
T-STAT3
p-STAT3
GAPDH

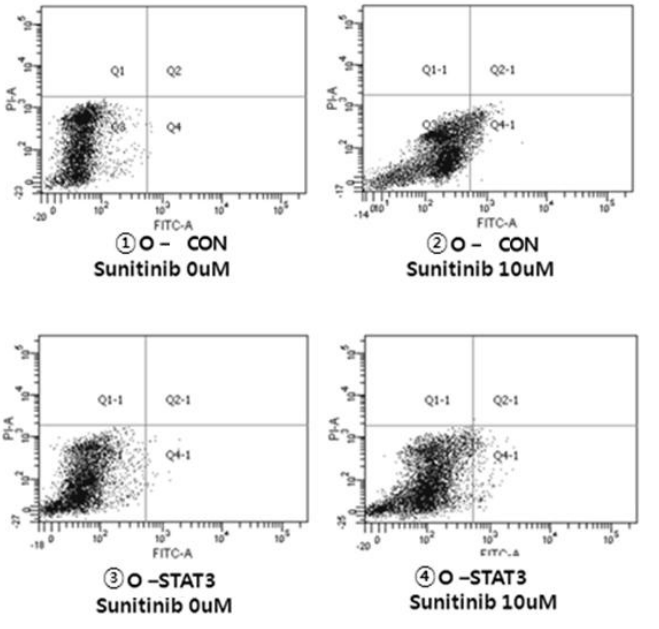
B


120%
100%
80%
60%
40%
20%
0%
Cell viability (%)
0
10
20
30
40
50
Sunitinib (μM)
OR-Con
O-STAT3
O-Con

C


O-Con
O-Stat3
O-Con
O-Stat3
O-Con
O-Stat3
O-Con
O-Stat3
Sunitinib (uM)
0 0 10 10 0 0 10 10
Cdk2
Bax
Bcl-2
a-tubulin
Cyclin E
Skp2
p53
p27
p21
a-tubulin

D


Q1
Q2
Q3
Q4
PI-A
FITC-A
① O – CON
Sunitinib 0uM
Q1-1
Q2-1
Q4-1
② O – CON
Sunitinib 10uM
③ O –STAT3
Sunitinib 0uM
④ O –STAT3
Sunitinib 10uM

E

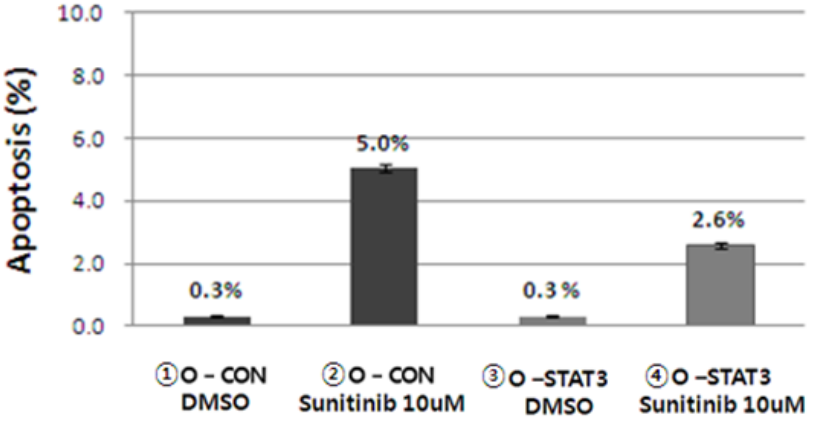

Apoptosis (%)
10.0
8.0
6.0
4.0
2.0
0.0
0.3%
5.0%
0.3 %
2.6%
① O – CON
DMSO
② O – CON
Sunitinib 10uM
③ O –STAT3
DMSO
④ O –STAT3
Sunitinib 10uM

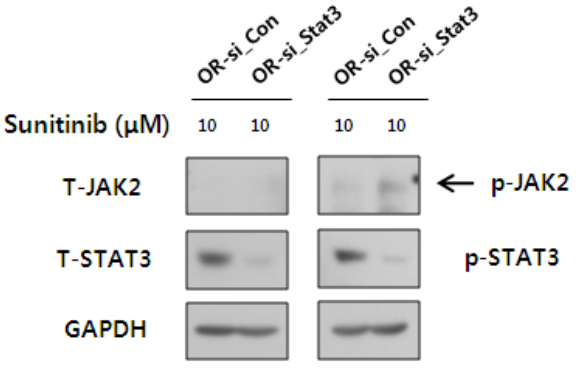
Figure 6. Effect of Stat3 overexpression on sunitinib resistance in O cells. (A, C) O cells were transiently transfected with either a Stat3-expressing (O-Stat3) or an empty vector (O-Con). After treatment with sunitinib for 24 h, the levels of the indicated proteins were evaluated by western blot analysis. GAPDH or α-tubulin was used as a loading control. (B) The MTT assay was performed for measurement of IC_{50}. Cells were plated in 96-well culture plates and treated with 10 μM of sunitinib for 3 days, after which the IC_{50} was measured. The cell viability was assessed according to the relative absorbance. Experiments were performed in triplicate. (D) To evaluate the effect of Stat3 overexpression on apoptosis, FACS analysis with annexin V/PI staining was performed. O-Stat3 and O-Con cells were treated with 10 μM sunitinib for 24 h. (E) Data are expressed as % apoptotic cells. The cell viability was represented with the relative absorbance. Experiments were performed in triplicate.

6. Stat3 knockdown sensitizes OR cells to sunitinib

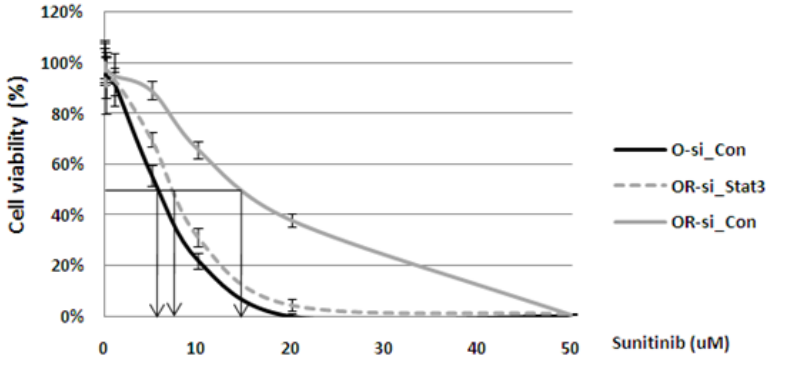
To evaluate whether Stat3 activation plays a critical role in sunitinib resistance, Stat3 was knocked down in OR cell lines using siRNA. Stat3 siRNA-transfected cells expressed decreased levels of phospho-Stat3, indicating that knockdown of Stat3 is sufficient to induce Stat3 down-regulation (Figure 7A). The knockdown of Stat3 markedly decreased the IC_{50} to levels that were similar to that of O cells, thus suggesting that Stat3 knockdown is sufficient to deprive sunitinib resistance in OR cells (Figure 7B). Next, the levels of proteins associated with cell cycle progression and apoptosis were measured (Figure 7C). Knockdown of Stat3 down-regulated the positive regulators of cell cycle (cyclin E, cdk2, and Skp2) and up-regulated the negative regulators (p21, p27, and p53). Similarly, the level of Bcl-2 was down-regulated, and the level of Bax was up-regulated. Knockdown of Stat3 markedly increased the proportion of apoptotic OR cells from 3.2% to 8.0% (Figure 7D and E).

These results show that Stat3 knockdown increased sunitinib-induced cell cycle arrest and apoptosis, leading to the deprivation of sunitinib resistance in OR cells. Stat3 activation may be an essential modulator of sunitinib resistance in cell lines that are resistant to the drug.

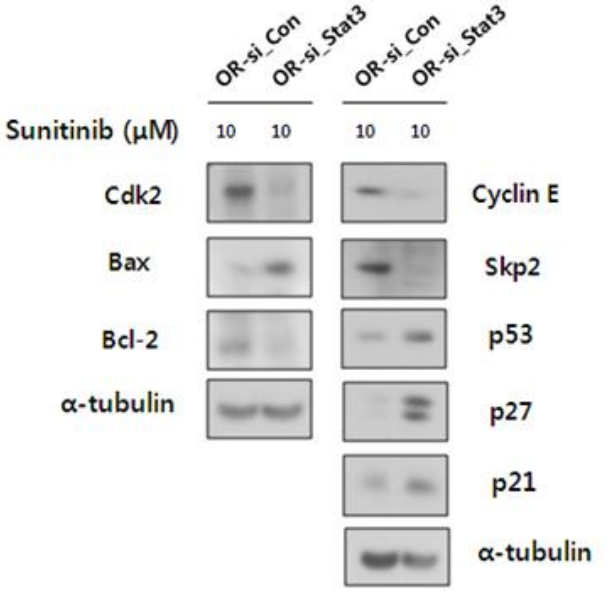
A.


OR-si_Con
OR-si_Stat3
OR-si_Con
OR-si_Stat3
Sunitinib (μM)
10
10
10
10
T-JAK2
p-JAK2
T-STAT3
p-STAT3
GAPDH

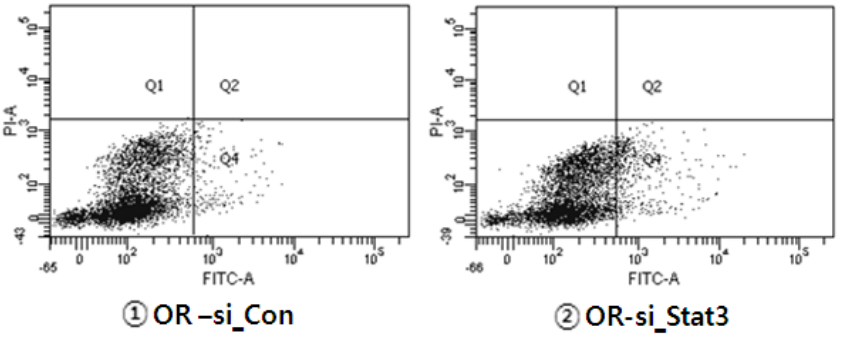
B.


120%
100%
80%
60%
40%
20%
0%
Cell viability (%)
0
10
20
30
40
50
Sunitinib (uM)
O-si_Con
OR-si_Stat3
OR-si_Con

C.


OR-si_Con
OR-si_Stat3
OR-si_Con
OR-si_Stat3
Sunitinib (μM)
10
10
10
10
Cdk2
Cyclin E
Bax
Skp2
Bcl-2
p53
α-tubulin
p27
p21
α-tubulin

D.


Q1
Q2
Q4
PI-A
FITC-A
① OR –si_Con
② OR-si_Stat3

E.

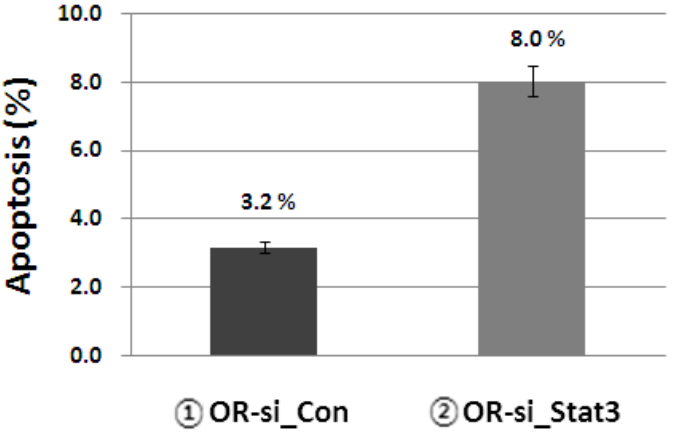

10.0
8.0
6.0
4.0
2.0
0.0
Apoptosis (%)
3.2 %
8.0 %
① OR-si_Con
② OR-si_Stat3

Figure 7. Effect of Stat3 knockdown on sunitinib sensitivity in OR cells. (A, C) OR cells were transiently transfected with siRNA against Stat3 (OR-si_Stat3) or control scrambled siRNA (OR-si_Con). Cells were maintained in the presence of 10 μM sunitinib, and the levels of the indicated proteins were evaluated by western blot analysis. GAPDH or α-tubulin was used as a loading control. (B) The MTT assay was performed for the measurement of IC_{50}. OR-siStat3, OR-si_Con, and control scrambled siRNA-transfected O cells (O-si_Con) were plated in 96-well culture plates and treated with 10 μM sunitinib for 3 days, after which the IC_{50} was measured. The cell viability was assessed according to the relative absorbance. Experiments were performed in triplicate. (D) To evaluate the effect of Stat3 knockdown on apoptosis, FACS analysis with annexin V/PI staining was performed. In OR-si_Stat3 and OR-si_Con cells that were under maintainence of 10 μM sunitinib were analyzed. (E) Data are expressed as % apoptotic cells. The results represent a relative cell number using means ± SD of percent OD. Experiments were performed in triplicate.

7. Increased IL-6 expression in OR cells and IL-6/JAK2 pathway as an escape pathway to sunitinib

We showed that the JAK2/Stat3 signaling pathway was activated in OR cells (Figure 5B). To evaluate the upstream signaling factors that may be involved, cytokines and growth factors associated with glycoprotein 130 (gp130), from which JAK2/Stat3 pathway gets the growth signal, were evaluated[27]. IL-6, which activates the JAK2 pathway via a receptor that forms a complex with gp130, is known to play a role in cancer cell survival[28-31]. In OR cells, the mRNA expression of IL-6 was high, compared with that in O cells. No significant changes in the levels of other cytokines and growth factors were observed (Figure 8A). These results suggest that IL-6 secretion in OR cells may play a role in the activation of JAK2/Stat3 signaling via autocrine and paracrine manners.

To address whether IL-6/JAK2 signaling transduction is affected by sunitinib, we evaluated the inhibitory effect of sunitinib on IL-6-induced JAK2 phosphorylation using western blot analysis. IL-6-induced JAK2 and Stat3 phosphorylation was not completely inhibited even in the presence of sunitinib, whereas overall JAK2 phosphorylation was decreased by sunitinib without IL-6 stimulation (Figure 8B). These results provide the possibility that IL-6/JAK2 signaling may serve as an alternative pathway that inhibits the anti-cancer effects of sunitinib.

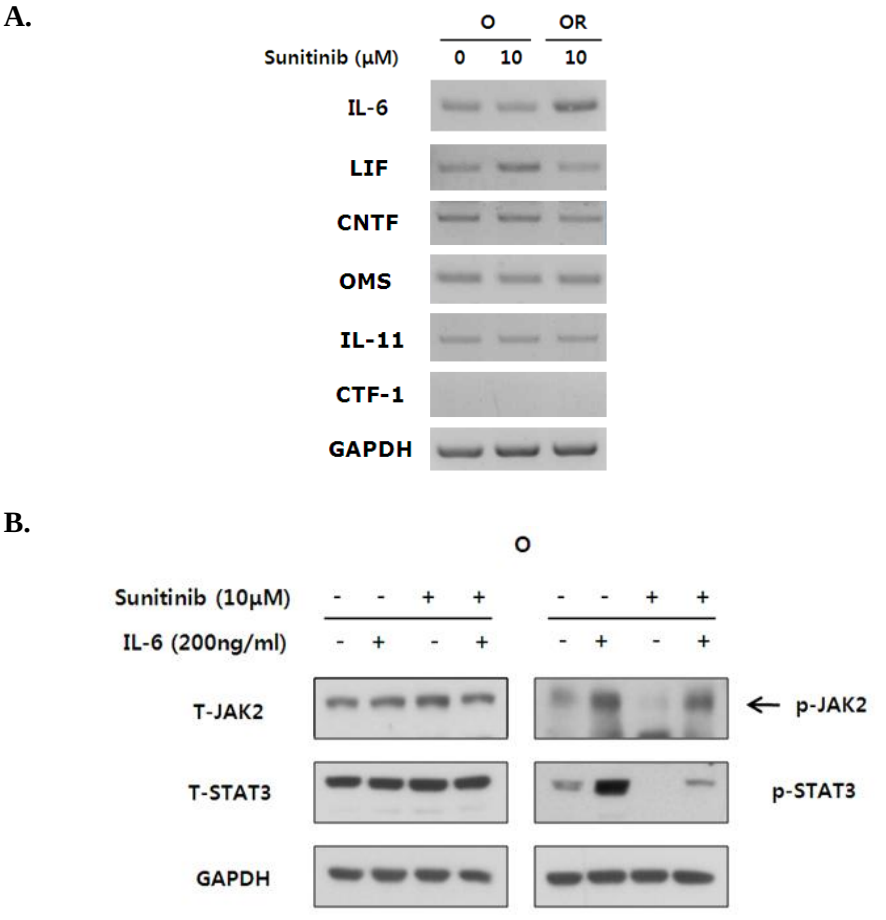


Figure 8. Effect of sunitinib on IL-6-induced JAK2 phosphorylation. (A) The mRNA levels of candidate gp130-associated cytokines and growth factors were analyzed by RT-PCR in O and OR cells. Interleukin-6 (IL-6), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), interleukin-11 (IL-11), and cardiotrophin-1 (CTF-1) were evaluated. (B) O cells were treated with sunitinib for 24 h, after which they were incubated with 200 ng/ml recombinant human IL-6 to stimulate JAK2 for 30 min. The levels of JAK2 and Stat3 proteins were evaluated by western blot analysis. GAPDH was used as a loading control.

8. Increased nuclear translocation of PKM2 in OR cells

PKM2 is one of the protein kinases that phosphorylate Stat3, which is translocated to the nucleus by cell growth signals[25]. PKM2 levels were measured in nuclear (NE) and cytoplasmic (Cyto) fractions of O and OR cells (Figure 9). Treatment with sunitinib (10 μM) decreased nuclear PKM2 levels in O cells and maintained high levels of nuclear PKM2 in OR cells. Interestingly, the nuclear levels of phosphorylated Stat3 were consistent with the nuclear PKM2 levels in both O and OR cells. This suggests that increased nuclear PKM2 may act as a protein kinase to phosphorylate Stat3 in OR cells.

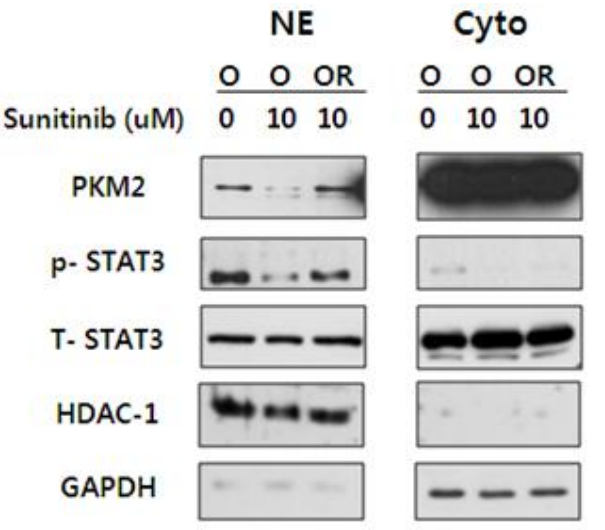


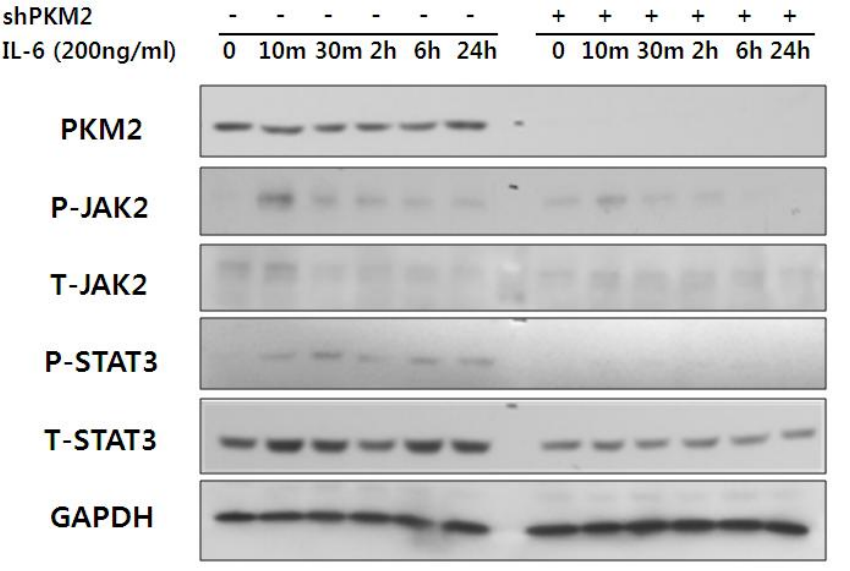
Figure 9. Stat3 phosphorylation and PKM2 nuclear translocation in sunitinib-resistant cells. Western blot analysis was performed, and protein levels were measured in nuclear (NE) and cytoplasmic (Cyto) fractions. The levels of HDAC-1 and GAPDH were used as nuclear and cytoplasmic loading controls, respectively.

9. Induction of JAK2 phosphorylation, followed by PKM2 phosphorylation and nuclear translocation

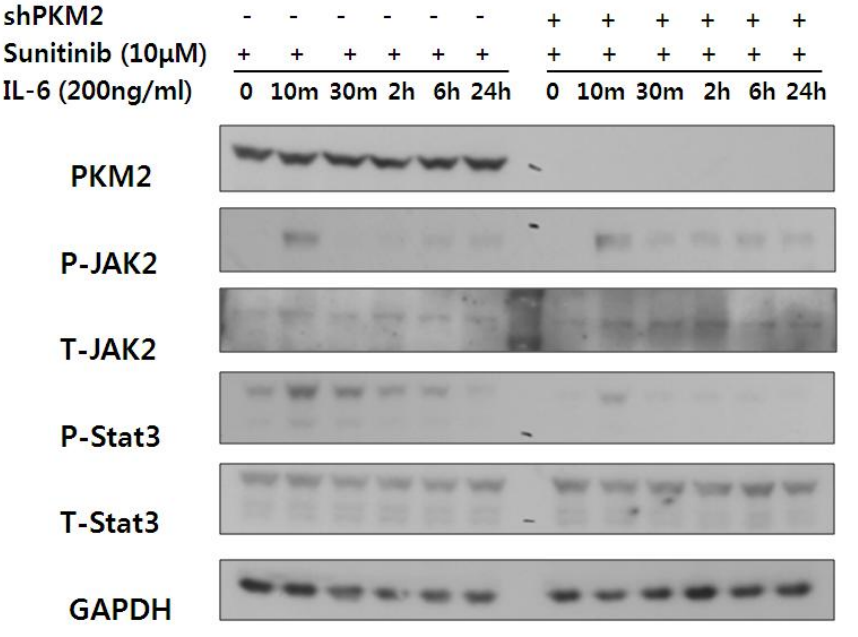
JAK2 activation by growth stimulation promotes the phosphorylation and nuclear translocation of PKM2[25]. We evaluated the relationships between JAK2, PKM2, and Stat3 activation. The general responses of PKM2 and Stat3 protein to IL-6/JAK2 stimulation in O and OR cells were similar. IL-6 induced the phosphorylation of JAK2 within 10 min. Phosphorylation of Stat3 was also increased after IL-6 treatment. However, PKM2 knockdown in O and OR cells prevented IL-6-stimulated Stat3 phosphorylation, even in cases in which JAK2 was phosphorylated (Figure 10A-1 and A-2). PKM2 knockdown also significantly reduced the IC_{50}, resulting in the increased sensitivity to sunitinib in OR cells (Figure 10B).

In addition, we prepared the nuclear extract to address whether phosphorylated PKM2 translocates to the nucleus and increases Stat3 phosphorylation under IL-6/JAK2 stimulation. In the control group, PKM2 was strongly phosphorylated by IL-6/JAK2 stimulation, and it translocated to the nucleus in both O and OR cells. Nuclear Stat3 phosphorylation was increased in the same pattern as that of PKM2 phosphorylation and nuclear translocation. On the contrary, PKM2 knockdown significantly inhibited the IL-6-induced phosphorylation of Stat3 (Figure 10C). These findings indicate that PKM2 acts as a transcriptional co-activator of Stat3 by maintaining Stat3 phosphorylation. Furthermore, this suggests that the increased nuclear level of PKM2 contributes to sunitinib resistance by increasing the expression of Stat3-target genes.

A-1 **O**


shPKM2
IL-6 (200ng/ml)
0 10m 30m 2h 6h 24h
0 10m 30m 2h 6h 24h
PKM2
P-JAK2
T-JAK2
P-STAT3
T-STAT3
GAPDH

A-2 **OR**


shPKM2
Sunitinib (10μM)
IL-6 (200ng/ml)
0 10m 30m 2h 6h 24h
0 10m 30m 2h 6h 24h
PKM2
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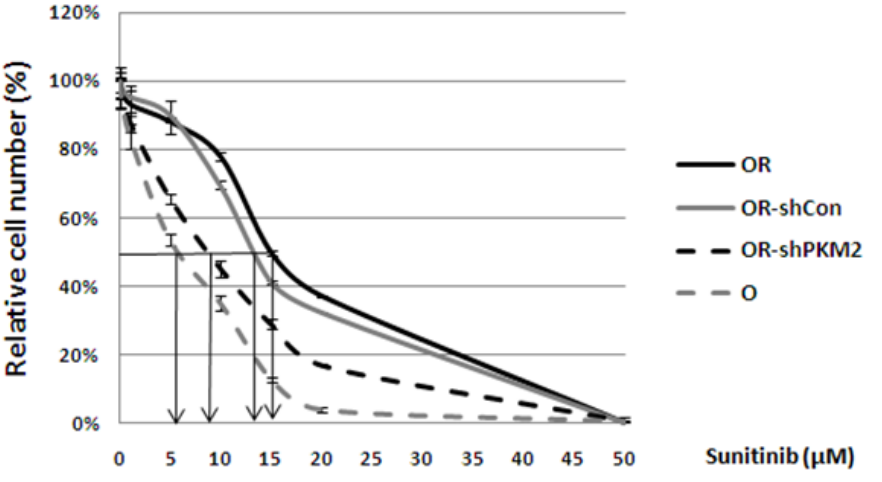
T-JAK2
P-Stat3
T-Stat3
GAPDH

B


120%
100%
80%
60%
40%
20%
0%
Relative cell number (%)
0
5
10
15
20
25
30
35
40
45
50
Sunitinib (μM)
OR
OR-shCon
OR-shPKM2
O

C

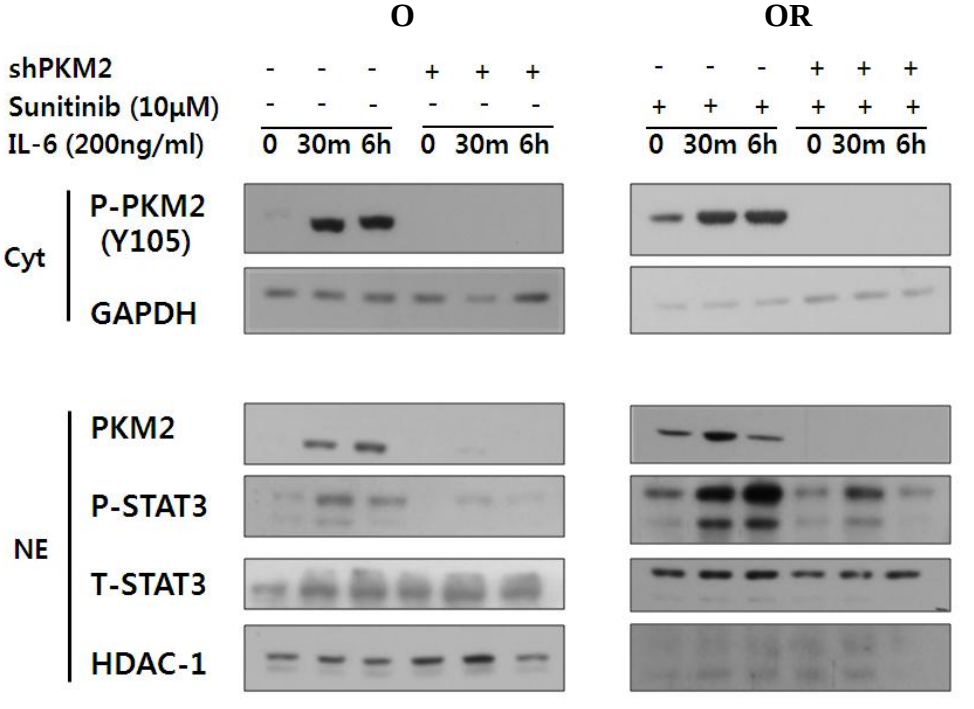

O
OR
shPKM2
Sunitinib (10μM)
IL-6 (200ng/ml)
0 30m 6h 0 30m 6h
0 30m 6h 0 30m 6h
Cyt
P-PKM2 (Y105)
GAPDH
NE
PKM2
P-STAT3
T-STAT3
HDAC-1

Figure 10. Induction of JAK2 phosphorylation, followed by PKM2 phosphorylation and nuclear translocation. Knockdown of PKM2 in O and OR cells were generated by virus infection using shPKM2 RNA. (A-1,2,C) Cells were treated with recombinant human IL-6 for different durations to stimulate JAK2. Western blot analysis was performed, and protein levels were measured in whole cell lysates, nuclear extracts (NE) or cytoplasmic extracts (Cyto). The levels of HDAC-1 and GAPDH were used as nuclear and cytoplasmic loading controls, respectively. (B) The MTT assay was performed to evaluate the effect of PKM2 expression on sunitinib resistance. The cell viability was assessed according to the relative absorbance. Experiments were performed in triplicates.

10. Stat3 activation contributes to sunitinib resistance by regulating cell proliferation and apoptosis via alternative escape IL-6/JAK2/Stat3 pathway which modulated by PKM2 as a Stat3 transcriptional co-activator.

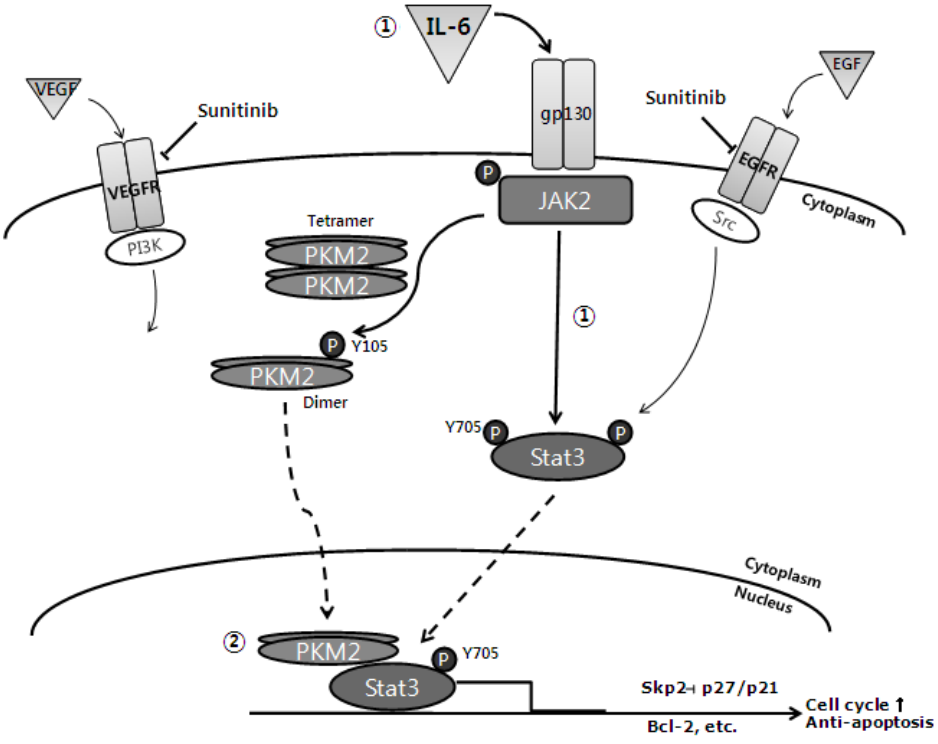


Figure 11. A schematic of the proposed mechanism of sunitinib resistance in the renal cell carcinoma cell line. ① Alternative escape pathway involving IL-6/JAK2/Stat3 activation and ② positive modulation of Stat3 transcriptional activity by PKM2 are depicted.

IV. DISCUSSION

In this study, we found that Stat3 was activated by IL-6/JAK2, and this was accompanied by an increase in the nuclear level of PKM2, which contributes to sunitinib resistance in the clear cell renal cell carcinoma cell line. The JAK2/Stat3 signal was uniquely inhibited by sunitinib treatment in O cells, whereas it was maintained at high levels in the sunitinib-resistant cell line. Subsequent experiments showed that activated Stat3 regulated cell cycle-associated proteins (skp2, p21, p27, cyclin E, and cdk2) and apoptosis-associated proteins (p53, Bax, and Bcl-2). In addition, it promoted cell proliferation and prevented apoptosis in the sunitinib-resistant cell line. The findings that Stat3 knockdown in sunitinib resistant cell line sensitized the sunitinib resistant cell line to sunitinib strongly suggest that Stat3 activation is important for sunitinib resistance.

The mechanisms of sunitinib resistance in cancer cells are not well known. It has been previously suggested that, during anti-angiogenic treatment, the up-regulation of an alternative growth signaling pathway may be responsible for acquired drug resistance. This is due to the recruitment of alternative angiogenic circuits or direct tumor-stimulating activity[32]. Piscazzi et al. demonstrated that the EGF-mediated activation of ERK is relevant for the escape of thyroid cancer cells from the inhibitory effect of sunitinib[78]. Similarly, our study showed that activation of IL-6/JAK2/Stat3 acted as an escape mechanism against sunitinib in the sunitinib-resistant cell line. We revealed that IL-6 expression in sunitinib-resistant cells was greater than that of parental cells, and the JAK2/Stat3 activation pathway was less inhibited by sunitinib in the sunitinib-resistant cell line. This suggested that the IL-6/JAK2/Stat3 signaling pathway could be an alternative escape pathway for cancer survival signals in sunitinib-resistant cell lines, and activation of this signaling pathway contributes to sunitinib resistance in the renal cell carcinoma cell line.

IL-6 plays an important role in cancer progression and metastasis[28-31], and it is also a chemo-resistant factor in various cancers[33-35]. Previously, Nishioka et al. reported an increase in IL-6 expression in the sunitinib-resistant acute myeloblastic leukemia cell line. They showed that sunitinib fails to inactivate JAK2 signaling in the sunitinib-resistant cell lines unlikely in the parental cell lines. They confirmed that enhanced up-regulation of the IL-6/JAK2/Stat5 compensatory pathway contributes to sunitinib resistance in the sunitinib-resistant cell line[36].

Stat3 is constitutively active in various signaling pathways, and activated Stat3 contributes to the survival of various cancers[11,18-20]. Although Stat3 signaling is known to play an oncogenic role in many cancers, it also participates in the resistance to anti-cancer drugs. Wang et al. reported that Stat3 mediates the resistance of breast cancer stem cells to tamoxifen. Constitutively activated Stat3 modulates cell cycle and apoptotic processes. Knockdown of Stat3 in the resistant cell line also increases the sensitivity to tamoxifen[37]. In addition, Chiu et al. showed that suppression of Stat3 activity sensitizes gefitinib-resistant non-small cell lung cancer cells (NSCLC), and Stat3 inhibition increases doxorubicin sensitivity in NSCLC cells by regulating the p53-mediated apoptotic process[38]. Sims et al. demonstrated that doxorubicin resistance can be reversed by preventing Stat3 phosphorylation in doxorubicin-resistant melanoma and breast cancer cells[39]. These studies suggest that Stat3 inhibition could be an alternative strategy to treat cancer patients who demonstrate resistance to first-line anti-cancer drugs.

The role of Stat3 activation is not well-characterized in sunitinib resistance, especially in renal cell carcinoma. In this study, we demonstrated that Stat3 activation was a key modulator of sunitinib resistance in a renal cell carcinoma cell line, where it promoted cell proliferation and reduces apoptosis. This suggests that targeting Stat3 may be a new strategy to overcome the resistance to the anti-cancer cell effect of sunitinib.

The elevated nuclear level of PKM2 following JAK2 activation in the sunitinib-resistant cell line is also thought to play an important role in the maintenance of Stat3 phosphorylation in the nucleus. PKM2 is known to be a glycolysis enzyme, pyruvate kinase. In a cancer cell, PKM2 has dual roles in cancer metabolism. First, it regulates the ratio of energy/precursor substances and affects the cell proliferation speed. The conversion from dimer to tetramer states, which is present in many cancer cells, allows PKM2 to act as a protein kinase. This change may regulate the glycolysis rates and provide carbon sources for biosynthesis from accumulated glycolytic intermediates. As a result, cancer proliferation speed is regulated[40]. Second, PKM2 can act as a multifunctional signaling molecule in the nucleus. Gao et al. have shown that PKM2 can phosphorylate Stat3 and promote the transcription of MEK5[25]. PKM2 is also a transcriptional co-activator for HIF-1α, which is important for cancer cell growth[41]. It has also been reported to positively modulate the transcriptional activation of octamer-binding transcription factor 4[42].

The role of PKM2 in drug resistance has been rarely documented. We found that the sunitinib-resistant cell line expressed higher nuclear levels of PKM2 and increased IL-6/JAK2 activation, compared with that of sunitinib treated parental cells. The level of Stat3 phosphorylation was positively correlated with the nuclear level of PKM2. However, in both resistant and parental cell lines, similar increasing patterns of PKM2 nuclear translocation and Stat3 phosphorylation were noticed under conditions in which IL-6/JAK2 was stimulated. From these results, we could assume that the protein kinase ability of PKM2 in the parental and resistant cell lines were not different from each other, and the influence factor for increased Stat3 phosphorylation in resistant cell line was an increase in nuclear PKM2 levels, which was present only in the resistant cell line.

We also showed that PKM2 knockdown in the sunitinib-resistant cell line resulted in decreased levels of phosphorylated Stat3 in the nucleus, compared

with that in control. A previous study has shown that nuclear translocation form of PKM2 (R399E) directly binds to GST-phospho-Stat3[25]. Overall, nuclear PKM2 is thought to act as a transcriptional co-activator of Stat3 through maintaining the phosphorylation of Stat3 or inhibiting the dephosphorylation of Stat3.

Our findings suggest that Stat3 activation by IL-6/JAK2 activation is a key modulator of the sunitinib-resistant mechanism against its direct-anti-cancer cell effect in renal cell carcinoma cells. Moreover, the increased nuclear level of PKM2 also acts as a transcriptional co-activator of Stat3 to contribute to sunitinib resistance (Figure 8). The combined treatment with Stat3 inhibition may increase the sensitivity to sunitinib in sunitinib-resistant renal cell carcinoma patients. The limitations of this study are the absence of pathologic data and *in vivo* confirmation. Future *in vivo* experiments and the linkage of clinical data are required.

V. CONCLUSION

Our findings suggest that up-regulation of IL-6/JAK2 pathway and the increased nuclear PKM2 levels are the key modulators of constitutive Stat3 activation, which in turn contributes to the resistance against the direct anti-cancer effects of sunitinib in RCC cell lines as an escape mechanism.

REFERENCES

1. Lam JS, Shvarts O, Leppert JT, Figlin RA, Belldegrun AS. Renal cell carcinoma 2005: new frontiers in staging, prognostication and targeted molecular therapy. J Urol 2005;173:1853-62.
2. Rini BI. Vascular endothelial growth factor-targeted therapy in renal cell carcinoma: current status and future directions. Clinical cancer research 2007;13:1098-106.
3. Athar U, Gentile TC. Treatment options for metastatic renal cell carcinoma: a review. Can J Urol 2008;15:3954-66.
4. Gotink KJ, Verheul HM. Anti-angiogenic tyrosine kinase inhibitors: what is their mechanism of action? Angiogenesis 2010;13:1-14.
5. Faivre S, Demetri G, Sargent W, Raymond E. Molecular basis for sunitinib efficacy and future clinical development. Nat Rev Drug Discov 2007;6:734-45.
6. Andrae N, Kirches E, Hartig R, Haase D, Keilhoff G, Kalinski T, et al. Sunitinib targets PDGF-receptor and Flt3 and reduces survival and migration of human meningioma cells. Eur J Cancer 2012;48:1831-41.
7. Piscazzi A, Costantino E, Maddalena F, Natalicchio MI, Gerardi AM, Antonetti R, et al. Activation of the RAS/RAF/ERK signaling pathway contributes to resistance to sunitinib in thyroid carcinoma cell lines. J Clin Endocrinol Metab 2012;97:E898-906.
8. Seandel M, Shia J, Linkov I, Maki RG, Antonescu CR, Dupont J. The activity of sunitinib against gastrointestinal stromal tumor seems to be distinct from its antiangiogenic effects. Clin Cancer Res 2006;12:6203-4.
9. Aceves-Luquero CI, Agarwal A, Callejas-Valera JL, Arias-Gonzalez L, Esparis-Ogando A, del Peso Ovalle L, et al. ERK2, but not ERK1, mediates acquired and "de novo" resistance to imatinib mesylate:

implication for CML therapy. PLoS One 2009;4:e6124.

10. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 2012;486:532-6.
11. Xin H, Zhang C, Herrmann A, Du Y, Figlin R, Yu H. Sunitinib inhibition of Stat3 induces renal cell carcinoma tumor cell apoptosis and reduces immunosuppressive cells. Cancer Res 2009;69:2506-13.
12. Ravaud A, Gross-Goupil M. Overcoming resistance to tyrosine kinase inhibitors in renal cell carcinoma. Cancer Treat Rev 2012;38:996-1003.
13. Rini BI, Atkins MB. Resistance to targeted therapy in renal-cell carcinoma. Lancet oncology 2009;10:992-1000.
14. Nishioka C, Ikezoe T, Yang J, Yokoyama A. Long-term exposure of leukemia cells to multi-targeted tyrosine kinase inhibitor induces activations of AKT, ERK and STAT5 signaling via epigenetic silencing of the PTEN gene. Leukemia 2010;24:1631-40.
15. Makhov PB, Golovine K, Kutikov A, Teper E, Canter DJ, Simhan J, et al. Modulation of Akt/mTOR signaling overcomes sunitinib resistance in renal and prostate cancer cells. Mol Cancer Ther 2012;11:1510-7.
16. Yu H, Jove R. The STATs of cancer--new molecular targets come of age. Nat Rev Cancer 2004;4:97-105.
17. Song L, Turkson J, Karras JG, Jove R, Haura EB. Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. Oncogene 2003;22:4150-65.
18. Wei Z, Jiang X, Qiao H, Zhai B, Zhang L, Zhang Q, et al. STAT3 interacts with Skp2/p27/p21 pathway to regulate the motility and invasion of gastric cancer cells. Cell Signal 2013;25:931-8.
19. Xiong H, Zhang ZG, Tian XQ, Sun DF, Liang QC, Zhang YJ, et al. Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells.

Neoplasia 2008;10:287-97.

20. Fang Z, Tang Y, Fang J, Zhou Z, Xing Z, Guo Z, et al. Simvastatin inhibits renal cancer cell growth and metastasis via AKT/mTOR, ERK and JAK2/STAT3 pathway. PLoS One 2013;8:e62823.
21. Niu G, Wright KL, Ma Y, Wright GM, Huang M, Irby R, et al. Role of Stat3 in regulating p53 expression and function. Mol Cell Biol 2005;25:7432-40.
22. Ara T, Nakata R, Sheard MA, Shimada H, Buettner R, Groshen SG, et al. Critical Role of STAT3 in IL-6-Mediated Drug Resistance in Human Neuroblastoma. Cancer Res 2013;73:3852-64.
23. Gao X, Wang H, Yang JJ, Chen J, Jie J, Li L, et al. Reciprocal regulation of protein kinase and pyruvate kinase activities of pyruvate kinase M2 by growth signals. J Biol Chem 2013;288:15971-9.
24. Hitosugi T, Kang S, Vander Heiden MG, Chung TW, Elf S, Lythgoe K, et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. Sci Signal 2009;2:ra73.
25. Gao X, Wang H, Yang JJ, Liu X, Liu ZR. Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. Mol Cell 2012;45:598-609.
26. Kim HE, Bae E, Jeong DY, Kim MJ, Jin WJ, Park SW, et al. Lipin1 regulates PPARgamma transcriptional activity. Biochem J 2013;453:49-60.
27. Xu S, Neamati N. gp130: a promising drug target for cancer therapy. Expert Opin Ther Targets 2013;17:1303-28.
28. Na YR, Lee JS, Lee SJ, Seok SH. Interleukin-6-induced Twist and N-cadherin enhance melanoma cell metastasis. Melanoma Res 2013;23:434-43.
29. Nguyen DP, Li J, Tewari AK. Inflammation and prostate cancer: the role of interleukin-6. BJU Int 2013.

30. Tawara K, Oxford JT, Jorcyk CL. Clinical significance of interleukin (IL)-6 in cancer metastasis to bone: potential of anti-IL-6 therapies. Cancer Manag Res 2011;3:177-89.
31. Zarogoulidis P, Yarmus L, Darwiche K, Walter R, Huang H, Li Z, et al. Interleukin-6 cytokine: a multifunctional glycoprotein for cancer. Immunome Res 2013;9:16535.
32. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 2008;8:592-603.
33. Dijkgraaf EM, Welters MJ, Nortier JW, van der Burg SH, Kroep JR. Interleukin-6/interleukin-6 receptor pathway as a new therapy target in epithelial ovarian cancer. Curr Pharm Des 2012;18:3816-27.
34. Wang Y, Niu XL, Qu Y, Wu J, Zhu YQ, Sun WJ, et al. Autocrine production of interleukin-6 confers cisplatin and paclitaxel resistance in ovarian cancer cells. Cancer Lett 2010;295:110-23.
35. Yamada D, Kobayashi S, Wada H, Kawamoto K, Marubashi S, Eguchi H, et al. Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. Eur J Cancer 2013;49:1725-40.
36. Nishioka C, Ikezoe T, Yang J, Yokoyama A. Multitargeted tyrosine kinase inhibitor stimulates expression of IL-6 and activates JAK2/STAT5 signaling in acute myelogenous leukemia cells. Leukemia 2009;23:2304-8.
37. Wang X, Wang G, Zhao Y, Liu X, Ding Q, Shi J, et al. STAT3 mediates resistance of CD44(+)CD24(-/low) breast cancer stem cells to tamoxifen in vitro. J Biomed Res 2012;26:325-35.
38. Chiu HC, Chou DL, Huang CT, Lin WH, Lien TW, Yen KJ, et al. Suppression of Stat3 activity sensitizes gefitinib-resistant non small cell lung cancer cells. Biochem Pharmacol 2011;81:1263-70.
39. Sims JT, Ganguly SS, Bennett H, Friend JW, Tepe J, Plattner R.

Imatinib reverses doxorubicin resistance by affecting activation of STAT3-dependent NF-kappaB and HSP27/p38/AKT pathways and by inhibiting ABCB1. PLoS One 2013;8:e55509.

40. Wu S, Le H. Dual roles of PKM2 in cancer metabolism. Acta Biochim Biophys Sin (Shanghai) 2013;45:27-35.

41. Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 2011;145:732-44.

42. Lee J, Kim HK, Han YM, Kim J. Pyruvate kinase isozyme type M2 (PKM2) interacts and cooperates with Oct-4 in regulating transcription. Int J Biochem Cell Biol 2008;40:1043-54.

ABSTRACT (IN KOREAN)

신세포암 세포주에서 Stat3 signaling pathway에 의한

sunitinib 내성 매개 기전

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최 경 화

Sunitinib 은 전이성 투명 신세포암에서 1차 치료로 사용되는 multi-targeted tyrosine kinase inhibitor 이나 최근 sunitinib 약제 내성이 중요한 임상적 문제로 대두되고 있다. 본 연구에서는 6개월 간 sunitinib의 처리 농도를 증가시켜 배양하는 방법으로 786-O (O) 투명 신세포암 세포주를 이용한 sunitinib 내성 투명 신세포암 세포주 (OR)를 구축하여 sunitinib 내성 기전을 밝히기 위한 연구를 진행하였다. OR 세포주에서는 특징적으로 sunitinib 에 의한 Stat3와 상위 JAK2의 인산화 억제가 관찰되지 않았다. 또한 OR 세포주에서 이렇게 활성화된 Stat3에 의해 세포 주기 관련 단백 (skp2, p21, p27, Cyclin E,

Cdk2) 과 세포사멸 관련 단백 (p53, Bax, Bcl-2) 이 조절되며, 이를 통해 세포 성장이 촉진되고 세포 사멸이 억제되는 현상을 보임을 확인하였다. O 세포주에서 Stat3 과발현 시 세포고사가 감소하고 sunitinib 에 대한 내성을 획득하였으며, 반대로 OR 세포주에서 Stat3의 발현을 억제하였을 시 sunitinib 에 대한 내성을 잃고 O 세포주 수준으로 약제 감수성이 증가함을 확인하였다. 이를 통해 JAK2/Stat3 신호의 활성화가 sunitinib 내성에 중요한 역할을 함을 확인하였다. 또한 OR 세포주에서 JAK2/Stat3를 활성화하는 상위 세포 성장 신호인 IL-6의 mRNA 발현이 증가됨을 확인하였으며, 이러한 IL-6 에 의한 JAK2/Stat3 활성화 경로는 sunitinib에 의한 억제 효과를 덜 받는 회피 경로임을 확인하였다. OR 세포주에서 확인한 IL-6/JAK2 활성화와 이에 따른 PKM2 인산화 및 핵내 이동, Stat3 인산화 증가의 연쇄적인 과정이 PKM2 발현 억제로 인해 약화되고, sunitinib 에 대한 감수성을 증가시킴을 확인하였다. 이를 통해 핵내 PKM2가 Stat3 의 인산화를 유지시킴으로써 JAK2/Stat3 신호 활성화에 중요한 역할을 함을 확인할 수 있었다.

본 연구를 통하여 IL-6/JAK2 활성화와 이에 따른 Stat3의 활성화가 sunitinib의 신세포암 세포 자체 내성 메커니즘에 기여하였음을 확인하였고, PKM2 의 핵 내 이동 증가로 인한 Stat3의 전사 활동 강화도 sunitinib 내성에 중요한 역할을 하였음을 확인하였다.

핵심되는 말 : 신장암, sunitinib, 내성, Stat3 단백