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Curcumin and (−)-epigallocatechin-3-gallate attenuate acrylamide-induced proliferation in HepG2 cells

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ABSTRACT

Acrylamide, a proven rodent carcinogen, is present in carbohydrate-rich food heated at high temperatures. It can be metabolized into glycidamide mainly by cytochrome P450 2E1 (CYP2E1). The fact that acrylamide is a potential carcinogen to human-beings draws public attention recently. This study aimed to elucidate the effect of acrylamide at low doses on proliferation of HepG2 cells, and to test whether the two well-studied chemopreventive agents, curcumin and (−)-epigallocatechin-3-gallate (EGCG), would have antagonistic effects against acrylamide. The results showed that lower concentration of acrylamide (⩽100 μM) significantly increased the proliferation of HepG2 cells, but not of the other cancer cells (MDA-231, HeLa, A549, and PC-3). Only in HepG2 cells, low concentration of acrylamide was able to induce CYP2E1 expression significantly. Knockdown of CYP2E1 restrained acrylamide to increase viability of HepG2 cells. In addition, acrylamide raised expression of epidermal growth factor receptor (EGFR), cyclin D1 and nuclear factor-κB (NF-κB), which contributed to cell proliferation. Both curcumin and EGCG effectively reduced acrylamide-induced proliferation, as well as protein expression of CYP2E1, EGFR, cyclin D1 and NF-κB. All these results suggest that low concentration of acrylamide may contribute to progression of hepatocellular carcinoma (HCC). Curcumin or EGCG could prevent acrylamide triggering this effect.

1. Introduction

Acrylamide is widely used in many fields from industrial manufacturing to laboratory personnel work. Since 2002, it has been reported that low levels of acrylamide can be formed in carbohydrate-rich food during high-temperature cooking processes such as frying, roasting and baking (Tareke et al., 2002). After exposure, acrylamide can be rapidly distributed to all tissues and be transformed into the more toxic form glycidamide (accounts for approximately 12% of the total urinary metabolites in humans (Fennell et al., 2005)) by CYP2E1, and is able to reach the fetus after passing the placental barrier in experimental animals (Parzefall, 2008). These findings raise human concerns about the risks of acrylamide for the general population and children.

Acrylamide has been shown to have various effects in vivo and in vitro. Studies demonstrate that acrylamide is neurotoxic and genotoxic (Baum et al., 2005; Hashimoto and Aldridge, 1970; He et al., 1989; Manjanatha et al., 2006), and possesses the ability to cause toxic effects on development and reproduction (Dearfield et al., 1988) to rats and mice. Besides, acrylamide has been classified as "probably carcinogenic to humans" (Group 2A) by the International Agency for Research on Cancer according to its carcinogenicity to rodents (IARC, 1994).

The carcinogenic mechanisms of acrylamide in experimental mice and rats have been investigated recently. The lowest carcinogenic effective dose observed in these studies was 1–2 mg/kg bw/day. Some of the cancer types (e.g., thyroid, scrotal mesotheliomas, mammary gland) indicate a possibility that acrylamide causes tumor in a hormone-based way (Beland et al., 2013; Dearfield et al., 1988; Friedman et al., 1995; Johnson et al., 1986; NTP, 2012). The other mechanisms are likely to be connected with its DNA damage effects and clastogenic properties (Banerjee and Segal, 1986; Park et al., 2002; Tsuda et al., 1993; Watzek et al., 2012; Yamasaki et al., 1996). Moreover, the molecular mechanisms of the carcinogenicity of acrylamide may involve its up-regulating the expression of cyclooxygenase-2 (COX-2), transcription activator-1 (AP-1) and NF-κB (Lim et al., 2011).

However, only a few studies proved the positive relationship between dietary acrylamide and human cancer, such as endometrial and ovarian cancer (Lipworth et al., 2012a). That may be the effective concentration of acrylamide after dietary intake in general population is extremely low. And the effective internal doses of acrylamide are much lower after biological metabolism. One

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study analyzing hemoglobin adducts formation, which is usually measured for the internal effective doses of acrylamide (Bergmark et al., 1993), with a daily high intake of 11 μg acrylamide per kg body weight for 4 days, is shown to just give an acrylamide-adduct increment of approximately 15 pmol/g in blood samples (Vikstrom et al., 2011). Besides, the effect of dietary acrylamide on tumor progress is not known, either.

In recent decades, phytochemicals have been aroused attention because of their cancer chemopreventive potentials. Many studies in vitro have demonstrated the antagonistic effect of several kinds of phytochemicals such as curcumin, hydroxytyrosol, resveratrol, tea polyphenols, and diallyl trisulfide against toxicity of acrylamide (Cao et al., 2008; Rodriguez-Ramiro et al., 2011; Xie et al., 2008; Zhang et al., 2009). For example, curcumin (2.5 μg/mL), a yellow pigment presented in turmeric, significantly reduces acrylamide-induced ROS production, DNA fragments, micronuclei formation, and cytotoxicity in HepG2 cells (Cao et al., 2008). It is reported that green tea polyphenols can inhibit the formation of acrylamide-induced DNA-adducts and recover acrylamide-induced inactivation of creatine kinase. Besides, tea polyphenols are able to increase GST activity remarkably (Xie et al., 2008). However, no data so far have demonstrated the antagonistic effect of either curcumin or tea polyphenols on the other effect of acrylamide.

Therefore, we focused on the effect of low concentration of acrylamide on cancer cells proliferation. HepG2 cells were used, because this cell line is derived from human hepatocellular carcinoma and has the xenobiotic-metabolizing properties similar to normal hepatic cells, and is widely used as a model for studies of liver metabolism and toxicity of xenobiotics and detection of hepatocarcinogenesis (Mersch-Sundermann et al., 2004). The antagonistic effect of curcumin and EGCG (a major green tea polyphenol) against acrylamide was also investigated.

2. Materials and methods

2.1. Cell culture and reagents

HepG2, A549, HeLa and PC-3 cells were purchased from Cell Centre of Chinese Academy of Medical Sciences (Peking, China). MDA-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). HepG2, A549 and HeLa were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/mL, Gibco), and streptomycin (100 μg/mL, Gibco). PC-3 cells were in RPMI Medium 1640 (Gibco, USA) and MDA-231 in Leibovitz's L-15 Medium (Gibco, USA). These cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Acrylamide (purity > 99.5%, dissolved in ddH_2O in a concentration of 1000 mM/L), curcumin (dissolved in DMSO in a concentration of 50 mM/L) and EGCG (dissolved in ddH_2O in a concentration of 2 mM/L) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pyrrolidine dithiocarbamate (PDTC) was purchased from Beyotime (Shanghai, China).

2.2. MTT assay

Cell viability was assessed by the methyl thiazol tetrazolium bromide (MTT) assay. Cells were seeded in a 96-well plate at a density of 4×10^3 cells per well in a final volume of 180 μL of medium. After indicated treatment, the cells were incubated with MTT solution (5 mg/mL) at 37 °C for 4 h. The formed formazan crystals were dissolved in DMSO at room temperate for 10 min. Then the absorbance was read at 490 nm with a microplate reader (Tecan /Infinite M200, Switzerland).

2.3. Western blot analysis

The experiments were prepared from seeding with 5×10^5 cells. After indicated treatments, the cells were washed twice with PBS and suspended in a lysis buffer including 1 mM DTT, 0.1% protease inhibitor and 5 mM PMSF (KeyGEN BioTECH, China). Protein concentrations were measured by BCA Protein Assay Kit (Beyotime, China). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate 60 μg of protein, which was then transferred to PVDF membrane (Millipore Corporation, USA). The primary antibodies included rabbit anti-EGFR monoclonal antibody (Cell Signaling technology, USA), rabbit anti-NF-κB/p65 monoclonal antibody (Cell Signaling technology, USA), rabbit anti-cyclin D1 polyclonal antibody (Santa Cruz Biotechnology, USA), rabbit anti-CYP2E1 monoclonal antibody (Epitomics, USA) and mouse anti-β-actin monoclonal antibody (BOSTER, China). Second antibodies included HRP-Conjugated AffiniPure Goat Anti-rabbit IgG (ZSGB-BIO, China) and HRP-Conjugated AffiniPure Goat Anti-mouse IgG (ZSGB-BIO, China). Immunoreactive proteins were visualized using ECL Western blotting detection reagents (GE Health-care, Buckinghamshire, UK). The bands were quantified with the Image J software.

2.4. EdU fluorescence staining

The 5-ethynyl-2′-deoxyuridine (EdU) fluorescence staining was used to detect the newly synthesized DNA in HepG2 cells (seeding with 4×10^3 cells per well in a 96-well plate) after the indicated treatment. All steps performed following the manufacturer's instructions of Cell-LightTM EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China).

2.5. siRNA transfection

HepG2 cells (2.5×10^5 or 4×10^3) were transfected with predesigned human CYP2E1 siRNA or siRNA control (50 nM) (RiboBio, Guangzhou, China) in a six-well plate or a 96-well plate for 4 h with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif, USA) in a serum-free and antibiotic-free media. All steps performed following the manufacturer's instructions.

2.6. Statistical analyses

The results were presented as the mean ± standard deviation (SD) compared with the controls. A one-way analysis of variance (ANOVA), which was followed by a Tukey HSD test for the multiple comparisons, was used to detect the differential effects of acrylamide on HepG2 cells. A p value < 0.01 was considered statistically significant.

3. Results

3.1. Effect of acrylamide on HepG2 cells proliferation

To investigate the effect of acrylamide on HepG2 cells viability, MTT assay was carried out. As shown in Fig. 1a, when HepG2 cells were treated with the lower concentrations of acrylamide (from 1 μM to 100 μM), cell viability was increased in a dose-dependent manner. After acrylamide (100 μM) treatment for 48 h, the cell viability was increased by 23.5% as compared with the control ($p < 0.001$). To further ascertain the proliferation-accelerating effect of this dose of acrylamide, EdU fluorescence staining assay was performed to detect the newly synthesized DNA in HepG2 cells. Results showed that the EdU-positive cells (red fluorescence staining) increased distinctly after acrylamide treatment, indicating a cell proliferation-promoting effect by acrylamide (Fig. 1b). However, HepG2 cells viability was inhibited when the cells were treated with the higher concentration (500 μM) of acrylamide for 48 h (Fig. 1a). This was consistent with the previous evidences, of which acrylamide (>250 μM) showed cytotoxicity in HepG2 cells (Cao et al., 2008; Zhang et al., 2009). Interestingly, treatment of acrylamide to the other cancer cells (including MDA-231, HeLa and PC-3) did not increase the cell viability, as Fig. 1c showed. Acrylamide ($\geqslant$100 μM) had an inhibitory effect on A549 cells viability (Fig. 1c). The reason may be that A549 is highly sensitive to acrylamide.

3.2. Role of CYP2E1 in acrylamide increased cell viability

Since acrylamide exhibits its potential carcinogenic effect mainly through glycidamide metabolized by CYP2E1, we assumed if there would be a difference between HepG2 cells and the other cancer cells in basal CYP2E1 expression. Fig. 2a showed that the expression of CYP2E1 appeared to be much higher in HepG2 cells compared with the cell lines else. Moreover, low dose of acrylamide, especially at 100 μM, increased CYP2E1 levels distinctly in HepG2, but not in MDA-231, HeLa, A549 or PC-3 cells (Fig. 2b and c).

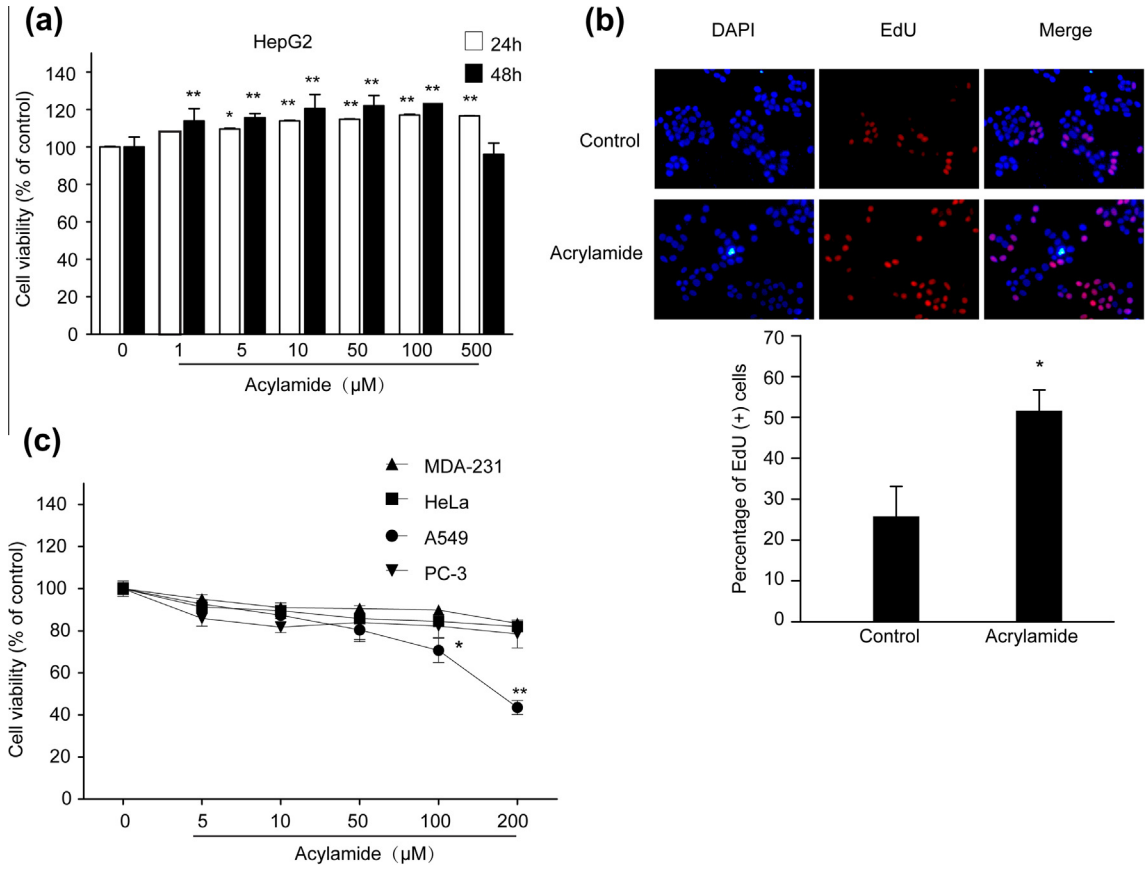

Fig. 1. Effect of acrylamide on proliferation of HepG2, MDA-231, HeLa, A549 and PC-3 cells. Acrylamide ranging from 1 to 100 μM increased the viability of HepG2 cells in a concentration-dependent manner after 24 h or 48 h of treatment, as analyzed by MTT assay (a). The EdU-positive cells (red fluorescence staining) increased distinctly after acrylamide (100 μM) treatment for 24 h measured by EdU fluorescence staining assay (b). Treatment of acrylamide (5, 10, 50, 100, 200 μM) for 24 h did not increase the viability of MDA-231, HeLa, A549 and PC-3 cells (c). Results are presented as the mean ± SD from three independent experiments (in triplicate for each experiment). Statistical differences to the controls are shown as $^{*}p < 0.01$, and $^{**}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To further investigate the role of CYP2E1 in viability increasing of HepG2 cells induced by lower concentration of acrylamide, knockdown of CYP2E1 in HepG2 cells was approached by transfection of predesigned siRNA targeting CYP2E1. Once CYP2E1 was knocked down, low concentration (100 μM) of acrylamide was not able to induce CYP2E1 expression (Fig. 2d). Also, knockdown of CYP2E1 resulted in failing to stimulate HepG2 cells viability (Fig. 2e).

3.3. Effect of acrylamide on proliferation factors

To examine if the common proliferation signaling molecules, such as EGFR and cyclin D1, would be stimulated by low concentration of acrylamide, HepG2 cells were treated with acrylamide (0, 10, 50, 100 μM) for 24 h. Protein levels of EGFR and cyclin D1 were then detected by Western blot analysis. As shown in Fig. 3a, acrylamide markedly elevated both protein levels in a dose-dependent manner. Meanwhile, the NF-κB transcription factor was also upregulated by acrylamide (Fig. 3b). Next, the role of NF-κB signaling in acrylamide-increased cells viability was investigated. HepG2 cells were treated with acrylamide in the absence or presence of NF-κB specific inhibitor PDTC. As shown in Fig. 3c, a significant reduction of NF-κB expression was found when HepG2 cells were pretreated with PDTC before acrylamide exposure, compared to that of the group with only acrylamide treatment. The target protein cyclin D1 and HepG2 cells viability were also blocked (Fig. 3c and d).

3.4. Effect of curcumin and EGCG on acrylamide-induced proliferation

Curcumin and EGCG alone significantly decreased HepG2 cells viability (Fig. 4a and b). To determine whether edible plant polyphenols would prevent acrylamide to mediate HepG2 cells proliferation, HepG2 cells were pretreated with curcumin (10 μM) or EGCG (10 μM) for 2 h, then incubated with acrylamide (100 μM) for an additional 24 h. Cell proliferation was then determined by MTT and EdU fluorescence staining assay. Both curcumin and EGCG had a significant reduction of cell viability (Fig. 4c). Furthermore, both curcumin and EGCG effectively reduced acrylamide to trigger cell proliferation (Fig. 4d).

3.5. Effect of curcumin and EGCG on acrylamide-induced CYP2E1 and proliferation factors

To further explore the mechanisms of the inhibitory effect of curcumin and EGCG on acrylamide-induced proliferation, CYP2E1 and the important proliferation factors were analyzed. As shown in Fig. 5a, CYP2E1 expression was inhibited obviously in the group with curcumin (10 μM) pretreatment for 2 h compared to the control group without curcumin. Meanwhile, the ability of acrylamide to induce EGFR, cyclin D1 and NF-κB overexpressions was also significantly restrained by curcumin (Fig. 5a and c). Likewise, EGCG (10 μM) displayed the similar effects on preventing acrylamide from activating CYP2E1, EGFR, cyclin D1 and NF-κB (Fig 5b and d).

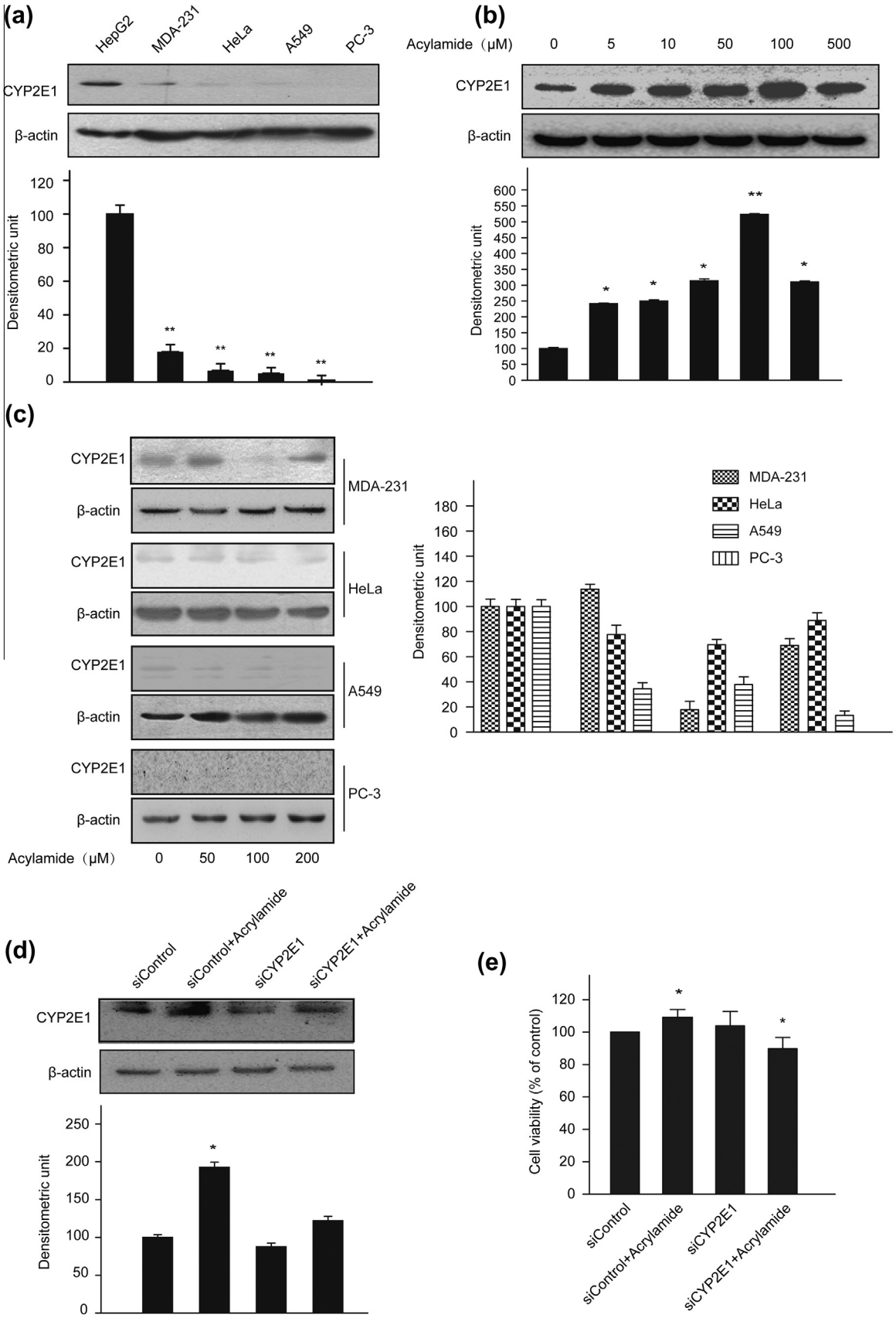

Fig. 2. Role of CYP2E1 in acrylamide increased cell viability. In comparison to HepG2, the other cancer cells showed low or negative expression of CYP2E1 (a). Acrylamide increased CYP2E1 expression distinctly in HepG2 cells after 24 h treatment, especially at concentration of 100 μM, but not in the other cell lines (b and c). CYP2E1 knockdown with CYP2E1 siRNA transfection (50 nM) for 28 h significantly inhibited acrylamide to increase CYP2E1 expression and HepG2 cells viability (d and e). Results are presented as the mean ± SD from three independent experiments (in triplicate for each experiment). Statistical differences to the controls are shown as $^{*}p < 0.01$, and $^{**}p < 0.001$.

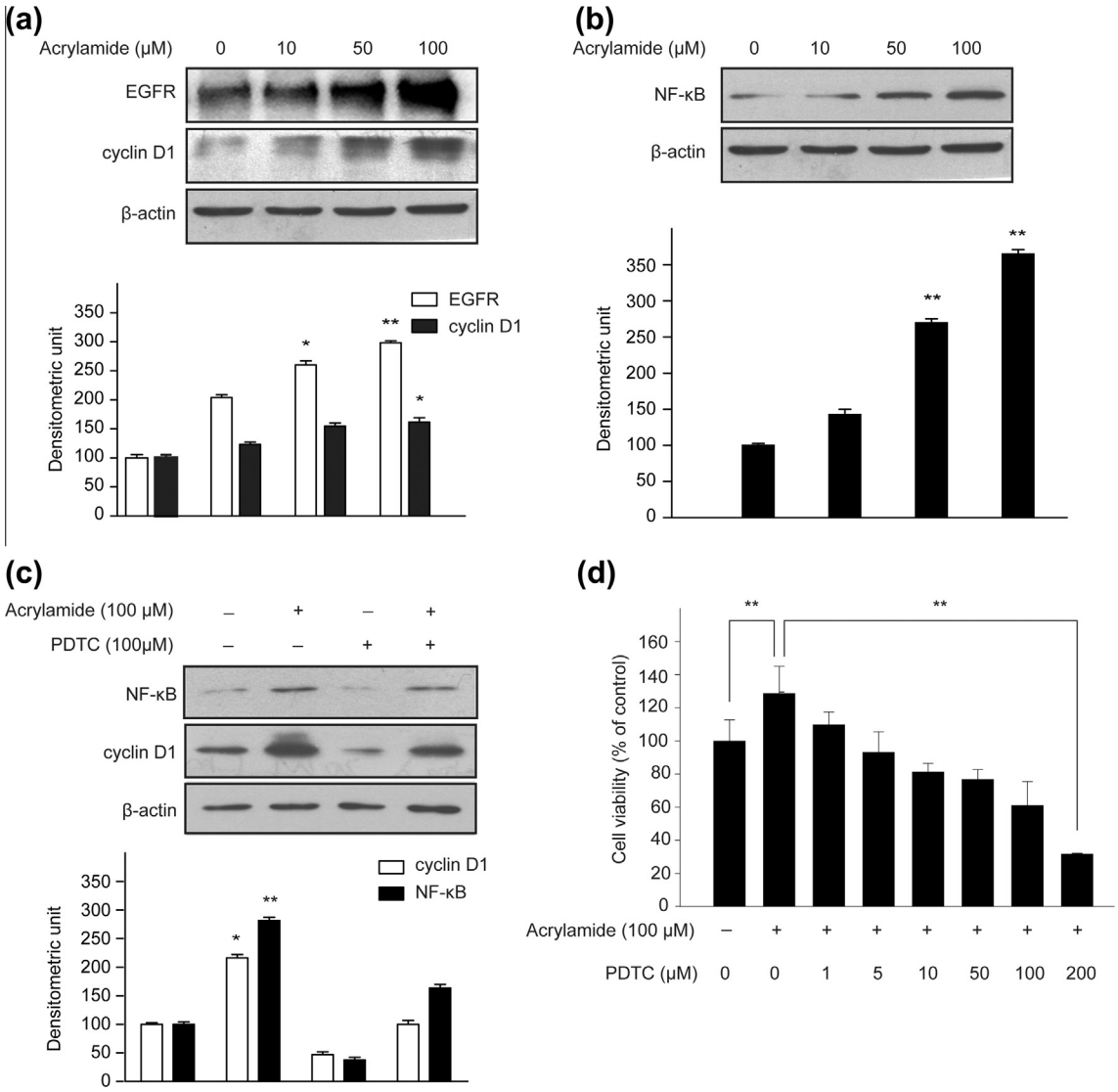

Fig. 3. Effect of acrylamide on proliferation factors. After 24 h of acrylamide treatment, expression of EGFR, cyclin D1 and NF-κB was increased, in a dose-dependent manner (a and b). PDTC (100 μM) pretreatment decreased acrylamide-induced NF-κB expression and its target protein cyclin D1 expression (c). PDTC (1–200 μM) pretreatment inhibited acrylamide-increased HepG2 cells viability in a dose-dependent manner (d).

4. Discussion

Acrylamide is a potential carcinogen. Previous studies indicate that the carcinogenesis of acrylamide is mainly involved in the genotoxicity induced by glycidamide (Hogervorst et al., 2010; Klaunig, 2008). The different outcomes of acrylamide exposure between experimental rodents and humans may be due to the concentrations of acrylamide applied to rats and mice being generally higher than that of human dietary exposure. In the present study, we focused on the effect of low concentration of acrylamide. To our knowledge, this is the first report demonstrating that low doses of acrylamide are able to increase the proliferation of HepG2 cells through upregulating CYP2E1 and certain malignant proliferation factors (EGFR, cyclin D1 and NF-κB).

CYP2E1 is a key enzyme in the metabolic activation of a variety of toxicants such as ethanol, acrylamide, nitrosamines and benzene (Guengerich et al., 1991). Some of these toxicants can even induce CYP2E1 overexpression, resulting in enhanced activation of procarcinogens to carcinogens (Gonzalez, 2007; Seitz and Osswald, 1992) and induction of oxidative stress which may stimulate carcinogenesis (Seitz and Stickel, 2007). Studies have also shown the positive relationship between CYP2E1 and tumor progress. For example, alcohol, the common substrate of CYP2E1, is able to mediate both CYP2E1 induction and proliferation increasing in HCC cells in vitro (Brandon-Warner et al., 2010). Considering such observations, we initially hypothesized that acrylamide might lead to elevated CYP2E1 as well as HCC cells proliferation. As expected, low concentration of acrylamide-induced CYP2E1 showed a strong correlation with HepG2 cells proliferation in this study though acrylamide did not increase cell viability in the other cancer cell lines. In A549 cells, the low doses of acrylamide even decreased the cell viability. However, these effects of acrylamide on cell viability were consistent with CYP2E1 level, indicating that the proliferation-increasing effect of low concentration of acrylamide largely depended on CYP2E1 expression.

Low dose of acrylamide exposure in human primary hepatocytes does not induce genes with carcinogenic potential. Instead, it activates genes involved in detoxification (Ehlers et al., 2013). In addition, only several studies have demonstrated the positive relationship between acrylamide and human cancer, among those people who have high acrylamide intake (mean intake, 40.2 μg/day) (Hogervorst et al., 2007; Lipworth et al., 2012b). Thus, the

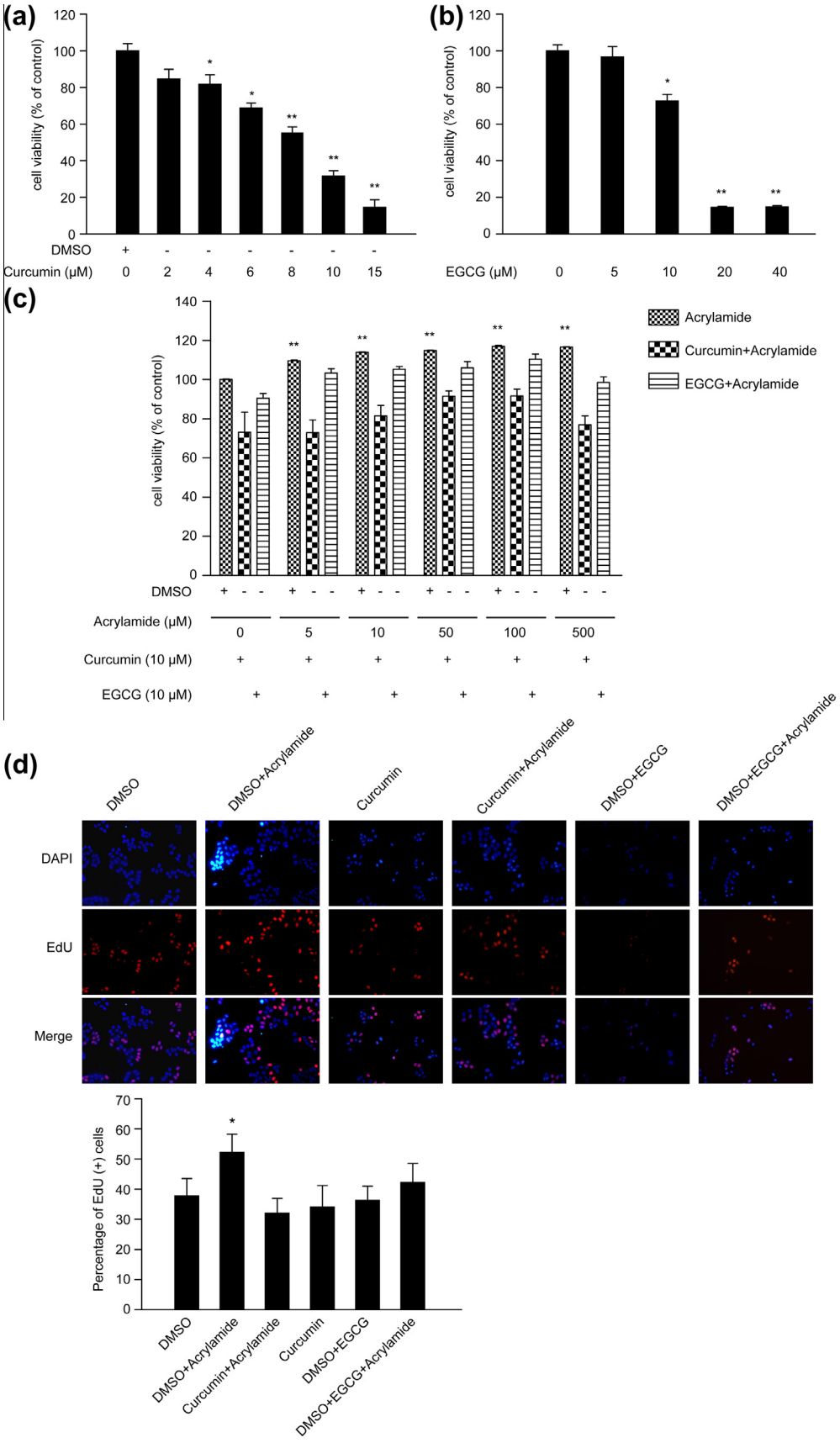

Fig. 4. Effect of curcumin and EGCG on acrylamide-induced proliferation of HepG2 cells. MTT assay showed that curcumin (2, 4, 6, 8, 10, 15 μM) and EGCG (5, 10, 20, 40 μM) alone inhibited HepG2 cells viability (a and b). Pretreatment with curcumin (10 μM) or EGCG (10 μM) for 2 h could distinctly prevent acrylamide (5, 10, 50, 100, 500 μM) to increase HepG2 cells viability (c). Curcumin (10 μM) and EGCG (10 μM) abolished the effect of acrylamide on HepG2 cells proliferation with the EdU assay (d). Results are presented as the mean ± SD from three independent experiments (in triplicate for each experiment). Statistical differences to the controls are shown as $^{*}p < 0.01$, and $^{**}p < 0.001$.

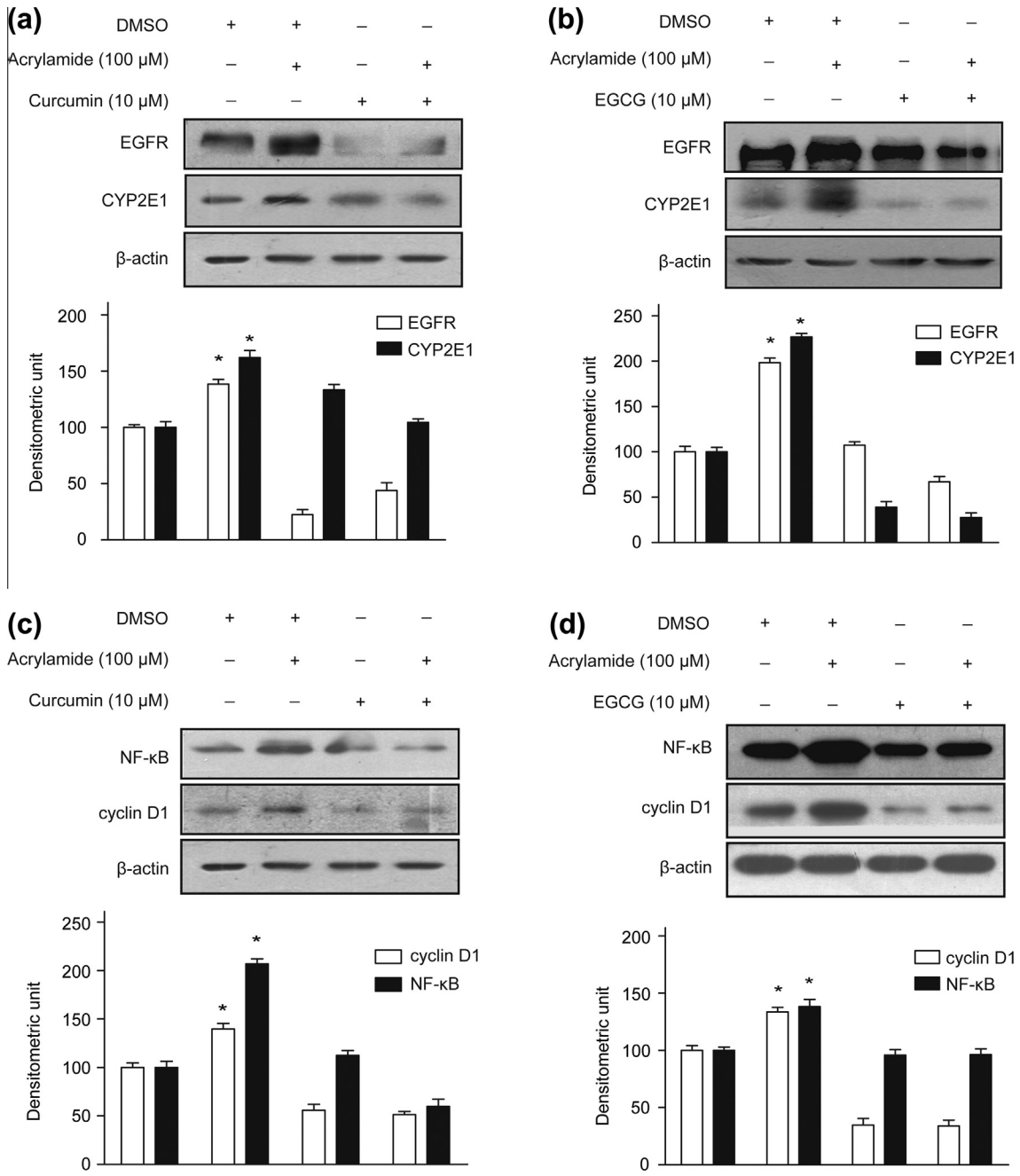

Fig. 5. Effect of curcumin and EGCG on acrylamide-induced CYP2E1, EGFR, cyclin D1 and NF-κB expression. Pretreatment with curcumin (10 μM) for 2 h inhibited acrylamide to induce CYP2E1, EGFR, cyclin D1, and NF-κB expression (a and c). EGCG, at the concentration of 10 μM, substantially repressed CYP2E1, EGFR, cyclin D1 and NF-κB induced by acrylamide (b and d).

carcinogenicity of dietary acrylamide to human-beings is not sure. The results from this study indicating that low concentration of acrylamide could increase HepG2 cells proliferation suggested that dietary acrylamide may affect the progress of hepatocellular carcinoma in human, which should be verified in the future.

EGFR signaling is able to enhance the activity of NF-κB transcription factor (Chen et al., 2001). NF-κB pathway contributes to cell proliferation mainly through constitutively activating certain target genes such as cyclin D1 promoter (Karin, 2006). In the current study, both curcumin and EGCG disturbed overexpression of EGFR which is associated with tumor progression and poor prognosis in many epithelial neoplasms, including hepatocellular carcinoma (Altimari et al., 2003; Ito et al., 2001; Lee et al., 2007; Tang et al., 1998), hence contributing to the inhibition of immoderate cell proliferation. NF-κB, an important transcriptional factor regulated by tumor necrosis factor-α (TNF-α) in hepatocytes, is shown to be in connection with liver neoplastic progression through regulating multiple genes involved in cellular transformation, proliferation, and survival (He and Karin, 2011). It has been shown that the treatment of chlormethiazole (CMZ), a CYP2E1 inhibitor, in ethanol-fed rats significantly blocks the ethanol-induced NF-κB activation (Ye et al., 2012). Likewise, apparent blockage of acrylamide-induced CYP2E1 as well as NF-κB and cyclin D1 by curcumin and EGCG was observed in our study. These data provide useful information that

curcumin and EGCG may be potential inhibitors of CYP2E1. However, in comparison to EGCG, curcumin presented the more significant inhibitory effect on acrylamide-promoting proliferation and NF-κB, but not CYP2E1, EGFR and cyclin D1, which indicated curcumin and EGCG might have different mechanisms in vivo and in vitro. To overcome the problem of the poor bioavailability of curcumin, which may result from its poor water solubility and pharmacokinetic profile, researchers have developed several improved forms like Theracurmin and liposome encapsulation of curcumin without adverse effects (Hasan et al., 2013; Kanai et al., 2013). Although these data have an insight into the possible mechanisms whereby curcumin and EGCG may slow the rate of HCC progression, further studies are required to determine whether these mechanisms are equally important in vivo.

In conclusion, data presented in this study demonstrate that curcumin and EGCG inhibit CYP2E1, EGFR/NF-κB signaling and cell proliferation stimulated by low doses of acrylamide in a human HCC cell line in vitro. Namely, reasonable supplement of curcumin and EGCG, which are natural antagonists of acrylamide, can avoid the side effects caused by improper diet like fried food which leads to inevitable acrylamide exposure.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

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