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IGF-1 receptor is down-regulated by sunitinib induces MDM2-dependent ubiquitination

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ABSTRACT

The insulin like growth factor receptor subtype 1(IGF-1R) plays an important role in cancers transformation and progression. The aim is to investigate the effects of sunitinib on IGF-1R cell signaling transduction, especially on receptor phosphorylation and ubiquitination. In HEK293 cells, IGF-1R signaling pathways are activated in response to IGF-1, which induces obvious phosphorylations of receptor tyrosine and Akt, ERK. However, the phosphorylations of receptor tyrosine, Akt and ERK were significant inhibited by sunitinib. We found that both IGF-1 and sunitinib obviously down regulated the IGF-1R expression. For analysis the ubiquitination, HEK293 cells were simulated with 100 ng/ml IGF-1 or 10 nM sunitinib for 10 min after serum starvation for 24 h. Both IGF-1 and sunitinib could obviously induce the IGF-1R ubiquitination at 10 min compared with control (only serum free, no stimulation), indicating IGF-1 and sunitinib down-regulate the IGF-1R by increasing the receptor degradation through ubiquitination dependent proteasome pathway. We also found that MDM2 combined to IGF-1R in response to sunitinib stimulation. To confirm it, HEK293 cells were transfected with human HA-MDM2 (+MDM2) or siRNA to MDM2 (−MDM2). Following 24 h serum starvation, cells were stimulated with 10 nM sunitinib for 10 min. In over-expressed MDM2 cells, IGF-1R was more ubiquitinated than that in mock-transfected cells (control), and no ubiquitination in −MDM2 cells. These results mean that sunitinib mediates ubiquitination of IGF-1R dependent on MDM2. In summary, sunitinib could block signaling transduction and mediate degradation of IGF-1R.

1. Introduction

The insulin like growth factor type 1receptor (IGF-1R) belongs to transmembrane, receptor tyrosine kinases families, which is known plays a crucial role in the development and progression of human cancers. Overexpression of IGF-1R is observed in many human malignancies [1], often involved in worse prognosis [2,3] The IGF-1R is a prominent target for anti-cancer therapy and the downregulation of its activity has been shown to inhibit the growth of many types of human tumor cells. Many researches on IGF-1R function inhibition have been investigated during the past years. The IGF-1R monoclonal antibodies, which mediate receptor downregulation, have been encouraging in cancer cell lines [4,5] and xenografts [6,7]. Another attempt to inhibit IGF-1R is the use of small molecules such as picropodophylin to inhibit kinase activity [8]. Ubiquitin was discovered in the 1970s to eliminating dysfunctional proteins, but it is known to be involved in numerous cellular processes like DNA repair, cell cycle, gene expression, regulation of signaling and protein internalization and trafficking recently. IGF-1R is also a substrate for ubiquitination [9–11], however, there is few research focusing on ubiquitination of the receptor.

Sunitinib is a small molecule and multi-target tyrosine kinase inhibitor, approved for treating the advanced and/or metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST). Sunitinib inhibits vascular endothelial growth factor receptors (VEGFR of −1, −2 and −3) [12] and the platelet-derived growth factor receptors (PDGFR in-α and-β) [12,13]. These receptors are implicated in angiogenesis and tumor progression [14–16]. In addition, sunitinib could inhibit colony-stimulating factor 1 receptor [17], stem-cell factor receptor [14], fms-like tyrosine kinase 3 [18], and glial cell line-derived neurotrophic factor [19], which play important roles in vascular endothelial cell growth and migration, vascular permeability, pericyte recruitment, lymphangiogenesis and tumor cells survival [20]. However, there is no any research focused on sunitinib wether inhibits the IGF-1R tyrosine kinase or not.

In this study, we aim to investigate the effects of sunitinib on IGF-1R cell signaling transduction. Especially we focus on whether

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sunitinib could inhibit the phosphorylation and induce the ubiquitination or not.

2. Materials and methods

2.1. Reagents

Anti-IGF-1R, anti-MDM2 and anti-ubiquitin antibodies were purchased from Santa Cruz Biotechnology Inc. The antibodies of phospho-IGF-1R tyrosine 1311, phosph-MAPK and phosph-Akt were from cell signaling Technology. Sunitinib (sutent11248) was from Pfizer Inc. All other reagents were from Sigma.

2.2. Cell cultures

HEK293 cells were cultured in Dulbecco's modified Eagle's medium with sodium supplemented with 10% FBS.

2.3. Transient transfection

The HEK293 cells were put at 80–90% confluent density in 6-well plates, which was transiently transfected with 4 μg/ml DNA plasmids containing Mdm2 (HA-MDM2) using Lipofectamine 2000 (Invitrogen). After 24 h, the transfected cell were split into 24-well plates and cultured at serum free medium for another 24 h. Cells then were stimulated with 50 ng/ml IGF-1 and/or 2.5 nM sunitinib. Protein extracts were prepared for immunoprecipitation or Western blot.

2.4. Small Interfering RNA

Mdm2 siRNA targeting human MDM2 mRNA (5′-AAG CCA UUG CUU UUG AAG UUA-3′) supplied by Dharmacon. siRNA (300 pmol) was transfected into cells using RNAimax reagent (Invitrogen) according to the instructions of the manufacturer. A nonsilencing RNA duplex (5′-AAUUCUCCGAACGUGUCACGU-3′), (Dharmacon) was used as a control.

2.5. Immunoprecipitation

The isolated cells were lysed with IP lysis buffer supplied by Invitrogen, using 500 μl lysis buffer with 10 mM *N*-ethylmaleimide, 50 μM MG132, and protease inhibitor cocktail tablet (Roche) per well in the 6-well plates. One microgram antibody were added to each sample and incubated for 3 h at 4 °C on a rocker platform. Then fifteen microliters of Dynabeads Protein G were added. After overnight incubation at 4 °C on a rocker platform, the immunoprecipitated complexes were collected by magnet(Invitrogen). The pellet was washed thrice with IP lysis buffer and then dissolved in a sample buffer for SDS–PAGE whereupon the samples were heated for 10 min at 95 °C and further analyzed by western blot.

2.6. SDS–PAGE and western blotting

Protein samples were dissolved in a sample buffer containing 1 mM β-mercapethnol. Samples were analyzed by SDS–PAGE with a 12% separation gel. After SDS–PAGE, the proteins were transferred to nitrocellulose membranes (GE Healthcare) at 4 °C for 1 h and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in PBS, pH 7.5. Incubation with appropriate primary antibodies overnight at 4 °C. This was followed by washes with PBST and incubation with a horseradish peroxidase-labeled secondary antibody (Pierce) for 1 h at room temperature. The detection was made with ECL (Pierce). The films were exposured; and then developed and fixed.

3. Results

3.1. IGF-1R signaling is activated in response to ligand stimulation

HEK293 cells were stimulated with 50 ng/ml IGF-1 for 2, 5, 10, 30 and 60 min after 24 h serum starvation. As expected, IGF-1R was phosphorylated at tyrosine residues Y1131 from 2 min to 60 min in response to IGF-1 stimulation (Fig. 1). Simultaneously, we used the same samples to detect the phosphorylation of the main signaling proteins, Akt and ERK. We found that ERK was phosphorylated from 5 min and max at 5 to 10 min and Akt was phosphorylated maximum at 30 min, as been shown in Fig. 1.

3.2. IGF-1R tyrosine phosphorylation is inhibited by sunitinib

As shown in Fig. 2, following serum starvation for 24 h, cells were treated 50 ng/ml IGF-1 or first treated 2.5 nM sunitinib for 1 h and then simulated with 50 ng/ml IGF-1 for 2, 5, 10, 30 and 60 min. Compared with single IGF-1, sunitinib significantly decreased the IGF-1R tyrosine phosphorylation, even there was no phosphorylation after IGF-1 stimulation for 1 h.

3.3. Sunitinib inhibits IGF-1R signaling transduction

HEK293 cells were treated as above. To detect whether sunitinib affects the IGF-1R signaling, we used IGF-1 induced Akt and ERK phosphorylation as a measurement of PI3K and MAPK pathways activation, seperately. As can be seen in Fig. 2, Akt and ERK proteins were phosphorylated after IGF-1 stimulation without sunitinib. However, in cells treated with with 2.5 nM sunitinib for 1 h and then simulated with 50 ng/ml IGF-1, both Akt and ERK phosphorylation was largely reduced, indicating that sunitinib inhibits the IGF-1R signaling pathway.

3.4. IGF-1 down-regulates IGF-1R and induces IGF-1R ubiquitination

To determine IGF-1R degradation, HEK293 cells were treated 100 ng/ml IGF-1 in serum free medium for 1, 3, 6, 12 and 24 h.

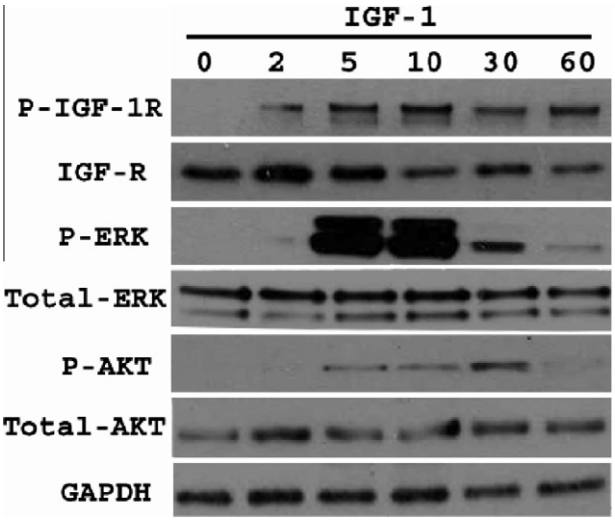

Fig. 1. IGF-1 receptor is activated by IGF-1. HEK293 cells plated into 24-well plates were stimulated with 50 ng/ml IGF-1 for 0, 2, 5, 10, 30 and 60 min after serum starvation for 24 h. IGF-1R was phosphorylated at tyrosine residues Y1131 from 2 min to 60 min in response to IGF-1 stimulation. Simultaneously, the main signaling proteins, ERK was phosphorylated from 5 min and max at 5 to 10 min whereas Akt maximum at 30 min. The experiments were performed three times indepently.