Epigallocatechin gallate inhibits cell growth and regulates miRNA expression in cervical carcinoma cell lines infected with different high-risk human papillomavirus subtypes

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Abstract. The aim of the present study was to investigate the inhibitory effects of the polyphenol epigallocatechin-3-gallate (EGCG) on the growth of cervical carcinoma cell lines infected with different high-risk human papillomavirus (HPV) subtypes, as well as the associated regulation of microRNA (miR) expression. Cell proliferation was measured using an MTT assay. The effects of 7 different concentrations of EGCG (100, 80, 60, 40, 20, 10 and 0 μg/ml) on HeLa cell proliferation were assessed. HeLa cell growth was significantly inhibited by EGCG in a dose- and time-dependent manner (P<0.05), and the IC_{50} was 90.74 and 72.74 μg/ml at 24 and 48 h, respectively. The expression of miR-210, miR-29a, miR-203 and miR-125b in HeLa (HPV16/18+), SiHa (HPV16+), CaSki (HPV16+) and C33A (HPV-) cell lines was measured using quantitative polymerase chain reaction analysis. In CA33 cells, miR-203 (all P<0.001) and miR-125b (P<0.01 and <0.0001) were significantly downregulated by EGCG, and miR-210 was significantly upregulated with 40 and 60 μg/ml EGCG (P<0.0001). miR-125b was significantly downregulated (P<0.001 and <0.0001), and miR-210 and miR-29 were significantly upregulated by ≤80 μg/ml EGCG in HeLa cells (all P<0.0001). In CaSki cells, miR-210, miR-29a (all P<0.001) and miR-125b (P<0.01-0.0001) were significantly upregulated by EGCG. In SiHa cells, miR-125b (both P<0.001) and miR-203 (P<0.01 and <0.0001) were significantly upregulated by EGCG. In conclusion, the results of the present study suggest that EGCG suppresses cervical carcinoma cell growth, possibly via regulating the expression of miRs, suggesting their potential as therapeutic targets for the control and prevention of cervical cancer. Additionally, EGCG may be considered a novel anti-cervical cancer drug in the future.

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Introduction

Statistics provided by the American Cancer Society revealed that 1,735,350 new cancer cases and 609,640 cancer deaths are projected to occur in the USA in 2018 (1). Cervical cancer is the fourth most common and deadly cancer that affects women worldwide, after breast, colorectal and lung cancer. An estimated 70% of global cervical cancer cases occur in underdeveloped areas and developing countries (2). However, patients with cervical cancer should not be subject to high rates of mortality even in developing countries. Therefore, it is desirable to decrease the mortality rate of cervical cancer. According to The National Central Cancer Registry of China, there were estimated to be 4.29 million new cases of cancer and 2.81 million cancer-associated mortalities in 2015 in China (3). For women, two of the 10 most common cancers are gynecologic cancers, with breast cancer (268,600 new cases) being the most prevalent and cervical cancer (98,900 new cases) being the seventh most common cancer in China, which is the largest formerly developing country (3). Therefore, research to identify novel therapeutic and preventative treatments for cervical cancer is underway in China (3).

Human papillomavirus (HPV) infection is one of the major risk factors for cervical cancer worldwide (4). High-risk HPV, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68, is the major cause of cervical cancer. It has been reported that different cervical cancer cell lines were initiated by different HPV subtypes. Cervical cancer cell lines with different HPV statuses/types, including HeLa (HPV16/18+), SiHa (HPV16+), CaSki (HPV16+) and C33A (HPV-), are commonly used to study cervical cancer (5,6). The HeLa cell line, which was derived from cervical cancer patient Henrietta Lacks at Johns Hopkins Hospital in 1951, is most commonly used. Tissue specimens harvested during her diagnosis and treatment were the first successfully isolated and cultured cervical cancer cells. The HeLa cells were later confirmed to be HPV16/18+. In the case of one drug, dehydrocostus lactone, which has been identified to inhibit the proliferation and

invasion of HeLa cell lines, this effect was associated with a time- and dose-dependent reduction in Akt phosphorylation. These inhibitory effects were enhanced when treatment was combined with specific phosphatidylinositide-3 kinase (PI3K)/Akt inhibitors, suggesting that the drugs may act via pathways including including PI3K/Akt (7). In the present study, the potential inhibitory effects of epigallocatechin-3-gallate (EGCG) on the proliferation of cervical cancer cells were assessed.

MicroRNAs (miRNAs/miRs) are small non-coding RNA molecules that regulate the expression of protein-coding target genes in eukaryotes (8). It has been reported that miRNAs are important regulatory factors involved in numerous crucial biological processes, including metabolism, migration, invasion, apoptosis and cell proliferation (9). However, a number of studies have indicated that epigenetic alterations also have a key role in carcinogenesis and metastasis. An increasing number of studies have focused on non-coding RNAs, including miRNAs and long non-coding RNAs (10,11). miRNAs may be used as biomarkers to assess disease progression or characteristics and may therefore have an application in the treatment of various cancer types (12). Furthermore, aberrant DNA methylation and histone modification in cervical cancer have drawn attention and have been extensively studied. An increasing number of studies have focused on non-coding RNAs, including miRNAs and long non-coding RNAs in particular. miRNAs may be used as biomarkers to assess disease progression or characteristics, and may therefore have an application in the treatment of various cancer types (13). It has been recognized that genetic mutation is a factor in the development of cervical carcinoma (14). In recent years, a number of studies have focused on investigating the molecular basis and mechanisms of epigenetic modifications in cervical cancer, including the role of DNA methylation and histone modification, and the diagnostic, prognostic and therapeutic potentials of miRNAs (15,16).

Previous studies have reported that certain miRNAs are downregulated in cervical cancer, while others are upregulated (17,18). It has been confirmed that miR-203 expression is typically downregulated in cervical cancer cells and tumor tissue (19). A recent study by our group demonstrated that miR-125b suppresses tumor growth by inhibiting PI3K/Akt/mammalian target of rapamycin (mTOR) activity, and may thus be an effective therapeutic target for cancer treatment (20). In addition, a microarray analysis indicated that miR-210 was upregulated in cervical carcinoma, while miR-29a was downregulated in grade II-III HPV16+ cervical intraepithelial neoplasia tumors (21). Green tea, a popular drink in China, has been thought to have beneficial health effects for thousands of years. It is mentioned in 'Hua yang guo zhi', a document written in the Eastern Jin Dynasty (317-420 AD). Tea has been demonstrated to inhibit tumor growth *in vitro* and *in vivo* (22,23). Cervical carcinoma is commonly initiated by differential high-risk subtypes of human papillomavirus, while the genesis of the majority of tumors may be suppressed by green tea, green tea extract and green tea polyphenols, particularly EGCG (24). The aim of the current study was to reveal the mechanism by which EGCG inhibits the proliferation of cervical cancer cell lines and the regulation of miRNA expression in different HPV subtypes. The current study demonstrated that EGCG may become a novel substance to prevent and treat cervical cancer.

Materials and methods

Cell culture. The human cervical cancer cell lines HeLa (HPV16/18+), SiHa (HPV16+), CaSki (HPV16+) and C33A (HPV-) were provided by the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were electroporated with engineered HPV types 16 and 18 and grown in a 1:1 (v:v) mixture of Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and Ham's F12 nutrient mix (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2.

Green tea compound. EGCG (Sigma-Aldrich; Merck KGaA) was dissolved in double distilled H_2O at a concentration of 5 mg/ml to prepare a stock solution. The solution was stored in an atmosphere of N_2 at -80˚C and diluted compound solutions were freshly prepared prior to the experiments.

MTT assay. The viability of HeLa cells was measured using an MTT-based Cell Growth Determination kit (Sigma-Aldrich; Merck KGaA). Cells were seeded into 96-well flat-bottomed plates (10,000 cells in 100 μl/well), incubated overnight at 4˚C and treated with different concentrations of EGCG (100, 80, 60, 40, 20 and 10 μg/ml) in triplicate for 0, 24 and 48 h, respectively. Cell culture medium with DMSO (final concentration, 0.1%; Invitrogen; Thermo Fisher Scientific, Inc.) was used as the negative controls. After drug treatment, 10 μl MTT solution was added to each well and the plates were incubated at 37˚C for another 2 h. The supernatant was subsequently removed and DMSO was added to each well to dissolve the formazan. The absorbance/optical density (OD) was read at 492 nm using a Thermo MK3 spectrophotometric microplate reader (Thermo Fisher Scientific, Inc.). The inhibition rate of HeLa cells by EGCG was calculated using the following formula: Inhibition rate (%)=(1-$OD_{drug\ exposure}/OD_{control}$) x100%. The half inhibition concentration (IC50) value of EGCG in inhibiting the growth of HeLa cells was measured by the MTT assay. Values were determined from the inhibition rate vs. drug concentration graphs using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of miRNAs. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), complementary (c)DNA was synthesized by stem-loop RT-qPCR using the Two Step Stemaim-it miR qRT-PCR Quantitation kit (SYBR Green; cat. no. LM-0101A; Novland Biopharma, Shanghai China). qPCR analyses of *Homo sapiens* (hsa)-miR-210, hsa-miR-29a, hsa-miR-203 and hsa-miR-125b was performed with an MX3000P Detection System (Agilent Technologies, Inc., Santa Clara, CA, USA) following the manufacturer's protocol. U6 was used as the endogenous control, with the expression of targeted miRNAs normalized to that of U6. cDNA was

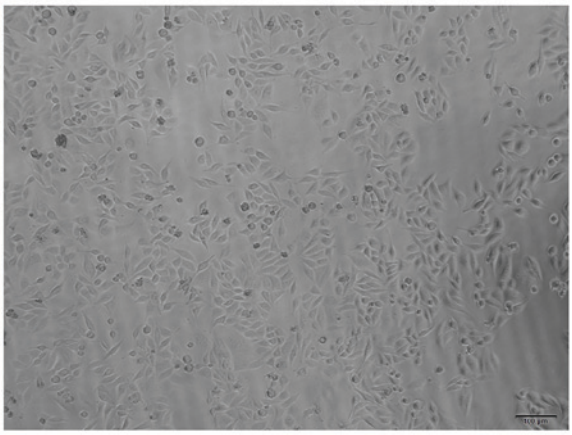

Figure 1. Assessment of the proliferation of HeLa cells using an MTT assay. Untreated control group; the cells exhibited a normal growth state with an inhibitory rate of 0%. (scale bar, 100 μm).

synthesized from 2 μg of total RNA. RT was performed using the following conditions: 30 min at 16˚C, 30 min at 42˚C and 5 min at 85˚C, followed by a hold at 4˚C. All RT reactions, including no-template controls and RT minus controls, were run in duplicate. The PCR amplifications using the Two Step Stemaim-it miR qRT-PCR Quantitation kit (SYBR Green) were performed in a 96-well plate with the following thermocycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions were run in triplicate. The threshold cycle (Cq) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The Cq values were calculated automatically and the quantity of expressed miRNA was calculated using the following formulae: ΔCq=Cq(target)-Cq(reference: U6), $\Delta\Delta Cq=Cq_{test\ miRNA}-Cq_{control}$ and Relative Expression Quantity of miRNA=$2^{-\Delta\Delta Cq}$ (25,26). Primers used in the present study were as follows: U6 small nuclear RNA forward, 5'-ATTGGAACGATACAGAGAAGATT-3' and reverse, 3'-GGAACGCTTCACGAATTTG-5'; hsa-miR-210 forward, 5'-GTGCAGGGTCCGAGGT-3' and reverse, 3'-TGTGCGTGTGACAGCGGC-5'; hsa-miR-29a forward, 5'-GTGCAGGGTCCGAGGT-3' and reverse, 3'-CGTAGCACCATCTGAAATCGG-5'; hsa-miR-203 forward, 5'-GTGCAGGGTCCGAGGT-3' and reverse, 3'-CGCGTGAAATGTTTAGGACC-5' hsa-miR-125b forward, 5'-GTGCAGGGTCCGAGGT-3' and reverse, 3'-CGTCCCTGAGACCCTAACTTGT-5'.

Statistical analysis. Values are expressed as the mean ± standard deviation. All statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Differences were analyzed by one-way analysis of variance followed by the Sidak test for multiple comparisons and multiple linear regression analysis was performed. P<0.05 was considered to indicate a statistically significant difference.

Results

EGCG inhibits HeLa cell proliferation. Growth retardation of HeLa cells was observed by using an MTT assay. Compared with that in the control group, the growth of HeLa cells was inhibited by EGCG at concentrations ranging from 10 to 100 μg/ml and treatment times of 24 and 48 h. Prior to drug the administration, the cells exhibited a normal growth behavior the inhibition rate was also considered to be the lowest (0%; Fig. 1). The cell growth was also inhibited to a certain extent after 24 h of incubation with EGCG (Fig. 2A-F), and cell growth was significantly inhibited after 48 h (Fig. 3A-F), and the inhibition was positively associated with the drug concentration. The cell growth inhibition by EGCG exhibited time-dependent (P<0.01) and dose-dependent (P<0.01) characteristics according to GraphPad Prism 6 analysis. The IC_{50} at 24 and 48 h of treatment was 90.74 and 72.74 μg/ml, respectively (Fig. 4).

EGCG treatment affects the expression of hsa-miR-210, hsa-miR-29a, hsa-miR-203 and hsa-miR-125b in cervical carcinoma cell lines. The results revealed that EGCG treatment regulated the expression of various miRNAs in different cervical carcinoma cell lines. Significant results are compared with the control cells. miR-203 (all P<0.0001) and miR-125b (P<0.01-0.0001) were significantly downregulated by EGCG treatment, while miR-210 was significantly upregulated by EGCG especially at 40 and 60 μg/ml (both P<0.0001; Fig. 5A). miR-29 did not follow a trend in C33A cells. In HeLa cells, miR-210 was significantly upregulated by 40, 60 and 80 μg/ml EGCG, miR-29 was significantly upregulated by 60 and 80 μg/ml EGCG (all P<0.0001; Fig. 5B). miR-203 was significantly downregulated by 20, 40 and 100 μg/ml EGCG (all P<0.0001) and was significantly upregulated by 10 μg/ml EGCG (all P<0.0001), therefore the expression of miR-203 did not follow a trend in HeLa cells. The expression of miR-125b was significantly downregulated by 20, 40, 60 and 100 μg/ml EGCG (P<0.001-0.0001), however it was significantly upregulated at 10 μg/ml EGCG (P<0.0001).

miR-210 and miR-29 was significantly upregulated at all concentrations in CaSki cells (all P<0.0001; Fig. 5C). miR-203 was also significantly upregulated at 40 and 100 μg/ml, but significantly downregulated at 10, 60 and 80 μg/ml EGCG (all P<0.0001). miR-125b expression was significantly upregulated with 10-80 μg/ml EGCG, especially at 40 μg/ml (P<0.0001) in CaSki cells. In SiHa cells, miR-210 was significantly upregulated by 10, 40, 60 and 80 μg/ml EGCG (all P<0.0001), while miR-29 was significantly upregulated at 10 and 40 μg/ml (both P<0.0001; Fig. 5D). miR-203 was significantly upregulated at all concentrations (P<0.001-0.0001) and miR-125b was significantly upregulated with 80 and 100 μg/ml (both P<0.0001).

Discussion

The latest data from Globocan 2018 clarified that the cancer incidence rate and mortality rate in China is the highest in the world (27). Among the 18 million new cancer cases and 9.6 million cancer-associated mortality worldwide, 3.804 million and 2.296 million were in China, respectively (28). According to a recent report from the Chinese authoritative official news agency Xinhuanet cervical cancer is the second most common cancer among women, following breast cancer, with 530,000 new cases reported worldwide every year (29). China has very high incidence and mortality rates, with ~98,900 new cases reported in 2015 and 30,500 mortalities. In other words, at least three Chinese

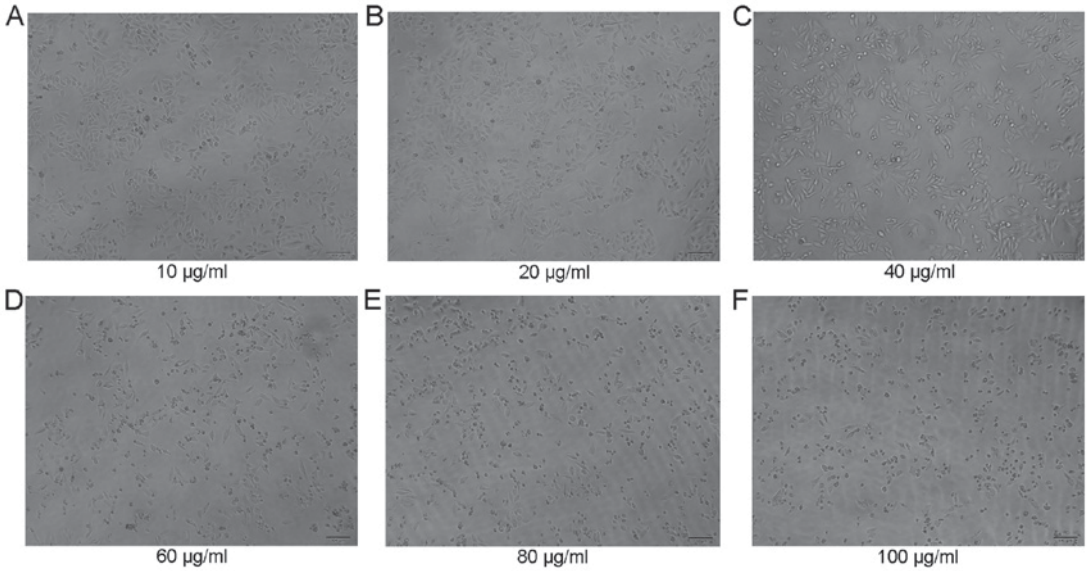

Figure 2. Effect of epigallocatechin gallate on HeLa cells after 24 h of incubation assessed using an MTT assay. (A) 10 μg/ml; inhibitory rate, 11.73%; (B) 20 μg/ml; inhibitory rate, 19.43%; (C) 40 μg/ml; inhibitory rate, 24.62%; (D) 60 μg/ml; inhibitory rate, 33.29%; (E) 80 μg/ml; inhibitory rate, 45.45%; (F) 100 μg/ml; inhibitory rate, 55.74% (scale bar, 100 μm).

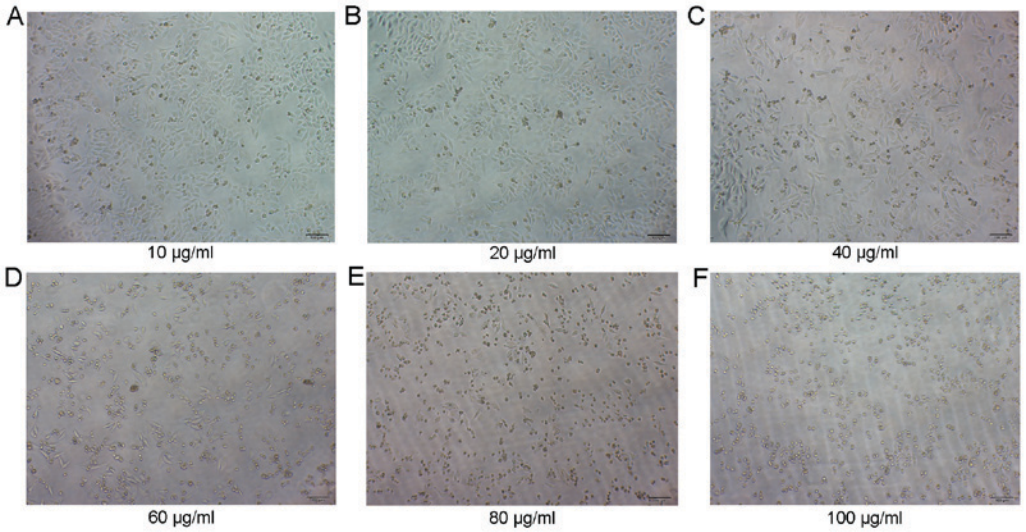

Figure 3. Effect of epigallocatechin gallate on HeLa cells after 48 h of incubation assessed using an MTT assay. (A) 10 μg/ml; inhibitory rate, 12.25%; (B) 20 μg/ml; inhibitory rate, 20.09%; (C) 40 μg/ml; inhibitory rate, 28.15%; (D) 60 μg/ml; inhibitory rate, 34.32%; (E) 80 μg/ml; inhibitory rate, 58.37%; (F) 100 μg/ml; inhibitory rate, 68.44% (scale bar, 100 μm).

women succumbed to cervical cancer every hour (30). Thus, the Chinese government has made efforts towards the prevention and treatment of cervical cancer, for example HPV E6/E7 mRNA testing has been applied to assess its potential as a predictive biomarker (31) and the recombinant human interferon α2a vaginal suppository has been used for the treatment of cervical cancer (32). Even so, a more cost effective method remains necessary. The regular consumption of vegetables and fruit reduces the risk of various cancer types, and the consumption of tea, particularly green tea, is associated with a reduced cancer risk (33,34). Cervical cancer is no exception as stated previously (22). Studies have demonstrated that natural Chinese medicine monomers and their derivatives can induce cancer cell apoptosis (35), autophagy (36) or regulatory signaling pathways (37) by regulating miRNAs. In addition,

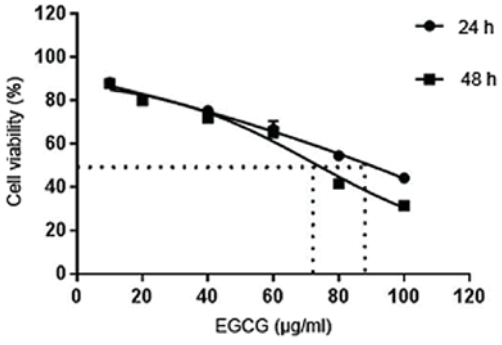

Figure 4. Multiple linear regression analysis of the results of the MTT assay. The cell growth inhibition by epigallocatechin gallate on HeLa cells was time-dependent (P<0.01) and dose-dependent (P<0.01). The IC_{50} for 24 and 48 h was 90.74 and 72.74 μg/ml, respectively.

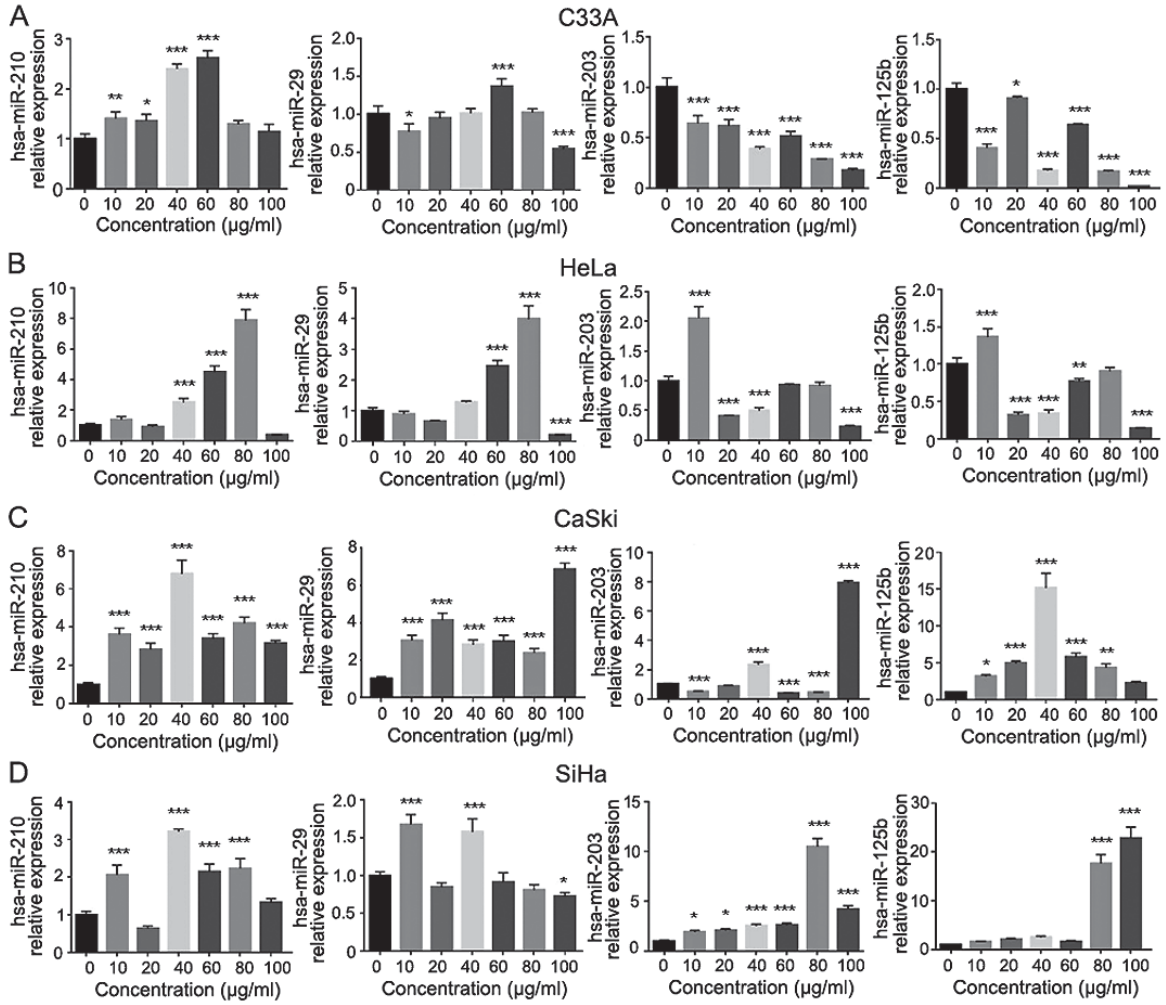

Figure 5. Polymerase chain reaction analysis of the effect of epigallocatechin gallate on miRs in cervical cancer cell lines. The miR expression of miR-210, miR-29, miR-203 and miR-125b in (A) CA33, (B) HeLa, (C) CaSki cells and (D) SiHa cells. *P<0.01, **P<0.001, ***P<0.0001 vs. control. hsa, *Homo sapiens*; miR, microRNA.

the monomers and their derivatives may also affect the sensitivity of chemotherapy drugs (38).

Shanghai, one of the most developed and wealthy cities in China, is located in the Yangtze River Delta and is the major production area of green tea. Therefore, the present study attempted to seek a way to improve the national conditions in the field of cervical cancer and HPV infection, to ultimately provide an economical and appropriate treatment.

Cervical cancer is characterized by epigenetic modifications, including DNA methylation, aberrant expression of non-coding RNAs and histone modification, which occur throughout the process of cancer progression and carcinogenesis A previous study has reported aberrant regulation of miRNAs in gynecological cancers; for instance, hsa-miR-126, hsa-miR-143 and hsa-miR-145 were downregulated, while hsa-miR-15b, hsa-miR-16, hsa-miR-146a and hsa-miR-155 were upregulated (39,40). The potential effect of green tea catechins has been revealed by a number of previous studies. EGCG is the major active component of green tea and it has been reported to block carcinogenesis by affecting a wide array of signal transduction pathways, including mitogen-activated protein kinase, PI3K/Akt, Janus kinase/signal transducer and activator of transcription, mTOR, epithelial growth factor and Notch/Wnt *in vitro*. EGCG has potential as an effective treatment for cancer intervention and prevention due to its anti-tumor bioavailability, safety and cost-effectiveness. EGCG is also able to regulate vascular endothelial growth factor, matrix metalloproteinases, urine plasminogen activator, insulin-like growth factor-1, EGF receptor and cyclin-dependent kinases (CDKs), as well as inhibit the activity of telomerase, degrade DNA methyl transferases, and inhibit cancer cell growth, proliferation, metastasis and angiogenesis (41,42). It has been postulated that cyclin d1-CDK4/6 complexes (D-type cyclins forming complexes through interaction with CDKs) are mainly responsible for driving the cell cycle transition from G1 to S phase (43). Catechin hydrate was reaveled to demonstrate inhibitory activity on SiHa cell proliferation by mediating apoptosis compared with cells without treatment (44). A recent study indicated that catechinmetabolites EGCM7, and EGC-M9, produced from EGCG, inhibit the proliferation of the HeLa (HPV16/18+) cell lines; however, the inhibitory mechanisms of catechin metabolites, produced by intestinal microbiota against cervical cancer cells, have not been clarified (45). However, the mechanism underlying cell cycle arrest and apoptosis induction mediated by EGCG remains to be elucidated.

Previous studies have shown that miR-203 upregulation has been identified in colon (46) and ovarian (47) cancers, whereas miR-203 downregulation was observed in mucosa-associated hepatocellular carcinoma (48), central nervous system tumors (49) and lymphoid tissue lymphoma (50). In cervical cells, ectopic expression of hsa-miR-203 decreased their rates of proliferation and anchorage-independent growth (51). miR-125b was downregulated in ovarian cancer, oral cancer, cervical cancer and hepatic carcinoma (52). Furthermore, miR-125b can increase the chemosensitivity and decrease the proliferation of breast cancer cells; the mechanism for this was be identical to that reported in previous studies that investigated apoptosis through the HAX-1 signaling pathway (53). In gallbladder cancer, miR-125b-5p enhances chemosensitivity by downregulating Bcl-2 (54). A previous study by our group, miRNA-125b was demonstrated to regulate the PI3K/Akt/mTOR pathway by targeting phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit δ (PIK3CD), thus reducing HeLa cell apoptosis via suppression of B-cell lymphoma 2 (19). Another previous study by our group from 2013 established that miR-203 is downregulated in cervical cancer; in addition, it was demonstrated that miR-203 suppresses cervical cancer cell proliferation and angiogenesis by targeting VEGFA (20). The results of the present study demonstrate that EGCG inhibits the proliferation of cervical cancer cell lines via a mechanism involving regulation by miRNAs. HeLa cells were treated with EGCG and a time- and dose-dependent effect on cell proliferation was observed. In addition, miR-203 and miR-125b were downregulated by EGCG treatment in C33A cells, while miR-210 was upregulated; the same result was observed in HeLa cells. miR-210, miR-29 and miR-125b were all upregulated in CaSki cells, while miR-210, miR-203 and miR-125b were upregulated to different expression levels in SiHa cells.

According to ancient Chinese literature, green tea has been used in China for ~3,000 years. It is the largest producer of tea worldwide, with an annual output of ~2.58 million tons in 2017, including 1.44 million tons of green tea. High-quality varieties are concentrated in east China. EGCG and EGC are highly bioactive compared with other tea catechins, and their mechanisms of action include roles in angiogenesis. EGCG has the potential to inhibit HPV and treat cervical cancer possibly by regulating the expression of associated miRNAs, so it may be considered an effective treatment drug to the cervical cancer in the future. Further investigation using these agents and research regarding the factors affecting the diagnosis and treatment of cervical cancer may yield a novel, cost-effective and safe drug with good efficacy for the treatment of cervical cancer.

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Availability of data and materials

The datasets used or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and LM designed experiments; QY, WaZ, PY, QG and JW performed the experiments; and YH analyzed the experimental results. ML analyzed the sequencing data and developed analysis tools. LM assisted with the supervision of the research. YZ and YH wrote the manuscript. WeZ performed a final check and gave approval of the version of the manuscript to be submitted.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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