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Inhibition of CIP2A determines erlotinib-induced apoptosis in hepatocellular carcinoma

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ABSTRACT

Erlotinib is a small-molecular inhibitor of epidermal growth factor receptor (EGFR). Here, we identify that cancerous inhibitor of protein phosphatase 2A (CIP2A) is a major determinant mediating erlotinib-induced apoptosis in hepatocellular carcinoma (HCC). Erlotinib showed differential effects on apoptosis in 4 human HCC cell lines. Erlotinib induced significant apoptosis in Hep3B and PLC5 cell lines; however, Huh-7 and HA59 T cell lines showed resistance to erlotinib-induced apoptosis at all tested doses. Down-regulation of CIP2A, a cellular inhibitor of protein phosphatase 2A (PP2A), mediated the apoptotic effect of erlotinib in HCC. Erlotinib inhibited CIP2A in a dose- and time-dependent manner in all sensitive HCC cells whereas no alterations in CIP2A were found in resistant cells. Overexpression of CIP2A upregulated phospho-Akt and protected Hep3B cells from erlotinib-induced apoptosis. In addition, silencing CIP2A by siRNA restored the effects of erlotinib in Huh-7 cells. Moreover, adding okadaic acid, a PP2A inhibitor, abolished the effects of erlotinib on apoptosis in Hep3B cells; and forskolin, a PP2A agonist enhanced the effect of erlotinib in resistant HA59 T cells. Combining Akt inhibitor MK-2206 with erlotinib restored the sensitivity of HA59 T cells to erlotinib. Furthermore, in vivo xenograft data showed that erlotinib inhibited the growth of PLC5 tumor but had no effect on Huh-7 tumor. Erlotinib downregulated CIP2A and upregulated PP2A activity in PLC5 tumors, but not in Huh-7 tumors. In conclusion, inhibition of CIP2A determines the effects of erlotinib on apoptosis in HCC. CIP2A may be useful as a therapeutic biomarker for predicting clinical response to erlotinib in HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the fifth most common cancer worldwide [1]. HCC remains a difficult cancer to treat for several reasons: it often is at an advanced stage at presentation and/or commonly arises on a background of chronic liver cirrhosis, making curative treatments a challenge; and HCC cells are frequently resistant to conventional cytotoxic therapeutics and radiation [1]. Developing tolerable and effective targeted agents is, therefore, mandatory to improve treatment for advanced HCC [2]. The success of sorafenib, a multi-targeted receptor tyrosine kinase inhibitor (TKI), in two randomized controlled phase III trials [3,4] supports the use of molecularly targeted therapies in the treatment of advanced HCC.

The fact that HCC commonly emerges on a background of persistent liver injury, inflammation and hepatocellular proliferation suggests that growth and survival-related pathways are key players in HCC development [5]. Among these pathways the epidermal growth factor receptor (EGFR) signaling pathway is one of the best studied and plays an important role in cell proliferation, motility, adhesion, invasion, survival, and angiogenesis [6,7]. EGFR

Abbreviations: HCC, hepatocellular carcinoma; PP2A, protein phosphatase 2A; CIP2A, cancerous inhibitor of PP2A; PI3 K, phosphatidylinositol-3-kinase; PDK1, phosphatidylinositol-3-kinase dependent 1; PARP, poly (ADP-ribose) polymerase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; s.c., subcutaneous.

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can be activated by a broad family of ligands, and can also engage in extensive cross talk with other signaling pathways, including the ras/raf/MEK/MAPK, protein kinase C (PKC), PI3 K/Akt and STAT pathways [5]. Accumulating evidence suggests that constitutive active EGFR signaling contributes to HCC carcinogenesis [5,8–10]. EGFR expression could be detected in up to 85% of HCC tissues, and one of its ligand, TGF-α, was upregulated during hepatocarcinogenesis. [8,9]. Moreover, EGFR has been shown to be overexpressed in a large variety of epithelial cancers including HCC and is typically correlated with more aggressive clinical behavior [11–13]. The identification of EGFR as an oncogene has led to the development of EGFR-targeted therapeutics, such as the small molecule TKIs gefitinib and erlotinib for non-small cell lung cancer (NSCLC), and monoclonal antibody cetuximab for colon cancer [14].

Erlotinib (Tarceva®) competes with ATP to bind to the EGFR kinase domain [15]. Erlotinib selectively inhibits EGFR tyrosine kinase activity with an IC_{50} of 2 nM in in vitro cell-free assay, and suppresses EGFR autophosphorylation with an IC_{50} of 20 nM in lung cancer cell lines [16]. Erlotinib has been shown to inhibit proliferation of cancer cells, including HCC cells, and block cell-cycle progression at the G1 phase [15–17]. Erlotinib has also been shown to induce mitochondrial-mediated apoptosis through activation of BAX and BAK in human H3255 NSCLC cells (which harbor EGFR L858R mutation) [18]. Clinically, erlotinib is currently an approved drug for treatment for NSCLC and pancreatic cancer [15]. In patients with advanced HCC, erlotinib as a single agent showed modest clinical efficacy with acceptable toxicity (most commonly skin rash, diarrhea, and fatigue) in two phase II clinical trials [7,19]. Accumulating evidence demonstrates that EGFR status (kinase domain activating mutations or gene amplifications) closely correlate with clinical response to erlotinib in patients with NSCLC [20–22]. However, these EGFR mutations and/or gene gains are not always observed in patients who respond to erlotinib, suggesting alternative mechanisms might also confer sensitivity to EGFR-targeting agents [20]. Moreover, it is notable that EGFR mutations are rare in HCC samples [10,23–25]. Interestingly, the two phase-II trials presented limited data regarding the correlation between response to erlotinib and EGFR status in HCC patients; one study did not explore this correlation due to limitations [7] and the other showed no statistical significance between EGFR expression and overall survival [19]. Therefore, the exact anti-tumor mechanisms of erlotinib in HCC warrant further elucidation.

As stated above, Akt is one of the key players in cancer cell survival and apoptosis regulation and can be activated through EGFR or through constitutive activation of PI3 K. This downstream oncogenic pathway has been shown to be constitutively activated and correlated with worse prognosis in HCC [26]. Our previous studies also demonstrated that downregulation of p-Akt is a major molecular determinant of bortezomib-induced apoptosis in HCC cells [27]. Alternatively, negative regulation of Akt signaling can be achieved by phosphatases, such as phosphatase and tensin homologue deleted on chromosome ten (PTEN), and protein phosphatase 2A (PP2A). PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3) at the 3-position, thereby countering the action of PI3 K and inhibiting the activation of Akt. In contrast, PP2A is a serine/threonine protein phosphatase that can directly dephosphorylate p-Akt and p-ERK. PP2A is composed of catalytic C subunit (PP2Ac), structural A subunit (PR65) and regulatory B subunits [28]. PP2A can also regulate apoptosis through inactivation of anti-apoptotic Bcl-2 or activation of pro-apoptotic Bad [29]. PP2A also regulates the cell cycle, cell survival and proliferation by either directly or indirectly inhibiting cdc2, MAPK and Akt kinases [29]. Therefore, PP2A can act as tumor suppressor [30]. In this regard, our recent data also indicated that the proteasome inhibitor bortezomib enhances PP2A activity thereby downregulating p-Akt and inducing apoptosis in HCC cells [31]. Moreover, several cellular inhibitors of PP2A have been identified, including SET[32], and CIP2A [33]. CIP2A has emerged as a novel oncoprotein and a growing number of reports have shown its overexpression in many human malignancies, including HCC [34–41]. CIP2A has been shown to stabilize the oncoprotein c-Myc by inhibiting PP2A activity toward c-Myc, thus promoting anchorage-independent cell growth and in vivo tumor formation [33]. Recently, we further demonstrated that CIP2A, through inhibition of PP2A-dependent p-Akt inactivation, mediates the apoptotic effect of bortezomib in HCC cells [42].

In this study, we discovered that erlotinib, apart from being a selective EGFR inhibitor, has a novel drug mechanism (CIP2A-dependent PP2A activation and p-Akt downregulation) in some of HCC cells. We found that erlotinib differentially induced apoptosis in some but not all tested HCC cells. Erlotinib inhibited CIP2A in a dose- and time-dependent manner in all sensitive HCC cells whereas no alterations in CIP2A were found in resistant cells. We confirmed that CIP2A, through inhibiting PP2A activity toward p-Akt, is the predominant mediator of erlotinib-induced apoptosis in HCC cells. Moreover, a combination of the Akt inhibitor MK-2206 and erlotinib restored the sensitivity of resistant HCC cells to erlotinib. Importantly, this CIP2A-dependent p-Akt inhibitory mechanism that mediates the efficacy of erlotinib was confirmed in an in vivo nude mouse model. Our results not only disclose erlotinib's novel anti-tumor mechanism but also suggest that CIP2A may be useful as a therapeutic biomarker for predicting the clinical response of erlotinib in HCC treatment.

2. Materials and methods

2.1. Reagents and antibodies

Erlotinib was kindly provided by Roche Pharmaceuticals. For *in vitro* studies, erlotinib at various concentrations was dissolved in DMSO and then added to cells in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). The final DMSO concentration was 0.1% after addition to medium. Okadaic acid and forskolin were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies for immunoblotting such as anti-Akt1, -Bcl-2, -Bcl-xL, -PARP and -PP2A-C, were purchased from Santa Cruz Biotechnology (San Diego, CA). Other antibodies including anti-caspase-8, -caspase-9, -CIP2A, -P-Akt (Ser473), -Mcl-1, -Bax, -Bak and -Bad were from Cell Signaling (Danvers, MA).

2.2. Cell culture and western blot analysis

The Hep3B and PLC5 cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA). The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (HSRRB; Osaka, Japan; JCRB0403). HA59 T was obtained from Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). All cells were immediately expanded and frozen down such that all cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. No further authentication was conducted in our lab. Cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B in a 37 °C humidified incubator and an atmosphere of 5% CO_2 in air. Lysates of HCC cells treated with drugs at the indicated concentrations for various periods of time were prepared for immunoblotting of caspase-8, caspase-9, PARP, P-Akt, Akt, etc. Western blot analysis was performed as previously reported [42].