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Oral administration of EGCG solution equivalent to daily achievable dosages of regular tea drinkers effectively suppresses miR483-3p induced metastasis of hepatocellular carcinoma cells in mice†

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The effect of non-cytotoxic doses of epigallocatechin-3-gallate (EGCG) on the metastatic capability of human hepatocellular carcinoma (HCC) cells was investigated *in vitro* and *in vivo*. miR483-3p, a microRNA whose expression correlates inversely with survival and positively with disease progression in HCC patients, was found to promote HCC cell migration and invasion *in vitro* as well as lung metastasis in nude mice established by the tail-vein injection of HCC cells. The induction of reactive oxygen species (ROS) and downregulation of antioxidant defense factors Nrf2 and SOD2 appeared to be an important underlying mechanism and treatment with a non-cytotoxic dose of EGCG effectively reversed the miR483-3p-induced enhancement of HCC cell migration and invasion *in vitro*. Moreover, administration through drinking water at doses (0.1% and 0.5% EGCG solution, respectively) equivalent to the intake of regular to heavy tea drinkers could also significantly inhibit lung metastasis of HCC cells based on the estimation from the USDA Database for the Flavonoid Content of Selected Foods and FDA guidelines for the conversion of animal dose to human equivalent dose. EGCG also significantly counteracted the miR483-3p-induced alteration in the expression of epithelial–mesenchymal transition (EMT) markers, E-cadherin and vimentin, and downregulated the endogenous expression of miR483-3p in HCC cells through an epigenetic mechanism that led to the hypermethylation of the miR483-3p promoter region. The data from our study illustrate that miR483-3p promotes HCC metastasis likely through the induction of oxidative stress and uncover a novel role of EGCG for protection against miR483-3p-mediated HCC metastasis *via* the epigenetic modulation of miR483-3p expression. These findings therefore provide further evidence supporting that regular tea consumption may contribute to protection against miR-483-3p-induced ROS and the associated HCC progression.

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1. Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver, and the third leading cause of cancer-related deaths worldwide. The poor overall prognosis and high mortality rate of HCC have been mainly attributed to the high rates of metastasis.[1,2] Therefore, elucidation of the underlying mechanism of HCC metastasis and development of strategies targeting critical events associated with the metastatic process are a highly sought-after approach for improving the clinical outcomes of HCC.

HCC cells metastasize in a series of steps, in which the epithelial–mesenchymal transition (EMT) plays a significant role in cell migration and invasion.[3,4] E-cadherin, encoded by the CDH1 gene, is regarded as a hallmark of epithelial cells[5,6] and

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is located at the basolateral membrane in adherens junctions.[7] E-cadherin ubiquitination and degradation are tightly associated with the loss of hepatocyte polarity in HCC progression.[8] Vimentin, an intermediate filament protein, plays an important role in facilitating the migration and invasion of malignant epithelial cells undergoing EMT.[9,10] Reactive oxygen species (ROS) including superoxide anion, hydroxyl radical and nonradical molecules (such as hydrogen peroxide) have been amply implicated in cancer cell metastasis.[11,12] Compared to normal cells, cancer cells frequently have elevated ROS due to their increased ROS production and/or reduced ROS-scavenging capacity.[13] Elevated ROS can initiate NF-κB dependent activation of Snail to induce EMT in epithelial cells.[14] In HCC cells, ROS was found to upregulate the expression of ADAM 9 (a disintegrin and metalloprotease 9), whose levels in HCC patients exhibit significant negative correlation with the levels of E-cadherin and positive correlation with the levels of vimentin.[15] Consistent with its important role in driving EMT, repression of ROS generation inhibited the EMT and metastasis of HCC cells.[16] Elevated ROS is also closely associated with the aberrant expression of microRNAs (miR). For example, miR21, an oncomir known to be overexpressed in most human tumors, promotes ROS formation by targeting the antioxidant enzymes SOD2 and SOD3.[17] miR200a was reported to target the Keap1 3′-untranslated region (3′-UTR), leading to Keap1 mRNA degradation, which contributed to the dysregulation of the activity of Nrf2, the master regulator of antioxidant defense. Epigenetic therapy targeting miR200a could restore Keap1 expression and reactivate the Nrf2-dependent antioxidant defense.[18] miR483-3p is overexpressed in many types of cancers, including HCC,[19] esophageal squamous cell carcinoma,[20] and pancreatic cancer.[21] However, whether it plays a role in regulating ROS production in HCC cells and driving HCC metastasis is still unknown.

In this study, we discovered that miR483-3p, which is upregulated in the advanced stage (stage IV) of HCC and inversely correlated with patient survival, greatly promoted ROS formation in HCC cells. Thus, we hypothesized that miR483-3p and its associated ROS induction may be a promising target for the suppression of HCC metastasis. To prove our hypothesis, we evaluated the effect of miR483-3p overexpression on the migratory and invasive capabilities of HCC cells *in vitro* and *in vivo*. We also assessed its impact on the levels of ROS and the expression of key antioxidant defense genes in HCC cells. Epidemiological studies tend to support an inverse association between tea consumption and the risk of primary liver cancer, and a meta-analysis of relevant databases, including PubMed, EMBASE, ISI Web of Sciences, and the Chinese Biomedicine Database also supported the potential beneficial effect of tea in this regard.[22–24] Epigallocatechin-3-gallate (EGCG) is the most abundant and purportedly the most active catechin in green tea, accounting for 50–80% of the total catechins.[25–27] EGCG is capable of modulating different molecular mechanisms underlying HCC, primarily through its strong antioxidant activity, and its anti-cancer activity has been demonstrated in various *in vitro* and *in vivo* settings.[28] With respect to HCC metastasis, although many studies have shown that EGCG could modulate the expression of the related transcription factors (*e.g.* fibroblast growth factor, hypoxia-inducible factor, and matrix metalloproteinases) to repress the metastasis,[28] the underlying mechanism associated with its antioxidant activity still needs further investigation. Having observed a significant inductive effect of miR483-3p on ROS generation and metastasis of HCC cells, we thus further investigated whether EGCG could counteract these malignant events. Our findings demonstrate that EGCG suppresses miR483-3p-induced metastasis of HCC cells through ROS inhibition and the epigenetic modulation of miR483-3p expression, highlighting a novel mechanism for the beneficial effect of the most abundant polyphenol in one of the most popular beverages worldwide.

2. Materials and methods

2.1. Cell culture and reagents

Human HCC cell lines HepG2 and Hep3B were purchased from American Type Culture Collection (Manassas, VA). The cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin) in an incubator maintained under 5% CO_2 at 37 °C. A trypsin–EDTA solution (0.25%) was used to digest the cells from the culture dish for passaging.

EGCG (purity >97%) was purchased from Sigma-Aldrich. Stock solutions of EGCG were prepared in DMSO for subsequent dilution to the concentrations specified elsewhere.

2.2. miRNA expression

For stable cell lines, HCC cells were transduced with a miR483-3p or an empty control vector. Lentivirus expressing miR483-3p or a scrambled nucleotide sequence was constructed from a parental lentiviral vector GV209 (Genechem) by replacing GFP with puromycin.

2.3. Cell migration and invasion assays

Twelve-well chemotaxis chambers were purchased from Neuro Probe (Gaithersburg, MD). Briefly, HCC cells in FBS-free media were seeded in the upper chamber (with or without Matrigel coating), while the lower chamber carried media with 10% FBS. After incubation for 24 h, cells in the upper chamber were removed and the membrane was fixed with methanol, followed by staining with 10% Giemsa (Sigma-Aldrich, St Louis, MO). The numbers of cells in different fields of view were counted to get an average sum of cells migrated.

2.4. Western blot and qPCR

For immunoblotting, the cellular protein extracts were subjected to 6–10% SDS–polyacrylamide gel electrophoresis (PAGE). Then the proteins in the gel were transferred onto nitrocellulose membranes (BioRad, California, USA). The membranes were probed with primary antibodies followed by incubation with the corresponding secondary antibody. The

antibodies probed included SOD2 (sc-133134, Santa Cruz), Nrf2 (sc-365949, Santa Cruz), E-cadherin (sc-8426, Santa Cruz, Dallas, TX, US), vimentin (sc-6260, Santa Cruz), and GAPDH (sc-32233, Santa Cruz).

Total RNA was extracted using RNAzol (Molecular Research Center, OH, US) according to the instructions. qPCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, US) in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The primers for qPCR are listed in the ESI.†

2.5. Luciferase reporter assay

Transient transfection of the HCC cell lines was performed with lipofectamine3000 (Invitrogen, Carlsbad, CA). For luciferase activity detection, a luciferase plasmid was transfected into the cells. The pRL-TK renilla luciferase plasmid was used as the internal control. The cells were harvested 48 h after transfection and analyzed with a Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI). The luciferase activity was detected with a luminometer fluorescence reader (Promega, Madison, WI).

2.6. ROS measurement

The attached cells were trypsinized, washed with PBS and stained with 2 μmol L^{-1} dichlorodihydrofluorescein diacetate (DCFH-DA). The stained cells were observed under a microscope or analyzed by flow cytometry. Quantification of the ROS levels was performed using a fluorescence spectrophotometer at an excitation wavelength of 480 nm and an emission wavelength of 535 nm. The mitochondrial superoxide anion levels were determined by staining with 5 mmol L^{-1} MitoSOX Red for 30 minutes at 37 °C, and their fluorescence intensity was measured by flow cytometry.

2.7. Immunofluorescence assay

Cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.1% Triton X100 and blocked with 1% BSA. Then the cells were incubated with primary antibodies, anti-E-cadherin or anti-vimentin (Cell Signaling Technology), followed by secondary antibody conjugation to Alexa-Fluor. After counterstaining with DAPI (Invitrogen), the cells were analyzed by fluorescent microscopy.

2.8. Animal studies

The animal studies were approved by the Committee of the Use of Live Animals in Teaching and Research at Shenzhen University (Protocol no. SYXK2014-0140) and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (permit number: SYXK2014-0140-SZU). The animals were fed in an SPF barrier environment and the animal facility number is HT1201A. Six-week-old female nude BALB/c mice weighed ~21 g were injected intravenously with 1×10^6 luciferase-labeled HepG2 cells (control or miR483-3p overexpression). The mice designated for injection with miR483-3p-overexpressed cells were divided into 3 sub-groups, which were given *ad libitum* 0%, 0.1%, or 0.5% EGCG solution, respectively, for two weeks before intravenous HCC cell injection, and then for two more months after injection. Metastasis of the HCC cells in the mice was assayed by bioluminescent imaging using an IVIS200 Imaging System (Caliper Life Sciences).

2.9. DNA methylation blocking and bisulfite conversion and sequencing

Plasmids containing dCAS9-Tet1 and sg-RNA targeting miR483-3p promoters were transferred into the cells virally to block DNA methylation. The bisulfite conversion of DNA was performed using the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. The resulting modified DNA was amplified by PCR using loci specific PCR primers. The PCR program was as follows: 95 °C for 4 min; 94 °C for 1 min; 55 °C for 2 min; 72 °C for 2 min; repeat steps 2–4 30 times; and 72 °C for 5 min; hold at 4 °C. The resulting amplified products were purified, sub-cloned into a TA cloning vector (Invitrogen), and sequenced. The primers used for PCR are listed in the ESI.†

2.10. Statistical analysis

All experiments were performed in at least three independent trials. The Student's *t*-test was employed in the analysis. *P*-Values smaller than 0.05, 0.01 and 0.001 were denoted by "*", "**" and "***", respectively. *P*-Values <0.05 were considered to be statistically significant.

3. Results

3.1. miR483-3p modulates the expression of EMT markers and increases the ROS levels in HCC cells

To evaluate the clinical relevance of miR483-3p in HCC, we analyzed the correlation between miR483-3p expression and patient survival in GSE31384 data sets. The high miR483-3p expression is associated with shorter overall survival (Fig. 1A). The TCGA data on the relative expression levels of miR483-3p through the course of HCC development also revealed the upregulation of miR483-3p in the advanced stages (Fig. 1B), suggesting that miR483-3p might be involved in HCC progression. To test this hypothesis, Hep3B and HepG2 cells were stably transduced with miR483-3p by lentiviral infection for overexpression and empty vector transduced cells were used as control. Matrigel-coated (for invasion) and uncoated (for migration) trans-well assays showed that miR483-3p overexpression augmented the mobility and invasiveness of HepG2 and Hep3B cells (Fig. 1C & D). A metastatic model in BALB/c nude mice established by the tail vein injection of HCC cells was further employed and bioluminescent imaging supported that overexpression of miR483-3p greatly promoted ($P < 0.001$) lung metastasis of HCC, thus reinforcing the role of miR483-3p in enhancing the metastatic potential of HCC cells (Fig. 1E).

Immunofluorescent microscopy showed that miR483-3p overexpression upregulated the mesenchymal marker vimentin

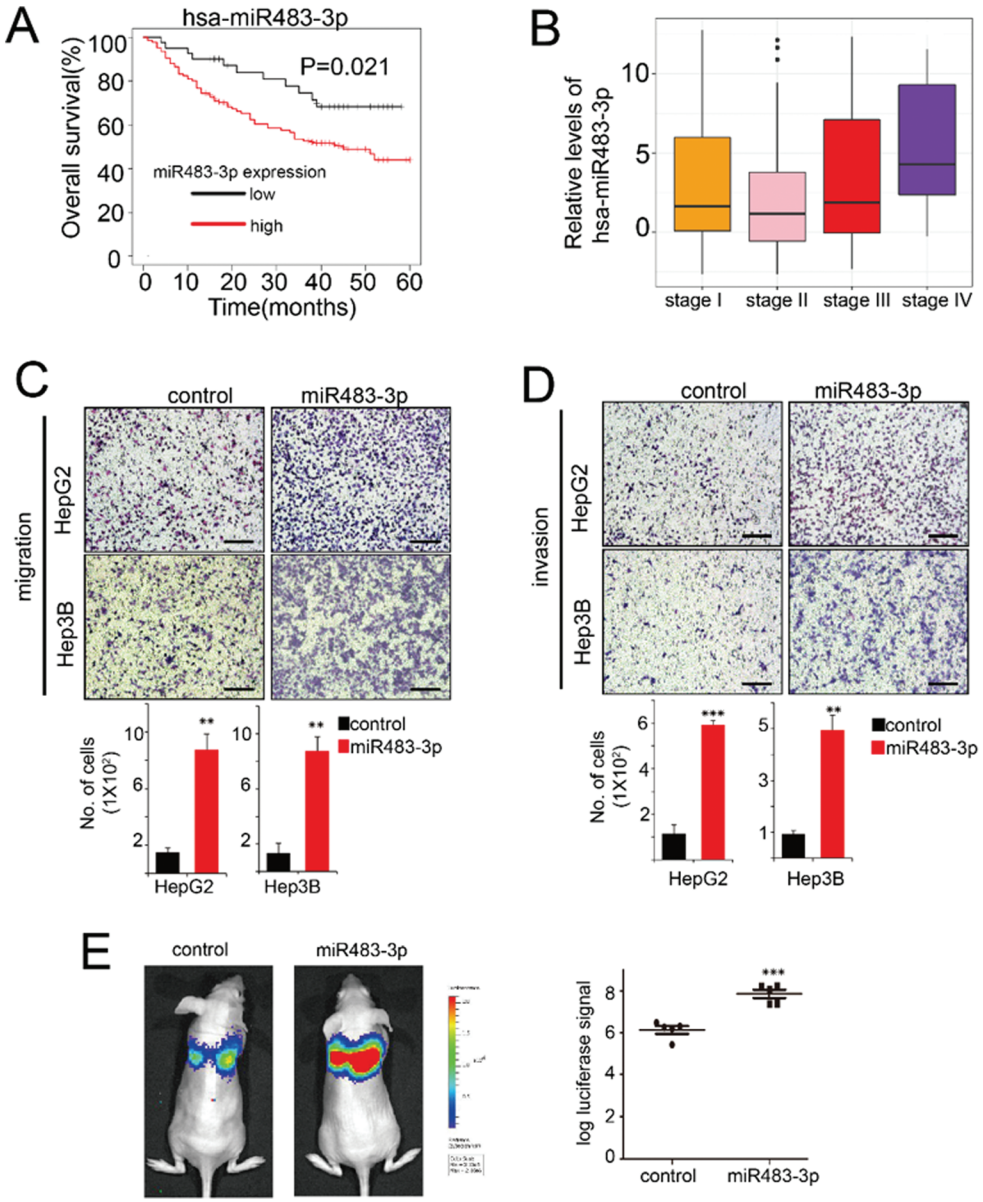

Fig. 1 miR483-3p upregulation is associated with HCC metastasis. (A) Overall survival analysis with a Kaplan–Meier estimator for miR483-3p in HCC patients according to the GSE31384 data set. (B) miR483-3p level is upregulated from stage I to IV in HCC patients according to the TCGA data set. (*P* value determined by Kruskal–Wallis test across tumor stages is 0.08). (C and D) Representative images of cell migration and invasion. Quantitative analysis is presented in the histograms. HCC cell migration and invasion were enhanced by miR483-3p overexpression. $*P < 0.05$, compared to control, scale bar = 100 μm. (E) Luciferase-labelled HepG2 cells were injected into the tail vein of mice (1×10^6 per mouse), and at the end point, the mice were intraperitoneally injected with 150 mg kg^{-1} D-luciferin 10 min before bioluminescent imaging using an IVIS200 Imaging System for the quantitative analysis of luciferase signals. (F) Dot-plot representation of luciferase signals. Data are shown as mean ± S.D. (n = 5 for each group). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, compared to control (the empty vector without miR483-3p expression).

and downregulated the epithelial marker E-cadherin in HepG2 and Hep3B cells (Fig. 2A), consistent with the phenotypic alterations shown in Fig. 1C–E. This was accompanied by the significantly higher levels of total cellular ROS as well as the superoxide anions in HepG2 and Hep3B cells with miR483-3p overexpression compared to the control (Fig. 2A–D). It also downregulated the expression of SOD2 and NRF2 (ESI Fig. 1†), whose binding sites in the 3′UTR were validated by the luciferase assay (Fig. 2E, ESI Fig. 2†). These data together suggest that miR483-3p overexpression favors EMT of HCC cells, which is likely related to its induction of ROS and suppression of antioxidant defense.

3.2. EGCG attenuates HCC metastasis induced by miR483-3p

Next, EGCG, a well-known potent antioxidant derived from green tea, was used to test if the scavenging of ROS could prevent the miR-483-3p-induced EMT of HCC cells. To pre-

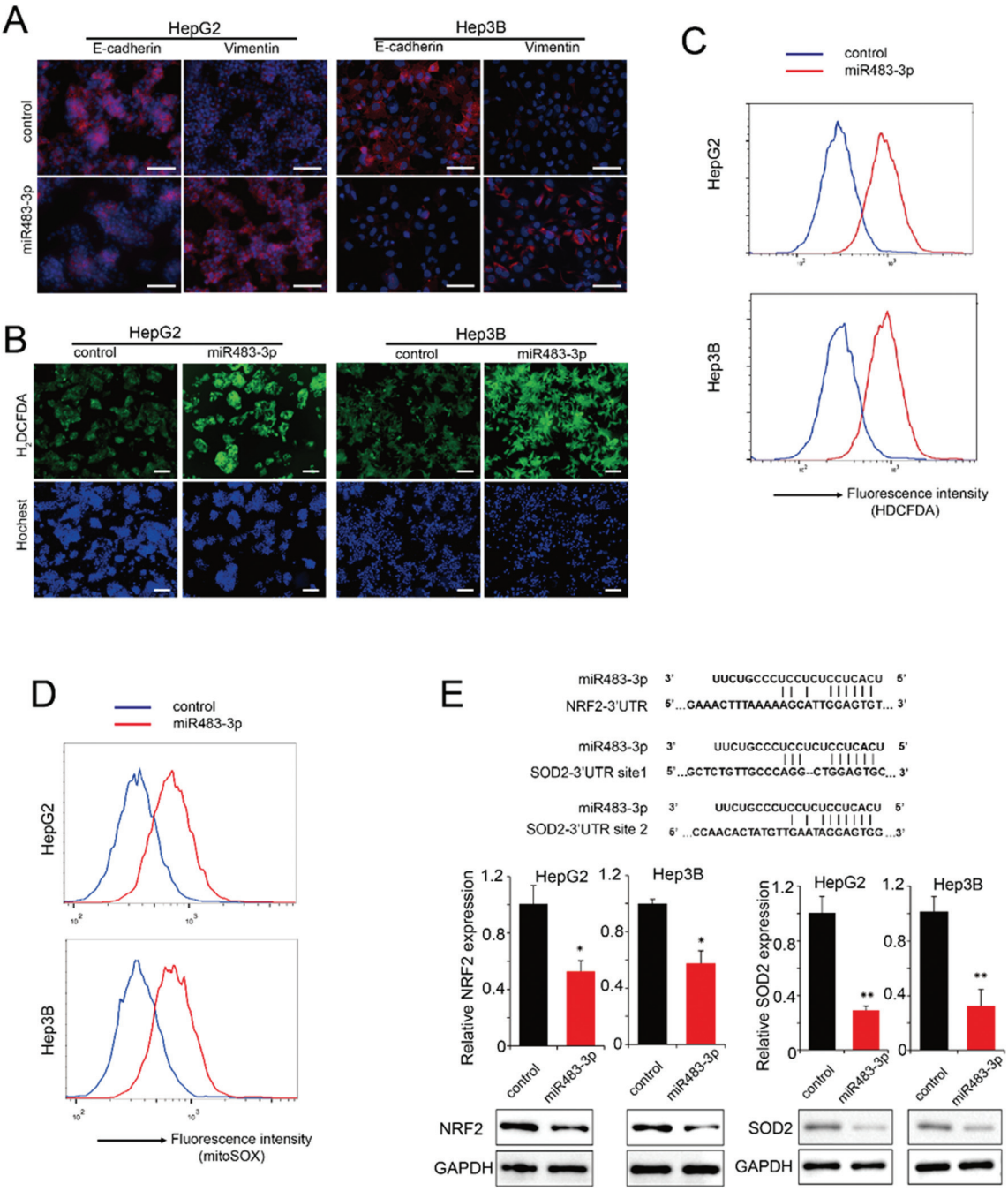

Fig. 2 ROS level in HCC cells is augmented by miR483-3p overexpression. (A) Expression of E-cadherin and vimentin detected by immunofluorescence assay in the indicated cell lines. Scale bar = 50 μm. (B and C) Control and miR483-3p-overexpressed cells were stained with DCFH-DA and Hoechst 33342, and examined by fluorescence microscopy and flow cytometry. Scale bar = 50 μm. (D) Mitochondrial superoxide anion analysis by flow cytometry of MitoSOX Red stained cells. (E) A schematic map of the binding sites of miR483-3p on the 3′ UTR of SOD2 and NRF2. The relative expression levels of SOD2 and NRF2 measured by QPCR and western blotting. Each bar represents the mean ± S.D. from triplicate experiments. *P < 0.05, **P < 0.01, compared to control (the empty vector without miR483-3p expression).

clude the interference from the potential cytotoxicity of EGCG on HCC cells, an MTT assay was performed with a range of doses (data not shown). In agreement with the literature, EGCG exhibited weak cytotoxic activity in the HCC cell lines.[29,30] It was found that 10–30 μM of EGCG dose-dependently reduced cellular ROS (20–75%) and further increase up to 70 μM did not lead to any stronger suppression of ROS levels (ESI Fig. 3†). Hence, 30 μM (non-cytotoxic dose) of EGCG was adopted in the subsequent experiments. The effective suppression of miR483-3p-induced ROS by EGCG in HCC cells was also confirmed by fluorescence microscopy and flow cytometry analyses (Fig. 3A–D).

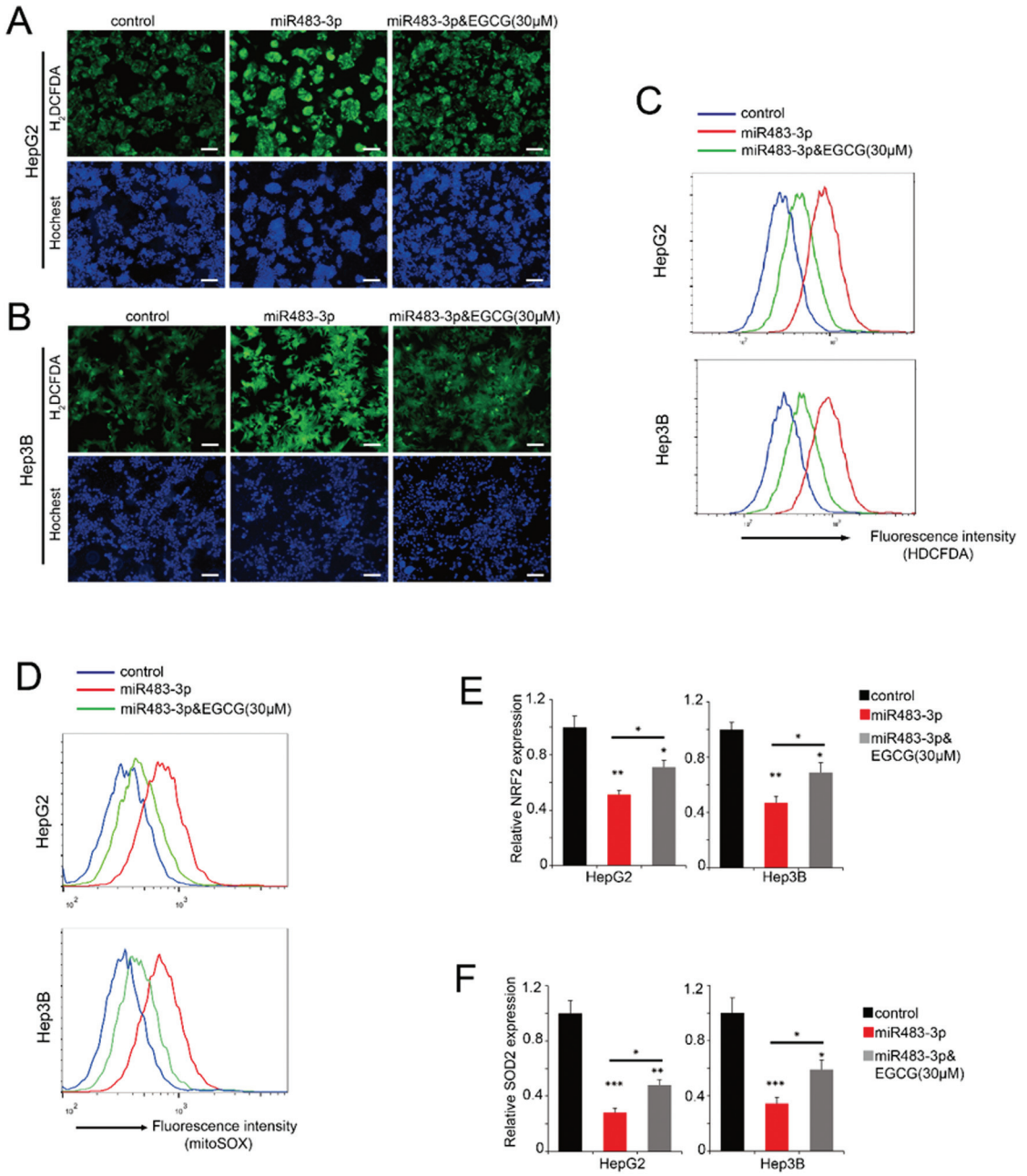

Fig. 3 EGCG upregulates the antioxidant defense and attenuates the ROS levels in miR483-3p-overexpressed HCC cells. (A–C) HCC cells were stained with DCFH-DA and Hoechst 33342, and analyzed by fluorescent microscopy and/or flow cytometry. EGCG attenuated miR483-3p-induced ROS in HCC cells. Scale bar = 50 μm. (D) Mitochondrial superoxide anion analysis by flow cytometry of MitoSOX Red stained cells. (E and F) Relative expression levels of SOD2 and NRF2 measured by QPCR in the indicated groups. EGCG partially restored the suppressed transcription levels of SOD2 and NRF2 caused by miR483-3p overexpression. Each bar represents the mean ± S.D. from triplicate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, compared to the control and indicated groups.

Considering the finding that miR483-3p overexpression significantly downregulated the expression of Nrf2 and SOD2 in the HCC cell lines (Fig. 2D), QPCR was performed to test if EGCG could reverse it. The results showed that EGCG partly restored the repressed expression of these two redox modulators (Fig. 3E & F), which together with its intrinsic ROS-scavenging capacity, might contribute to its strong protective effect against miR483-3p-induced oxidative stress in HCC cells. Correspondingly, the enhanced cellular migratory and invasive capacity caused by miR483-3p overexpression was greatly attenuated (60–70%) by EGCG treatment (Fig. 4A), which was also evidenced by immunofluorescence assay (Fig. 4B & C) and Western blotting (ESI Fig. 4†) of the relative expression levels of E-cadherin and vimentin in control and miR483-3p stably transduced cells with or without intervention by EGCG.

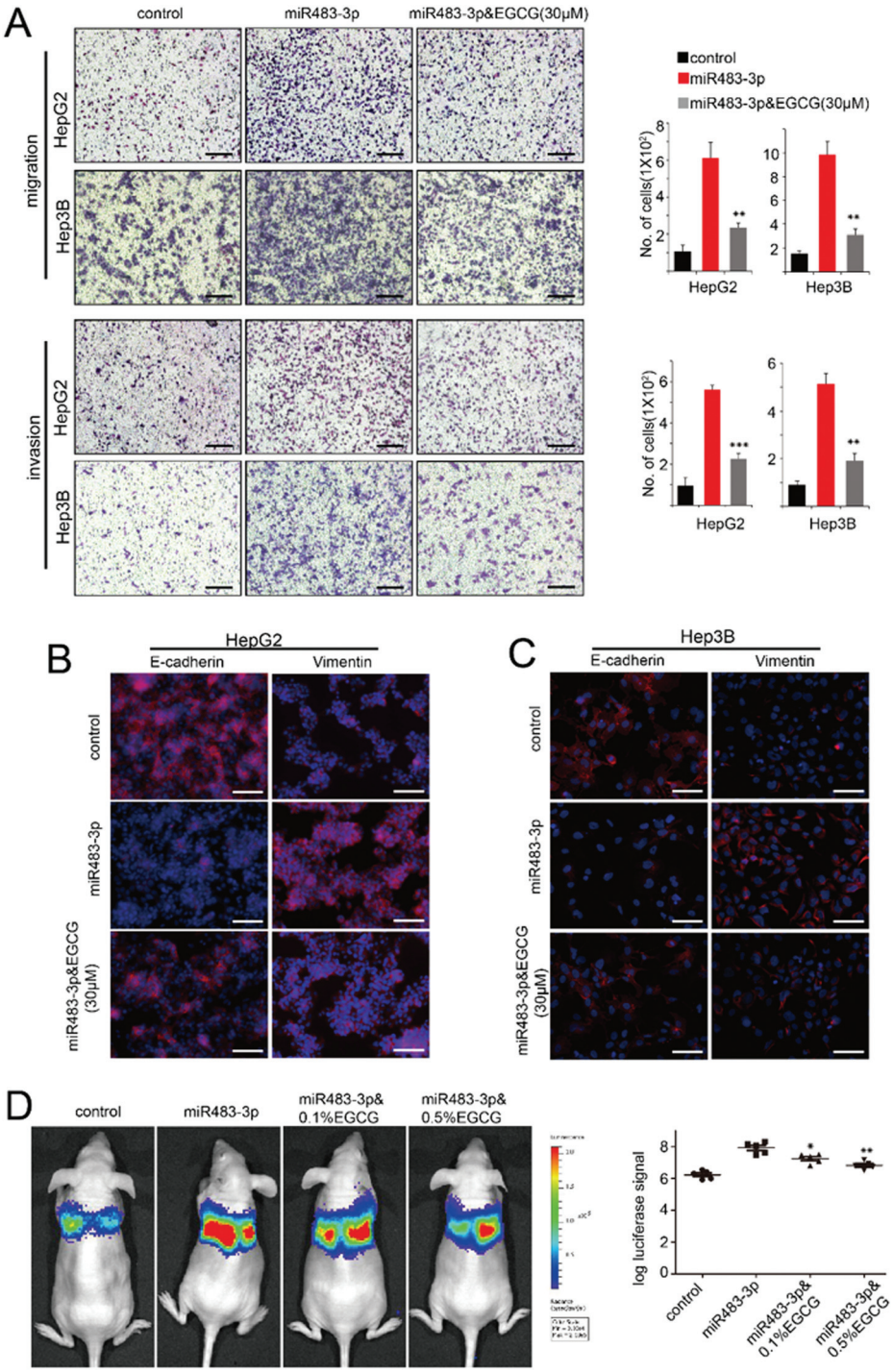

Fig. 4 EGCG attenuates the enhanced metastatic capacity of HCC cells induced by miR483-3p *in vitro* and *in vivo*. (A) Representative images of cells from migration and invasion assay. HCC cell migration and invasion enhanced by miR483-3p overexpression were suppressed by EGCG treatment. $^{**}P < 0.01$ and $^{**}P < 0.001$, compared to the miR483-3p group. Scale bar = 100 μm. (B and C) Changes in E-cadherin and vimentin expressions determined by immunofluorescence assay. Scale bar = 50 μm. (D) Luciferase-labelled HepG2 cells were injected into the tail veins of mice (1×10^6 per mouse). The miR483-3p groups were treated with the indicated doses of EGCG to evaluate the effect on miR483-3p-induced metastasis *in vivo versus* vehicle control. Luciferase signals were measured using an IVIS200 Imaging System for quantitative analysis. Dot-plot representation of luciferase signals. Data are shown as mean ± S.D. ($n = 5$ for each group). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the miR483-3p-overexpressed group.

A lung metastasis model in nude mice further confirmed the anti-metastatic potential of EGCG. Prior to tail-vein injection, the mice were acclimatized to the drinking water which contained 0%, 0.1%, or 0.5% EGCG for two weeks. As shown in Fig. 4D, miR483-3p overexpression strongly induced lung metastasis of HepG2 cells, which was attenuated significantly

by EGCG in a dose-dependent fashion. Of note, the inhibitory effect of 0.5% EGCG solution against lung metastasis of HCC cells induced by miR483-3p was almost double that of 0.1% EGCG solution.

3.3. Suppression of HCC cell motility and invasiveness by EGCG is not mediated solely through its antioxidant activity

The induction of ROS appeared to play an important role in driving HCC cell migration and invasion as a non-cytotoxic dose of EGCG largely canceled the inductive effect of miR483-3p overexpression in these events and restored the perturbed expression of E-cadherin and vimentin. To clarify whether ROS-scavenging alone was sufficient to overcome the EMT-driving momentum of miR483-3p, *N*-acetylcysteine (NAC), a widely used ROS inhibitor, was used to see if it could recapitulate the effect produced by EGCG. A much higher concentration (4 mM) of NAC was needed to generate a similar inhibitory effect to that of 30 μM EGCG on miR483-3p-induced ROS (Fig. 5A). Subsequently, the use of equipotent ROS-scavenging doses of EGCG and NAC revealed that EGCG possessed a significantly stronger activity than that of NAC against miR483-3p-induced HCC cell migration and invasion (Fig. 5B & C, ESI Fig. 5†). A QPCR analyses found that EGCG significantly repressed miR483-3p levels in HepG2 and Hep3B cells, while NAC did not show any appreciable effect (Fig. 5D, ESI Fig. 6†). These data partly explain the much weaker effect of NAC on miR483-3p-mediated cell migration and invasion than that of EGCG and suggest the existence of an ROS-independent mechanism underlying EGCG's effect. It has been reported that miR483-3p overexpression could target ERp29 to suppress its expression, leading to increased HCC cell migration.[31] This was likely due to the inhibitory effect of ERp29 on EMT and thus cell invasion and metastasis.[32] Herein, we also observed the decreased expression of ERp29 in miR483-3p-overexpressed HCC cells (ESI Fig. 7A†). Correspondingly, EGCG could partly restore the repressed expression of ERp29 (ESI Fig. 7B†) probably through the suppression of endogenous miR483-3p, which might contribute to its strong inhibitory effect against miR483-3p-induced cell motility.

3.4. EGCG regulates miR483-3p expression epigenetically

EGCG dose-dependently (10–60 μM) decreased miR483-3p expression in HCC cells (Fig. 6A). The analysis of the promoter methylation status of miR483-3p in stage I and stage IV of HCC in the TCGA data set showed that most patients in stage IV had lower methylation levels (Fig. 6B). This indicates that epigenetic alteration (demethylation) in the miR483-3p promoter region may be an important factor that drives its upregulation during HCC progression.

Having established EGCG's dose-dependent suppression of miR483-3p expression in HCC cells, we sought to determine its impact on the methylation levels of the miR483-3p promoter region. Bisulfite sequencing showed that EGCG enhanced the miR483-3p promoter methylation of HCC cells (Fig. 6C).

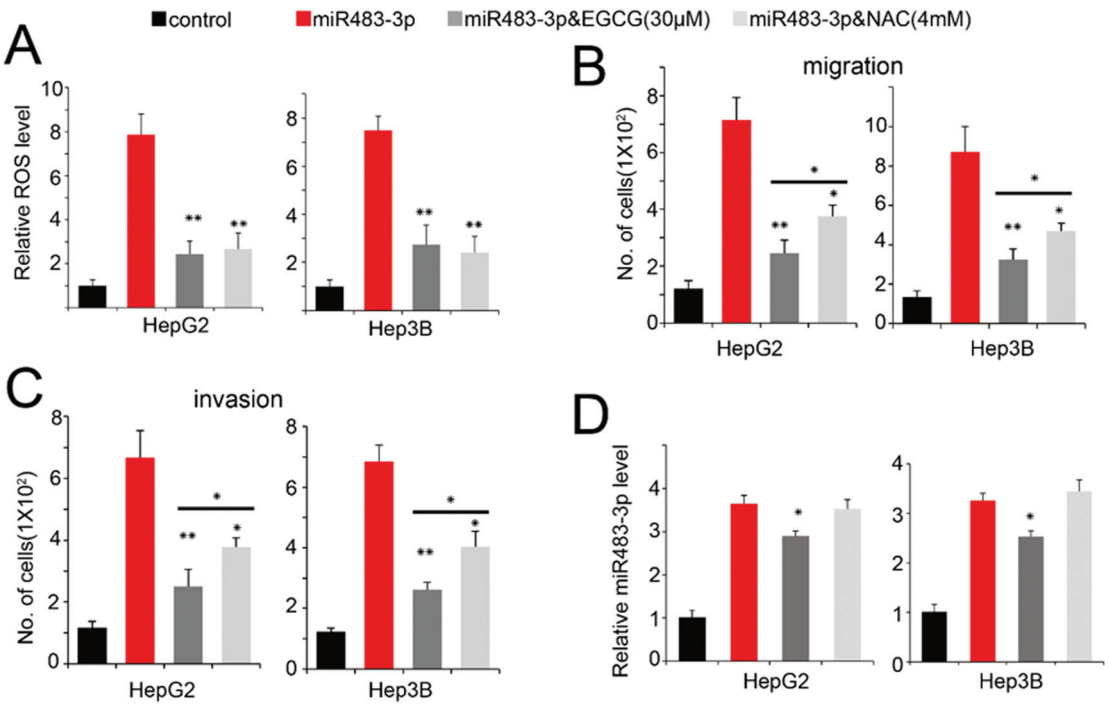

Fig. 5 EGCG inhibits miR483-3p-mediated HCC cell migration and invasion partly through ROS scavenging. (A) Relative ROS levels in the HCC cells of the indicated groups. (B and C) Inhibition of miR483-3p-induced HCC cell migration and invasion by EGCG (30 μM) and NAC (4 mM). (D) Relative expression levels of miR483-3p in the indicated groups. Data are showed as mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001, compared to the miR483-3p group or between miR483-3p&EGCG and miR483-3p&NAC.

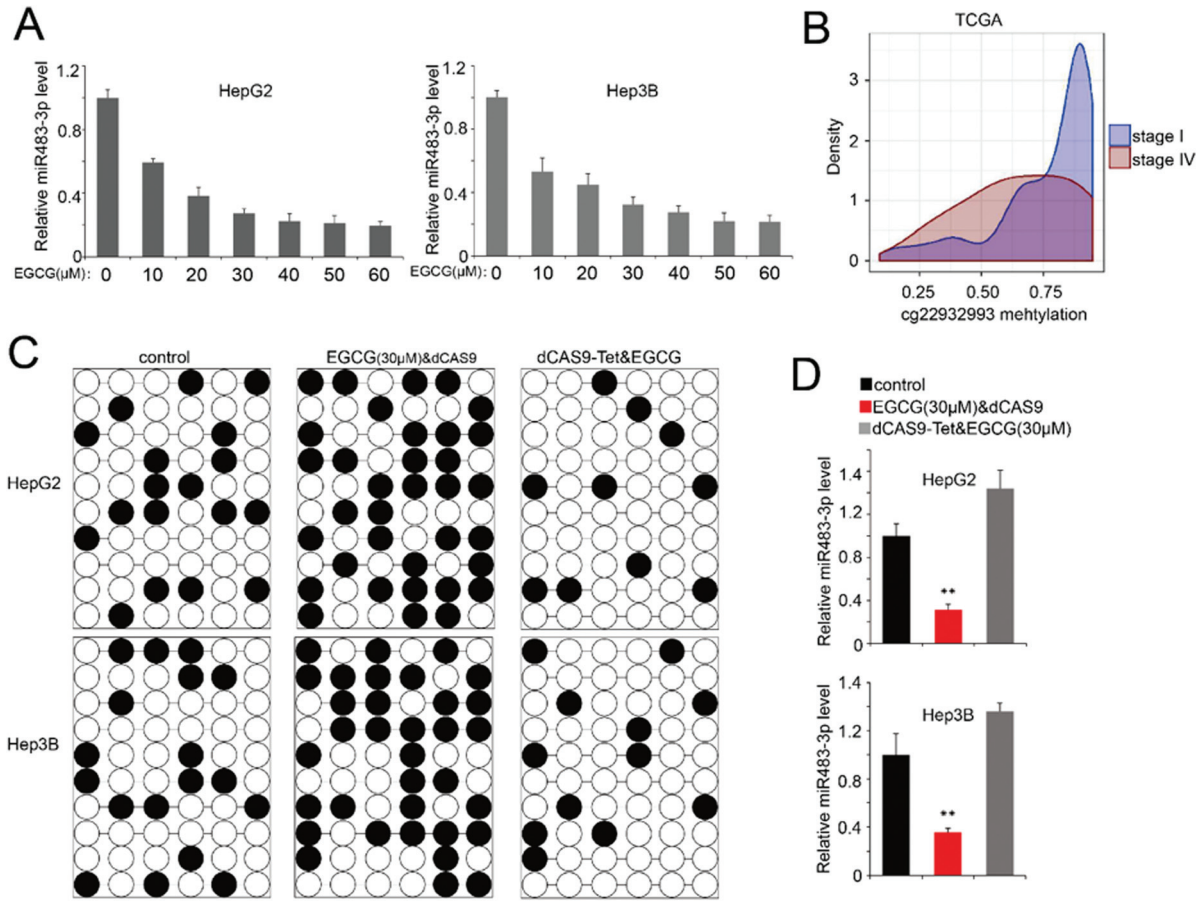

Fig. 6 EGCG suppresses miR483-3p expression through epigenetic modulation. (A) EGCG dose-dependently reduced the expression of miR483-3p in HepG2 and Hep3B cells determined by QPCR assay. (B) Methylation levels of the miR483-3p promoter in stage I and stage IV HCC patients. (C) Changes in the methylation status of the miR483-3p promoter region. EGCG enhanced methylation of miR483-3p promoter and dCas9-TET targeting miR483-3p promoter recovered the hypomethylation status. (D) Relative expression of miR483-3p in the indicated groups. Each bar represents mean ± S.D., $n = 3$, $^{**}P < 0.01$, compared to control.

To verify this phenomenon, HepG2 and Hep3B cells were transfected with dCAS9-TET and sgRNA targeting miR483-3p promoter regions to counteract the effect of EGCG. As expected, EGCG-induced methylation was abrogated by dCAS9-TET-mediated demethylation (Fig. 6C). Correspondingly, the dCAS9-TET targeting miR483-3p promoter completely cancelled EGCG's suppression of miR483-3p expression (Fig. 6D).

4. Discussion

During EMT, epithelial cells gradually lose their epithelial features, exemplified by the absence of cell–cell adhesion and cell polarity, downregulation of adherens junction marker proteins such as E-cadherin,[8] and progressive acquisition of migratory and invasive capabilities to transform into mesenchymal cells with the upregulation of mesenchymal-specific markers such as vimentin.[3,4,7] Notably, the maintenance of mesenchymal phenotypes in carcinomatous epithelial cells is vimentin-dependent and vimentin-upregulated HCC cells exhibit augmented motility and stability.[33,34] ROS has been amply implicated as an important modulatory factor in HCC metastasis,[35] and elevated ROS is also closely associated with the aberrant expression of miRs.[18] Overexpression of miR483-3p leads to significantly elevated ROS in HCC cells, and this effect is likely mediated by its targeting of the 3′UTR of SOD2 and NRF2. A meta-analysis of databases together with *in vitro* and *in vivo* evidence strongly supports the pro-metastatic role of miR483-3p in HCC.

The anticancer efficacy of green tea has been frequently attributed to its phenolic antioxidants, especially EGCG. Given the favorable correlation between green tea consumption and primary liver cancer risk and the fact that tea is one of the two most consumed beverages in the world,[36] it would be important to assess if EGCG was able to abrogate miR-483-3p-induced ROS and EMT in HCC cells. Although EGCG only exhibited a moderate cytotoxic effect on HCC cells, a non-cytotoxic dose was able to strongly suppress cell invasion and migration, besides the attenuation of ROS levels. Moreover, administration through drinking water (0.1% and 0.5% drinking water, respectively) could also significantly inhibit lung metastasis of HCC cells. According to the USDA Database for

the Flavonoid Content of Selected Foods, the mean EGCG content of brewed green tea is 77.8 mg per 100 ml, and can be as high as 203.20 mg per 100 ml.[37] Thus, on average, a 350 mL cup of brewed green tea would contain 272.3 mg of EGCG, and may reach 711.2 mg of EGCG. Based on the FDA guidelines for the conversion of animal dose to human equivalent dose (HED),[37] these dosages could be achieved by regular to heavy tea drinkers. These findings therefore provide further evidence supporting that regular tea consumption may contribute to protection against miR-483-3p-induced ROS and the associated HCC progression.

It is worth noting that the expression of miR483-3p inversely correlated with the overall survival rate of HCC patients, and positively correlated with HCC progression. The data herein also support a role of miR483-3p in driving HCC metastasis. Hence, it could be inferred that controlling its expression level could be the key (if not more effective than antioxidation) to counteract its metastasis-driving effect. Apart from antioxidant activity, accumulating evidence also indicates that miRs are promising molecular targets of polyphenols.[38,39] While EGCG dose-dependently decreased miR483-3p expression in HCC cells, NAC did not have any significant impact. It was suggested that the high methylation levels of the regions flanking the miR-coding sequence suppress miR expression, and that the removal of DNA methylation from these miR loci could upregulate their expression.[40] Bisulfite sequencing together with dCAS9-TET and sgRNA transfection targeting the miR483-3p promoter region confirmed that EGCG could enhance the methylation of the promoter region. These data reinforced the notion that epigenetic modulation of miR483-3p promoter methylation likely underlays EGCG's regulatory effect on its expression in HCC cells.

5. Conclusion

In conclusion, the present study demonstrates that miR483-3p overexpression enhances the metastatic potential of HCC cells both *in vitro* and *in vivo*. Induction of oxidative stress is identified to be an important underlying mechanism which is supported by: (1) the upregulation of ROS and downregulation of the antioxidant defense elements SOD2 and NRF2 and (2) the attenuation of the enhanced metastatic capacity by the ROS-scavenging agents EGCG and NAC. Apart from ROS scavenging, the finding that an equipotent ROS-scavenging dose of NAC generates a much weaker inhibitory effect than that of EGCG on miR483-3p-induced HCC cell migration and invasion reveals a novel mechanism of this predominant bioactive tea polyphenol, namely the epigenetic repression of miR483-3p expression.

Abbreviations

EGCG Epigallocatechin-3-gallate
HCC Hepatocellular carcinoma
miR microRNA
EMT Epithelial–mesenchymal transition
ROS Reactive oxygen species

Conflicts of interest

The authors declare no conflicts of interest.

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