Induction of apoptosis via inactivating PI3K/AKT pathway in colorectal cancer cells with the aged Hakka stir-fried green tea

Xin-yue Zhang  
Wuyi University

Hai-ying Huang  
Meizhou Academy of Agriculture and Forestry Sciences

Shi-li Sun  
Guangdong Academy of Agricultural Sciences

Dong-li Li  
Wuyi University

Ling-li Sun  
Guangdong Academy of Agricultural Sciences

Qiu-hua Li  
Guangdong Academy of Agricultural Sciences

Ruo-hong Chen  
Guangdong Academy of Agricultural Sciences

Xing-fei Lai  
Guangdong Academy of Agricultural Sciences

Zhen-biao Zhang  
Guangdong Academy of Agricultural Sciences

Xi Zheng  
Wuyi University

Wing-Leung Wong  
Wuyi University

Shuai Wen (✉ wyu2017ws@163.com)  
Guangdong Academy of Agricultural Sciences

Research Article

Keywords: Cell Apoptosis, Cell Cycle Progression, PI3K/AKT Signalling, Hakka Stir-Fried Green Tea, Storage Time

Posted Date: October 17th, 2022
Abstract

Human colorectal cancer is notorious for its high morbidity and mortality; however, the lack of effective and low-toxic drugs has currently been a bottleneck for the treatment of the disease. The present study reported a new functional food of Hakka stir-fried green tea (HSGT) aged with different years, including those stored starting from 2003 (03Y), 2007 (07Y), 2011 (11Y), 2015 (15Y) and 2019 (19Y) respectively, for their effective therapeutic activity against colorectal cancer. The major active ingredients including polyphenols, catechins, amino acids, catechins, gallic acid and caffeine found in the lyophilized powder of these aged HSGT were analyzed with high performance liquid chromatography. Our results showed that, at the cellular level, all these aged HSGT inhibited significantly the proliferation of colon cancer cells (HT-29) in a concentration-dependent manner. In particular, the batch of 15Y and 19Y exhibited the highest inhibition rate for 48 hours treatment. Further, all these aged HSGT examined were able to enhance the apoptosis of HT-29 cells and block the transition of G1/S phase population to G2/M phase. Western blotting results also showed that the aged HSGT inhibited CDK2, CDK4 and CylinB1 protein expression, as well as increased PRAP expression and Bax/Bcl2 ratio in HT-29 cells. In addition, an upstream signal, PI3K/AKT signaling, was found involving in this regulation, as evidenced by the inhibition of phosphorylated PI3K and AKT by the aged HSGT. Therefore, our study reveals that the aged HSGT may inhibit colon cancer cell proliferation, cell cycle progression and promoted apoptosis of colon cancer cells by inactivating PI3K/AKT signalling. The therapeutic effects of the HSGT aged with different years were also investigated.

Introduction

Human colon cancer is a malignant tumor with extremely high morbidity and mortality worldwide. The disease thus threatens human health seriously. Although colon cancer mostly occurred in developed countries before, the incidence and mortality of this disease have gradually increased in developing countries including China probably due to the social development and lifestyle changes in the recent decade [1, 2]. According to the newly released data, a 5-year survival for patients with metastatic colon cancer is less than 20% and more than 50,000 patients are estimated died from colon cancer in 2020 [3]. Surgical resection and chemotherapy are conventional treatments for colon cancer, but postoperative recurrence and adverse drug side effects bring great pain and nightmares to patients [4]. Therefore, novel and effective therapeutic strategies with low side effects are essential to treat the disease and improve patient outcomes.

Uncontrolled growth of colon cancer cells depends on persistent activation of corresponding intracellular proliferative signals. Under the stimulation of various extracellular growth factors, receptor tyrosine kinases (RTKs) are activated to stimulate the subsequent increase in the phosphorylation and activity of phosphatidylinositol 3-kinase (PI3K). PIP3 is phosphorylated by PI3K that binds to AKT and promotes phosphorylation at Thr308 of AKT [5, 6]. Some recent studies have shown that the activation of PI3K/AKT pathway plays a positive role in the carcinogenesis, cell survival, migration, and metabolism of colon cancer [7–9]. Intervention by inhibitors or RNA interference technology or PI3K/AKT pathway and
related upstream and downstream sites may block the pathway, inhibit cell proliferation and cycle, and promote cell apoptosis. For example, it was reported that berberine inhibited PI3K and AKT expression and induced apoptosis and cell cycle arrest in SW480 cells [10]. Small interfering RNA and molecular inhibitors of mismatch repair gene MutL Homolog 1 (MLH1) suppressed colon cancer sensitivity to cetuximab treatment via PI3K/AKT signaling [11]. As a downstream signal of PI3K/AKT, the progression of cell cycle is necessary for colon cancer proliferation. Several small-molecule inhibitors, such as botulin or periplocymarin, blocked cell cycle progression in colon cancer cells by silencing PI3K/AKT signaling, accompanied by the expression of cycle-related proteins such as cyclin-dependent kinase (including CDK2, CDK4 or CDK6) and cyclins (Cyclin B1 or D1) [12–14]. In addition, the pharmacological inhibition of PI3K/AKT may promote colon cancer cell apoptosis by regulating apoptosis-related proteins PARP, Bcl2 and Bax, and show effective anti-tumor effects [12, 15]. The effective means to target PI3K/AKT is thus the prospect of developing colon cancer therapeutics.

Tea is a traditional functional beverage in which the active ingredients, such as catechins, tea polyphenols, caffeine, and flavonoids, are widely considered to be effective in inhibiting tumor cell proliferation and development [16]. For example, camellia ptilophylla extract significantly promoted colon cancer cell HCT116 apoptosis, which was attributed to a decrease in AKT phosphorylation [17]. However, different varieties of tea, fermentation time or aged time (years of storage) may have great differences in the production of active ingredient contents and anti-cancer effects. Hakka stir-fried green tea (HSGT) is a traditional historical tea in Guangdong, China, and its potential role in anti-cancer treatment and the influence of different production processes have not been investigated systematically. Herein, we compared comprehensively the active components of HSGT aged with different years and studied their functions in the regulation of cell proliferation, cell cycle and apoptosis in colon cancer cells (HT-29). Our results showed for the first time that that the traditional HSGT after aging for years markedly reduced the activity of PI3K/AKT signaling and its downstream target gene clusters from cell cycle or apoptosis.

Materials And Methods

Cells and freeze-dried powder of tea extract

Colon cancer cell line HT-29 were purchased from National Collection of Authenticated Cell Cultures of Chinese Academy of Sciences (Shanghai, China), and cultured in the medium containing 90% McCoy's 5A Medium (Invitrogen, CA, USA) and 10% FBS (Gibco, CA, USA). The tea samples of Hakka stir-fried green tea (HSGT) stored since year 2003 (03Y), 2007 (07Y), 2011 (11Y), 2015 (15Y) and 2019 (19Y) were purchased from Meizhou Junbao Industrial Co., Ltd. An appropriate amount of HSGT was ground and mixed with 90°C boiling water at a solid-to-liquid ratio of 1:20. The tea soup was leached for 30 min and immediately filtered, repeated 3 times. The three tea soups were combined and steamed to 1/10 of the original volume, and freeze-dried to obtain lyophilized HSGT powder.

Analysis Of Active Ingredients In Tea Extract
According to GB/T 8313 − 2018 and GB/T 8314 − 2013 standards, we detected the content of tea polyphenols and free amino acids in lyophilized HSGT powder. The content of soluble sugar and flavonoids was determined by the anthrone-sulfuric acid colorimetric method and the aluminum trichloride colorimetric method. The monomer components of catechin, gallic acid and caffeine were measured by a high-performance liquid chromatograph (Agilent 1200 Series, CA, USA). The chromatographic column was a phenomenex C18 column (150×4.6 mm, 5 µm), and the mobile phase included phase A (containing 0.5% acetic acid, 1% acetonitrile and 2% methanol) and phase B (containing 0.5% acetic acid, 10% acetonitrile and 20% methanol). In the first 30 min of elution, phase A decreased from 72.5–20%, while phase B increased from 27.5–80%. When the elution time was in 30–35 min, phase A increased from 20–72.5%, and phase B decreased from 80–27.5%, and then continued to 40 min. The injection volume was 10 µL, the flow rate was 1.0 mL/min, and the constant temperature was 28°C. Quantitative analysis was performed by the external standard method according to the peak area at a wavelength of 280 nm.

**Cell Viability Assay**

The concentration of HT-29 cells in log phase was adjusted to $5 \times 10^4$ cells/mL, and then added to 96-well plates at 100 µL/well. After 24 hours, the culture medium of adherent HT-29 cells was discarded, and 100 µL/well of HSGT solutions of different concentrations (lyophilized powder dissolved in McCoy’s 5A Medium) were added respectively. As a control well, McCoy’s 5A Medium without FBS was added at 100 µL/well. Each group was repeated 4–6 times and cultured at 37°C 5% CO$_2$ for 24 h or 48 h. At the time of detection, 10 µL/well MTT solution (Beijing MYM Biotechnology Co., Ltd.) was added and co-cultured for 3–4 h. Subsequently, the medium in each well was aspirated, 150 µL/well of MDSO (Biosharp, Anhui, China) solution was added and incubated for 10 min. A TriStar LB941 multifunctional microplate reader (Berthold Technologies, Baden Württemberg, Germany) was used to measure OD value at a wavelength of 490 nm and obtained the cell viability.

**Cell Cycle Assay**

HT-29 cells were treated with 0.2 mg/mL HSGT solutions with different storage years for 24 h/48 h. Trypsinized cells were resuspended and centrifuged in 1 mL of ice-cold PBS. Then, 1 mL of 70% ethanol in an ice bath was added to the cell pellet, mixed by pipetting, and fixed for 12–24 h. After washing with ice PBS, cells were added to 0.5 mL propidium iodide (PI) staining solution (C1052 Cell Cycle and Apoptosis Detection Kit, Beyotime Biotechnology, Shanghai, China) for 30 min at 37°C in the dark. The cell suspension was then filtered through a filter and placed on ice. A flow cytometer (Accuri C6 Plus, BD, USA) was used to detect the fluorescence signal at the excitation wavelength 488 nm channel. Flow cytometry data was processed by FlowJo-V10 software.

**Cell Apoptosis Assay**
HT-29 cells were treated with 0.2 mg/mL HSGT solution of different storage years for 24 h or 48 h, in which 5 µM cisplatin (MedChemExpress, NJ, USA) was used as a positive control for apoptosis. 1×10^5 digested HT-29 cells were resuspended with 195 µL Annexin V-FITC binding solution (C1062L Annexin V-FITC Apoptosis Detection Kit, Beyotime Biotechnology), and then 5 µL fluorescein isothiocyanate (FITC) and 10 µL PI staining solution was added. The cell suspension was incubated at room temperature (20–25°C) for 10–20 min in the dark, then filtered through a filter and placed on ice. Under the excitation wavelength of 488 nm flow cytometer (Accuri C6 Plus, BD, USA), the FITC fluorescence signal at 515 nm and the PI fluorescence signal at 560 nm were detected. FlowJo-V10 software was used to process and analyze the data.

**Western Blotting Assay**

RIPA lysate (Beyotime Biotechnology) was used to extract proteins from HT-29 cells treated with HSGT solution. The protein concentration was determined with BCA protein detection kit (Thermo Fisher Scientific, CA, USA), followed by purified water and 4×loading buffer (Solarbio, Beijing, China) to adjust the protein concentration. Protein samples were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (Millipore, MA, USA). 5% non-fat dry milk (Bio-FROXX, Einhausen, Germany) was used to block PVDF membrane and then incubated with primary antibodies overnight at 4°C. Primary antibodies incubated included: p-PI3K (Tyr458) antibody (CST 4228, Cell Signaling Technology, MA, USA), PI3K antibody (CST 4292), p-AKT (Ser473) antibody (CST 4060), AKT antibody (CST 4691), Bax antibody (Abcam ab32503, Cambridge, UK), Bcl-2 antibody (Abcam ab117115), PARP antibody (CST 9542), CDK2 antibody (CST 2546), CKD4 antibody (CST 12790S), Cyclin B1 (CST 4138) and β-actin antibody (CST 4970). The PVDF membrane was washed and incubated with secondary antibodies, including HRP-labeled goat anti-rabbit IgG antibody (KPL 074-1506, SeraCare Life Sciences, MA, USA) and HRP-labeled goat anti-mouse IgG antibody (KPL 074-1806, SeraCare Life Sciences) at room temperature for 50 min. Blot signals on membranes were imaged by a chemiluminescent gel imaging system (Tanon 5200, Shanghai, China). Band grayscale was analyzed with Image J software.

**Data Statistics And Analysis**

All data are presented as mean ± standard deviation (SD), and each experimental data was repeated at least three times under the same conditions. Data analysis was performed using SPSS 20.0 and GraphPad Prism 8.0 software. One-way ANOVA analysis was performed between multiple tea samples treatments, with different lowercase letters in the same row showing significant differences at p < 0.05 level.

**Results**
Identification of active ingredients in Hakka stir-fried green tea aged for different years

Active ingredients such as polyphenols, catechins and amino acids in tea are believed to be helpful for anti-cancer treatment. We first analyzed the Hakka stir-fried green tea (HSGT) aged for different years, including five batches that have been stored since 2003 (03Y), 2007 (07Y), 2011 (11Y), 2015 (15Y) and 2019 (19Y), and the content of main active ingredients obtained from their lyophilized powders. As shown in Table 1, for the conventional composition of the HSGT, the content of tea polyphenols found in batches of 11Y and 15Y was significantly higher than other. The amino acid and soluble sugar contents found in 03Y and 19Y HSGT were significantly higher than those in other years. For the ratio of phenol to ammonia and flavonoids, the lyophilized powder of 11Y HSGT showed the highest content.

Table 1
Analysis of conventional components from the freeze-dried powder of the HSGT aged for different years.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>03Y</th>
<th>07Y</th>
<th>11Y</th>
<th>15Y</th>
<th>19Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea polyphenols / %</td>
<td>39.26 ± 0.24</td>
<td>32.39 ± 0.33</td>
<td>41.65 ± 0.09</td>
<td>39.91 ± 0.22</td>
<td>38.27 ± 0.01</td>
</tr>
<tr>
<td>Free amino acid / %</td>
<td>5.35 ± 0.04</td>
<td>4.40 ± 0.00</td>
<td>4.19 ± 0.02</td>
<td>5.01 ± 0.08</td>
<td>5.51 ± 0.09</td>
</tr>
<tr>
<td>Ratio of TP to FAA</td>
<td>7.44 ± 0.06</td>
<td>7.38 ± 0.30</td>
<td>9.93 ± 0.05</td>
<td>8.14 ± 0.06</td>
<td>7.04 ± 0.01</td>
</tr>
<tr>
<td>Soluble sugar / %</td>
<td>26.84 ± 0.05</td>
<td>23.69 ± 0.62</td>
<td>24.34 ± 0.53</td>
<td>23.64 ± 0.26</td>
<td>26.65 ± 0.29</td>
</tr>
<tr>
<td>Flavonoids / %</td>
<td>2.60 ± 0.04</td>
<td>3.01 ± 0.03</td>
<td>3.25 ± 0.05</td>
<td>2.85 ± 0.03</td>
<td>2.59 ± 0.01</td>
</tr>
</tbody>
</table>

Notes: Values represent means ± SD (n = 4). Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same row. TP, Tea polyphenols; FAA, Free amino acid.

For the analysis of catechins, gallic acid and caffeine, there were no significant differences in the content of total catechins, ester catechins and non-ester catechins between these aged HSGTs (Table 2). However, the content of catechin (C), catechin gallate (CG), galocatechin (GC), galallocatechin-3-gallate (GCG), gallic acid (GA), and caffeine (CAFF) in the freeze-dried powder of HSGT with long aging time (03Y or 07Y) was found increased significantly compared with other groups. Epigallocatechin (EGC) and epicatechin (EC) showed higher content in the short aging time (11Y, 15Y or 19Y) compared to 03Y or 07Y. Epicatechin-3-gallate (ECG) showed the highest content from the 11Y HSGT. Moreover, there was no significant difference found for epigallocatechin-3-gallate (EGCG) among the groups. Taken together, the data obtained may reveal differentially active components in the HSGT aged with different years. Their therapeutic effect on colon cancer cells was investigated in detail in the following sections.
Table 2
Quantitative analysis of catechins, gallic acids and caffeines in HSGT freeze-dried powders.

<table>
<thead>
<tr>
<th>Ingredients (mg/g)</th>
<th>03Y</th>
<th>07Y</th>
<th>11Y</th>
<th>15Y</th>
<th>19Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>2.64 ± 0.05 a</td>
<td>1.67 ± 0.04 b</td>
<td>1.65 ± 0.14 ab</td>
<td>0.76 ± 0.04 b</td>
<td>1.08 ± 0.08 ab</td>
</tr>
<tr>
<td>ECG</td>
<td>29.73 ± 0.96 b</td>
<td>33.74 ± 0.37 ab</td>
<td>33.94 ± 0.81 a</td>
<td>30.34 ± 1.18 b</td>
<td>26.51 ± 0.21 b</td>
</tr>
<tr>
<td>GCG</td>
<td>16.47 ± 0.39 a</td>
<td>10.45 ± 0.48 b</td>
<td>11.44 ± 0.21 b</td>
<td>11.13 ± 0.51 b</td>
<td>12.24 ± 0.24 b</td>
</tr>
<tr>
<td>EGCG</td>
<td>137.55 ± 1.04 a</td>
<td>141.79 ± 0.04 a</td>
<td>146.77 ± 0.85 a</td>
<td>131.21 ± 0.98 a</td>
<td>131.20 ± 0.63 a</td>
</tr>
<tr>
<td>EC</td>
<td>9.65 ± 0.11 b</td>
<td>9.31 ± 0.25 b</td>
<td>12.41 ± 0.21 a</td>
<td>13.52 ± 0.47 a</td>
<td>14.37 ± 0.15 a</td>
</tr>
<tr>
<td>C</td>
<td>6.17 ± 0.03 a</td>
<td>4.48 ± 0.13 ab</td>
<td>6.49 ± 0.02 a</td>
<td>5.46 ± 0.14 ab</td>
<td>4.35 ± 0.26 b</td>
</tr>
<tr>
<td>GC</td>
<td>69.02 ± 0.00 b</td>
<td>86.00 ± 1.28 a</td>
<td>51.71 ± 0.85 c</td>
<td>52.05 ± 1.87 c</td>
<td>44.13 ± 0.73 c</td>
</tr>
<tr>
<td>EGC</td>
<td>38.65 ± 1.25 c</td>
<td>33.02 ± 0.77 c</td>
<td>45.03 ± 0.09 b</td>
<td>58.59 ± 1.82 a</td>
<td>63.67 ± 0.36 a</td>
</tr>
<tr>
<td>GA</td>
<td>10.88 ± 0.03 a</td>
<td>9.79 ± 0.17 b</td>
<td>6.40 ± 0.22 c</td>
<td>5.54 ± 0.33 c</td>
<td>3.45 ± 0.03 d</td>
</tr>
<tr>
<td>CAFF</td>
<td>92.67 ± 0.25 a</td>
<td>90.45 ± 1.47 a</td>
<td>75.85 ± 0.10 b</td>
<td>88.99 ± 1.06 ab</td>
<td>71.96 ± 0.01 b</td>
</tr>
<tr>
<td>Ester catechins</td>
<td>185.59 ± 4.96 a</td>
<td>184.01 ± 2.66 a</td>
<td>180.13 ± 7.42 a</td>
<td>188.57 ± 7.20 a</td>
<td>169.85 ± 2.02 a</td>
</tr>
<tr>
<td>Non-ester catechins</td>
<td>120.91 ± 2.13 a</td>
<td>132.37 ± 0.72 a</td>
<td>115.26 ± 0.15 a</td>
<td>129.62 ± 4.30 a</td>
<td>128.21 ± 2.61 a</td>
</tr>
<tr>
<td>Total catechins</td>
<td>306.50 ± 5.45 a</td>
<td>328.23 ± 2.40 a</td>
<td>308.18 ± 15.38 a</td>
<td>303.29 ± 11.49 a</td>
<td>298.06 ± 4.89 a</td>
</tr>
</tbody>
</table>

Notes: Values represent means ± SD (n = 4). Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same row. CG, catechin gallate; ECG, Epicatechin-3-gallate; GCG, gallocatechin-3-gallate; EGCG, epigallocatechin-3-gallate; EC, epicatechin; C, catechin; GC, gallocatechin; EGC, epigallocatechin; GA, gallic acid; CAFF, caffeine.

The Aged Hakka Stir-fried Green Teas Inhibit The Proliferation Of Colon Cancer Cells

We evaluated the effect of aged HSGT extracts on the proliferation of HT-29 cancer cells. The HT-29 cells were treated with the aged HSGT solutions at different concentrations for 24 and 48 hours and followed
by MTT assays to identify cell viability. The results showed that the HSGT extract at high concentration (1.0 mg/mL) significantly inhibited the viability of HT-29 cells at either 24 or 48 hours of treatment compared to the lower concentration (0.2 mg/mL) or the control group with buffer. While treated for 24-hour at each specific HSGT concentration, no significant effect on cell viability was observed for the HSGT aged with different years (Fig. 1A). Nonetheless, the results obtained for 48-hour treatment were obviously varied. As shown in Fig. 1B, the 19Y HSGT aged starting from 2019 generally had lower cell viability than 03Y or 07Y at the treatment concentration of 0.2 and 0.4 mg/mL, respectively. These results also indicated that 19Y HSGT significantly inhibited the viability of HT-29 cells in a concentration-dependent manner. Moreover, the results may suggest that the HSGT with shorter aging time (15Y and 19Y) could be more advantageous in the inhibition of cell viability under 48-hour treatment conditions.

The Aged Hakka Stir-fried Green Teas Promote Cell Apoptosis

We further investigated whether the HSGT with different aging time could increase apoptosis in HT-29 cells, which could be a possible factor causing the decrease of HT-29 cell viability. Since significant differences in cell viability inhibition were observed for the HSGT with different years of aging at 0.2 mg/mL for 48-hour treatment (Fig. 1B), we thus selected this concentration for subsequent experiments. For the HT-29 cells after treated with 0.2 mg/mL of different HSGT for 24 hours, it was found that the apoptosis rate of 03Y and 19Y HSGT was significantly increased compared with the control group. It was noteworthy that 03Y HSGT exhibited a very comparable effect to the positive control using cisplatin (Fig. 2A-B). For the 48-hour treatments, compared to the control, all the aged HSGT examined generally enhanced the apoptosis significantly in the HT-29 cells. Among different aged-HSGT, 03Y HSGT was found to be the highest apoptosis-promoting effect and it was also significantly higher than the positive control (Fig. 2C-D). These results may confirm that a significant enhancement in HT-29 cell apoptosis induced by the aged HSGT tested. In particular, 03Y HSGT was found to be the most potent one.

The Aged Hakka Stir-fried Green Teas Slow Down Cell Cycle Progression

We then examined whether the aged HSGT could regulate cell cycle progression in HT-29 cells. In the assays, the 03Y, 07Y, 11Y, 15Y, 19Y HSGT and control were investigated with HT-29 cells at a concentration of 0.2 mg/mL for 24- and 48-hour, respectively. Cell cycle analysis was then performed with flow cytometry for comparison (Fig. 3A-B). Statistical results showed that all these aged HSGT were able to increase the proportion of G1 and S phases and decrease the proportion of G2/M phase in the HT-29 cell population, regardless of treatment time (Fig. 3C-D). In particular, the ratio of G1 phase and S phase for the 19Y HSGT-treated cells was significantly higher in than that of other aged HSGT (Fig. 3D). Therefore, we concluded that the aged HSGT could block the transition of the G1/S phase population of HT-29 cells to G2/M phase, thereby reducing cell viability.
The aged Hakka stir-fried green teas reduce the expression of cell cycle-related proteins in colon cancer cells

Due to the inhibitory role of the aged HSGT in cell cycle progression, we examined further several major cell cycle regulatory proteins. The CDK2, CDK4 and CylinB1 levels in HT-29 cells treated with 0.2 mg/mL of the aged HSGT for 48 h were examined and compared with the Western blot results (Fig. 4A). Quantitative analysis of protein bands revealed that the aged HSGT generally causes significant decreases in CDK2, CDK4 and CylinB1 protein levels (Fig. 4B-D). The results indicate that the aged HSGT may block effectively the cell cycle progression by inhibiting the expression of CDK2, CDK4 and CylinB1 proteins.

The Aged Hakka Stir-fried Green Teas Inactivate Pi3k/akt Signaling And Enhance Apoptotic Pathways

Activation of PI3K/AKT signaling is essential for cancer cell viability including colon cancer and also maintains downstream inhibitory signals of apoptosis. We therefore examined the levels of phosphorylated PI3K (p-PI3K), phosphorylated AKT (p-AKT), PRAP, Bax and Bcl2 in HT-29 cells treated with 0.2 mg/mL HSGTs for 48 hours by Western blotting (Fig. 5A). Quantitative analysis of protein bands revealed that the expression of p-PI3K and p-AKT in HT-29 cells were markedly reduced after the treatment with the aged HSGT, demonstrating an inhibitory effect on PI3K/AKT signaling (Fig. 5B-C). For the apoptotic pathway, the aged HSGT increased PRAP expression and the Bax/Bcl2 ratio in HT-29 cells (Fig. 5D–E). In particular, in cells treated with 15Y and 19Y HSGT, p-AKT expressions were significantly lower than in other aged-HSGT (Fig. 5C) and their Bax/Bcl2 ratio was also significantly higher than others (Fig. 5E). These results demonstrate the inhibitory effect of the aged HSGT on PI3K/AKT signaling and a sustained enhancement in the apoptotic pathway.

Discussion

As one of the severe and refractory cancer types, the pathogenesis of colon cancer and its effective treatment options are constantly being explored. It is generally believed that the pathogenesis of colon cancer is related to the accumulation of gene mutations in colon epithelial cells and disturbance of the immune microenvironment [18, 19]. In response to these characteristics, advanced drug treatment options including immunotherapy (anti-PD1 or anti-CTLA4 therapy) and targeted drugs (such as bevacizumab and cetuximab) have already been used in the treatment of patients or undergoing clinical trials [20, 21]. However, these drugs still cannot avoid tumor recurrence, and the side effects lead to a serious decline in the life quality of patients. The main purpose of the present study is to search for active and low toxic natural substances from traditional Chinese herbs such as HSGT to ensure both the efficacy and safety against colon cancer.

Our study identified the main components from the extracts of the traditional HSGT aged with different years. The in vitro activity of the aged HSGT against the proliferation of colon cancer cell was
systematically investigated and compared for the first time. In fact, previous studies have confirmed the beneficial effects of different kinds of tea extracts in colon cancer treatment [22–24]. In a study of Japanese men, it was reported that green tea may possibly reduce the risk of colon cancer [24]. Among the bioactive components studied in green tea, EGCG was found down-regulated phosphorylated STAT3 to induce apoptosis in colon cancer cells (SW480) [22]. In addition, catechins (mainly EGC and EGCG) was able to restrict the proliferation of HCT116 colon cancer cells [23]. These results were also validated in the present study and we found that 15Y and 19Y HSGT showed the greatest inhibitory effect on cell viability among different aged HSGT. The HPLC analysis confirmed that the extract of 15Y and 19Y HSGT contained high levels of EGC and EC. Given that there was no significant difference found in the EGCG content across different aging years in our results, we thus reasoned that the effective anticancer activity against HT-29 could be probably mediated by the EGC and EC contents of the aged HSGT.

A previous study found that the activation of AMPK cascade signaling by HSGT protected against liver injury in a high-fat diet-induced obesity model [25]. However, the therapeutic potential of HSGT in anticancer was not reported. In the present study, we provided evidence that the aged HSGT in colon cancer therapy exhibited biological growth inhibition of HT-29 cells regardless the time of aging. However, for apoptosis and cell cycle regulation, there is variability in the HSGT aged for different years. For example, with an effective treatment time of 48 hours, among the different aged HSGT, 03Y HSGT induced the most apoptosis and 19Y HSGT had the most severe arrest in the G1/S phase of the cell cycle. The results may indicate that the time for HSGT aging is important for improving the potency of the tea in anticancer treatment. Moreover, many teas including Keemun black tea, Qingzhuan tea and Pu-erh tea have different active ingredients depending on the number of years they are aged [26–30]. The changes of active components in tea due to aging time may result in significant differences in cancer therapy or other diseases. For example, the content of statins and polyphenols in Pu-erh tea fermented for a short period of 42 days was higher than that with longer fermentation time, which is beneficial for relieving cardiovascular disease caused by hyperlipidemia [31]. Given the different apoptosis and cell cycle regulation of the teas with different aging time in our study, the aged HSGT combined with cyclin inhibitors or apoptosis-promoting molecules can be utilized to develop an effective management strategy for colon cancer in different scenarios.

For the 03Y and 19Y aged HSGT, both contain amino acid and soluble sugar contents that are significantly higher than other aged HSGT. In terms of difference of the 03Y and 19Y aged HSGT, GA in 03Y group was found significantly higher than that in 19Y group, while EGC was higher in 19Y group. We therefore hypothesized that GA in the 03Y group mediated apoptosis, while EGC in 19Y group might affect cell cycle regulation. It was reported that GA induced the apoptosis of HCT-15 colon cancer cells in a ROS-dependent manner [32]. Furthermore, the natural plant-derived GA inhibited PI3K/AKT phosphorylation and increased colon cancer cell apoptosis [33]. In terms of cell cycle regulation, it was previously found that EGC kept Lovo colon cancer cells in G1 phase [34] and inhibited the expression of cyclin D1 and CDK4 in tumor cells [35]. Thus, EGC is also believed to be involved in the activation of p-PI3K and p-AKT-induced tumor growth signals [36]. Taken together, all these results may point to the fact
that the active ingredients in the aged HSGT activates PI3K/AKT signaling to achieve the inhibition of colon cancer cell growth.

In short, the HSGT aged with different years has the certain effect that inhibit colon cancer cell growth, arrest in the G1/S phase of the cell cycle, and induce apoptosis, which via suppressing PI3K/AKT signaling pathway, down-regulating CDK2, CDK4 and CylinB1 proteins, as well as up-regulating PARP protein and ratio of Bax/Bcl-2.

Declarations

Acknowledgement

The research was supported by the "14th Five-Year Plan" team-building projects of Guangdong Academy of Agricultural Sciences [202126TD]; Guangdong Basic and Applied Basic Research Foundation [2020A1515011266, 2021A1515010958]; Guangzhou Science and Technology Plan Projects [202002030202, 202102020047]; Key-Area Research and Development Program of Guangdong Province [2020B0202080003]; Innovation Fund projects of Guangdong Academy of Agricultural Sciences [202115, 202035]; Special fund for scientific innovation strategy-construction of high level Academy of Agriculture Science [R2019PY-JX004].

Author contributions

Xinyue Zhang: Methodology, Data curation, Formal analysis, Visualization, Writing-original draft. Haiying Huang: Investigation, Formal analysis, Funding acquisition. Shili Sun: Investigation, Writing-original draft, Funding acquisition. Dongli Li: Formal analysis, Methodology. Lingli Sun: Formal analysis, Methodology. Qiuhua Li: Formal analysis, Methodology. Ruohong Chen: Formal analysis, Methodology. Xingfei Lai: Formal analysis, Methodology. Zhenbiao Zhang: Formal analysis, Methodology. Xi Zheng: Formal analysis, Methodology. WingLeung Wong: Conceptualization, Visualization, Writing-original draft, Writing – review & editing. Shuai Wen: Conceptualization, Writing-original draft, Visualization, Writing – review & editing.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

References


Figures
Figure 1

Inhibitory activity of HSGT extracts with various storage years on HT-29 cell viability. HT-29 cells were treated with 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL HSGT extracts of each storage year (including 03Y, 07Y, 11Y, 15Y and 19Y) for 24 hours A and 48 hours B, respectively, and cell viability was detected by MTT assay. Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same graph.
The promotion of apoptosis of HT-29 cells by HSGT extracts with various storage years. HT-29 cells were treated with 0.2 mg/mL HSGT extract of each storage year (including 03Y, 07Y, 11Y, 15Y and 19Y) for 24 hours (A and B) and 48 hours (C and D), respectively. 0.2 mg/mL cisplatin was used as a positive control (Pos group). The proportion of apoptotic cells in each group was examined and counted by flow cytometry.
Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same graph.

Figure 3

HSGT extracts with various storage years inhibited HT-29 cell cycle progression. HT-29 cells were treated with 0.2 mg/mL HSGT extract of each storage year (including 03Y, 07Y, 11Y, 15Y and 19Y) for 24 and 48 hours.
hours, respectively A and B. The phase ratios of total cell cycle in each group were examined by flow cytometry C and D. Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same graph.

**Figure 4**

HSGT extracts with various storage years inhibited expression of cell cycle-related proteins. A HT-29 cells were treated with 0.2 mg/mL HSGT extract of each storage year for 48 hours, and then CDK2, CDK4 and CyclinB1 expressions were detected by western blot. B-D Relative quantification of CDK2, CDK4 and CyclinB1 protein levels in each group was shown, with actin as a reference. Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same graph.
Figure 5

HSGT extracts with various storage years inactivated PI3K/AKT signaling and enhanced apoptotic pathways. **A** HT-29 cells were treated with 0.2 mg/mL of each storage year HSGT extract for 48 hours, followed by detection of p-PI3K, p-AKT, PRAP, Bax and Bcl2 expression by western blotting. **B-E** Relative quantification of p-PI3K, p-AKT, PRAP protein levels (with actin as references) and Bax/Bcl2 ratio in each
group was shown. Significant difference between groups was indicated with the different lowercase letters (a, b, c, et al) in the same graph.