Function of Deubiquitinating Enzyme USP14 as Oncogene in Different Types of Cancer

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Abstract

**Background/Aims:** Non-small cell lung cancer (NSCLC) tissues overexpress USP14, which promotes tumor cell proliferation and is associated with shorter overall survival time. **Methods:** The expression of USP14 was assayed in many types of cancers. USP14 was up-and down-regulated using appropriate plasmid or lentiviral vector constructs and its effects on proliferation, cell colony number, and apoptosis rate were measured. A human NSCLC cell line was inoculated into nude mice and the survival rates were recorded. **Results:** We found USP14 amplification and overexpression in many different cancers. The overexpression of USP14 in USP14 low-expression cell lines promoted cell proliferation and migration, whereas USP14 downregulation suppressed tumor cell proliferation, decreased tumor cell colony number, increased apoptosis rate, and decreased cell migration and invasion. **Conclusion:** USP14 plays an oncogenic role in various types of cancer, and may thus represent a new cancer therapy target.

Introduction

Deubiquitinating enzymes (DUBs) remove covalently attached ubiquitin from proteins, thereby controlling substrate activity and/or abundance [1]. The USP family of DUBs, specifically, plays a crucial role in various cellular processes and signaling pathways [2-11]. Mammalian proteasomes are associated with three DUBs: RPN11, UCH37, and USP14 [2, 4]. USP14, in particular, can inhibit proteasomes in vitro as well as protein turnover in cells [2].
We demonstrated in a previous study that USP14 is up-regulated in NSCLC tumor tissues, and as such is associated with shorter patient survival times [12]. To the best of our knowledge, our report was the first to show that USP14 plays a role in NSCLC.

The exact mechanisms through which USP14 affects NSCLC and other types of cancer are unknown, however all cancers result from the accumulation of genetic changes that occur in the DNA sequence of the genomes of cancer cells, so in order to create a compendium of genetic changes in specific cancers, it is important to obtain the complete DNA sequence of large numbers of cancer genomes. The Cancer Genome Atlas (TCGA) project is a large-scale collaborative effort to characterize the genomic changes inherent to cancer; the TCGA has profiled and analyzed rather large numbers of human tumors in order to catalog molecular aberrations at DNA, RNA, protein, and epigenetic levels. The TCGA data allows us to build an integrated understanding of commonalities, differences, and emergent themes across tumor lineages [13].

We investigated the role of USP14 in various types of cancer in this study. Based on a search through the TCGA database, we first analyzed the oncogenic role of USP14 in different types of cancer and found that its up-or down-regulation affects cell proliferation and apoptosis an array of cancer cell lines. The results of this study altogether indicate a possible oncogenic role of USP14.

Materials and Methods

Cell Culture

Human Embryonic Kidney 293 cells HEK293T, human normal lung fibroblast cell line MRC-5, human NSCLC cell lines (SK-MES-1, H1299, SPC-A-1, LTEP-A-2 and A549), human breast cancer cell lines (MCF-10A, MCF-7, MDA-231), hepatic cell line L02, human hepatocarcinoma cell lines (HepG2, Huh-7), human glioma cell lines (U251), human pancreatic carcinoma cell line (PANC, SW1990), human prostate cancer cell lines (DU145, PC3), and human gastric adenocarcinoma cell lines (AGS, MKN45) were purchased from the American Type Culture Collection (ATCC) and cultured in either Dulbecco's modified eagle medium (DMEM) or 1640 medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin (Bio Light, Shanghai, China) as described in our previously published study [12].

Mice and Treatment

Nude mice were obtained from the Animal Center of the Chinese Academy of Science (Shanghai, China), and maintained in the Mice Care Center of the Second Military Medical University. A549 cells were inoculated into nude mice subcutaneously at a density of 1 x 10^7 cell/ml and volume of 100 ml. After A549 cell inoculation, all mice were carefully monitored and their tumor volumes were measured every week. The survival rates of the nude mice were recorded during a 160-day follow-up period.

USP14 Amplification and Expression in Different Types of Cancer

USP14 amplification data and graph were obtained from the cBioPortal for Cancer Genomics [14, 15]. The USP14 expression in tumor data came from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/).

Immunohistochemistry Analysis

We obtained 45 LC tissues, 60 BC tissues, and 37 PDAC tissues from Changhai Hospital, Second Military Medical University (Shanghai, China). All patients provided signed, informed consent for their tissues to be used for scientific research. The Ethical Committee of Changhai Hospital, Second Military Medical University (Shanghai, China) approved our procedures. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. Tissues were obtained before chemotherapy and radiotherapy and immediately frozen and stored at ~80°C. Immunohistochemistry images were analyzed in Image Pro-plus.
RNA Extraction and Real-time qPCR
We extracted RNA with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol, then performed cDNA synthesis and real-time qPCR using the Qiagen system [12]. Real-time quantitative PCR analysis was performed using standard protocols on an Applied Biosystem’s 7500 HT sequence Detection System, and primer design was based on standard protocols [16] as synthesized by Shengong Company (Shanghai, China).

Plasmid Transfection
USP14 overexpression plasmids (Plasmid 22569: Flag-HA-USP14) [1] were constructed by Shengong Company (Shanghai, China). Flag-HA-USP14 were transfected into cells (6 × 10^4 cells/ well) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), then collected after 48h for confirmation and further assay.

Lentivirus Vectors
The USP14 lentivirus vectors and scramble control (Src) were generated by Shengong Company (Shanghai, China). Cells were transfected with 1mL of lentiviral supernatant containing equal doses (4 × 10^8 PFU) for 2 h at a multiplicity of infection of 1:5, followed by incubation for 2 h at 37℃ according to previously published methods [12, 17, 18]. Gene transfer efficiency was evaluated by GFP expression, which was detected by luminescence microscope (Leica, Berlin, Germany).

Cell Growth Assay
For the cell growth assay, 5 × 10^3 cells per well were seeded in triplicate into a 96-well plate with complete growth medium. Cells were then counted over five days using the MTT assay (Promega, Fitchburg, WI, USA) as described previously [12, 19-21].

Migration and Invasion Assay
We performed migration assays in 24-well plates (Transwell, Costar; 6.5 mm diameter and 3.0 µM) (Cambridge, MA, USA) by adding 700 µl fetal bovine serum to the lower chamber and 100 µl of cell suspension (10^6/ml) to the upper chamber; then assessing cell numbers on the bottom (migr. Cells) and on the membrane (not migrated cells) 2 h later under an optical microscope. Matrigel invasion assays were performed with Biocoat Matrigel invasion chambers (Cambridge, MA, USA) as previously described [22]. Chambers were plated and assayed in the same manner. Well examinations were repeated in triplicate and the invaded/migrated cells were quantified per field of view and statistically analyzed.

Statistical Analysis
Data are presented as the mean ± SEM from at least three independent experiments. The difference between groups were analyzed using two-tailed Student’s t-test when only two groups were compared; the differences between groups were analyzed using ANOVA when three or more groups were compared. Mice survival rates and times were determined by Kaplan-Meier analysis. Statistical analyses were performed using SPSS software (Version 17.0) and P < 0.05 was considered statistically significant.

Results
USP14 Amplification in Various Cancers
We found in a previous study that there are higher levels of USP14 in NSCLC tissues, which was associated with shorter survival time overall. Further, the overexpression of USP14 promotes tumor cell proliferation [12]. Clinical study and data analysis revealed that USP14 alterations, (especially amplification,) exist in a variety of cancers including bladder cancer, lung cancer (LC), breast cancer (BC), and pancreatic ductal adenocarcinoma (PDAC) (Fig. 1). We hypothesized, to this effect, that USP14 plays an oncogenic role in many cancers.
In order to evaluate the expression of USP14 in cancer tissues, we examined 45 LC tissues, 60 BC tissues, and 37 PDAC tissues via immunohistochemistry (IHC) staining. We found that the USP14 protein levels in LC, BC, and PDAC tissues were higher compared to corresponding normal control tissue (Fig. 2A). First, we summarized the mean relative USP14 levels in all LC, BC, and PDAC tissues and arbitrarily defined the mean expression of USP14 in matched normal control samples as 100% (Fig. 2B). Next, we analyzed the expression of USP14 in different types of cancer by exploring expression data in the Gene Expression Omnibus (GEO) database repository, a public gene expression resource. We found that in PDAC, breast cancer, and gastric cancer, the USP14 expression level is significantly higher in the tumor tissues than in the matched normal tissues; similarly, in the mammary tumorigenesis MMTV-neu model, the level of USP14 is higher in tumors than in normal tissue. In melanoma, USP14 level in the metastatic site is higher than in the primary tumor site, suggesting that USP14 may promote tumor metastasis (Fig. 2C). We then assayed the levels of USP14 mRNA in various cancer cell lines, and found that USP14 is up-regulated in most of the cancer cell lines analyzed though the specific levels varied among lines (Fig. 2D). The more malignant cell lines, PANC1, MDA-231, U251, and DU145, show higher levels of USP14 expression, so we surmised that USP14 also plays a role in tumorigenesis.

**USP14 Over-expression Effects and USP14 Low-expressing Cell Lines**

The cancer cell lines H1299 and MCF7 show a relatively low expression of USP14 compared to other cell lines, thus, these cell lines were selected to further study the effects of USP14 overexpression by plasmid transfection (Fig. 3A). Cell proliferation analysis via an MTT assay 48h post-transfection showed that USP14 up-regulation promotes H1299 and MCF7 cell proliferation (Fig. 3B). As anticipated, the relative colony number was increased by USP14 overexpression in both H1299 and MCF-7 (Fig. 3C) and, interestingly, the migration capacities of both cell lines were also increased by USP14 overexpression (Fig. 3D).
USP14 Down-regulation Suppresses Tumor Cell Function

As shown in Fig. 2D, cell lines A549, MDA-231, Panc-1 and DU145 are USP14 high-expressing cells, so we selected them to investigate the effects of USP14 down-regulation transfection of shRNA lentiviral vectors. Analysis by real-time qRT-PCR showed both the USP14 shRNA-1 and the USP14 shRNA-2 lentiviral vectors down-regulated the level of USP14 in the four cell lines analyzed (Fig. 4A). The MTT cell proliferation assay conducted 48h post transfection showed that transfection of USP14 shRNA-1 and USP14 shRNA-2 vectors suppress cells proliferation to a greater extent than control vector Src-shRNA (Fig. 4B). Additionally, transfection of the USP14 shRNA-1 vector decreased the colony number.
of the four cell lines analyzed (Fig. 4C). Apoptosis analysis by FACS revealed that the down-regulation of USP14 results in increased apoptosis rate of A549, MDA-231, Panc-1, and DU145 cell lines (Fig. 4D). Furthermore, migration (Fig. 4E) and invasion (Fig. 4F), assayed to examined USP14 in terms of key features of malignant tumor cells, both decreased due to USP14 down-regulation in the A549 and MDA-231 cell lines.

**In Vivo Effect of USP14 Down-regulation**

To evaluate in the vivo effect of USP14 down-regulation, we investigated whether the levels of USP14 expression were associated with overall survival of nude mice inoculated with A549 cells transfected with USP14 shRNA or control shRNA vector. The expression of USP14 in A549 cells was first down-regulated by shRNA transfection, then transfected A549 were inoculated into the nude mice subcutaneously. We found that measureable tumors first emerged in the control mice, and that mice treated with USP14 shRNAs showed lower-volume emerging tumors at the same time point (Fig. 5A). We also measured the survival times of these mice during a period of 160-days post-transfection, and found that the downregulation of USP14 effectively protected the mice, as there were more deceased mice in the control group as their tumors progressed. The Kaplan-Meier curves indicated that mice treated with cells in which USP14 was down-regulated had a significantly lengthier overall survival time than those treated with control cells (Fig. 5B).

**Discussion**

In this study, under guidance by the TCGA project, we investigated the role of USP14 in various types of cancer and found that USP14 plays an important role in tumorigenesis. The TCGA has applied promising emerging technologies to the analysis of specific tumor types, and its disease-specific approach has revealed novel oncogenic drivers and genes contributing to functional changes [23-25], established definitions of molecular subtypes
and identified new biomarkers on the basis of genomic, transcriptomic, proteomic, and epigenomic alterations. In this study, we found that USP14 amplification causes major alteration in various types of cancer (Fig. 1), suggesting that USP14 is involved in many different types of cancer. Gene Expression Omnibus (GEO) analysis also indicated a higher level of USP14 is expressed in a variety of cancers, with prostate cancer as a notable exception (Fig. 2C); this observation is consistent with the fact that USP14 deletion is a major USP14 alteration inherent to prostate cancer (Fig. 1). We also found that in the mammary tumorigenesis MMTV-neu model, USP14 level in tumors is also higher than in normal tissue.

This study was a preliminary effort to begin to understand the functional relevance of USP14 in cancer. In a clinically relevant experiment, we did successfully identify USP14 overexpression in several different types of cancer, however, changes in USP14 level among different clinical cancers and cancer cell lines was highly variable. It is worth noting that USP14 amplification in liver cancer and its expression in the hepatocarcinoma cell line are [26-32], and identified new biomarkers on the basis of genomic, transcriptomic, proteomic, and epigenomic alterations. In this study, we found that USP14 amplification causes major alteration in various types of cancer (Fig. 1), suggesting that USP14 is involved in many different types of cancer. Gene Expression Omnibus (GEO) analysis also indicated a higher level of USP14 is expressed in a variety of cancers, with prostate cancer as a notable exception (Fig. 2C); this observation is consistent with the fact that USP14 deletion is a major USP14 alteration inherent to prostate cancer (Fig. 1). We also found that in the mammary tumorigenesis MMTV-neu model, USP14 level in tumors is also higher than in normal tissue.

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![Fig. 4. Down-regulation of USP14 reduces tumor cell proliferation and induces cell apoptosis. Cancer cell lines A549, MDA-231, Panc-1 and DU145 were transfected with shRNA lentiviruses vectors. The USP14 levels in the cells were assayed by qRT-PCR 48h post-transfection, and transfection of an empty plasmid was used as a blank control, the value of which was arbitrarily defined as 100% (A). A549, MDA-231, Panc-1, and DU145 were infected with USP14 shRNA lentiviruses at density of 5 x 10^5 cells/well; MTT assay was performed 24h later and OD values recorded. Data are the mean ± SEM of three separate experiments (B). After USP14 shRNA-1 infection, the colony numbers of A549, MDA-231, Panc-1, and DU145 were counted and the colony numbers of the control arbitrarily defined as 1; data are the mean ± SEM of three separate experiments (C). After USP14 shRNA-1 lentivirus infection, the apoptosis cells were stained (Annexin V) and counted by FACS, and the percentage of apoptosis cells in the control arbitrarily defined as 100%; data are the mean ± SEM of three separate experiments (D). The migrated cells of A549 and MDA-23 after 3h were also collected and counted, and data expressed as the mean ± SEM of three separate experiments (E). The migrated cells of A549 and MDA-23 were also collected and counted, and data expressed as the mean ± SEM of three separate experiments (F), * P < 0.05.](image)
relatively low, suggesting that USP14 might not be involved in liver cancer tumorigenesis – this may, in fact, hint toward the mechanism involved in USP14’s effects on cancer. In a previous study, we found that β-catenin protein levels sharply decrease in conjunction with USP14 silencing in A549 cells [12], researchers have likewise found that β-catenin, a key member in the Wnt pathway that promotes proliferation in various tumors [33-38], is controlled through ubiquitination [39-41]. Given this information, SOX1, a developmental gene, may function as a tumor suppressor by interfering with Wnt/β-catenin signaling in the development of hepatocellular carcinoma. Together, these observations support the hypothesis that low expression of USP14 in hepatocellular carcinoma is correlated with SOX1 function.

As discussed above, we found that USP14 exhibited oncogenic roles in various types of cancer. It is thus possible that USP14 inhibition suppresses tumor proliferation. A small-molecule inhibitor of USP14 has been developed and shown to enhance proteasome activity [2], likely because USP14 regulates proteasomal ATPases [42]. We hope that USP14 inhibition will show therapeutic effects, which we plan to investigate in future studies.

In conclusion, our data proved the role of USP14 in tumorigenesis and suggests a potential molecular therapy target for future cancer treatment development.

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Disclosure Statement

The authors have declared that no competing interests exist.

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