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Potentiation of treatment efficacy against colon cancer of dopamine via elevating KLF2 expression in tumor vascular endothelial cells

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Abstract. – OBJECTIVE: To investigate the anti-tumor effect and potential mechanism of dopamine combined with 5-FU in colon cancer.

MATERIALS AND METHODS: Babl/c F1 generation male mice (N = 60) were inoculated with mouse C26 colon cancer cells below the pit to construct colon cancer model. Tumor-bearing mice were then divided into 4 groups (N = 8 each): model control group, dopamine group, 5-FU group and dopamine combined with 5-FU group. Dopamine (100 mg/kg/d) was intraperitoneally injected into mice. The tumor-suppressing rate was calculated. Serum VEGF concentration was gained. Tumor tissues were subjected to HE staining. KLF2 expression was determined by immunohistochemical (IHC) staining. *In vitro* cultured C26 cells which treated with dopamine and cell apoptosis were analyzed by flow cytometry.

RESULTS: Compared with model control group, all treatment groups showed significantly decreased tumor weight and volume ($p < 0.01$), increased tumor necrosis ($p < 0.05$), reduced serum VEGF concentration ($p < 0.05$), and enhanced KLF2 expression in microvessels ($p < 0.05$). Combined treatment in terms of dopamine combined with 5-FU had the most pronounced effect compared with both dopamine and 5-FU treatment groups individually. Dopamine single t 5-FU and 5-FU groups showed a similar proportion of viable C26 cells ($p > 0.05$).

CONCLUSIONS: Dopamine exerts anti-tumor effects by modulating tumor vascular homeostasis through the KLF2 signaling pathway, and potentiates the treatment efficacy of anti-tumor drug 5-FU. Our study discovered clinical significance concerning the novelty of therapeutic strategy against colon cancer.

Key Words:
Colon cancer, Dopamine, KLF2, Tumor blood supply.

Introduction

Colon cancer ranks the fourth most common cancer worldwide. The incidence of colon cancer kept increasing in the past several decades[1]. In China, although colon cancer has a relatively low incidence in males aged 40-70 years old, recent epidemic studies have reported an increasing tendency in terms of the incidence rates of the malignancy, especially in patients who are under 30 years old[2]. At the stage, surgery is the main treatment for colon cancer. However, for different reasons, there are several patients who are not suitable for surgery and thus require chemotherapy treatment. Also, chemotherapy is still required after surgery in some cases at times. 5-FU is the standard drug for chemotherapy of colon cancer. However, there are some negative aspects such as slow drug pharmacokinetics into tumor lesion and an uneven distribution[3], which largely affect the chemotherapy efficacy against colon cancer. Therefore, it is significant to develop a novel strategy for effective delivery of 5-FU into tumor cells in order to generate the treatment efficacy of the anti-tumor drug[4].

Drug delivery into tumor lesion by blood supply system is a critical step of anti-tumor therapy, as appropriate blood supply can reduce tumor cell viability potentially by elevating blood oxygen level, and thus inhibit further growth of tumors[5]. Therefore, the improvement of tumor blood supply has become a new strategy in terms of the treatment of colon cancer. Dopamine, a classical blood vessel transmitter/drug, has been known to inhibit tumor survival[6]. Studies have suggested

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that dopamine stabilizes tumor blood vessels by up-regulating the expression of KLF2, a zinc finger transcription factor in tumor endothelial cells[7]. However, the effect and mechanism of dopamine in combination with 5-FU in colon cancer has not been investigated in the past. In this study, we constructed a colon cancer mouse model and evaluated the therapeutic effect of dopamine in combination with 5-FU. We also explored the molecular mechanism by which dopamine exerted the anti-tumor effect.

Materials and Methods

Materials

A total of 60 male Babl/c F1 generation mice weighted 18±2 g were purchased from Sitaike Animal Corp (Shanghai, China) and raised in an SPF grade Animal Center at Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine. Mouse C26 colon cancer cell line was purchased from Chinese Academy of Science (Shanghai, China). Dopamine (>98% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-FU (lot No. 20070802) was purchased from Haipu Biotech (Nanjing, China). Mouse VEGF ELISA kit was purchased from Biosources (Camarillo, CA, USA). Goat anti-mouse CD34 monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). The animal study was approved by the Animal Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine.

Cell Culture

C26 cells were cultured in 1640 medium containing 10% fetal bovine serum and 1% streptomycin/penicillin at 37°C, 5% CO2 incubator. C26 cells (passage 4) were divided into 4 groups which are: control group, dopamine (1 mg/mL) group, 5-FU (0.5 mg/mL), and dopamine combined with 5-FU group. After 7-days incubation, cell apoptosis was analyzed by flow cytometry.

Construction of Mouse Colon Cancer Model and Dopamine Interference

C26 cells at exponential phase were inoculated below the mouse pit (5 × 106 cells per animal). After 7-10 days, C26 cells formed visible tumors. C26 tumor tissues were homogenized into 1-mm3 pieces. Under sterile conditions, C26 cell suspension was inoculated below the mouse pit using a 1-ml syringe. Three passages were performed as described above. Stable mouse colon cancer model was successfully constructed when the tumor body reached 100 mm3. Colon cancer mice were randomly divided into 4 groups (N = 8 each) which are: model control group, dopamine group, 5-FU group and dopamine combined with 5-FU group. For dopamine and dopamine combined with 5-FU groups, dopamine (100 mg/kg/d) was injected daily into the peritoneal cavity for 1 week. For 5-FU and dopamine combined with 5-FU groups, 5-FU (1 mg/kg/d) was injected daily into the tail vein of mice for 1 week. The model group received a daily intraperitoneal injection of an equal volume of saline. At day 8, mice were sacrificed by cervical dislocation. Blood samples were collected, and colon cancer lesion was resected. Tumor length, width, and weight were measured. Tumor specimen was fixed in 30% formaldehyde and cut into 4-μm sections.

Calculation of Tumor-Suppressing Rate

The tumor volume was calculated as previously described in last section[7]. Tumor volume (TV) = ab2/2, where a and b stand for tumor length and width, respectively. Using the model control group as reference, the tumor volume suppressing rate and tumor weight suppressing rate in all three treatment groups was calculated as follows: tumor volume suppressing rate = (1-average tumor volume in drug treatment group / average tumor volume in model control group) × 100%, and tumor weight suppressing rate = (1- average tumor weight in drug treatment group / average tumor weight in model control group) × 100%.

ELISA

Blood samples were collected from orbital vein plexus. Serum was collected after the completion of centrifugation. VEGF expression was measured using VEGF ELISA kit following the manual instruction. A standard curve was plotted by a software. The serum VEGF concentration in all samples was calculated.

Hematoxylin and Eosin (HE) Staining

Tissue cubes were fixed in 40 g/L 30% formaldehyde solution, embedded in paraffin, cut into 4-μm slices and observed under an inverted microscope (100 ×). Regions with tumor necrosis were collected. Necrosis area in three randomly selected visual fields (200 ×) was analyzed in a double-blinded manner. Image pro software was used to process images. A score was given based on the necrosis area: 0 for 5%, 1 for 5%-25%, 2 for 25%-50%, and 3 for >50%.

Table I. Tumor weight, volume and suppression rate in all groups of rats (N=8, mean±SD).

Group	Weight (g)	Weight suppression rate (%)	Volume (cm^3)	Volume suppression rate (%)
Model control	0.73±0.22	-	1.00±0.17	-
Dopamine	0.40±0.22	45.8	0.50±0.30	51.2
5-FU	0.29±0.09	60.3	0.40±0.12	62.8
Dopamine + 5-FU	0.11±0.06	84.6	0.16±0.09	81.6

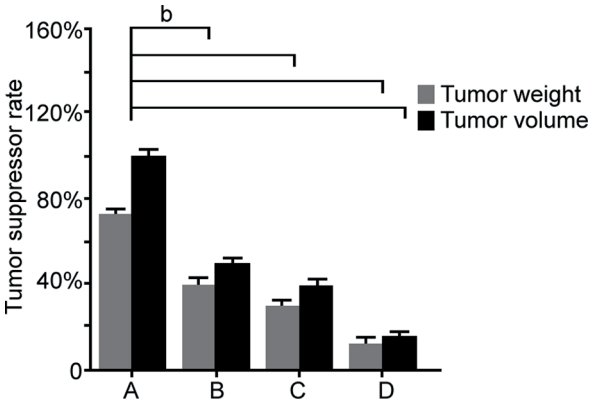

Figure 1. Tumor weight and volume suppression rates of all drug treatment groups. ***A,*** Model control group; ***B,*** Dopamine group; ***C,*** 5-FU group; ***D,*** Dopamine + 5-FU group. b, $p < 0.01$ compared to model control group.

Immunohistochemical (IHC) Analysis of KLF2 Expression in Tumor Microvessels

Paraffin-embedded sections were dewaxed with xylene and dehydrated with ethanol. Slices were processed to remove endogenous peroxidase, followed by antigen retrieval, serum blocking, antibody binding, DAB staining, rising, hematoxylin staining, HCl-ethanol immersing, gradient ethanol dehydration, xylene immersing and coverslip mounting. Goat anti-mouse KLF2 monoclonal antibody (1:50) biotin-labeled secondary IgG (1:200), and horseradish peroxidase-labeled streptavidin (1:200) were also utilized. KLF2-positive microvessels in dark brown color were counted under an inverted microscope.

Flow Cytometry

C26 cells were collected after 7-days cell culture and cell apoptosis was analyzed by flow cytometry. According to manufacture's instructions, C26 cells were prepared into 105/mL cell suspension. Cell suspension, reaction buffer, and FITC-Annexin V reagent were mixed at a ratio of 250:50:1, and incubated at room temperature for 15 minutes in the dark. C26 cells were assayed for phosphatidylserine expression under the wavelength of 625 nm. Caspase-3 activity was measured using caspase-3 kit following the manual. Briefly, C26 cells were lysed and incubated with chromogenic substrate at 37°C for 30 minutes. The absorbance at 490 nm was measured by flow cytometry.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were analyzed by Student's *t*-test. Statistical significance was defined as $p < 0.05$.

Results

Dopamine Inhibited Colon Cancer and Potentiated the Treatment Efficacy of 5-FU

The tumor volume of subcutaneous transplant tumors was determined at 7 days after model preparation. Although there was no significant difference in pretreatment tumor volume amongst all groups, tumor lesion size in all treatment group was significantly lower compared with model control group ($p < 0.01$, Table I, Figure 1 and 2). The tumor weight suppressing rates in dopamine, 5-FU and dopamine combined with 5-FU groups were 45.8%, 60.3%, and 85.6%, respectively, and tumor volume suppressing rates were 51.2%, 62.8%, and 81.6%, respectively, suggesting that dopamine exerted anti-tumor effects against co-

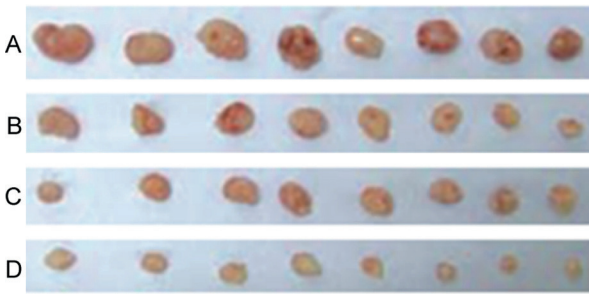

Figure 2. Rat tumor volume. ***A,*** Model control group; ***B,*** Dopamine group; ***C,*** 5-FU group; ***D,*** Dopamine + 5-FU group.

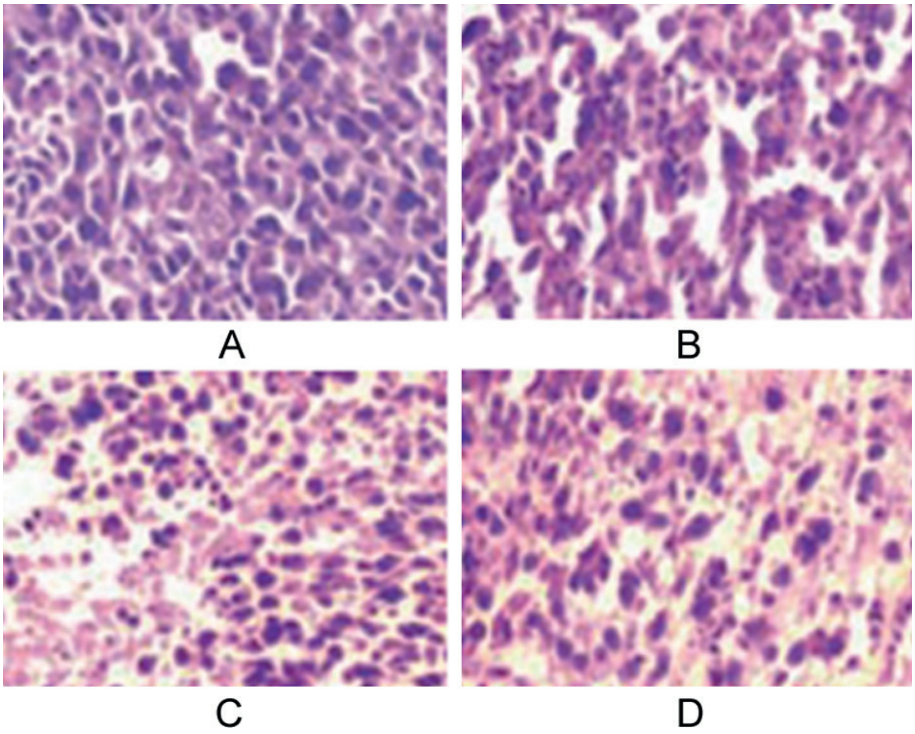

Figure 3. Tumor body necrosis of all drug treatment groups of rats (X400). ***A,*** Model control group; ***B,*** Dopamine group; ***C,*** 5-FU group; ***D,*** Dopamine + 5-FU group.

lon cancer. More importantly, dopamine potentiated the treatment efficacy of the classical drug 5-FU.

Dopamine Increased Tumor Tissue Necrosis of Colon Cancer

Upon the completion of 7-days treatment, the tumor tissue necrosis was evaluated by HE-staining. No necrosis was observed in model control group. In contrast, all treatment groups exhibited moderate to severe necrosis with cell nuclear atrophy, deformation, rupture, or sparse arrangement (Figure 3). The necrosis score in dopamine combined with 5-FU group was significantly higher compared with both dopamine and 5-FU groups ($p < 0.05$, Table II), indicating that dopamine potentially facilitated colon cancer necrosis.

Dopamine Regulated VEGF to Stabilize the Tumor Vessel Status

The serum VEGF concentration in all treatment groups was significantly lower compared with model control group ($p < 0.01$). The VEGF suppressing rates in dopamine, 5-FU, and dopamine combined with 5-FU groups were 15.8%, 34.2%, and 44.8%, respectively (Table III). These results indicated that exogenous dopamine injection had regulated the expression of VEGF to stabilize the tumor vessel status in colon cancer, and thus potentiated the efficacy of 5-FU.

Table II. Tumor necrosis of all drug treatment groups.

	Necrosis score of all groups (N)				
Group	**0**	**1**	**2**	**3**	**Total**
Model control	13	11	0	0	11
Dopamine	7	13	3	1	25
5-FU	2	7	12	3	44
Dopamine + 5-FU	1	2	10	11	55

Table III. Serum VEGF concentration (N=8, mean±SD). N/A, no suppression.

Group	Serum VEGF concentration (ng/L)	Suppression rate
Model control	415.4±14.2	N/A
Dopamine	313.0±12.6	15.8%
5-FU	302.0±12.7	34.2%
Dopamine + 5-FU	272.0±14.5	44.8%

Dopamine Elevated KLF2 Expression in Tumor Vessels

IHC analysis showed a significant elevation in terms of KLF2 expression of the tumor microvessels in all treatment groups ($p < 0.05$). Moreover, the KLF2 expression in dopamine combined with 5-FU group was significantly higher compared with both dopamine and 5-FU groups ($p < 0.05$, Figure 4), indicating that dopamine might stabilize the status of tumor microvessels via KLF2 related signals.

Dopamine Slightly Promoted the Apoptosis of In Vitro Cultured Colon Cancer Cells

Flow cytometry showed that the proportion of viable tumor cells in model control and dopamine groups were 87.23% ± 4.46%, and 78.23% ± 7.45%, respectively ($p < 0.05$, Figure 5), suggesting that dopamine only slightly inhibited the activity of in vitro cultured colon cancer cells. Furthermore, the proportion of viable tumor cells in dopamine combined with 5-FU group was slightly higher than that in 5-FU group, suggesting that dopamine had not effectively increased the treatment efficacy of 5-FU under *in vitro* conditions ($p < 0.05$).

Discussion

Our study showed that dopamine not only exerted anti-tumor effects against colon cancer, but also potentiated the treatment efficacy of the classical anti-tumor drug 5-FU. More importantly, we found that dopamine stabilized the tumor

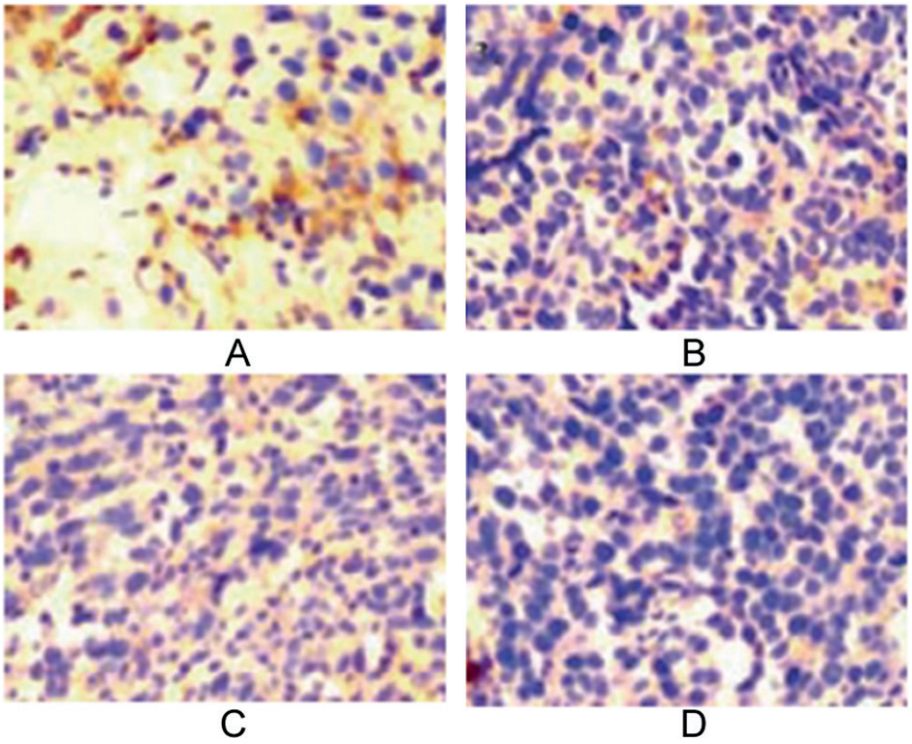

Figure 4. Growth condition of mouse colon cancer microvessel (SP × 200). ***A,*** Model control group; ***B,*** Dopamine group; ***C,*** 5-FU group; ***D,*** Dopamine + 5-FU group.

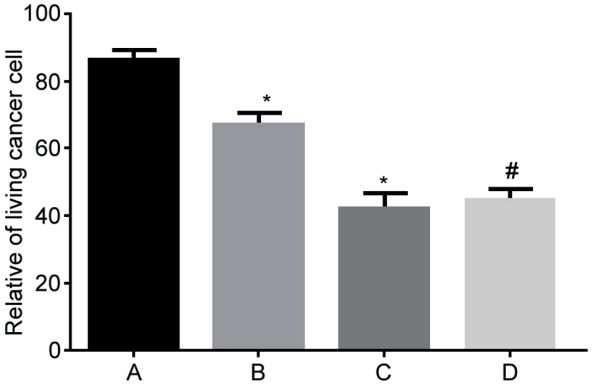

Figure 5. Tumor cell survival ratio of in vitro cultured mouse colon cancer cells after drug treatment. ***A,*** Model control group; ***B,*** Dopamine group; ***C,*** 5-FU group; ***D,*** Dopamine + 5-FU group. *, $p < 0.05$ compared to group A; $^{\#}$, $p < 0.05$ compared to group C.

vessel status via up-regulating KLF2 expression in tumor microvessels, which means there's potential treatment by targeting the facilitator of drug delivery into tumors through blood supply.

As an important catecholamine neurotransmitter, dopamine plays pluripotent biological functions including recognition, emotion, appetite and motor reflex. More importantly, dopamine plays a crucial role in the maintenance of tissue blood supply[8-10]. Studies have suggested that moderately increased blood supply in tumor tissue can manage tumor cell viability and improve drug concentration in tumor region due to the reason that tumor cells are more responsive under hypoxic condition[11]. Although the anti-tumor effects of dopamine have been previously reported, the mechanism underlying the anti-tumor effects of dopamine remains unknown. Our paper suggested that the anti-tumor effects of dopamine might be associated with its ability to stabilize the tumor blood supply.

Dopamine has been confirmed to have anti-tumor effects[12,13]. In this work, we found that dopamine significantly inhibited colon cancer cell viability, and facilitated tumor necrosis. We also found that dopamine affected tumor blood supply via modulating VEGF expression, which is in consistency with previous studies regarding the effect of dopamine on tumor blood supply[14,15]. More importantly, our study revealed that dopamine potentiated the treatment efficacy of the classical drug 5-FU, suggesting that the combined therapy of 5-FU and dopamine is promising in the treatment of colon cancer.

Further, we investigated the regulatory mechanism of dopamine in blood supply of colon cancer. KLF2, a crucial factor that helps to stabilize microvessels, has been proven as an important downstream target of the dopamine-related signal pathway[15,16]. Previous researches[17,18] have demonstrated the regulatory effects of dopamine on KLF2 in colon cancer. Down-regulation of KLF2 frequently accelerates tumor cell proliferation, which results in tumor tissue aggravation[19,20]. The mechanism involves the transition of blood vessel homeostasis which leads to tumor growth, including internal hypoxia of tumors, activation of oncogene and impeding of drugs into tumors[21]. In this study, we found that KL2 expression was significantly increased in colon cancer, and has been effectively suppressed in tumor microvessels in all treatment groups afterward. Moreover, dopamine treatment effectively decreased serum VEGF level in colon cancer mice. Meanwhile, that the experience also indicated that dopamine slightly promoted the cell apoptosis of in vitro cultured colon cancer cells, and had not improved the treatment efficacy of 5-FU in these cells, indicating that dopamine required blood supply system to exert its anti-tumor effects as well as its ability to potentiate the 5-FU. All together, we believed that dopamine inhibited tumor growth and potentiated the treatment efficacy of 5-FU via stabilizing tumor microvessels and enhancing blood drug concentration inside tumors.

Conclusions

In summary, dopamine not only exerts anti-tumor effects, but also potentiates the treatment efficacy of 5-FU via stabilizing tumor microvessels through KLF2 signaling. The combined therapy of dopamine and 5-FU might provide a novel therapeutic strategy against colon cancer. In the meantime, we expect further clinical investigations to validate our findings.

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Conflict of interest

The authors declare no conflicts of interest.

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