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## Induction of Tca8113 tumor cell apoptosis by icotinib is associated with reactive oxygen species mediated p38-MAPK activation

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Icotinib, a selective EGFR tyrosine kinase inhibitor (EGFR-TKI), has been shown to exhibit anti-tumor activity against several tumor cell lines. However, the exact molecular mechanism of icotinib's anti-tumor effect remains unknown. This study aims to examine the cytotoxic effect of icotinib on Tca8113 cells and its potential molecular mechanism. Icotinib significantly resulted in dose-dependent cell death as determined by MTT assay, accompanied by increased levels of Bax and DNA fragmentation. Icotinib could also induce Reactive Oxygen Species (ROS) generation. Further studies confirmed that scavenging of reactive oxygen species by N-acetyl-L-cysteine (NAC), and pharmacological inhibition of MAPK reversed icotinib-induced apoptosis in Tca8113 cells. Our data provide evidence that icotinib induces apoptosis, possibly via ROS-mediated MAPK pathway in Tca8113 cells.

### 1. Introduction

Epidermal growth factor receptor (EGFR) is frequently over-expressed by regulating cancer cell proliferation and survival in multiple human malignancy (Hicks et al. 2006). Therefore, direct inhibition of the EGFR signaling pathway may have significant antitumor effects and establish a strategy for cancer therapy (Yu et al. 2011). Several studies have reported that EGFR-TKI, such as gefitinib and erlotinib, induce apoptosis in many cell lines through the activation of intrinsic apoptosis pathways (Okamoto et al. 2012; Michikoshi et al. 2013; Yamaguchi et al. 2013). In addition, researchers demonstrated that the HDAC inhibitor vorinostat enhances the antitumor effect of gefitinib in CAL27 cells by modulating ErbB receptor expression (Bruzese et al. 2011).

Oxidative stress related to reactive oxygen species (ROS) is a classical stress response mechanism to modulate apoptosis in cancer cells (Manthe et al. 2010; Yu and Kim 2013), as is shown by the effects of several anti-cancer drugs. Phospho-NSAIDs, for example, have been found to act against cancer through oxidative stress mediated apoptosis and oxidative stress may be useful targets for anticancer strategies (Sun et al. 2011). Bacterial lipoprotein has also been found to induce TNF-alpha release and apoptosis in THP-1 monocyte cells through the P38 MAPK (O'Sullivan et al. 2009). Moreover, inhibition of EGFR has been proved to augment oridonin-induced A431 cell apoptosis by enhancing oxidative stress through blockage of JNK MAPK (Yu et al. 2012).

Icotinib hydrochloride, 4-[(3-ethinylphenyl)amino]-6, 7-benzo-12-crown-4-quinazoline hydrochloride, a novel and specific EGFR-TKI, is widely used to exert meaningful inhibitory activity in EGFR (Li et al. 2007; Yang et al. 2012). Icotinib has also been used in the treatment of advanced non-small cell lung

cancer patients (Song et al. 2013). Studies show that icotinib induced apoptosis by up-regulation of Cbl-b EGFR in mutation-positive non-small-cell lung cancer (Mu et al. 2013). Icotinib is also found to inhibit growth of human epithelial carcinomas of A431 cells through negatively regulating AKT signaling (Gao et al. 2013). Moreover, in a randomised, double-blind phase 3 non-inferiority trial, icotinib had less drug-related adverse events than gefitinib (Shi et al. 2013). All the above evidence shows that icotinib exhibits anti-tumor activity against several tumor cell lines.

However, the possible mechanism that works in the process remained unclear, and the relationship between ROS, tumor cell apoptosis and icotinib has not been reported yet. In this study we tested the effects of icotinib on the Tca8113 cell line. We found that icotinib led to apoptosis in Tca8113 cells, which is associated with oxidative stress mediated MAPK activation.

### 2. Investigations and results

#### 2.1. Icotinib inhibited the proliferation and induced apoptosis of Tca8113

To determine whether icotinib induces cell death in Tca8113, the cells were exposed to various concentrations of icotinib or vehicle and viability was measured by MTT experiments (Fig. 1A). A marked decrease in cell viability was observed in cells treated with icotinib for 24 h in the range of 5 to 20  $\mu$ M whereas exposure to 2.5  $\mu$ M icotinib or less had little impact on cell viability. Treatment with icotinib resulted in a significant enhancement of apoptosis when measuring the expression of Bax and DNA fragmentation (Fig. 1B-C).

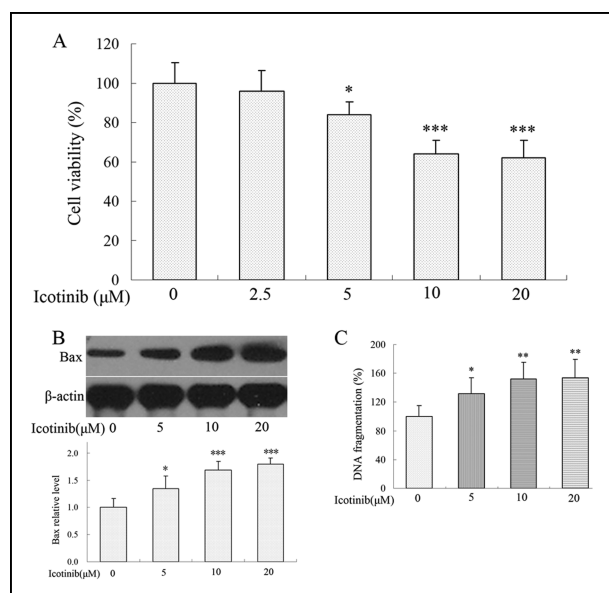


Fig. 1: Treatment with icotinib decreased the cell viability and induced apoptosis. Cell viability significantly decreased from the concentration of 5  $\mu\text{M}$  to 20  $\mu\text{M}$  after icotinib treatment for 24 h (A). Icotinib induced apoptosis in the expression of Bax (B) and DNA fragmentation (C) treated with 5, 10 or 20  $\mu\text{M}$  icotinib for 24 h. Data are expressed as mean  $\pm$  SEM,  $n=5$  for each group. \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$  compared with control group.

## 2.2. Treatment with icotinib increased the level of intracellular ROS and induced apoptosis in Tca8113 cells, which was reversed by the ROS scavenger

To investigate whether ROS accumulation is related to the icotinib-induced apoptosis, we analyzed the effect of ROS accumulation on the Tca8113 cells exposed to icotinib using the ROS-indicator CM-H2DCFDA. Icotinib significantly increased the level of ROS, which was blocked by the ROS scavenger, N-acetyl-L-cysteine (NAC) (Fig. 2A). Furthermore, the DNA fragmentation induced by icotinib was almost completely suppressed when the cells were co-treated with icotinib and NAC (Fig. 2B). These results suggest that the apoptosis induced by icotinib is related to ROS generation.

## 2.3. Icotinib-induced apoptosis action involved MAPK activation

Activation of MAPK is known to induce apoptosis and promote cell death (Venugopal et al. 2007). To investigate whether MAPK signaling was involved in the icotinib-induced apoptosis action, we determined the effects of icotinib on MAPK in Tca8113 cells. The results showed that icotinib significantly enhanced the relative level of p-p38/p38 MAPK in Tca8113 cells compared with the vehicle controls (Fig. 3A). NAC significantly blocked icotinib activated p-p38/p38 (Fig. 3B). We also found that the up-regulation of the relative level of p-p38/p38 MAPK induced by icotinib was markedly suppressed in the MAPK inhibitor SB203580 (Fig. 4A). SB203580 also inhibited icotinib-induced apoptosis (Fig. 4B). However, SB203580 in cells treated with icotinib had no effects on ROS production in the relative level of p-ErbB4/ErbB4 (Fig. 4C).

## 3. Discussion

Although accumulating data have substantiated that EGFR-TKI induced apoptosis in many tumor cells, the molecular mechanisms of the relationship between icotinib, ROS, and apoptosis are still not fully understood. In the present study, we found

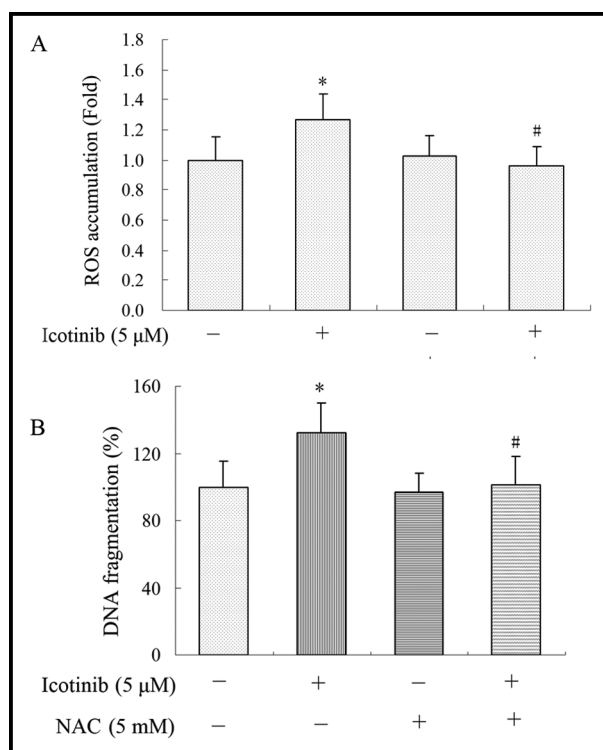


Fig. 2: Treatment with icotinib increased the accumulation of intracellular ROS in Tca8113 cells. A, Tca8113 cells were treated with DMSO, icotinib (5  $\mu\text{M}$ ), with or without 5 mM NAC for 24 h, and the intracellular ROS (A) and the DNA fragmentation (B) were measured. Data are expressed as mean  $\pm$  SEM,  $n=5$  for each group. (\*: significant as compared with Veh-treated control group, \* $P<0.05$ . #: significant as compared with icotinib-treated group, # $P<0.05$ ).

that icotinib could inhibit cell proliferation and induce apoptosis in Tca8113, and that the inhibition of icotinib was related to MAPK-mediated ROS production. Therefore, icotinib could be a candidate for the treatment of human cancer.

The epidermal growth factor receptor family of receptor tyrosine kinases is frequently overexpressed in many cancers and plays an important role in tumor development and progression (Gan et al. 2012). Inhibition of EGFR by erlotinib induced autophagosome formation and the expression of the autophagy marker LC3B-II in FaDu and Cal-27 cells (Sobhakumari et al. 2013). An EGFR-targeting monoclonal antibody, mAb806, is remarkably effective in tumor regression in the EGFRvIII-driven murine lung cancers (Li et al. 2007). Previous research has shown that icotinib could inhibit the proliferation of lung cancer HCC827 cells (Mu et al. 2013), in squamous cell carcinoma cell line A431 (Gao et al. 2013). In the present data, we found that apoptosis was induced by icotinib in Tca8113 cells, which was confirmed by measurement of DNA fragmentation and Bax levels. This study with previous research suggested that icotinib could inhibit cell proliferation and induce apoptosis and be developed into drugs for oral cancer treatment.

Oxidative stress by excessive production of ROS has been shown to be associated with apoptotic cell death (Sun et al. 2011; Qu et al. 2013). In our study, we clearly established a role of ROS in the apoptosis induction by icotinib. We found that icotinib treatment induced ROS generation in Tca8113 cells. To provide evidence that oxidative stress was involved in icotinib-induced apoptosis, an SOD mimic, NAC was employed. Further studies confirmed that the ROS scavenger antioxidant (NAC) prevented the icotinib-induced ROS generation and subsequent caspase 3 expression induced by icotinib. The study provides evidence that the enhancement of apoptosis induced by icotinib is possibly dependent on the elevated ROS levels.

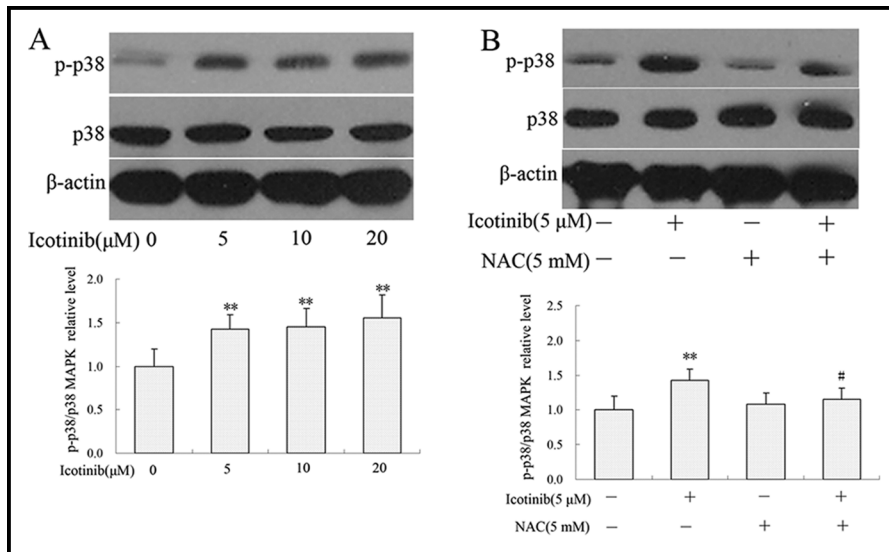


Fig. 3: Effect of icotinib on activation of MAPKs in Tca8113 cells. (A) The expression of p-p38 and p38 MAPK was measured by Western blot analysis after treatment with various concentrations of icotinib. (B) Tca8113 Cells were treated with 5 μM icotinib in the absence or presence of 5 mM NAC for 24 h. The levels of p-p38/p38 were analyzed by Western blot. Data are expressed as mean ± SEM, n=5 for each group. (\*: significant as compared with Veh-treated control group, \*\* $P < 0.01$ . #: significant as compared with Icotinib-treated group, #  $P < 0.05$ ).

Oxidative stress is known to modulate the p38 MAPK signaling pathway (Zhou et al. 2010; Yu et al. 2012). In our studies, the activation of MAPK induced by icotinib was supported by the increase of p-p38 MAPK. Experiments with the MAPK inhibitor SB203580 showed that inhibition of MAPK abolished icotinib-induced apoptosis. The relative level of p-p38/p38 MAPK was found to be reduced by treatment with NAC in experiments in which NAC was added after removal of icotinib. These results suggested that MAPK serves as a downstream mediator of icotinib-induced oxidative stress. We finally tested whether activation of MAPK was required for cells apoptosis induction by icotinib. Pharmacological inhibition of MAPK inhibited apoptosis induced by icotinib suggesting that icotinib induces apoptosis *via* regulating MAPK pathway.

In conclusion, our present study provides evidence that icotinib could induce apoptosis *via* ROS mediated p38-MAPK activation, suggesting that icotinib may be useful in the treatment of cancer.

## 4. Experimental

### 4.1. Materials

Icotinib was purchased from Zhejiang Beta PharmaInc (Zhejiang, China). Antibodies against Bax and p-p38 MAPK, p38 MAPK were purchased from Cell Signaling Technology. NAC were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and cell culture: The human tongue carcinoma cell line Tca8113 was cultured in RPMI 1640 medium supplemented

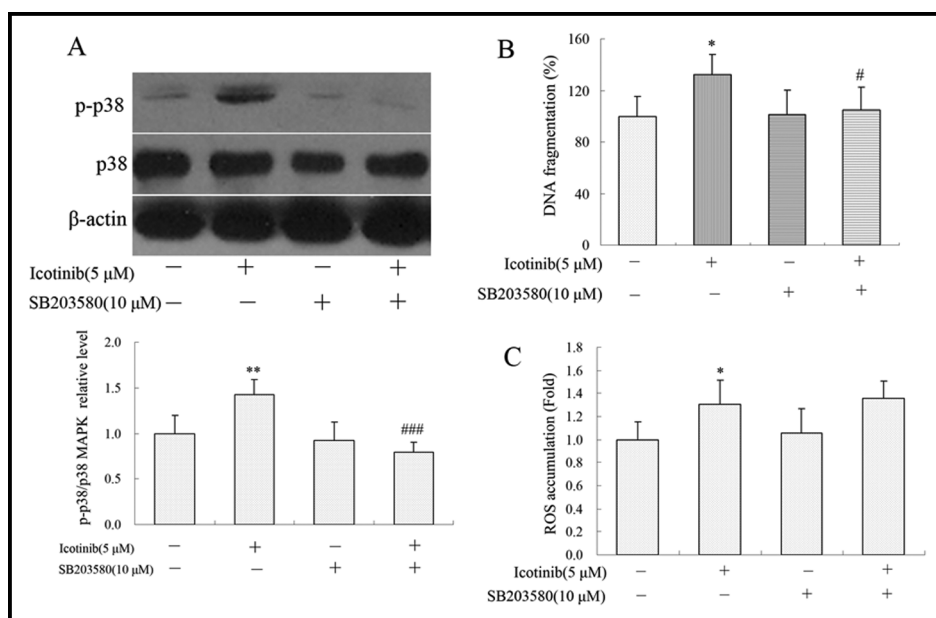


Fig. 4: The effect of MAPKs on apoptosis by icotinib-induced ROS in Tca8113 cells. (A) Tca8113 cells were treated with 5 μM icotinib in the absence or presence of p38-MAPK inhibitor, SB203580 (10 μM) for 2 h, then p-p38/p38 MAPK (A), DNA fragmentation (B) and ROS generation (C) were measured. Data are expressed as mean ± SEM, n=5 for each group. (\*: significant as compared with Veh-treated control group, \* $P < 0.05$  and \*\* $P < 0.01$ . #: significant as compared with the icotinib-treated group, #  $P < 0.05$  and ###  $P < 0.001$ ).

with 10% FBS plus penicillin and streptomycin at 37 °C in a CO<sub>2</sub> incubator.

#### 4.2. Determination of cell viability

Inhibition of cell proliferation by icotinib was measured by MTT assay as previously described (Chen et al. 2004).

#### 4.3. DNA Fragmentation analysis

DNA fragmentation was detected according to the manufacturer's instructions by a Cell Death Detection ELISAPLUS kit (Roche Diagnostics). Briefly, cells were collected after drug treatment. Then cells were washed with D-Hanks solution and incubated with 200 µl of lysis buffer for 30 min at 37 °C and centrifuged at 200 × g for 10 min at 4 °C. 20 µl supernatant was incubated in streptavidin-coated microplate containing anti-DNA-peroxidase and anti-histone-biotin. The anti-histone-biotin antibody was used to capture apoptotic nucleosomes via histone component and the anti-DNA-peroxidase was used to bind the DNA part of the nucleosomes. The level of peroxidase was quantified by 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS). The absorbance was read at 405 nm using microplate reader. The DNA fragmentation was assessed as percentage of the control group.

#### 4.4. ROS measurements

ROS levels were assayed by the fluorescent probe using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, USA) as previously described (Myhre et al. 2003). Briefly, cells were incubated in culture medium, DCFH-DA (10 mM) was added at 37 °C for 30 min after icotinib treatment, washed three times and assayed using a fluorescence microscope and a fluorescence spectrophotometer (excitation 488 nm, emission 530 nm).

#### 4.5. Statistical analysis

All data were expressed as mean ± SEM, and statistical analysis was performed using one-way ANOVA. The exact F statistic value and degrees of freedom were used to calculate probabilities and results were deemed significant if the *P* value was less than 0.05.

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