Apoptotic block in colon cancer cells may be rectified by lentivirus mediated overexpression of caspase-9

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Abstract

Background and aim : **At present, the inhibition of apoptosis during pathogenesis of colorectal cancer is widely recognized while the role of caspase-9 in this process remains controversial. We aimed to investigate the differential expression of caspase-9 and evaluate the therapeutic potential of expression intervention in this study.**

Methods : **We first examined the different expression of caspase-9 in normal colon mucosa, adenoma and cancer, investigating the relationship between its expression and clinico-pathological characteristics. Secondly, overexpression of caspase-9 was established in colon cancer cell lines by lentivirus infection to study the changes in growth, proliferation and apoptosis.**

Results : **Compared with normal colon mucosa, the expression of caspase-9 was higher in adenoma while lower in cancer both at mRNA and protein level ($P < 0.05$). In addition, the down-regulation of caspase-9 expression is more common in poorly differentiated cancers ($P < 0.05$). Concerning cell lines, overexpression cell groups showed higher expression of caspase-9, poorer colony formation and slower cell proliferation. In terms of apoptosis related indicators, caspase-9 overexpression leads to higher apoptosis rate and G0/G1 arrest, while up-regulating the expression of caspase-3 ($P < 0.05$). Interestingly, down-regulation of carcinoembryonic antigen secretion was also observed in caspase-9 overexpression cells ($P < 0.05$).**

Conclusion : **The change of caspase-9 expression from colon mucosa, adenoma to cancer suggested it may be involved in the carcinogenesis of colon cancer. The overexpression of caspase-9 exhibits an inhibitory role in cancer growth and proliferation while promoting apoptosis. However, a non-apoptotic role of caspase-9 facilitating differentiation was also implied.** (Acta gastroenterol. belg., **2013**, 76, **372-380**).

Key words : Caspase-9, overexpression, colon cancer, apoptosis, differentiation.

Introduction

Apoptosis, or programmed cell death is a mechanism exists in multi-cellular organisms. Recent discoveries showed that the defects in its regulation are associated with the pathogenesis of cancer and neuro-degeneration (1). In terms of colon cancer, the inhibition of apoptosis was reported to facilitate tumor growth, promote neoplastic progression, and confer resistance to cytotoxic anticancer agents (2).

Caspases, a family of cysteine proteases, are mainly responsible for the execution of apoptosis. Of these enzymes, caspase-9 is the initiator of the intrinsic or mitochondrial pathway (3). Upon pro-apoptotic signals of this pathway, cytochrome c is first released from mitochondria, followed by the oligomerization of Apaf-1 (apoptotic protease activating factor 1), the activation of caspase-9 and the formation of apoptosome (4). This will finally activate downstream effector, such as caspase-3 and -7, which carry out proteolysis to execute cell death (1). According to general understanding, the activation of caspase-9 is an upstream signaling event required for anticancer agent-induced apoptosis in various human tumors (5,6). However, some researchers believed that apoptosome is not an essential participant in tumor suppression, thus tended to question the role of caspase-9. They claimed that tumor cell could survive by switching their death program from apoptosis to a caspase-independent mode (7,8).

In the present study, firstly, we examined caspase-9 expression of colon mucosa, adenoma and cancer, discussing the relationship between its expression and clinicopathological characteristics. Secondly, owning to the observed low expression of caspase-9 in cancer tissue, we specifically established caspase-9 overexpression colon cancer cell lines using a lentivirus-mediated transduction system, investigating the alterations in colony formation, proliferation and apoptosis, cell cycle and carcinoembryonic antigen (CEA) secretion. Thus, the goal was to study the variation of caspase-9 expression, investigate the effect of overexpression intervention while evaluating the potential for future studies.

Material and Methods

Included patients and specimen preparation

Ninety-three patients with biopsy-proved colon cancer, who underwent radical resection at the Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, from September, 2005 to June, 2006 were included. Thirty-six patients with colon adenoma, who received colonoscopy and biopsy during the same period, were also included. The normal mucosa was sampled from uninvolved bowel 10 cm proximal to the primary tumor. All sampled cancer, adenoma and normal colon mucosa were placed in liquid nitrogen and stored at -80°C for future steps. After sampling, the surgical

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Table 1. — **Relationship between the expression of caspase-9 and clinicopathologic characteristics**

		Total	mRNA Down-regulation	Up-regulation	*P*	Protein Down-regulation	Up-regulation	*P*
Sex	Male	51	39	12	0.294	42	9	0.463
	Female	42	28	14		32	10	
Age	≤ 50	31	21	10	0.513	26	5	0.467
	> 50	62	46	16		48	14	
Infiltration	T1	6	4	2	0.887	4	2	0.522
	T2	31	23	8		24	7	
	T3	42	31	11		36	6	
	T4	14	9	5		10	4	
Node	N0	51	35	16	0.824	41	10	0.652
	N1	33	24	9		26	7	
	N2	9	7	2		6	3	
Differention	Well	23	13	10	0.032	14	9	0.023
	Moderate	53	38	15		44	9	
	Poor	17	16	1		16	1	

specimens were sent to routine postoperative pathology for detailed information in infiltration and node status according to TNM stage manual (9). The characteristics of included cancer cases are shown in Table 1.

Reverse transcription polymerase chain reaction (PCR)

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction, and, reverse transcription of RNA was conducted by First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as reference. The specific primers and probes for caspase-9 and GAPDH are listed in Table 2, all designed by Takara Biotech. BLAST searches were performed to confirm the gene specificity of the nucleotide sequences. Quantitative real-time PCR was operated on iCycler iQ system (BIO-RAD, Hercules, CA, USA), adopting the comparative threshold cycle (Ct) method (ΔΔCt) as described before (10). The amplification course was : 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 53°C, and 20 s at 72°C. In addition, a non-template control (ddH2O) was analyzed for each mastermix. Samples were amplified simultaneously in triplicate.

Immunohistochemistry

First, 4 um sections were cut from formalin-fixed, paraffin-embedded tissue blocks and transferred to glass slides disposed by APES (3-Aminopropyl-Triethoxysilane). Following deparaffinization in xylene and rehydration through graded ethanol, the sections were treated in citrate buffer for antigen retrieval using a pressure cooker. Then, these sections were stained immunohistochemically with caspase-9 (Cell Signaling, Beverly, MA, USA) monoclonal antibody, by an immunohistochemical (IHC) method using avidin-biotin-peroxidase complex (ZYMED, San Francisco, CA, USA) and visualized by diaminobenzidine. Binary antibody was applied to the sections at 37°C for 45 min. Negative control sections were stained with isotype IgG antibody PE-Cy7 (eBioscience, San Diego, CA, USA).

The IHC images were captured by Olympus DD70 BX51 system and analyzed by IMAGE-PRO plus v5.02 software. For each section, 8 random high-power fields were selected and the brown-stained particles within were chosen as the interest. The measured optical density represented intensity of staining for interest. Thus, the average integrated optical density (IOD) of 8 fields implicated the expression of caspase-9 protein.

Table 2. — **Primers and probes for reverse transcription PCR examination of caspase-9 and GAPDH**

Caspase-9	
Primer forward	5'-GGACATCCAGCGGGCAGG-3'
Primer reverse	5'-TCTAAGCAGGAGATGAACAAAGG-3'
Probe	5'-FAM-ATCAGGCCAGGCAGCTGATCATAGA-3'-TAMRA
GAPDH	
Primer forward	5'-CCTCAAGATCATCAGCAAT-3'
Primer reverse	5'-CCATCCACAGTCTTCTGGGT-3'
Probe	5'-FAM-ACCACAGTCCATGCCATCAC-TAMRA-3'

Table 3. — **Selected sequences for construction of target plasmid**

Primer	Sequence
CASP9-EcoRI-forward	TAGAGCTAGCGAATTCGCCACCATGGACGAAGCGGAT
CASP9-EcoRI-reverse	CCTTGTAGTCGAATTCTGATGTTTTAAAGAAAAGTTTTT
3Flag-forward	CATCAGAATTCGACTACAAGGATGAC
3Flag-reverse	CAGCGGCCGCGGATCCTTTGTCGTCATCATCCTTATAG

Cell culture

Human colon cancer cell lines SW480 and LoVo, with different degrees of malignancy, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained in RPMI-1640 and DMEM (Gibco, Grand Island, NY, USA) culture respectively and supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Human embryonic kidney (HEK) cell line 293T was also purchased from ATCC and maintained in DMEM.

Constructs of lentivirus expression vectors

The lentiviral vectors expressing short hairpin RNA (shRNA) were constructed as previously described. The caspase-9 (CASP9, NM_001229) and 3Flag sequences were amplified and connected by polymerase chain reaction (PCR). Selected primers were listed in Table 3. The CASP9-3Flag fragment and expression vector pCDH-EF1-MCS-T2A-Puro (SBI, Mountain View, CA, USA) were homologous recombined and transformed into E. coli competent cells following double digestion of the vector with EcoRI and BamHI. Bacterial colonies containing recombinant plasmids were identified by PCR and their cloning sequenced before plasmid extraction.

Lentiviral vector with or without target sequence were produced by four plasmids cotransfection of the human embryonic kidney (HEK) 293T cells. Supernatants containing lentiviruses were harvested 72 h later, filtered through a 0.45 mm cellulose acetate filter, then ultracentrifugated. The control lentiviral vectors were constructed similarly. The titer of lentiviruses was determined by quantitative PCR.

Establishment of stable expression cell lines

According to preliminary experiment of infection efficiency, SW480 and LoVo cells were infected by GFP-puro-L.V., CASP9-L.V. or empty lentiviral vectors using a multiplicity of infection (MOI) of 20. In brief, 8.8×10^4 cells per well were seeded in triplicate in 24-well plates. Next, viral suspension (according to examined titers by PCR) and 5 µg/ml polybrene (Sigma, St. Louis, MO, USA) were supplemented. After 24 h of culture, the media were replaced by fresh RPMI-1640 and DMEM. Three days after infection, the cells were digested by 0.25% (w/v) trypsin-EDTA, passaged onto 6 cm diameter petri dish and maintained under standard conditions. Following that, stable transductants were selected by 2 ug/ml puromycin dihydrochloride (Calbiochem, San Diego, CA, USA), and, photos were taken at the 5th and 12th day.

Western blot assay

Total protein was extracted using 1× lysis buffer (Santa Cruz Biotech, Santa Cruz, CA, USA). The concentration of protein was assayed using BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and adjusted to 2 µg/µl. This was incubated in 2× loading buffer and heated at 100°C for 10 min. Next, samples were loaded in lanes, underwent electrophoresis on a 10% SDS-PAGE gel, and transferred to a 0.2um polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% w/v nonfat dry milk and incubated overnight with a caspase-9 monoclonal antibody (Cell Signaling, Beverly, MA, USA). The blots were detected by the secondary antibody, horseradish peroxidase-linked immunoglobulin (Dako, Glostrup, Denmark), and visualized using the Amersham ECL Plus Western Blot detection System (GE Healthcare, Little Chalfont, Buckingham, UK).

Colony formation experiment

After digestion, wind and percuss log-phase cells into single cell suspension. The cells (300 per well) were seeded in triplicate into 6 cm diameter petri dishes and incubated at 37°C in a humidified incubator. Ten days after incubation, colonies were fixed with methanol and stained with 5% Giemsa. The colonies with a diameter of > 0.3 mm were counted under light microscope (Leica DMLS, Wetzlar, Germany) at 10× magnification, and, colony formation efficiency was determined by the count of colony per dish.

MTT colorimetric assay

Viable cells were quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Briefly, cells of different treatment were seeded in 96-well plates (1×10^4/ml). Each well was supplemented with 100 µg MTT (2 mg/ml solution) in complete media and incubated at 37°C for 4 h. The medium and MTT solution were then removed, and 150 µl of dimethyl sulfoxide was added to each well. Absorbance was read at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Reported data were the

average of 6 parallel wells, and, the experiment was repeated for 3 times with similar results.

TdT-mediated X-dUTP nick end labeling (TUNEL) assay

The TUNEL technique was performed to detect apoptotic cell using the In situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). For the 5-FU (5-fluorouracil) treated groups, cells were first cultivated with 100 ug/ml 5-FU for 24hr before TUNEL test. Briefly, cells were fixed in 4% paraformaldehyde, washed twice in PBS, then loaded on glass slides and permeabilized in a solution containing 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C. These were incubated with TUNEL reaction mixture for 1 h at 37°C. Following washing, the slides were incubated with peroxidase labeled anti-digoxin antibody for 30 min at 37°C. After that, slides were visualized by diaminobenzidine and lightly counterstained with hematoxylin. We recorded apoptotic index as the count of apoptotic cells divided by that of total cells observed from 50 fields at 40× magnitudes (Olympus Optical, Hamburg, Germany).

Cell cycle analysis by flow cytometry

Flow cytometry analysis was conducted on a FACS Calibur flow cytometer (BD Bioscience Pharmingen) and analyzed using Cell Quest software (BD Biosciences, San Jose, CA, USA). First, 1×10^6 cells were seeded in 10 cm diameter petri dishes and cultured for 18 h. These were washed twice with ice-cold PBS, treated with trypsin and fixed in 70% cold ethanol at 4°C for 30 min. Then, the cells were suspended in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase and 0.1% Triton X-100 at room temperature for 30 min before analyzed with a flow cytometer.

Quantitative enzyme-linked immunosorbent assay

First, 2×10^6 cells were seeded into a 25 cm^2 flask with complete media and incubated for 2 days. For CEA measurement, the medium was changed and incubation continued for another 3 days. Then, the supernatant was harvested after centrifugation and stored at -20°C. For caspase-3 measurement, lysis buffer was added to the harvested cells after cultivation with Z-VAD-FMK. The expression of caspase-3 and CEA was measured by quantitative enzyme-linked immunosorbent assay kit (Sigma, St. Louis, MO and Lifekey, Monmouth Jct, NJ, USA, respectively) according to the manufacturer's instructions. In brief, samples were incubated with a biotinylated and horseradish peroxidase-labeled monoclonal antibody in streptavidin-coated microstrips. After washing, buffered substrate was added to propel enzyme reaction. The color intensity of each well was determined by a microplate reader (Bio-Rad, Hercules, CA, USA) at 405 nm. Calibration curves were constructed for each assay and the concentrations of samples were read from the calibration curve and normalized to nanograms per 10^6 cells.

Statistical analysis

The relative expression of mRNA was analyzed with REST (relative expression software tool) software on the basis of the comparative threshold cycle method (ΔΔCt). Other data in this study was analyzed with SPSS 13.0 package (SPSS Inc., Chicago, IL, USA). In brief, the differences in continuous variables between groups were evaluated by one-way ANOVA test, while the relationship between caspase-9 expression and clinicopathological parameters were assessed by chi-square test.

A P value of < 0.05 was considered as significant.

Results

Different expression of caspase-9

In this study, the amplification efficiency for caspase-9 and GAPDH was 1.82 and 1.88, respectively. The highest expression of caspase-9 mRNA was seen in adenoma, while lowest in cancer. Compared with mucosa, the differences in expression were both significant (adenoma vs. mucosa, P = 0.001 ; cancer vs. mucosa, P = 0.003. Fig. 1a). Data of randomization test was not shown.

Fig. 2 showed the representative images of immunohistochemical staining. Usually, the cytoplasm and membrane of glandular cells were positively stained, but not the stromal components. Similar to the expression of caspase-9 mRNA, the highest IOD was observed in adenoma while lowest in cancer. The differences were both significant when compared with mucosa (adenoma vs. mucosa, P = 0.016 ; cancer vs. mucosa, P = 0.021. Fig. 1b).

Caspase-9 expression and clinicopathological characteristics

As shown in Table 1, a relationship between the change of caspase-9 expression and the differentiation of cancer was observed. Specifically, down-regulation of caspase-9 expression were more common in poorly differentiated cancers (P = 0.032, mRNA ; P = 0.023, protein). However, for sex, age, tumor infiltration and node status, the differences in caspase-9 expression were all not significant.

Caspase-9 overexpression cell lines

Twelve days after infection, transduction rates of more than 90 percent were observed in Lentivirus infected SW480 and LoVo cells (Fig. 3a, b). Thus, six transduced cell lines were successfully constructed. These include 4 control cell lines, SW480-L.V., SW480-GFP-puro-L.V., LoVo-L.V., LoVo-GFP-puro-L.V. and two caspase-9 overexpressing cell lines, SW480-CASP9-L.V. and LoVo-CASP9-L.V.

Compared with untreated controls, caspase-9 overexpression cell lines showed significant increment in the expression of caspase-9 mRNA (approximately 61 fold for SW480-CASP9-L.V. and 25 fold for LoVo-CASP9-

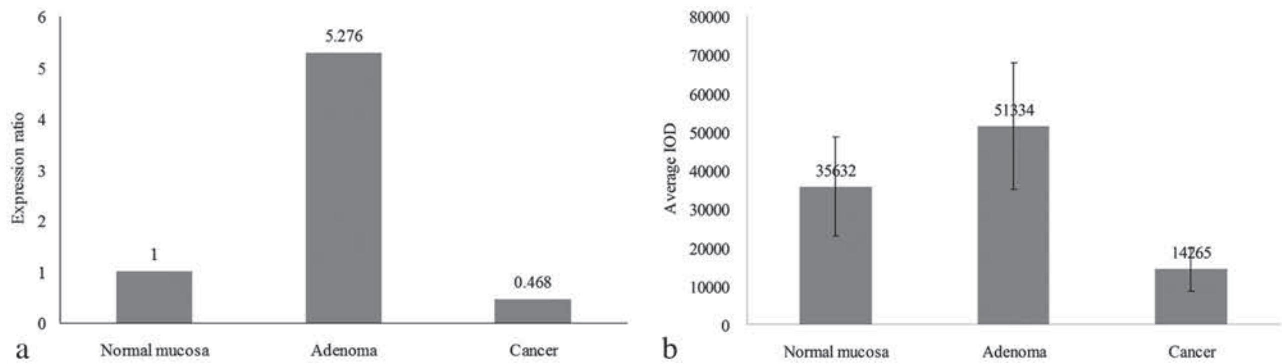

Fig. 1. — The expression of caspase-9 for normal colon mucosa, adenoma and cancer. (a) Relative expression of caspase-9 mRNA by quantitative reverse transcription polymerase chain reaction. The differences in expression were both significant (adenoma vs. mucosa, $P = 0.001$; cancer vs. mucosa, $P = 0.003$. REST analysis) (b) Average integrated optical density (IOD) of caspase-9 protein by immunohistochemical staining. The differences were both significant (adenoma vs. mucosa, $P = 0.016$; cancer vs. mucosa, $P = 0.021$. ANOVA analysis).

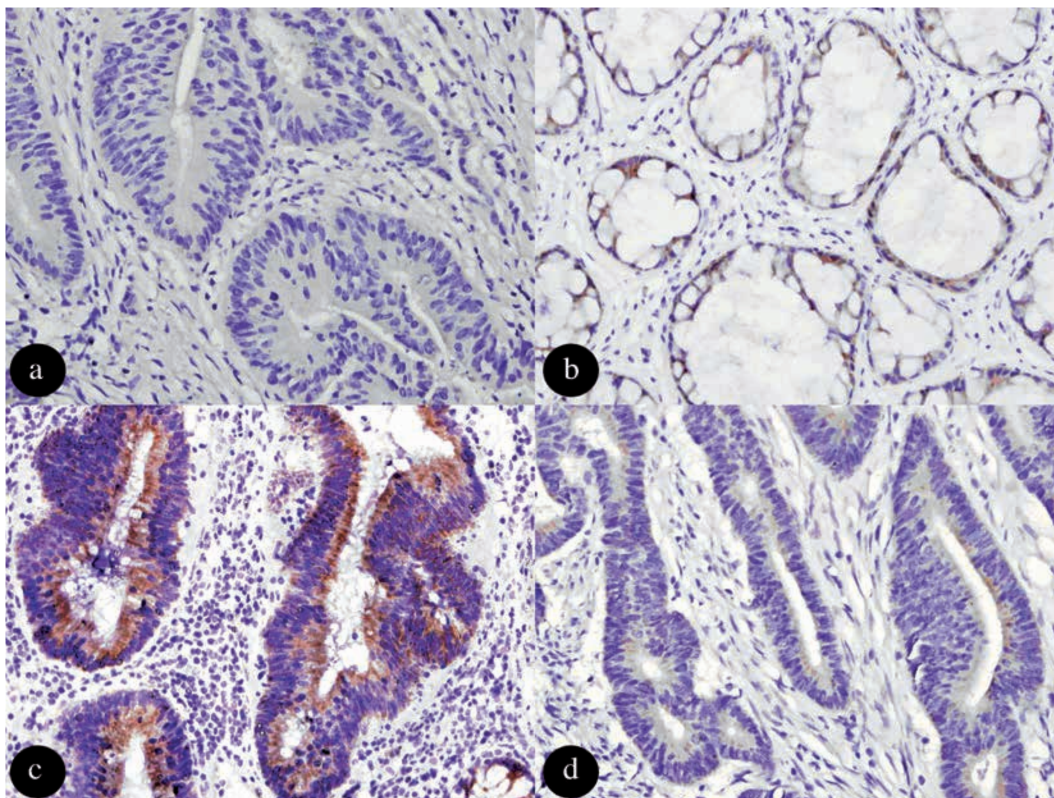

Fig. 2. — Immunohistochemical staining of caspase-9 for colon mucosa, adenoma and cancer (SP, ×40). (a) Negative control. (b) Normal colon mucosa. The cytoplasm and membrane of gland cells are brown stained while stromal cells remain negative (IOD = 38581). (c) Adenoma. Strong positive staining was observed. The stained particles gather in groups within the acinar lumina (IOD = 55418). (d) Cancer. Light staining mainly located in the acinar lumina (IOD = 11143).

L.V. respectively). However, the differences between transduced and untreated controls were all not significant (Fig. 3c, 3d). Similarly, Western Blot also showed a marked increment in the expression of caspase-9 protein for the study groups, while no significant difference was observed between controls ($P > 0.05$, Fig. 3e).

Overexpression of caspase-9 affects colony formation

Next, soft agar colony formation assay was applied to examine clonogenicity of different cell groups. After 10 days of incubation, colonies were observed in all groups. However, compared with the untreated and

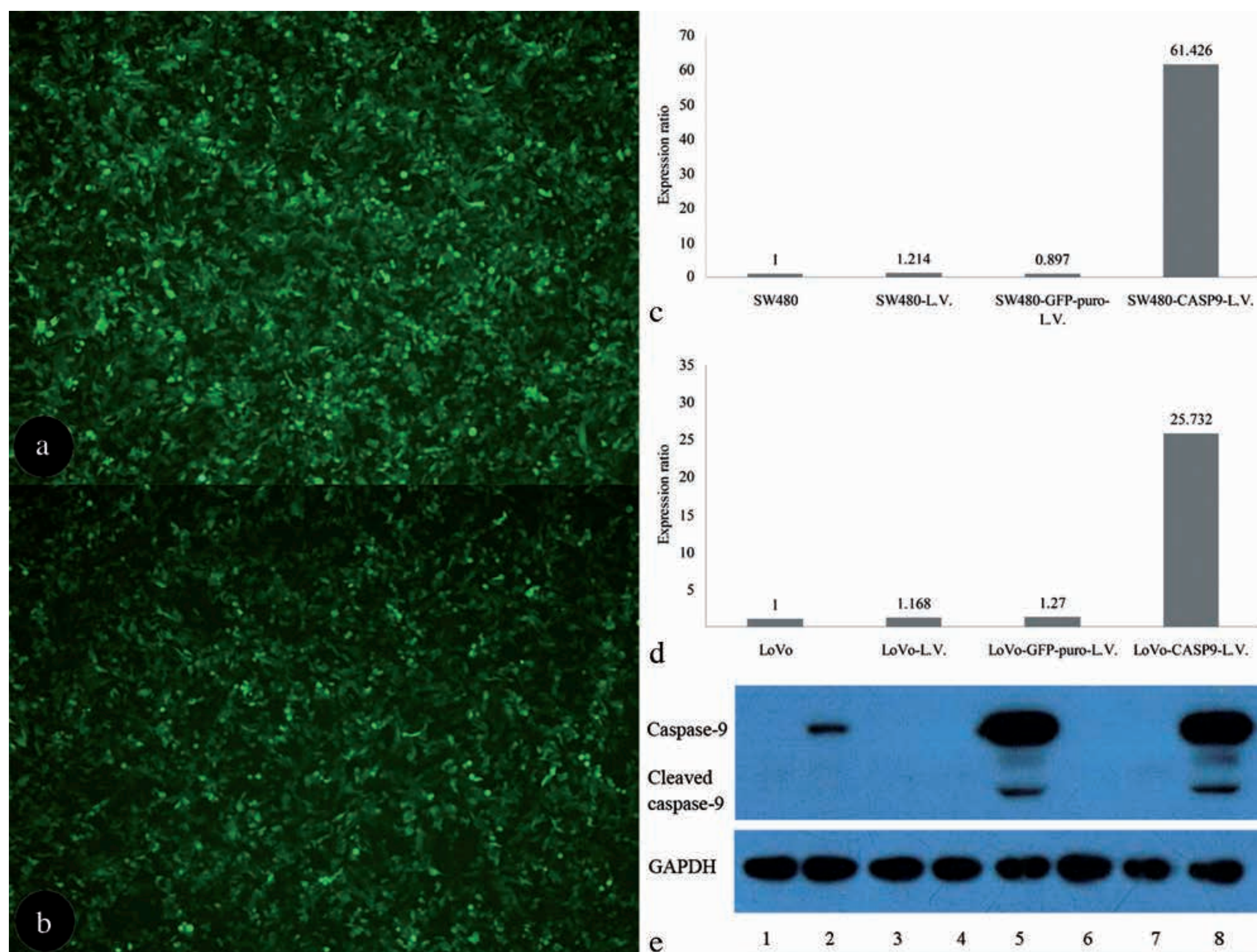

Fig. 3. — Stable cell lines with transduction. (a, b) Pictures were taken at day 12 after infection (Fluorescence, ×10). 3a : SW480-GFP-puro-L.V., 3b : LoVo-GFP-puro-L.V.. (c, d) Quantitative reverse transcription PCR detected statistically higher relative expression of caspase-9 mRNA for cells transduced with caspase-9 sequence ($P = 0.001$, REST analysis) while the differences between untreated and treated controls were all not significant ($P > 0.05$, REST analysis). 3c : SW480 cell groups, 3d : LoVo cell groups. (e) Western blot assay. Lane 1 : HEK-293T cell ; Lane 2 : Positive control, HEK-293T transduced with CASP9-L.V. ; Lane 3 : untreated SW480 cell ; Lane 4 : SW480-GFP-puro-L.V. cell ; Lane 5 : SW480-CASP9-L.V cell ; Lane 6 : untreated LoVo cell ; Lane 7 : LoVo-GFP-puro-L.V. ; Lane 8 : LoVo-CASP9-L.V.

treated controls, smaller colony size and fewer colony counts were observed in SW480-CASP9-L.V. and LoVo-CASP9-L.V. cultivation dishes. Concerning colony formation efficiency, the differences between caspase-9 overexpression cells and untreated controls were both significant (P = 0.022 and 0.013 for SW480 and LoVo cells, respectively).

Overexpression of caspase-9 inhibits proliferation

We used MTT assay to measure cell proliferation during 7 days of cultivation. Compared with untreated SW480 and LoVo cells, the caspase-9 overexpression groups showed smoother proliferation curve although the calculated difference was not significant ($P = 0.079$ and 0.117 for SW480 and LoVo cells, respectively. Fig. 4). The statistics between treated and untreated controls were all not significant (Data not shown).

Overexpression of caspase-9 induces apoptosis and affects cell cycle

Apoptosis was first examined by TUNEL array. Under a light microscope, brown stained granules localized in the nucleus were observed in an apoptotic cell. Usually, nuclear fragmentation, nuclear pyknosis and plasma membrane vesicles were typical morphological changes. As shown in Table 4, higher apoptosis rate was observed in caspase-9 overexpression cells when compared with controls ($P < 0.05$). However, the differences between untreated and treated controls were not significant (statistics not shown).

Secondly, flow cytometry was used to determine the effect of caspase-9 overexpression in cell cycle progression. As the result, more G0/G1 phase and less S phase cells were observed for the caspase-9 overexpression groups. Statistics were significant between the study

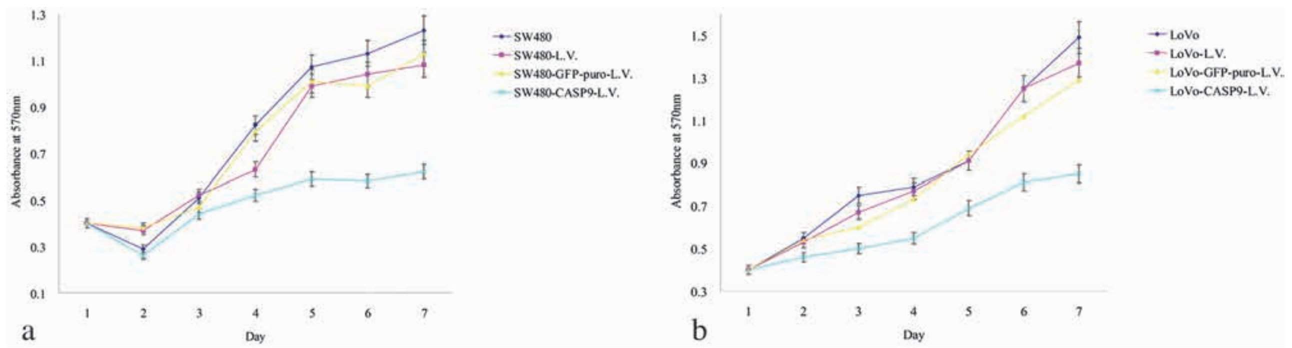

Fig. 4. — Growth curve for cells with or without caspase-9 overexpression. (a) SW480 cell groups. (b) LoVo cell groups. Reflected by absorbance at 570nm, the cells with caspase-9 overexpression exhibited smoother curves when compared with the controls. However, data showed that the differences between the study groups and untreated controls were not significant ($P = 0.079$ and 0.117 for SW480 and LoVo cells, respectively) within 7 days of observation.

Table 4. — **Apoptosis rate and cell cycle distribution of studied cell lines (%, x ± s)**

Cell line	Apoptosis rate		Cell cycle		
	Blank	5-FU	G0/G1	S	G2/M
SW480	2.9 ± 0.6*	10.3 ± 2.6*	39.2 ± 4.9*	43.1 ± 3.6*	17.7 ± 3.0
SW480-L.V.	2.7 ± 0.3*	12.6 ± 1.1*	36.0 ± 5.8*	37.1 ± 6.1	26.9 ± 9.1
SW480-GFP-puro-L.V	3.2 ± 0.7*	9.8 ± 3.0*	44.6 ± 4.4	42.1 ± 2.0*	13.3 ± 3.1
SW480-CASP9-L.V.	6.7 ± 1.3	21.4 ± 3.4	53.7 ± 7.1	27.5 ± 8.8	18.8 ± 15.2
LoVo	3.1 ± 0.6*	10.4 ± 2.8*	44.9 ± 8.1*	45.1 ± 7.2*	9.9 ± 2.3
LoVo-L.V.	4.4 ± 1.2*	13.0 ± 1.2*	49.0 ± 5.9*	33.3 ± 2.7*	17.7 ± 3.3
LoVo-GFP-puro-L.V.	4.7 ± 2.1*	10.4 ± 3.6*	40.4 ± 6.5*	35.0 ± 3.5*	24.6 ± 3.1
LoVo-CASP9-L.V.	7.7 ± 1.6	20.4 ± 4.6	61.3 ± 3.7	22.0 ± 3.9	18.7 ± 1.9

* The difference was significant when compared with caspase-9 overexpression cell group ($P < 0.05$).
Data shown here represents at least three times repetition of single experiment.

groups and untreated controls, but not between untreated and treated controls (data not shown). See table 4 and fig. 5.

Caspase-9 overexpression increase caspase-3 expression while decrease carcinoembryonic antigen (CEA) secretion

The influence of caspase-9 overexpression in caspase-3 expression and CEA secretion was examined by enzyme-linked immunosorbent assay. Compared with untreated controls, an approximately 3-fold increment in caspase-3 expression was observed in LoVo-CASP9-L.V. cells while CEA secretion decreased by one third (Table 5). Similar results were observed in SW480 cells although the difference in CEA secretion was not significant. However, the differences between untreated and treated controls were all not significant ($P > 0.05$, see table 5).

Discussion

In recent years, convincing data has indicated that colorectal neoplasia undergoes a continuous change from normal mucosa, to adenoma and eventually to cancer (11, 12). According to Bedi *et al.*, the transformation of colorectal epithelium to carcinoma was associated with a progressive inhibition of apoptosis (13). Thus, the variation of expression for a certain factor in normal tissue, benign tumor and malignancy may bring about hints of its function in carcinogenesis. In this study, we observed the highest expression of caspase-9 in adenoma both at mRNA and protein level. It suggests that the activity of apoptosis in adenoma is not inhibited but enhanced. Therefore, the overexpression of caspase-9 in colorectal adenoma might be viewed as a protective mechanism against increased proliferation. However, loss of this protective mechanism may contribute to tumor genesis which at last showed as inhibited apoptosis, including down-regulation of caspase-9, in cancer (14). Thus, it can be inferred that the down-regulation of capase-9 expression might be associated with the deregulation of apoptosis in colorectal cancer (15).

According to general belief, although mutation or loss of caspase-9 is not frequent, the blockage of its activities by inhibitor facilitates oncogenic transformation and tumor progression (16). This was supported by Soengas *et al.*, who observed that the inactivation of Apaf-1 or caspase-9 might substitute for p53 loss in promoting the oncogenic transformation of Myc-expressing cells (15). However, Marsden *et al.* reported the overexpression of

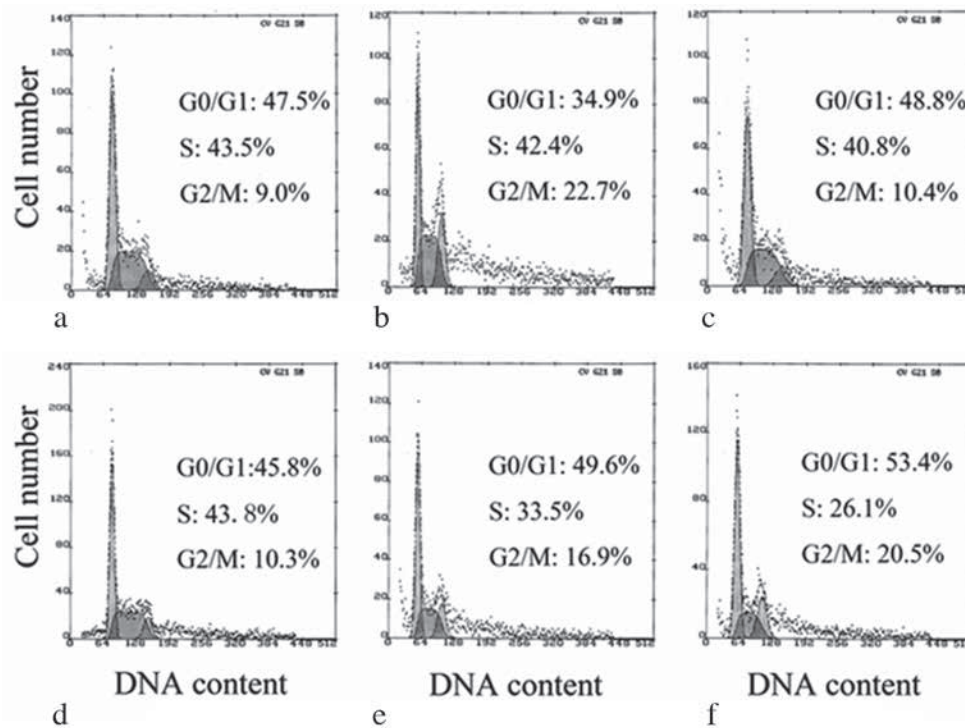

Fig. 5. — Cell cycle analysis of flow cytometry for cells with or without caspase-9 overexpression. (a) untreated SW480 cells. (b) SW480-L.V. cells. (c) SW480-CASP9-L.V. cells. (d) LoVo cells. (e) LoVo-L.V. cells. (f) LoVo-CASP9-L.V. cells. The differences in percentage of G0/G1 phase and S phase cells between the caspase-9 overexpression and untreated cells were both significant ($P < 0.05$).

Table 5. — **The caspase-3 and carcinoembryonic antigen (CEA) level examined by enzyme-linked immunosorbent assay**

	Caspase-3	*P*	CEA	*P*
SW480	1.93 ± 0.79		17.22 ± 0.59	
SW480-L.V.	2.20 ± 0.26	0.431	16.86 ± 0.58	0.306
SW480-GFP-puro-L.V.	1.69 ± 0.31	0.504	16.34 ± 1.24	0.149
SW480-CASP9-L.V.	4.91 ± 2.03	0.007	15.64 ± 2.04	0.099
LoVo	2.30 ± 0.26		23.25 ± 5.29	
LoVo-L.V.	2.01 ± 0.46	0.218	26.35 ± 3.64	0.264
LoVo-GFP-puro-L.V.	2.40 ± 0.23	0.494	21.11 ± 2.53	0.392
LoVo-CASP9-L.V.	7.45 ± 0.62	0.000	15.69 ± 3.48	0.015

Data shown here represents at least three times repetition of single experiment.

Bcl-2, an inhibitor of apoptosis by safeguarding mitochondrial membrane, could inhibit lymphocyte apoptosis while the absence of caspase-9 could not (17). Similar to this, Ekert *et al*. found that Apaf-1, caspase-2 and caspase-9 are required for rapid, efficient apoptosis of myeloid cells, but their loss does not enhance survival of cells (18). They tended to believe that caspase-9 is not required for programmed cell death, but merely affect its survival rate. Thus, the role of caspase-9 in carcinogenesis remains controversial.

In this study, we found that overexpression of caspase-9 inhibited proliferation, facilitated apoptosis and blocked cell cycle transition. Obviously, the results confirmed the apoptosis promoting role of caspase-9. Moreover, the finding was strengthened by the elevation in apoptosis effector caspase-3 production for caspase-9 overexpression cells. Although some researchers tended to question the trigger effect of any individual malfunction of caspases in carcinogenesis, the elevation of effector caspase-3 expression does mean more active apoptosis execution (19). Therefore, the overexpression of caspase-9 might be viewed as an actual reverse factor in carcinogenesis related apoptosis inhibition.

On the other hand, poorer colony formation, slower cell proliferation and less CEA secretion were observed in caspase-9 overexpression cells. According to Yang *et al*. and Chien *et al*., these were characteristics representing better differentiation (10,20). The results were consistent

with the histopathological analysis of colon cancer specimen which discovered a significant correlation between down-regulation of caspase-9 expression and poor differentiation of cancer. Due to the widely accepted relationship between tumor differentiation and patient survival, the finding was further supported by another study of ours which observed the prognostic value of caspase-9 in survival for colorectal cancer patients (21). In fact, except for a role in determining cell survival, more and more discoveries supported the non-apoptotic roles of caspases (22,23). Similar to us, Murray *et al*. reported the role of caspase-9 in muscle cell differentiation, where knockdown of caspase-9 prevented the differentiation of myoblasts into myotubes (24). Thus, the present study suggests a two-facet protective role of caspase-9, including not only mediation of apoptosis, but also promotion of differentiation.

In terms of current understanding, the activity of caspase is usually essential for the occurrence of apoptosis. The initiator caspases, such as caspase-8, -9 and -10, play an obligatory and unique role in the activation of this proteolytic cascade. This makes them good candidates for controlling attempt and targeted therapy. The first part of this study observed the variation of caspase-9 expression during tumor transformation and its relationship with cancer differentiation. This was followed by functional tests of caspase-9 overexpression cancer cells which resulted in poor colony formation, slowdown proliferation, enhanced apoptosis, cell cycle arrest and adjusted secretion. However, whether these will happen in a tissue background is still unclear. According to Matrisian *et al*. and Liotta *et al*., the result of mere cell line is not preferable since they may not express important surface molecules and secretory molecules related to the communication or interaction with the surrounding microenvironment (25,26). Thus, further investigation of the joint function with other members is needed in deciding the potential of caspase-9 in colon caner intervention.

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Conflict of interest

All authors have read and approved the final version of the manuscript. None of the authors has any type of financial interest to disclose.

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