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ORIGINAL ARTICLE

Anti-proliferative and anti-apoptotic potential effects of epigallocatechin-3-gallate and/or metformin on hepatocellular carcinoma cells: in vitro study

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Abstract

The effects of epigallocatechin-3-gallate (EGCG) and metformin single treatment have been tested against hepatocellular carcinoma (HCC). This study aimed to assess the combination effects of EGCG and metformin on proliferation and apoptosis of HepG2cells and identified new potential molecular targets. The effect of EGCG and metformin against cell proliferation in HepG2 was determined using MTT assay. Reverse transcription polymerase chain reaction was applied to examine the gene expression of cyclin D1, lncRNA-AF085935, caspase-3, survivin and VEGF. The level of protein expression of glypican-3 was assessed by western blot. In HepG2 cells, EGCG and metformin combination treatment exhibited high significant effect against tumor proliferation. It significantly reduced cyclin D1, lncRNA-AF085935, glypican-3 and promoted apoptosis through increasing caspase3 and decreasing survivin compared to control cells. Moreover, EGCG and metformin treated cells showed decreased expression levels of VEGF. Our study provided new insights of the anticarcinogenic effects of EGCG and metformin on HCC through their effects on glypican-3 and lncRNA-AF085935.

Keywords Hepatocellular carcinoma · EGCG · Metformin · Glypican-3 · LncRNA-AF085935

Introduction

Hepatocellular carcinoma (HCC) is reported to be one of the commonest cancers and the third leading cause of cancer deaths worldwide [1]. Apoptosis is a physiological process of cell death that works to control cell clusters [2].

Disturbance in regulation of cell apoptosis can lead to cell over-proliferation which can give rise to tumor development or tumorigenesis. The apoptotic signaling pathway is regulated by a diversity of factors and is established on the equilibrium between cell death and survival factors [3, 4].

Definitely, most HCC cells display strong resistance to stimuli that prompt apoptosis in other cells [5]. Consequently, disabling apoptotic resistance has become important for the development of current therapeutic lines for HCC treatment.

Epidemiological researches have shown that green tea which is a tremendously prevalent drink worldwide, has anticarcinogenic and anticancer effects [6]. A chief polyphenol of green tea, epigallocatechin-3-gallate (EGCG), is believed to be the most biologically effective compound in

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green tea and has been advised to be a possible anticancer agent against many cancers including HCC [7].

Metformin is anti-diabetic drug which belongs to biguanides. Various epidemiologic works have revealed that metformin could decrease incidence of different tumors in diabetic patients treated with it [8]. Increasing evidence has speculated that metformin might affect angiogenic pathways [9]. Furthermore, the underlying molecular mechanism of antineoplastic activity of EGCG and metformin is still largely unknown.

Glypican-3 (GPC3), one of the glypican family of heparan-sulfate proteoglycans (HSPGs) [10]. GPC3 is highly expressed in the majority of HCCs and it has been revealed as an worthy diagnostic marker for HCC, it was proved to be a more reliable tumor marker permitting earlier HCC diagnosis in comparison with serum alpha-fetoprotein [11, 12].

LncRNAs are type of newfound non-coding RNAs, who had a length more than 200 nucleotides [13]. Evidence has recognized an aberrant expression of lncRNA in numerous diseases, particularly in tumors, including HCC [14, 15]. LncRNA-AF085935 which is transcribed in antisense orientation of GPC3, has been proved to be a possible biomarker for HCC [16].

Inspite of the latest progresses in the medical management of liver cancer, the long-term prognosis of HCC is still discouraging. Developing new therapeutic strategies for HCC could be one of the pressing needs. In spite of the availability of a wide range of therapeutic options, the effectiveness of these methods and the survival of patients with hepatocellular carcinoma remains poor. So, the current study was planned to assess new insights of the anticarcinogenic effects of EGCG, metformin and their combination on HCC through their effects on GPC3 and lncRNA-AF085935 that may change the speed of proliferation and apoptosis of the cell.

Materials and methods

Cell culture and chemicals

The study was approved by the ethics committee of Faculty of medicine, Cairo University. Human hepatocellular carcinoma cells (HepG2) as liver cancer cell line was bought from the American Type Culture Collection (ATCC; Minnesota, USA), it was cultured in Dulbecco's Modified Eagle's Medium (DMEM) and enriched with fetal bovine serum (10%) and 1% concentration ratio of penicillin and streptomycin (Lonza, Verviers, Belgium). Culture of HepG2 cells were preserved in typical humidified incubator supplied with 5% CO_2, 95% air at 37 °C. EGCG and metformin were obtained from Sigma-Aldrich Chemical Co., St. Louis, Mo, U.S.A., they were freshly solubilized in dimethyl sulfoxide (DMSO).We used 2% DMSO solution as vehicle.

Determination of cytotoxicity effect by MTT assay

The anti-proliferative effect of EGCG, metformin and combination of both of them on HepG2 cells, were investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells were fixed with the frequency of 5000 cells into 96 well plates for 24 h, and the cells were treated with diverse concentrations of EGCG and metformin at doses 0, 0.01, 0.1, 1, 10, 100 µg/ml. The HepG2 cells were exhibited to EGCG and metformin at 24 h in different doses, (0.2 mg/mL) MTT reagent was supplemented to the wells in line with the manufacturer's instructions (Sigma-Aldrich Co.) and composite to all wells plate for 4 to 6 h. When the purple precipitate was clearly visible, detergent reagent was added (100 µl per well) to make the formazan dye soluble. Plates were left with cover in the dim for 2 to 4 h. The plate cover was detached and the absorbance in each well was assessed at a range from 490 to 630 nm using ELISA plate reader (Dynatech MRX 5000; Dynex, Chantilly, VA). For every concentration, eight readings were recorded and the average was calculated. Half maximal inhibitory concentration (IC50) values of EGCG and metformin were estimated using the Prism software version 4.0 (GraphPad Software Inc., San Diego, CA, USA).

Real time PCR

The effect of EGCG and metformin on gene expression was assessed using real time PCR. HepG2 cells at 1×10^5 cell/well were grown in a 6 well plate at IC50 concentration of EGCG and metformin. Cells were washed with cold PBS, trypsinized, harvested and centrifuged. Cells were suspended in 200 µl cold RNA lysis buffer with 5 µl RNase (20 µg/ml) for 15 min. The cells were chilled on ice and further subjected to RNA extraction and purification using Thermo Fisher Scientific Inc. Germany (GeneJET, Kit, #K0732) following the manufacturer's instructions. The yield of total RNA obtained was determined at 260 and 280 nm using Beckman dual spectrophotometer. Genes expression were measured using real time PCR (StepOne, version 2.1, Applied biosystem, Foster city, USA). For cDNA synthesis 1000 ng of the total RNA from each sample were used. Subsequently PCR amplification cycles using SensiFAST™ SYBR® Hi-ROX One-Step Kit, catalog no. PI-50217V, UK, were done. The thermal cycling profile was 15 min at 45 °C for cDNA synthesis followed by 5 min at 95 °C for reverse transcriptase inactivation and polymerase activation. Subsequently, 40 cycles of PCR amplification were done, which involved 15 s DNA denaturation at 95 °C, 20 s primers

annealing at 55 °C and 30 s at 72 °C for the step of amplification. Normalization of each target gene were made relative to the mean critical threshold (CT) values of 18 s RNA housekeeping gene by the ΔΔCt method. Table 1 demonstrated the sequences of primers for each gene.

Western blotting

HepG2 cells were lysed with NP-40 lysis buffer (50 mMTris, pH 7.4; 150 mMNaCl; 1% NP-40), and proteins from the whole cell lysate were separated in a 12% SDS-PAGE. The electrophoresed proteins were blotted onto a Amersham™ Hybond® P (GE10600021, SIGMA) Western blotting membranes, PVDF and incubated with the primary antibodies; rabbit anti-Glypican-3 (1:500) at 4 °C overnight, and mouse anti-β-actin (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. Quantification of the Western blot bands was performed via Image analysis software on the ChemiDoc MP imaging system (version 3) formed by Bio-Rad (Hercules, CA). Relative density of each band was evaluated and normalized with β-actin.

Statistical analysis

The data were planned, tabulated and analyzed statistically using the statistical version 18 of the SPSS software computer package (SPSS Inc, USA). For quantitative data, the mean, median, standard deviation (SD), and inter quartile range (IQR) were considered. Kolmogorov–Smirnov test (KS) test was performed as a test of normality. The variables were not-normally distributed, so Mann–Whitney U test was used as a test of significance. For clarification of results of tests of significance, significance was assumed at $p \leq 0.05$.

Results

In our study we investigated the effect of EGCG and metformin alone and in combination with each other on both proliferation and apoptosis of HepG2 cells through measuring levels of cyclin D1, lncRNA-AF085935, glypican-3, caspase-3, survivin and VEGF.

EGCG and metformin reduced cell viability in HepG2 cell lines

We assessed the cell viability in vitro by MTT assay. HepG2 treated with EGCG and metformin showed decreasing cell viability with dose dependent manner. IC50 for each treatment was calculated. IC50 values of EGCG and metformin exposure were 31.4 and 7.57 µg/ml, at 24 h exposure, respectively.

HepG2 cells treated for 24 h with both EGCG and metformin at altered concentrations 0, 0.01, 0.1, 1, 10, 100 µg/ml. EGCG was shown to decrease the viability of HepG2 cells at all tested concentrations in a dose-dependent manner. Percentages of cell viability were, 93.73%, 92.31%, 83.47%, 66.89%, 58.44% ,42.38%, at all concentration levels. While treatment with metformin produced more inhibition of cell viability with percentages of 93.73%, 87.53%, 73.34%, 57.45%, 47.67%, 38.45%, at all concentration levels (Fig. 1). Metformin produced significant reduction of cell viability than EGCG ($p < 0.05$ at 1 and 10 µg/ml concentrations). This suggests more potent anti-proliferative activity of metformin than EGCG in HepG2 cells.

Assessment of cell proliferation

The cell viability was compared between HepG2 cells without treatment (control) and with vehicle treated. No

Table 1 Primers sequence of all studied genes

Gene symbol	Primer sequence from 5′–3′	Gene bank accession number
Caspase-3	F: TGACAGCCAGTGAGACTTGG R: GACTCTAGACGGCATCCAGC	NM004346.3
Survivin	F: AGA ACTGGCCCTTCTTGGAGG R: CTT TTTATGTTCCTCTATGGGGTC	NG029069.1
Cyclin-D1	F: GGAAAGCTTCATTCTCCTTGTTG R: TCTAGGTAAACCTCTGAGGTCC	NM053056.2
VEGF	F: CGGGAACCAGATCTCTCACC R: AAAATGGCGAATCCAATTCC	NM003376.5
lncRNA-AF085935	F: CAGGGCAGCAAGGTGTTTTC R: TTGGTGGGTTGCCTGATACC	NG009286.1
18s RNA	F: CAGCCACCCGAGATTGAGCA R: TAGTAGCGACGGGCGGGTG	JX132355.1

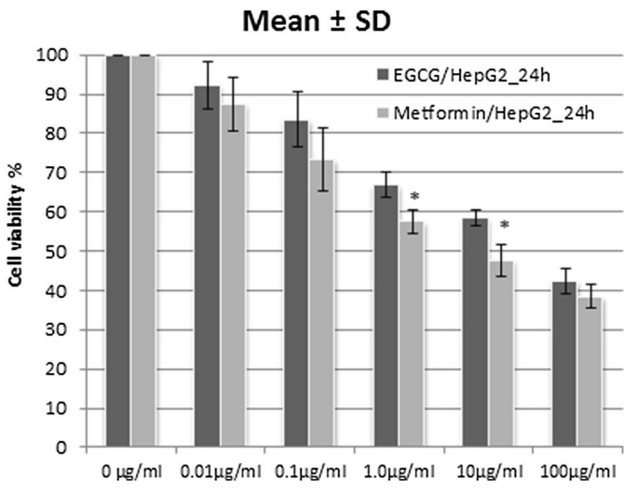

Fig. 1 Effect of increasing concentrations of EGCG and Metformin on the viability of HepG2 cells for 24 h, the cell viability was measured by MTT assay. Data show mean ± SD values. *p < 0.05 versus EGCG treated cells

statistical significance difference with p-value > 0.05 between both of them.

The supplementation of EGCG with IC50 value of 31.4 µg/ml and metformin with IC50 value of 7.57 µg/ml significantly inhibit cell proliferation (< 0.0001) compared to control (untreated) and vehicle treated cell line. Meanwhile, treatment of HepG2 cells with metformin resulted in more significant inhibition to cell proliferation compared to EGCG (p < 0.0001). However, when we determined the effects of using EGCG and metformin in combination on the growth of HepG2 cells, we found that the drug combination caused a stronger significant growth inhibition as compared with the single treatment with EGCG or metformin (p < 0.0001) (Fig. 2a). These results proposed that the combination of EGCG and metformin, synergistically inhibit cell proliferation in the HCC cell line.

In addition, we showed in our study that EGCG and metformin reduce cell proliferation by modulation of cell cycle progression. Cyclin D1 expression was analyzed, as indicated by RT-PCR. The cyclin D1 expression was significantly decreased in EGCG and metformin HepG2 treated cells compared with the control and vehicle treated cells (p < 0.0001 each). Besides, treatment of HepG2 cells with a combination of EGCG and metformin showed a significant reduction in cyclin D1 expression level compared to each drug alone (p < 0.05) (Fig. 2b).

To further examine the mechanism of anti-proliferative effect of EGCG and metformin on HepG2 cells we assessed lncRNA-AF085935 expression level in HepG2 cells by quantitative RT-PCR. As shown in Fig. 4, it was found that expression level of lncRNA-AF085935 was significantly down-regulated in EGCG-treated cells as well as metformin treated cells compared with its levels in control and vehicle treated cells (p < 0.0001 each). Metformin was significantly more effective than EGCG in reducing the level of lncRNA-AF085935 in HepG2 cells (p < 0.001). Additionally, significant reduction of lncRNA-AF085935 expression was detected in the cells treated with both EGCG and metformin more than in single treated cells with EGCG (p < 0.0001) or metformin (p < 0.05) (Fig. 2c). These data implied that the down-regulation of lncRNA-AF085935 induced by the combination of EGCG and metformin probably contributed to their inhibitory effect on cell proliferation in HepG2 cells.

Moreover, glypican-3 plays an important role in the liver cancer progression. It may contribute in the regulation of cell division and growth. Therefore, we determined the level of glypican-3 in HepG2 cells treated with EGCG and those treated with metformin by Western blot analysis (Fig. 3a). As shown in Fig. 3b the level of glypican-3 was significantly inhibited by EGCG and metformin in HepG2 cells (p < 0.0001 each) compared to control and vehicle treated cells. Moreover, metformin induces a more significant inhibition when compared to green tea treated cells (p < 0.0001). Also, the combination of EGCG and metformin treatment induces a significant supplementary inhibition compared to EGCG alone (p < 0.0001) or metformin alone (p < 0.05). These results showed that the antineoplastic activity of EGCG and metformin might be associated with their regulatory effect on glypican-3.

Apoptosis assessment

Apoptosis involves balance between pro-apoptotic protein and anti-apoptotic proteins. Our results suggest a potential role of EGCG and metformin on HepG2 cells. The rate of apoptosis was assessed by measuring the expression levels of caspase-3 and survivin by RT-PCR.

Caspase-3 levels was significantly increased in HepG2 cells treated with EGCG and those treated with metformin (p < 0.0001 each) compared to control and vehicle treated cells. Additionally, the HepG2 cells treated with metformin were found to exhibit a more significant elevation in caspase-3 level than those treated with EGCG (p < 0.0001). Moreover, concomitant treatment of HepG2 cells with both EGCG and metformin displays a more significant upregulation in caspase-3 expression level compared to its level in cells treated with each one alone (p < 0.0001) (Fig. 4a).

Regarding the expression level of anti-apoptotic protein survivin, our results showed that the cells treated with EGCG and cells treated with metformin show a significant decrease of survivin expression level (p < 0.0001 each) compared with control and vehicle treated cells. Meanwhile, survivin expression level was significantly decreased in metformin treated cells compared to EGCG treated cells (p < 0.0001). Moreover, using the

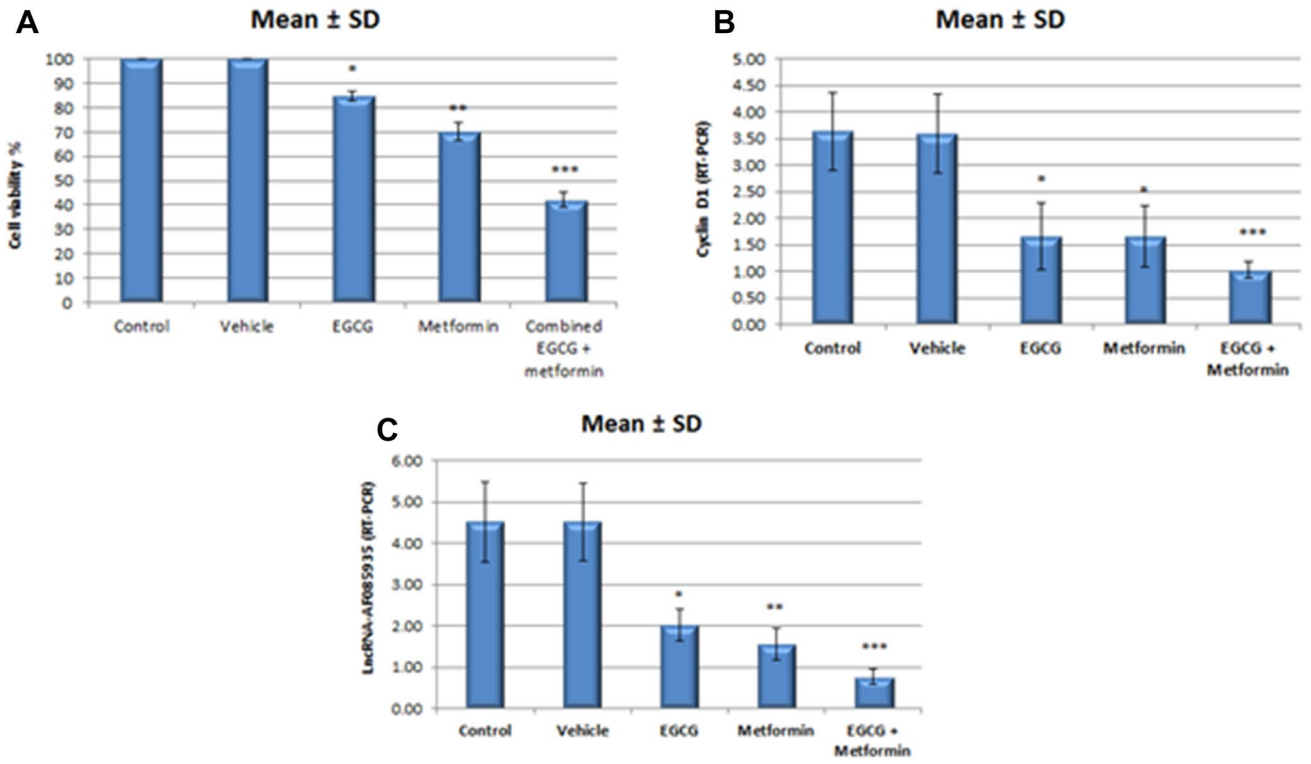

Fig. 2 EGCG and Metformin inhibit cell proliferation in HepG2 cells. HepG2 cells were treated with EGCG at concentration 31.4 µg/ml, metformin at concentrations 7.57 µg/ml and a combination of both. **a** The cell viability was assessed at 24 h of treatment by MTT assay proliferation kit. Data show mean ± SD values. *$p < 0.0001$ versus control (untreated) and vehicle treated cells, **$p < 0.0001$ versus control, vehicle treated as well as EGCG treated cells, $p < 0.0001$ versus control, vehicle treated, EGCG treated cells and metformin treated cells. **b** Relative expression of cyclin D1 in cells treated with 31.4 µg/ml EGCG, 7.57 µg/ml metformin and a combination of both, as well as in control and vehicle cells. Results are expressed as means ± SD. *$p < 0.0001$ versus control and vehicle treated cells, ***$p < 0.0001$ versus control and vehicle treated cells but $p < 0.05$ versus EGCG and metformin treated cells. **c** The effect of different treatments on the expression level of lnc RNA-AF085935 in HepG2 treated with EGCG at concentration 31.4 µg/ml, metformin at concentrations 7.57 µg/ml and a combination of both. Results are expressed as means ± SD. *$p < 0.0001$ versus control and vehicle treated cells, **$p < 0.0001$ versus control and vehicle treated cells but $p < 0.001$ versus EGCG treated cells, ***$p < 0.0001$ versus control, vehicle treated and EGCG treated cells while $p < 0.05$ versus metformin treated cells

combination of EGCG and metformin has more significantly decreased survivin level than using EGCG alone ($p < 0.05$), while, combination of both drugs was insignificantly different than metformin alone, as shown in Fig. 4b.

Angiogenesis

VEGF expression was reduced significantly in the cells treated with EGCG ($p < 0.001$) and in metformin treated cells ($p < 0.0001$) in contrast to control and vehicle treated control cells. Additionally, the cells treated with the combination of EGCG and metformin resulted in significant decrease in VEGF expression level compared to EGCG single therapy ($p < 0.05$) (Fig. 5).

Discussion

HCC is characterized by extensive carcinoma cell infiltration and metastasis and considered one of the risky types of tumor, especially in Asia and Africa [17]. The process of apoptosis is crucial for continuing the physiologic balance between cell death and cell growth. It is believed that cell proliferation and inhibition of apoptosis might play an important role in cancer development [18].

Although advances have been made in liver cancer therapies, the current effective treatments of HCC remain limited. Our study provides a novel insight to understand the anticancer activity of the EGCG and/or metformin. The antitumor activity of EGCG and/or metformin was evaluated in HepG2 cells. The uprising of EGCG and/

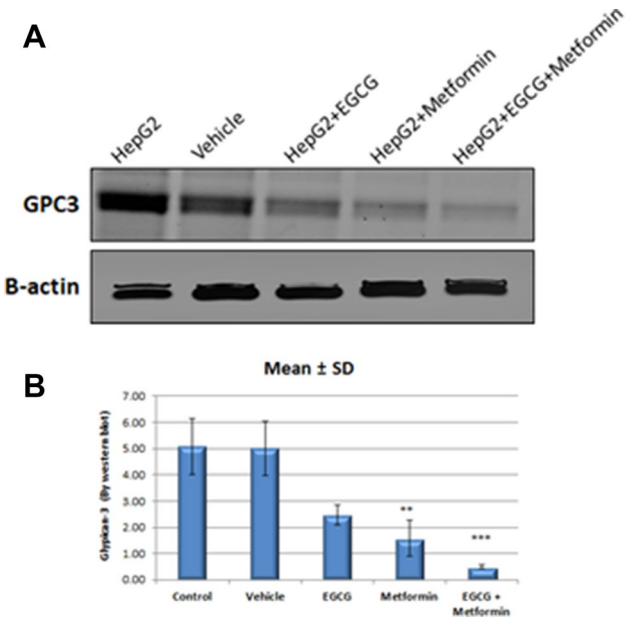

Fig. 3 The effect of EGCG treatment at concentration 31.4 µg/ml, metformin at concentrations 7.57 µg/ml and a combination of both on glypican 3 level (by western blot) in HepG2 cells. **a** Scanning densitometry for protein level expression GPC3 versus β-actin as housekeeping protein in all studied groups. **b** Results are expressed as means ± SD. *$p < 0.0001$ versus control and vehicle treated cells, **$p < 0.0001$ versus control, vehicle treated as well as EGCG treated cells, ***$p < 0.0001$ versus control, vehicle treated and EGCG, furthermore, $p < 0.05$ versus metformin treated cells

or metformin effect in inhibiting cell proliferation has been studied to explore their effect on HCC therapy. We established that EGCG and metformin single treatments exhibited anti-proliferative effect against HepG2 cells, in addition, combination of both of them reduced dramatically cell proliferation in HepG2 cells in comparison to untreated cells. These results concur with those from preceding studies which confirmed the inhibitory effect of EGCG on the growth of numerous HCC cell lines including HepG2 cells [18]. Kuo and Lin have displayed that EGCG inhibits proliferation in HepG2 cells by delaying cell cycle progression in G1 phase and by encouraging apoptosis [19]. Similarly, metformin was observed to inhibit growth in HepG2 cells and several types of human cancer cells, through several mechanisms such as activation of AMP-activated protein kinase (an important mediator of the LKB1 which is tumor suppressor) and transcription factor CCAAT/enhancer-binding protein delta (CEBPD) which prompted cell apoptosis [20].

To elucidate the molecular mechanisms underlying the physiological effect of EGCG and/or metformin on cell cycle, we observed significant decrease in cyclin D1 expression upon treatment with EGCG, metformin and a combination of both. Cyclin D1 promotes growth of cell as well as synthesis of DNA. Increased level of cyclin D1 helps cancer development [21]. However, down-regulation of cyclin D1 expression limits the progression of the cell cycle to the G0/G1 phase and prevents tumor cell growth [22]. Comparable results were correspondingly detected in preceding studies which suggested that EGCG decreases the expression of cyclin D1, inducing cell cycle arrest in various cancer cells [23]. Also, many studies revealed that EGCG trigger cell growth arrest pathways at G1 stage of cell cycle through regulation of cyclin D1, cdk4, cdk6, p21/WAF1/CIP1 and p27/KIP1, and induced apoptosis

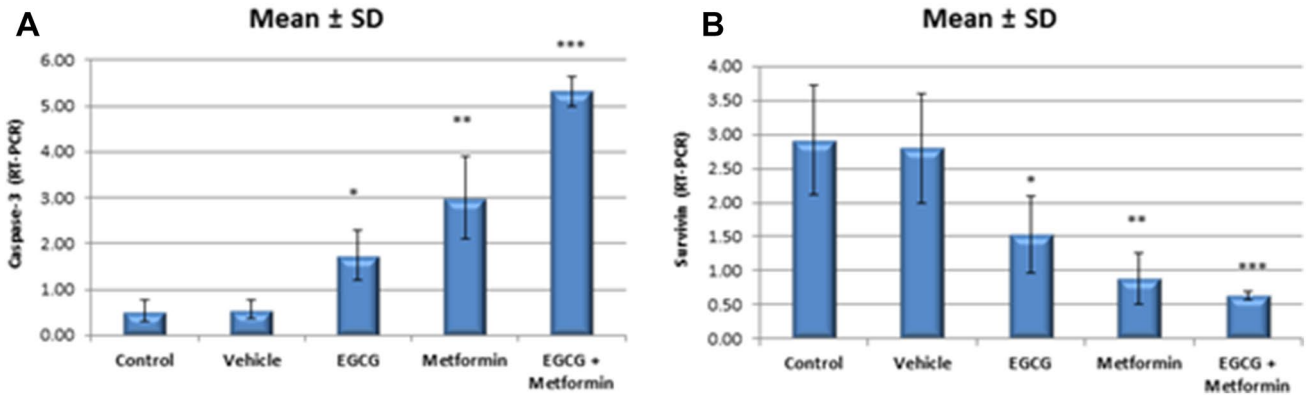

Fig. 4 **a** Effects of EGCG, Metformin and combination of both on caspase-3 expression in HepG2. The cells were treated with EGCG (31.4 µg/ml) and/or Metformin (7.57 µg/ml) for 24 h. The caspase-3 expression level was measured by RT-PCR, and expressed relative to the control cells. Each value represents the mean ± SD. *$p < 0.0001$ versus control and vehicle treated cells, **$p < 0.0001$ versus control, vehicle treated as well as EGCG treated cells, ***$p < 0.0001$ versus control, vehicle treated, EGCG and metformin treated cells. **b** The effect of EGCG and/ or metformin on survinin expression in HepG2. Cells were incubated with EGCG (31.4 µg/ml) and/or Metformin (7.57 µg/ml) for 24 h, the survinin expression was determined by RT-PCR, and expressed relative to the untreated and vehicle treated control cells. Data were expressed as mean ± SD. *$p < 0.0001$ versus control and vehicle treated cells, **$p < 0.0001$ versus control, vehicle treated as well as EGCG treated cells ***$p < 0.0001$ versus control and vehicle treated cells and $p < 0.05$ versus EGCG treated cells

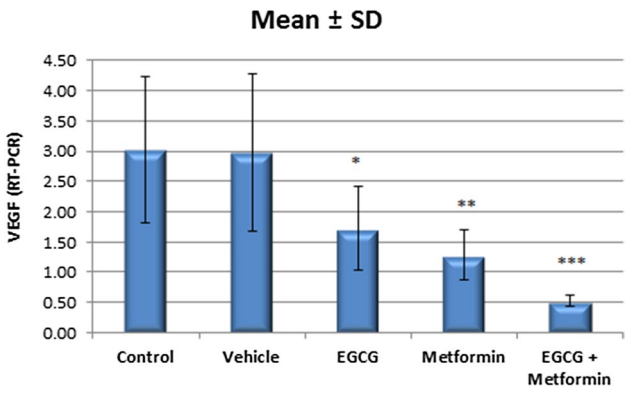

Fig. 5 Expression level of VEGF in HepG2 cells treated with 31.4 µg/ml of EGCG, 7.57 µg/ml of metformin and those treated with both of them relative to untreated cells. Data were expressed as mean ± SD. *$p < 0.001$ versus untreated and vehicle treated cells, **$p < 0.0001$ versus control and vehicle treated cells, ***$p < 0.0001$ versus control and vehicle treated cells and $p < 0.05$ versus EGCG treated cells

through generation of ROS and caspase-3 and caspase-9 activation [23].

Furthermore, Sur et al., found that EGCG down-regulates expression of cyclin D1 in hepatocyte progenitor cell/stem cell, restricting liver carcinogenesis via modulation of Wnt/Hh regulatory pathways [24]. Regarding metformin effect on expression of cyclin D1, our results are in agreement with the findings of CAI et al., who showed that treatment with metformin decreases the levels of cyclin D1 in HCC cells [25]. Furthermore, other studies stated that metformin may also inhibit HCC cell growth by down-regulating cell-cycle regulatory protein, cyclin D1 [26].

To our acquaintance our work is the first to discuss the influence of EGCG and/or metformin on the levels of GPC3 and lncRNA-AF085935, which is recorded orientation of GPC3 [27]. We demonstrated that EGCG and/or metformin treatments led to significantly decrease the expression level of GPC3 in HepG2 cells, in addition, we found that lnc RNA-AF085935 was significantly down-regulated in EGCG-treated cells, metformin treated cells as well as cells treated with combination of both drugs compared with its level in the untreated cells. This work may demonstrate a novel role of EGCG and/or Metformin on the regulation of GPC3 and lncRNA-AF085935 in HCC. Studies on the affiliation between lncRNAs and HCC only started in recent years; numerous lncRNAs including lncRNA-AF085935 have been discovered and are found to be abnormally expressed in HCC [16, 28]. lncRNA-AF085935 was demonstrated to enhance HCC cell growth and migration in vitro as well as proliferation of xenograft tumor in vivo via activating GPC3 [29], Furthermore, high lncRNA-AF085935 expression in HCC tissues predicts poor outcome in HCC patients, therefore, it was suggested that lncRNA-AF085935 may be targeted in HCC treatment.

GPC3 is significantly up-regulated in several HCCs nevertheless in ordinary mature tissues [30]. Moreover, upregulation of GPC3 is concomitant with bad prognosis in HCC [31]. Many studies confirmed the proliferative effect of GPC3 in HCC and recommended innovative therapeutic approaches, which are urgently needed for HCC, and could be generated by GPC3 targeting [32, 33].

To interpret the mechanism of EGCG and/or Metformin against HCC growth, we showed that EGCG and metformin induced apoptosis in HepG2 cells. We investigated the expression of caspase-3, a protein involved in the apoptotic pathway [34], which was observed in our study to be significantly up-regulated in response to EGCG and/or metformin treatments in HepG2 cells compared to untreated cells. Consistent with our current results, preceding researches presented the ability of EGCG to prompt caspase-3 activity in the gastric carcinoma cells [35].

Furthermore, Nishikawa et al., presented that EGCG reduced growth of HLE cells (HCC cell line), by the induction of apoptosis as a result of the activation of caspase-8, -9 and -3 [36]. Moreover, metformin was accelerating apoptosis in HepG2 and Huh7 via enhancing expression of caspase-3 [37]. Moreover, Xiong et al., reported significant elevation of caspase-3 level in Hep-G2 cells, inducing apoptosis in these cells [38].

We subsequently assessed the expression level of anti-apoptotic gene survivin, a principal inhibitor of apoptosis, highly expressed in most cancers and is closely related to increased tumor recurrence, multiple-drug resistance, and reduced survival of patients [39]. Previous studies suggested that survivin is overexpressed in HCC and participated in progression of HCC by affecting apoptosis, stromal angiogenesis and cell growth [40]. We found that EGCG and/or metformin treatments were able to down-regulate the expression level of survivin in HepG2 cells compared with untreated cells. Our results are in accordance with aforementioned finding that conveyed the effect of tea polyphenol on cell lines of breast cancer and suggested that it down-regulates survivin, accelerating the apoptosis [41].

Finally, we used VEGF as a marker to check whether or not the EGCG and/or metformin treatments exerted anti-angiogenic activity against HepG2 cells. We observed that EGCG and/or metformin treatments caused decrease in the VEGF level compared with the control. Previous studies have revealed that tumor proliferation and expansion can be prevented by inhibition of angiogenesis. It is well known that HCC is one of a hyper-vascular tumors in which VEGF expression is closely related to angiogenic activity and tumor progression [42, 43]. Our findings are in line with preceding studies which presented that EGCG reduced VEGF produced by cancer cells indicating its anti-angiogenic activity

[44, 45]. Moreover, EGCG was observed to down-regulate VEGF expression level in HepG2 cells [46]. Shirakami et al., demonstrated inhibition of the VEGF–VEGFR axis after EGCG treatment in HuH7 cells [47]. The downregulation in VEGF level with metformin treatment agrees with preceding results which revealed its inhibitory effect against VEGF expression in HCC cells after insufficient radiofrequency ablation [48]. Additionally, another study proved the inhibitory influence of metformin on VEGF expression, inhibiting HCC angiogenesis [49].

Conclusion

This is the first report widely describing the mechanism underlying the influence of EGCG, metformin and the combination of both on treatment of HepG2 cells. Our data revealed that both EGCG and metformin induced apoptosis by increasing gene expression of caspase-3 and decreasing that of survivin, they also inhibited cell proliferation by decreasing cyclin D1, lncRNA-AF085935 and GPC3. Our study demonstrated for the first time the role of EGCG and metformin on lncRNA-AF085935 and GPC3 in HCC cells. Moreover, the existing results suggest that using combined EGCG and metformin therapy could be more effective than the single usage of each in treatment of HCC cells in vitro.

There are some limitations of our results as regard deficit of in vivo experimental results and this could be recommended in future research work.

Compliance with Ethical Standards

Conflict of interest All authors declared that there was no conflict of interest.

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