

EGCG inhibits growth and induces apoptosis in renal cell carcinoma through TFPI-2 overexpression

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Abstract. EGCG (epigallocatechin gallate), the major catechin found in green tea, has been shown to inhibit proliferation and induce apoptosis in many tumors. EGCG can inhibit DNMT (DNA methyltransferase) activity, and cause CpG demethylation and reactivation of methylation-silenced genes. Tissue factor pathway inhibitor-2 (TFPI-2), a member of the Kunitz-type serine proteinase inhibitor family, is inversely related to an increasing degree of malignancy. Our previous study showed that the expression of TFPI-2 and invasiveness of renal cell carcinoma had a negative correlation. Overexpression of TFPI-2 may induce tumor cell apoptosis in renal cell carcinoma. Lower expression of TFPI-2 in renal cell carcinoma was partly due to hypermethylation of the gene promoter. Herein, using MTT and flow cytometry, we demonstrated that EGCG can inhibit growth and induces apoptosis in renal cell carcinoma cell line 786-0. In addition, Western blotting and real-time RT-PCR showed that EGCG can upregulate expression of TFPI-2. Before and after EGCG treatment, real-time methylation specific PCR could not detect methylation status of TFPI-2 gene promoter in cell line 786-0. *In vivo* invasiveness and metastasis test did not indicate any significant differences between control and treatment group. Our results suggest that EGCG inhibits growth and induces apoptosis in renal cell carcinoma through TFPI-2 overexpression. This is the first report showing that EGCG is likely to be an effective agent for renal cell carcinoma.

Introduction

Green tea is one of the most widely consumed beverages. Generally, a typical cup of green tea contains 100-150 mg catechins, including 50% of epigallocatechin-3-gallate

(EGCG) (1). Numerous studies have shown that EGCG possesses obvious antiproliferative, proapoptotic, anti-angiogenic, antimetastatic, and cell cycle regulation activities in various *in vitro* and *in vivo* tumor models. These experimental studies together with several epidemiological studies have suggested that EGCG harbors strong anticancer and cancer preventive effects in numerous human cancers.

Hypermethylation of DNA is a key epigenetic mechanism for the silencing of many genes including those for tumor suppressors, DNA repair enzymes, and receptors. In this aberrant methylation, the cytosine of the CpG island in or near the promoter region of the newly synthesized DNA strand is methylated by DNMT. A study has demonstrated that the gallic acid moiety of EGCG plays a crucial role in its high-affinity, direct inhibitory interaction with the catalytic site of the human DNMT1, and its binding with the enzyme is stabilized by Mg²⁺ (2). Our previous study showed that overexpression of TFPI-2 may induce tumor cell apoptosis in renal cell carcinoma. Lower expression of TFPI-2 in renal cell carcinoma was partly due to hypermethylation of the gene promoter. TFPI-2 is likely to be a tumor suppressor in renal cell carcinoma. Does EGCG upregulate expression of TFPI-2 through inhibition of DNMT? Is EGCG a novel anti-cancer agent against renal cell carcinoma?

In the present study, we tested the above hypothesis by examining the effect of EGCG on renal cell carcinoma cell line 786-0 and expression regulation of TFPI-2 by EGCG.

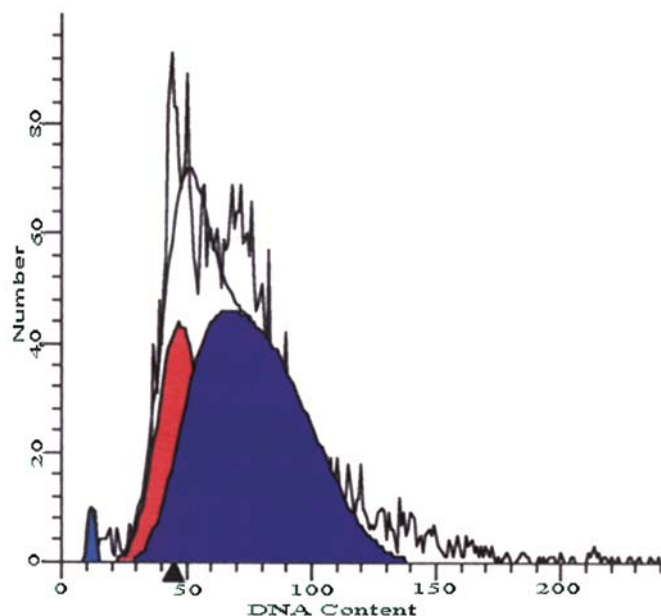
Materials and methods

Cell culture and treatment conditions. The human renal cell carcinoma cell line 786-0 was purchased from Institute of Biochemistry and Cell Biology, Shanghai, P.R. China. Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 µg/ml) and streptomycin (100 µg/ml) in a 5% CO₂ atmosphere. Cells were then incubated with various concentrations of EGCG (10 and 20 µM) (purchased from Boyun Biotechnology Ltd., Shanghai) or PBS buffer (as control) for 6 days. Each group was made up of 5 samples.

MTT assay. To determine the change of tumor cells number, colorimetric assay using MTT reagent was performed to detect their growth activity. The cells were incubated with MTT and dimethyl sulfoxide for 4 h and 10 min, respectively. Then the cells were quantified by measuring the absorbance

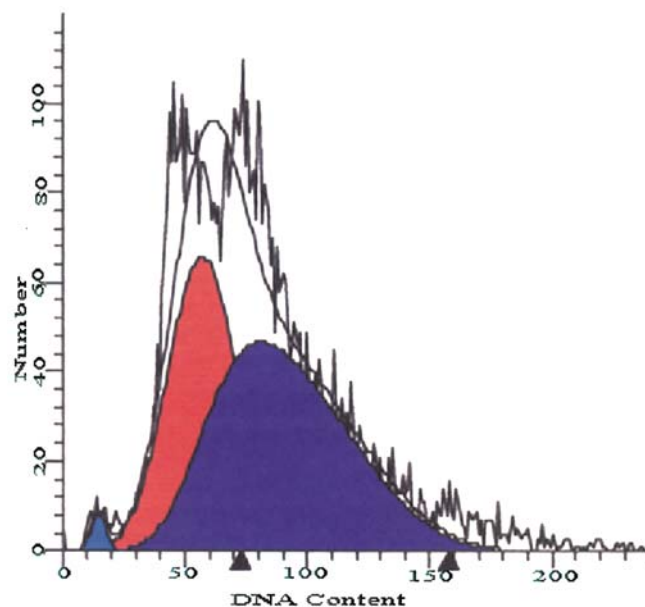
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Sample: 1. G0-G1: 22.10% at 47.00, G2-M: 0.00% at 105.61
Total S-phase: 77.90%, Apoptosis rate: 1.01%

Figure 1. Flow cytometry revealed cell cycle S-phase rate and apoptosis rate in PBS (as a control) treatment group.



Sample: 1. G0-G1: 40.79% at 57.25, G2-M: 3.09% at 115.08
Total S-phase: 56.12%, Apoptosis rate: 1.21%

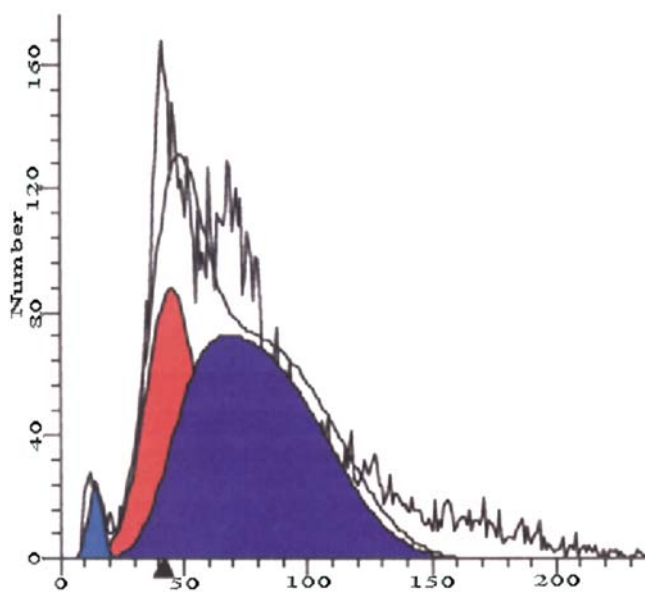
Figure 2. In EGCG (10 μ M) treatment group, flow cytometry revealed that cell cycle S-phase rate was lower, while apoptosis rate higher than that in PBS treatment group.

at 490 nm. Inhibition rate = $(1 - \text{OD value of treatment group} / \text{OD value of control group}) \times 100\%$.

Flow cytometry analysis. Cells were dehydrated with 95% alcohol followed by PBS. Trionx-100 (1 ml) was added to the cell line. After 10 min, cells were centrifuged for 5 min, 1500 rpm. Then RNase and propidium iodide were added. Sample was filtrated by nylon network. After stored for 20 min, cells were detected by flow cytometry (Becton Dickinson FACScalibur) which demonstrated information of cell cycle and apoptosis.

Western blotting. Total cellular protein was isolated from cells in lysis buffer containing proteinase inhibitors, and equal amount of protein were separated by SDS/PAGE and electroblotted to a nitrocellulose membrane. Immunoblotting was performed using primary rabbit anti-human TFPI-2 antibody (Santa Cruz Biotechnology, 1:400 dilution), then the protein was washed and incubated again with secondary anti-rabbit IgG (Santa Cruz Biotechnology, 1:2000 dilution) at room temperature. Protein was detected using enhanced chemiluminescence and autoradiography.

Real-time RT-PCR and real-time methylation specific PCR. Total cellular RNA was extracted by TRIZOL reagent according to manufacturer's instructions. RNA was resolved in isopropanol and kept in -20°C until cDNA synthesis. The final volume of the real-time RT-PCR reactions was 50 μ l and included 5 μ l cDNA, primers 0.5 μ l each, probe 0.5 μ l, dNTPs 0.5 μ l, and TaqMan enzyme 1 μ l. The sample was incubated at 50°C for 2 min, at 95°C for 10 min, followed by 40 cycles, 95°C for 15 sec, 60°C for 1 min. All the real-time RT-PCR reactions were performed on ABI-7300 platform.



Sample: 1. G0-G1: 29.14% at 43.78, G2-M: 5.44% at 104.72
Total S-phase: 65.43%, Apoptosis rate: 2.71%

Figure 3. In EGCG (20 μ M) treatment group, flow cytometry revealed that cell cycle S-phase rate was lower, while apoptosis rate higher than that in PBS treatment group.

GAPDH was used as control. The sequences of probes and primers were as follows: TFPI-2 probe: FAM-TCTGCC CCCAGCAGACAGAGGA-TAMRA; TFPI-2 primer (sense): 5'-TCACGGCCCCTCATGGT-3'; TFPI-2 primer (antisense): 5'-TCTGATCTCATGGCTTGACACA-3'.

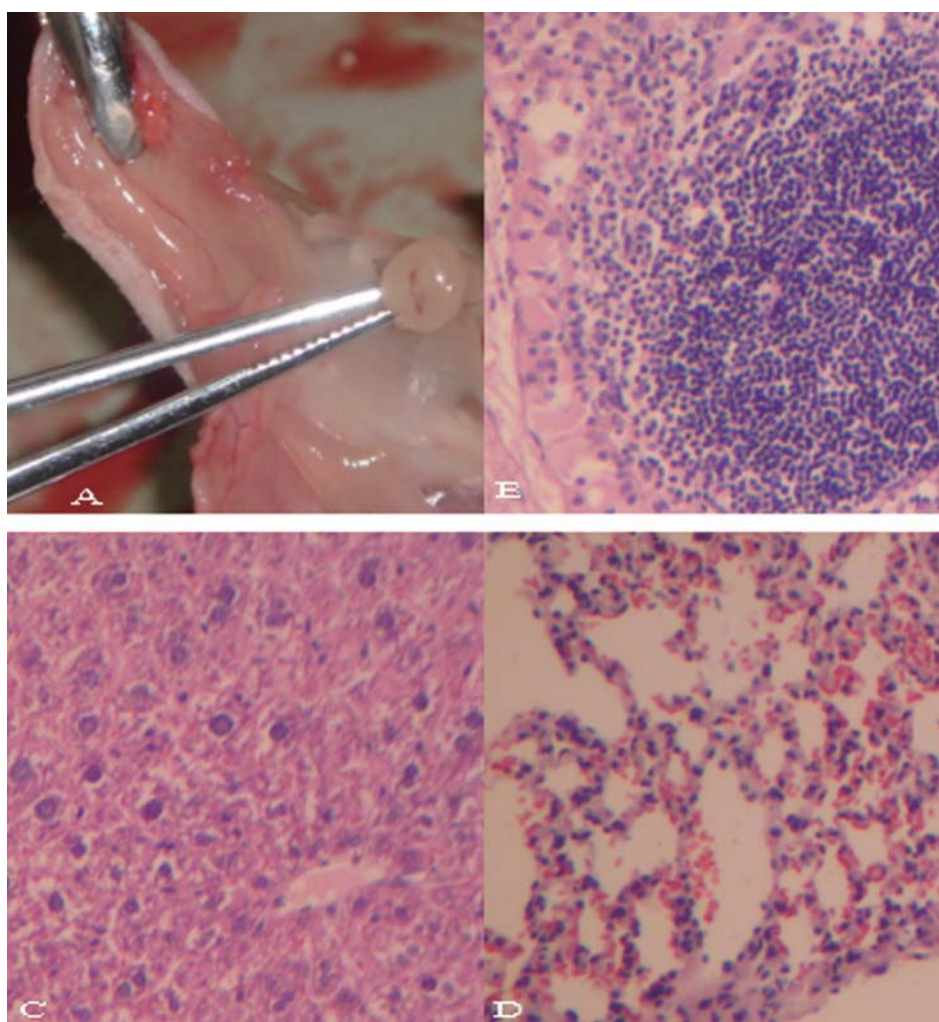


Figure 4. Gross feature of a subcutaneous tumor (A). In all three groups, H&E staining of a subcutaneous tumor (B), nude mouse liver (C) and lung (D) showed no invasiveness and metastasis of tumor.

GAPDH probe: FAM-AAGGTCATCCCTGAGCTGAA-TAMRA; GAPDH primer (sense): 5'-ACCCAGAAGAC TGTGGATGG-3'; GAPDH primer (antisense): 5'-TTCTAG ACGGCAGGTCAGGT-3'. TFPI-2 probe for real-time methylation specific PCR: FAM-TTCCAGCTGAACGAGC TCCAAGGC-TAMRA; TFPI-2 primer (sense) for real-time methylation specific PCR: 5'-CCTCTTGCCTTTGGACA TGG-3'; TFPI-2 primer (antisense) for real-time methylation specific PCR: 5'-TCTGCGGCATTTCTCTGGT-3'.

Nude mouse model. Fifteen four-week-old nude mice were maintained in a pathogen-free environment and handled according to the university's guidelines for animal care and use. Nude mice were divided into three groups. Nude mice were injected with 5×10^6 786-0 cells into the right flank. After 6 weeks, the tumor reached ~10 mm in diameter. Subcutaneous tumor tissue, liver and lung tissue were taken to perform H&E staining. We evaluated the invasiveness of tumor cells among three groups.

Results

EGCG inhibits proliferation and induces apoptosis of cell line 786-0. MTT and flow cytometry were used to examine

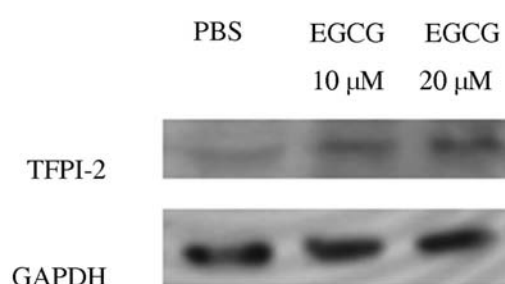


Figure 5. Western blotting revealed increased expression of TFPI-2 after EGCG treatment.

the proliferation status of the cell line 786-0. The mean OD value of 10 μ M EGCG group, 20 μ M EGCG group and control group were: 0.526, 0.441 and 0.577. The inhibition rate of 10 μ M and 20 μ M EGCG treatment groups were: 8.8 and 23.6%. S phase rate in EGCG treatment groups was lower than that in control group ($p < 0.05$) (Figs. 1-3). The mean S phase rate of 10 μ M EGCG group, 20 μ M EGCG group and control group were: 54.31, 60.50 and 71.32%, suggesting that EGCG inhibits growth of cell line 786-0.

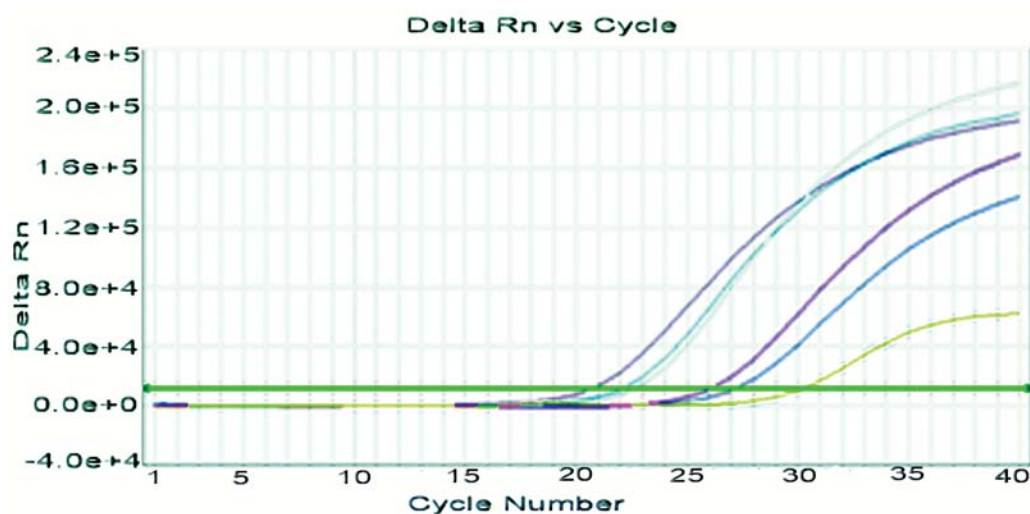


Figure 6. Overexpression of TFPI-2 mRNA after EGCG treatment was detected by using real-time RT-PCR.

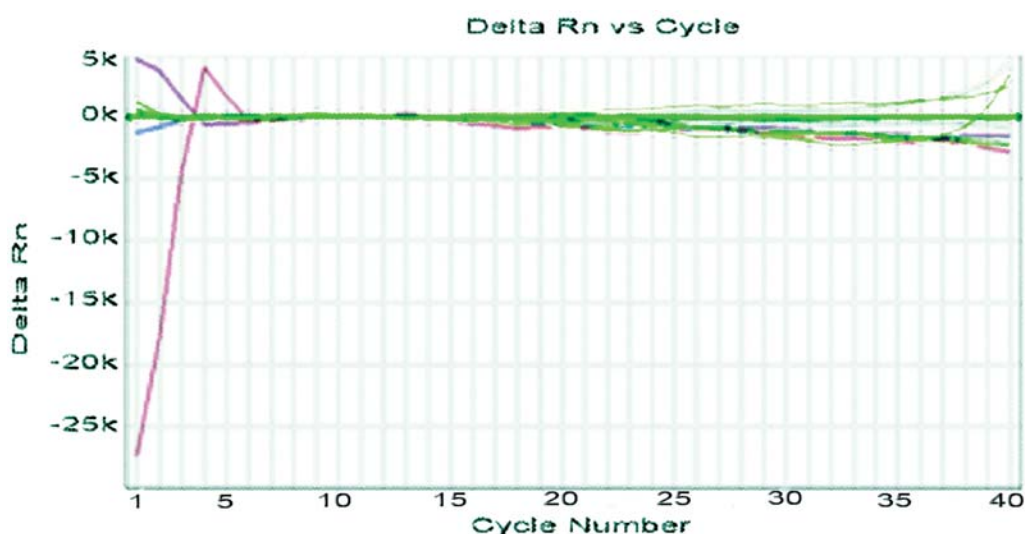


Figure 7. Before and after EGCG treatment, methylation of TFPI-2 gene promoter was undetected by using real-time methylation specific PCR.

Flow cytometry was also used to evaluate apoptosis status of cell line 786-0 (Figs. 1-3). The mean apoptosis rate of 10 μ M EGCG group, 20 μ M EGCG group and control group were: 1.09, 2.27 and 0.94%. Apoptosis rate in EGCG treatment groups was higher than that in the control group ($p < 0.05$).

Invasiveness of tumor cells in vivo. In subcutaneous tumor tissue of nude mice, H&E staining revealed no invasiveness of tumor cell. We did not find metastasis in nude mouse liver or lung tissue (Fig. 4).

EGCG upregulates expression of TFPI-2. After being treated with 10 μ M EGCG or 20 μ M EGCG, Western blotting revealed that TFPI-2 was upregulated by 72.9 and 103% ($p < 0.05$) (Fig. 5). Real-time RT-PCR demonstrated that the mean relative expression amount of TFPI-2 mRNA was: 2.33% (10 μ M EGCG), 4.16% (20 μ M EGCG) and 0.35% (control group) (Fig. 6). TFPI-2 mRNA expression in EGCG

treatment groups was significantly higher than that in the control group ($p < 0.05$).

Methylation status of TFPI-2 gene promoter. Real-time methylation specific PCR was performed to detect methylation status of TFPI-2 gene promoter. We could not detect methylation of TFPI-2 promoter in 10 μ M EGCG group, 20 μ M EGCG group or the control group (Fig. 7).

Discussion

The study of plant polyphenols as anticancer agents has increased substantially in recent years, due partly to their profound effects *in vitro* and *in vivo* on tumor cell growth, apoptosis and signaling pathways. In addition, epidemiological studies have revealed promising preventative and therapeutic roles for polyphenols. EGCG, the major catechin found in green tea, has been shown to inhibit proliferation



the apoptosis in many cell lines. In the present study, RT-PCR and flow cytometry, we are the first to show that EGCG can inhibit growth and induces apoptosis in renal cell carcinoma cell line 786-0.

Study has clearly demonstrated that EGCG can inhibit activity of DNMT which causes CpG demethylation and reactivation of methylation-silenced genes (2). Kinetic studies indicate that EGCG inhibits nuclear DNMT activity competitively with a K_i of $6.89 \mu\text{M}$. The dominant form of DNMT1 is the main cause, although DNMT 3 α and 3 β may also be present in smaller quantities in the nuclear extracts (3). Molecular modeling studies indicate that EGCG is well accommodated in a hydrophilic pocket of DNMT1. EGCG is effectively tethered within the DNMT1 binding site by at least four hydrogen bonds involving Ser1229, Glu1265, Pro1223 and Cys1225 (4). Many tumor suppressor and receptor genes have been reported to be hypermethylated and transcriptionally silenced during the development of different types of cancers. It is likely that inhibition of DNMT can also prevent the hypermethylation and silencing of these key genes and, therefore, this inhibition would contribute to the treatment and prevention of many tumors.

Besides epigenetic mechanism, the roles of EGCG on tumors include regulation of signal pathway and many proteins: COX-2 (5), MMP-7 (6), EGFR (7), estrogen receptor (8), FGF (9), FOXO3 (10), IGF (11), P57/KIP2 (12), HIF-1 α (13), VEGF (13).

Tissue factor pathway inhibitor-2 (TFPI-2), a member of the Kunitz-type serine proteinase inhibitor family, is a structural homologue of tissue factor pathway inhibitor (TFPI). The expression of TFPI-2 in tumors is inversely related to an increasing degree of malignancy, which may suggest that TFPI-2 can inhibit growth of neoplasms. TFPI-2 is an inhibitor of MMPs (matrix metalloproteinases) which can promote invasiveness of tumor through matrix degradation (14). The levels of TFPI-2 were very low or undetectable in many tumor cells but markedly upregulated in normal cells. TFPI-2 functions in the maintenance of the stability of the tumor environment and inhibits invasiveness and growth of neoplasms, as well as metastases formation. TFPI-2 has also been shown to induce apoptosis and inhibit angiogenesis, which may contribute significantly to tumor growth inhibition. When infected with Raav-TFPI-2, human glioblastoma cell line SNB19 showed decreased formation of microvessels (15). Upregulation of TFPI-2 expression in tumor tissue inhibits invasion, tumor growth and metastasis, which creates a novel possibility of cancer treatment.

Hypermethylation of TFPI-2 promoter CpG islands in human cancers and cancer cell lines was demonstrated to be responsible for down regulation of mRNA encoding TFPI-2 and decreased or abolished synthesis of TFPI-2 protein during cancer progression. The finding was associated with increased cancer growth and invasion. TFPI-2 methylation was detected in 29% metastatic melanoma specimens, while it was not detected in all primary melanoma specimens (16). Immunohistochemically, all melanoma specimens with TFPI-2 promoter methylation lacked immunoreactivity for TFPI-2 (16). In NSCLC (non-small cell lung cancer), decreased TFPI-2 expression and hypermethylation were more frequently associated with stage III and IV of the disease. Approximately

one half of NSCLC cases with silenced TFPI-2 gene were lymph node-positive (17). TFPI-2 methylation was observed in all cases of esophageal carcinoma cell lines (18). Aberrant methylation of TFPI-2 was detected in as many as 73% of pancreatic cancer xenografts and primary pancreatic adenocarcinomas, and was more likely in old patients with pancreatic cancer. Hypermethylation of TFPI-2 was significantly associated with progression of primary pancreatic ductal neoplasms (19). The aberrant methylation rate of TFPI-2 in pure pancreatic juice aspirated endoscopically from patients was as high as 100%, promoter methylation of TFPI-2 in the pure pancreatic juice may be an effective marker in diagnosis and progression of pancreatic cancer (20). TFPI-2 promoter methylation was detected in 80% of hepatocellular carcinoma cell lines and 47% of human hepatocellular carcinoma, overexpression of TFPI-2 significantly suppressed the proliferation and invasiveness of hepatocellular carcinoma cells, TFPI-2 is a candidate tumor suppressor gene in human hepatocellular carcinoma (21).

Besides epigenetic basis, TFPI-2 is also associated with genetic changes in different cancers (e.g., head and neck carcinomas, gastric, colon, prostate, vesicular, ovarian and testicular cancer, malignant melanoma, and glial neoplasms) (22).

Our previous study showed lower expression of TFPI-2 in renal cell carcinoma compared to normal kidney. The expression of TFPI-2 and stage of renal cell carcinoma showed a negative correlation. The relationship between expression of TFPI-2 and apoptosis index of renal cell carcinoma had a positive correlation. Expression of TFPI-2 mRNA and protein were significantly lower in methylated tumors than those in demethylated tumors.

Our present study demonstrated that EGCG can upregulate expression of TFPI-2 in the renal cell carcinoma cell line 786-0. Overexpression of TFPI-2 may inhibit growth and induce apoptosis in renal cell carcinoma. EGCG is likely to be an effective agent for renal cell carcinoma. The upregulation of TFPI-2 by EGCG may not be related to the gene promoter methylation status. The exact pathogenesis of upregulation needs to be further investigated.

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