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Inhibition of Invasion and Up-regulation of E-cadherin Expression in Human Malignant Melanoma Cell Line A375 by (-)-Epigallocatechin-3-gallate

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Summary: The inhibitory effects of (-)-epigallocatechin-3-gallate (EGCG) on the invasion of human malignant melanoma cell line A375 and the possible molecular mechanisms of this effect were investiaged. A375 cells were pretreated with 20 μg/mL EGCG for 24, 48 and 72 h respectively and the E-cadherin expression was detected by Western blot analysis. A375 cells were also pretreated with different concentrations of EGCG (1, 5, 10 and 20 μg/mL) for 72 h and the expression of E-cadherin was measured by RT-PCR. The adhesion and invasion of A375 cells were tested by cell-matrigel adhesion assay and matrigel invasion assay respectively. The results showed that EGCG could significantly up-regulate the expression of E-cadherin time- and concentration-dependently (both $P<0.05$). Statistical analysis showed that A375 cells invasion was inhibited by EGCG and correlated with the up-regulation of E-cadherin expression. It was suggested that EGCG strongly inhibited invasion of A375 cells, and the inhibition mechanism was possibly associated with the up-regulation of E-cadherin expression.
Key words: (-)-epigallocatechin-3-gallate; melanoma; E-cadherin; invasion

Malignant melanoma is a tumor with high invasion and metastasis ability, recurrence and mortality rate. Epithelial-mesenchymal transition (EMT) plays a critical role in tumor metastatic processes and has attract much attention[1]. A critical step in the disassembly of adherens junctions leading to EMT is inactivation of E-cadherin mediated adhesion[2, 3]. Loss of E-cadherin function may be caused by multiple mechanisms involving transcriptional repression and extracellular glycosylation that suppresses adhesion[4]. (-)-Epigallocatechin-3-gallate (EGCG) generally accounts for greater than 80% of the total polyphones and is the most widely studied polyphone for disease prevention[5]. It was reported that EGCG had anti-oxidative, anti-mutagenic, anti-inflammatory and anti-carcinogenic functions[6, 7]. In order to investigate the suppressive effects of EGCG on the invasion of human malignant melanoma cell line A375, and the possible molecular mechanisms of this effect, E-cadherin expression and cell adhesion, invasion ability were detected after cell line A375 was pretreated with different concentrations of EGCG.

1 MATERIALS AND METHODS

1.1 Materials

EGCG >95% pure (Sigma Chemical Co., USA). Mouse anti-human E-cadherin, HRP-labeled goat-anti-mouse IgG (Santa Cruz Co. USA). RPMI medium 1640, fetal bovine serum (FCS, GIBCO Co., USA). Matrigel gel (BD Co., USA). Boyden camber (Millipore-PCF type, Millipore Co., USA). Amplification primer (Shanghai Sangon Biological Engineering Technology & Services Co.Ltd, China).

1.2 Cell Line and Cell Culture

Human malignant melanoma A375 cells were provided by Doctor Taojuan and were cultured in RPMI medium 1640 supplemented with 10% heat inactivated FCS, 2 mmol/L glutamate, 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2.

1.3 Cell Adhesion Assay

Ten micrograms of Matrigel (30 μL) were added to each well of the 96-well culture plate and the plate was air-dried in the biohazard hood. Then 20 μL of 2% BSA was added into each well of the plate and the plate was incubated at 37°C for 1 h. Then 3×10^4 cells treated for 72 h with 0, 1, 5, 10, 20 μg/mL EGCG were plated in each well in quadruplicate. After incubation at 37°C for 1 h, the cells were washed three times with phosphate-buffered saline (PBS). The plate was incubated for other 4 h after addition of 20 μL MTT (5 mg/mL). MTT was then removed and 150 μL DMSO was added into each well. *A* at 570 nm was read by micro-titre plate reader. Cell adhesion inhibition ratio was calculated with following formula: Adhesion inhibition ratio=(*A* of control group–*A* of treatment group)/*A* of control group.

1.4 Invasion Assay

Invasion into the reconstituted basement membrane matrigel was assayed. The invasion assay was based on the method of Oshiro[8] with modifications. Briefly, cells were incubated with or without EGCG for 72 h and

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trypsinized to form single-cell suspension in serum-free RPMI 1640 medium containing different concentrations of EGCG at both the upper and lower compartments of the chamber. After 48-h incubation for invasion, non-invasive cells residing on the upper surface of the filter were thoroughly removed by wiping with several cotton swabs. Cells invading through the matrigel-precoated membrane filter were stained and counted under the microscopy. This experiment was repeated three times. The number of migrated cell was determined by the average cell number of 25 fields of view. Cell invasion inhibition ratio was calculated with following formular: Invasion inhibition ratio=(Number of invasive cells of control group–Number of invasive cells of treatment group)/Number of invasive cells of control group)×100%.

1.5 RT-PCR Analysis for E-cadherin Expression

Total RNA was extracted from A375 cells treated with different concentrations of EGCG. Trizol was used for the extraction of total RNA. The reverse transcription was carried out according to the kit instructions. PCR primer was designed according to the sequences of E-cadherin and GAPDH (used as endogenous standard control) by using Primer Express Version 1.0. E-cadherin primer: upstream: 5'-CTGGTTCAGATCAAATCCAAC-3'; downstream: 5'-TTCTCAGGCACCTGACCCTT-3'. The size of the product was 502 bp. The amplification conditions consisted of 94°C for 5 min for predenaturing, followed by 40 cycles at 94°C for 30 s for denaturing, at 58°C for 30 s for annealing and at 72°C for 30 s for extension, followed by re-extension at 10 min at 72°C. GAPDH primer: upstream: 5'-TGGTATCGTGGAAGG ACTCATGAC-3'; downstream: 5'-ATGCCAGTGAGC TTCCCGTTCAGC-3'. The size of the product was 190 bp. The PCR product was subjected to 1.0 % agarose gel electrophoresis and imaged with Gel Image Analysis System for the identification of target fragment. The ratio of E-cadherin/GAPDH represented the semi-quantitative level of E-cadherin mRNA.

1.6 Western Blot Analysis for E-cadherin Expression

A375 cells (exponential phase of growth, 1×10^6) were plated in a 6-well culture plate and treated with 20 μg/mL EGCG for 24, 48 and 72 h. The total protein was extracted. Fifty micrograms of the resolved proteins were subjected to SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane from the gel. The nitrocellulose blots were blocked for 1 h at 37°C using TBS buffer (PBS buffer plus 0.05 % Tween-20). The blots were then washed twice and incubated overnight with antibody against E-cadherin and Tubulin (marker protein) IgG (1:1000) (Santa Cruz Co., USA). The blots were then washed twice and incubated with HRP-labeled goat-anti-mouse serum (1:500 titre) for 60 min at room temperature. Then the blots were washed and visualized with enhanced chemiluninescence detection and imaged on IBAS2.0 film. *A* values of E-cadherin and marker protein Tubulin were determined by Gel-Pro Analyzer 4.0 software analysis. $A_{\text{E-cadherin}}/A_{\beta\text{-Tubulin}}$ represented the relative quantity of E-cadherin protein.

1.7 Statistical Analysis

All data were expressed as $\bar{x}\pm s$. SPSS 12.0 software package was used for ANOVA analysis and *t*-test. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Effect of EGCG on Adhesion of A375 Cells

After A375 cells were treated with different concentrations of EGCG for 72 h, the effect of EGCG on adhesion was examined. The results showed that EGCG inhibited the adhesion ability of A375 cells in a concentration-dependent manner. The adhesion inhibition ratio was (46.82±5.07)%, (51.71±5.24)% and (77.64±6.82)% ($P<0.05$) at concentrations of 5, 10 and 20 μg/mL respectively (table. 1) .

Table 1 A375 cells were treated with different concentrations of EGCG for 72 h

Groups	A_{570}	Cell adhesion inhibition ratio (%)	Invasion cell number	Cell invasiveness inhibition ratio (%)
Control	1.51±0.03	—	240.55±8.09	—
EGCG (μg/mL)				
1	1.40±0.09	4.03±0.39	234.90±2.02	6.97±0.98
5	1.18±0.03$^{\triangle *}$	46.82±5.07	180.21±4.08$^{\triangle *}$	34.16±2.72
10	0.82±0.03$^{\triangle *}$	51.71±5.24	113.53±5.19$^{\triangle *}$	40.98±3.04
20	0.40±0.02$^{\triangle *}$	77.64±6.82	30.40±2.99$^{\triangle *}$	62.61±5.93

$^{*}P<0.05$ as compared with control group; $^{\triangle}P<0.05$ as compared with each other among different concentration groups

2.2 Effect of EGCG on Invasion of A375 Cells

After exposure to EGCG, the invasion inhibition was determined and was shown in table 1. EGCG inhibited the A375 cells invasion in a concentration-dependent manner. The invasion inhibition ratio was (34.16±2.72)%, (40.98±3.04)%, (62.61±5.93)% ($P<0.05$) at concentrations of 5, 10, 20 μg/mL respectively. The number of invasive cells was reduced gradually with increased concentrations of EGCG.

2.3 Effect of EGCG on the E-cadherin mRNA Level in A375 Cells

The E-cadherin mRNA level in A375 cells was semi-quantified by reverse transcriptase polymerase chain reaction (RT-PCR) with an endogenous standard after the cells were exposed to EGCG for 72 h. Electrophoresis showed a band at 190 bp (GAPDH) appeared in

every group. The *A* ratio of E-cadherin/GAPDH was 1.02±0.09, 1.54±0.03, 1.73±0.04 and2.29±0.18 after pretreatment with EGCG at concentrations of 5, 10 and 20 μg/mL respectively. E-cadherin mRNA levels in the treated groups were significantly increased as compared with control group. The mRNA level was also increased with the increased concentrations of EGCG (fig. 1).

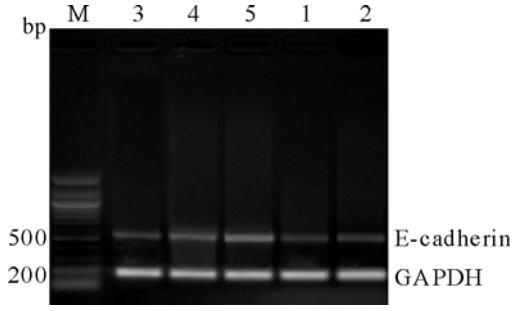

Fig. 1 Expression of E-adherin mRNA in A375 cells by RT-PCR analysis after the cells were treated with different concentrations of EGCG for 72 h
1: 0 μg/mL; 2: 1 μg/mL; 3: 5 μg/mL;
4: 10 μg/mL; 5: 20 μg/mL

2.4 Effect of EGCG on the E-cadherin Expression

Western blot was carried out to investigate the expression of E-cadherin in the A375 cells treated with 20 μg/mL EGCG at different time points (fig. 2). The results showed that the expression of E-cadherin was increased by incubation with EGCG in a time-dependent manner. $A_{\text{E-cadherin}}/A_{\beta\text{-Tubulin}}$ ratioalues was 0.15±0.01, 0.31±0.03, 0.68±0.05 and 1.23±0.02 at 0, 24, 48 and 72 h after incubation with EGCG. *P* values between each group were less than 0.05. The increased expression of E-cadherin was most obvious at 72 h after incubation.

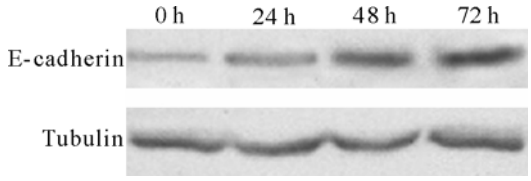

Fig. 2 Expression of E-cadherin protein in A375 cells by Western blot analysis after the cells were treated with EGCG for different time

3 DISCUSSION

During the complicated multi-step process of cancer metastasis, tumor cell invasion of the basement membrane is one of the earliest critical steps[5], which permits cells to invade surrounding tissue[9]. Simultaneously, the migration properties of cancer cells play a very important role in tumor metastasis and invasion[10]. E-cadherin is an important cell adhesion molecule and plays a key role in the early stage of tumor metastasis.

Loss of E-cadherin during carcinoma progression can take place through mechanisms operating at different levels, and both irreversible and reversible inactivating mechanisms appear to exist[2, 11]. Hypermethylation of the E-cadherin promoter resulting in E-cadherin expression silencing is probably a more frequent mechanism in the most common nondiffuse-type carcinomas. Hypermethylation causes a change in the chromatin structure and compaction of the DNA, thereby making the E-cadherin gene inaccessible for transcription factors and RNA polymerases, and then inhibiting its transcription. Hypermethylation would prevail at the initial invasion steps leading to transient down-regulation of E-cadherin, thereby allowing dissociation of individual cells from the primary tumour mass, while a decrease in methylation with subsequent reexpression of E-cadherin would occur in established metastatic foci[12]. EGCG can inhibit invasion and metastasis in many tumors[13-16]. According to some studies, EGCG can inhibit DNA methyltransferase and reactivate methylation-silenced genes in some cancer cell lines[17]. Our study showed that EGCG dose-dependently inhibited the adhesion and invasion of A375 cells. The results demonstrated that EGCG dose-dependently promoted the E-cadherin mRNA expression and time-dependently promoted the expression of E-cadherin in A375 cells, which was possibly contributed to the inhibitory effect of EGCG on the invasion and migration of A375 cells. Further investigation was needed to study how EGCG manipulated the expression of E-cadherin protein. In conclusion, this study demonstrated that EGCG inhibited the invasion and metastasis of A375 cells, which might be contributed to EGCG up-regulating the expression of E-cadherin. This finding also illustrated the importance of E-cadherin in tumor invasion, and could possibly contribute to the application of EGCG to clinical study and the design of effective anti-invasion drugs.

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