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Research Paper

Overexpression of ULK1 Represents a Potential Diagnostic Marker for Clear Cell Renal Carcinoma and the Antitumor Effects of SBI-0206965

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ABSTRACT

Background: Uncoordinated 51-like kinase 1 (ULK1) plays a vital role in autophagy. ULK1 dysregulation has recently been found in several human cancers.
Methods: mRNA expression levels of ULK1 and clinical information were analysed from The Cancer Genome Atlas data. ULK1 expression levels were verified in 36 paired fresh ccRCC tissue specimens by western blot analysis. Expression of ULK1 was knockdown by shRNA lentivirus. ULK1 activity was inhibited by SBI-0206965. The effect of inhibition of ULK1 was measured by detecting the apoptotic rate, autophagy, and the ratio of ROS and NADPH. The efficacy of SBI-0206965 *in vivo* was assessed by the murine xenograft model.
Findings: ULK1 mRNA expression was significantly upregulated in clear cell renal cell carcinoma (ccRCC) and overexpression of *ULK1* correlated with poor outcomes. We found that ULK1 was highly expressed in 66.7% of ccRCC tumours ($p < 0{\cdot}05$). Knockdown of ULK1 and selective inhibition of ULK1 by SBI-0206965 induced cell apoptosis in ccRCC cells. We demonstrated that SBI-0206965 triggered apoptosis by preventing autophagy and pentose phosphate pathway (PPP) flux. Furthermore, blocking the kinase activity of ULK1 with SBI-0206965 resulted in a level of anticancer effect *in vivo*.
Interpretation: Taken together, our results suggested that ULK1 was upregulated in ccRCC tumours and may be a potential therapeutic target. Therefore, SBI-0206965 should be further considered as an anti-ccRCC agent.
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Research in context

Evidence Before This Study

Elevated ULK1 expression has been observed in human cancers, including nasopharyngeal carcinoma, oesophageal squamous cell carcinoma, colorectal cancer, and hepatocellular carcinoma. Egan et al. developed SBI-0206965, a selective small molecule inhibitor of ULK1 kinase in 2015. They found that SBI-0206965 inhibits autophagy and enhances apoptosis in human glioblastoma and lung cancer cells. Recent studies showed that SBI-0206965 could suppress phosphorylation of the β1-Ser108 of AMP-activated protein kinase (AMPK), and induce cell apoptosis and enhance the sensitivity of cisplatin against non-small cell lung cancer cells. However, to date, the exact expression profile of ULK1 and the biological mechanism of SBI-0206965 in human ccRCC have not been determined.Added Value of This Study

In TCGA KIRC cohorts, ULK1 is highly expressed in renal clear cell carcinoma tissues, and its expression is positively correlated with the patient's survival time. We observed that ULK1 expression was upregulated in 66.7% of our frech ccRCC tissues ($p < 0{\cdot}05$). Knockdown of ULK1 and selective inhibition of ULK1 by SBI-0206965 under starvation conditions induced cell apoptosis in ccRCC cells. Then, we demonstrated that SBI-0206965 triggered apoptosis by preventing autophagy and pentose phosphate pathway (PPP) flux. Furthermore, SBI-0206965 resulted in a level of anticancer effect *in vivo* in a murine xenograft model.Implications of All the Available Evidence

Our results suggested that ULK1 may be a potential therapeutic target, and SBI-0206965 should be further considered as an anti-ccRCC agent.

1. Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common type of renal malignancy, being responsible for approximately 75% of all cases of renal cell carcinoma (RCC) [1]. Compared with that of other subtypes of RCC, ccRCC is characterized by high metastasis and rates of relapse. Almost 30% of new ccRCC cases have metastasised by the time of diagnosis and will suffer from recurrence after resection [2]. The 5-year overall survival rate of metastatic RCC is only 10%, which is in contrast to that of non-metastatic RCC with an estimated rate of over 55% [3]. Therapeutic agents such as sorafenib [4], sunitinib [5], everolimus [6], bevacizumab [7], and pazopanib [8] produce partial improvement for ccRCC patients, but the efficacy of these drugs for metastatic RCC remains limited [9, 10]. Therefore, there is a need to identify novel biomarkers for predicting the progression and prognosis of ccRCC and to develop novel treatment strategies.

Previous studies of budding yeast demonstrated that autophagy related 1 (ATG1) is one of the upstream components of the autophagy pathway [11, 12]. In mammals, ATG1 has two homologous [13], uncoordinated 51-like kinase 1 (ULK1) and ULK2, which initiate autophagy in response to starvation [14]. ULK1 and ULK2 also phosphorylate key glycolytic enzymes to promote additional carbon flux into PPP during times of nutritional stress in order to maintain redox homeostasis. Knockdown of ULK1 and ULK2 leads to decreased NADPH/NADP+ ratios and lower percentages of cell death [15].

Elevated ULK1 expression has been observed in human cancers, including nasopharyngeal carcinoma [16], oesophageal squamous cell carcinoma [17], colorectal cancer [18], and hepatocellular carcinoma [19] and is an independent predictor of poor survival for patients with these cancers. A recent study analysed the prognostic significance of five autophagy-related proteins in specimens from patients with metastatic RCC receiving mammalian target of rapamycin (mTOR) inhibitors as treatment and found that ULK1 expression correlates with the response to everolimus or temsirolimus [20]. Egan et al. developed SBI-0206965, a selective small molecule inhibitor of ULK1 kinase. SBI-0206965 inhibits autophagy and enhances apoptosis in human glioblastoma and lung cancer cells, suggesting it has therapeutic potential [21]. SBI-0206965 also suppresses phosphorylation of the β1-Ser108 of AMP-activated protein kinase (AMPK), which has been demonstrated to upregulate pro-survival pathways [22]. Recently, Tang et al. showed that SBI-0206965 induces cell apoptosis and enhances the sensitivity of cisplatin against non-small cell lung cancer cells [23]. However, to date, the exact expression profile of ULK1 and the biological mechanism of SBI-0206965 in human ccRCC have not been determined.

In this study, we investigated the expression pattern of ULK1 and the antitumor effects of SBI-0206965 on ccRCC. Upregulation of ULK1 at the protein level was confirmed in 36 freshly collected ccRCC samples. SBI-0206965 appeared to increase apoptosis by inhibiting cell autophagy and by increasing the levels of reactive oxygen species (ROS) in ccRCC cells. In a xenograft mouse model, SBI-0206965 inhibited tumour growth without producing any symptoms of toxicity. Results from this work revealed that ULK1 may be a novel prognostic marker and suggests that SBI-0206965 may be a potential therapeutic agent for ccRCC.

2. Materials and Methods

2.1. Analysis of the Cancer Genome Atlas (TCGA) Data of ccRCC

Published mRNA expression data for 72 normal kidney tissues and 524 ccRCC specimens were downloaded from TCGA (http://cancergenome.nih.gov) on July 2016. Differential gene expression was analysed using R and Bioconductor software. Kaplan–Meier survival curves were generated for ccRCC patients entered in the TCGA database (http://www.oncolnc.org/).

2.2. Clinical Specimens

Thirty-six ccRCC tissue specimens and their matched normal adjacent tissues located >2 cm from the edge of the cancer tissue were obtained from patients at Fuzhou General Hospital from November 2013 to November 2015. The collection and use of the tissue specimens were approved by the Human Research Ethics Review Committee of Fuzhou General Hospital (No. 2013–017). All patients provided written informed consent. Table 1 lists the demographic details of the patients.

2.3. Cell Culture

The ccRCC cell lines A498 and ACHN were obtained from GeneChem (Shanghai, China). Normal human lung cell line HKC and HEK293T were obtained from the Chinese Academy of Sciences Committee Typical Culture Collection Cell Bank (Shanghai, China). Cells were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1 × penicillin/streptomycin (Thermo Fisher Scientific, USA). All the cells were cultured at 37 °C in a humidified incubator with 5% CO2.

2.4. Plasmids and Transfection

Plasmid pEGFP-LC3 was derived from the Addgene plasmid 22,564. Lentiviral vectors pLKO.1-shRNA-ULK1 (clone ID TRCN0000000835) and SHC002 (pLKO.1-puro Non-Mammalian shRNA Control Plasmid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). For virus packaging, the non-targeting sequence control or shRNA constructs were co-transfected with pMD2.G and psPax2 into HEK293T cells using FuGENE HD (Promega, Madison, USA). A498 and ACHN cells were

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