Expression of α-, ß- and γ-synuclein in colorectal cancer, and potential clinical significance in progression of the disease

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Abstract. The synucleins (α-, ß- and γ-synuclein) are a small, soluble, highly conserved group of neuronal proteins that attracted considerable attention due to their involvement in both neurodegenerative diseases and cancer. In this study, we examined the synuclein exprsssion in colorectal cancer (CRC) tissues, tumor-matched non-neoplastic adjacent tissues (NNAT), and CRC cell lines, and then investigated clinical significance of synucleins. By using semi-quantitative RT-PCR, synuclein mRNA expression was detected in eight CRC cell lines. It was much higher in CRC samples than in NNAT samples (P<0.05). The results of Western blotting showed that the levels of synucleins protein expression in CRC cells approximately corresponded to the levels of synuclein mRNA expression. Immunohistochemical staining revealed that γ-synuclein protein expression was up-regulated in CRC samples compared to NNAT samples (P=0.022), and was significantly correlated with clinical stage and lymph node involvement of CRC (P<0.05). Although, there was no significant difference in either α- or ß-synuclein protein expression between tumor and normal samples (P>0.05), often more than one form of synuclein was expressed in a tumor sample. More ratios of later stage and lymph node-positive tumors expressed a least one type of synuclein protein, and more ratios showed positive for either α or γ-synuclein expression, as well as positive either for ß or γ-synuclein in more ratios of lymph node-positive tumors. These results show that α-, ß- and γ-synuclein are expressed in a high percentage of CRC. γ-synuclein protein is valuable for evaluation of progression of CRC, and it is more sensitive to predict advanced stage and lymph node invasion by detection of γ-synuclein protein combined with either α- or ß-synuclein protein or both than by detection of γ-synuclein only.

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Abbreviations: CRC, colorectal cancer; NNAT, tumor-matched non-neoplastic adjacent tissues; BCSG1, breast carcinoma specific gene 1; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate

Key words: synuclein, expression, colorectal cancer, clinicopathological factors

Introduction

The synucleins are a family of small, soluble, highly conserved neuronal proteins that consist of α-, ß-, and γ-synuclein. They are a natively unfolded group of proteins that are characterized by 5-6 repeats of amino acid motif (KTKEGV), constituting most of the N-terminal half of the proteins. These repeats result in the formation of conserved amphipathic A2-helices also characteristic of apolipoproteins, which mediate reversible binding to phospholipid membranes. This property supports the role of synucleins in vesicular release at presynaptic nerve terminals (1-4). Although their normal cellular functions have not been clearly defined, synucleins have attracted considerable attention due to their involvement in neurodegenerative diseases. α-synuclein is the major component of Lewy bodies in Parkinson's disease and has also been identified as the non-amyloid component of amyloid deposition, the hallmark of Alzheimer's disease (5,6). ß- and γ-synuclein are assumed to have a neuroprotective role by inhibiting α-synuclein aggregation and toxicity (7,8).

Synuclein expression is usually highly tissue-specific, and restricted to brain tissue and presynaptic terminals. However, aberrant expression of synucleins, particularly γ-synuclein, beyond neuronal system may be highly associated with human malignancies (4). γ-Synuclein [also referred to as breast carcinoma specific gene 1(BCSG1)] initially was cloned from infiltrating breast carcinoma cells by using the expressed sequence tag-based differential cDNA sequencing approach in 1997 (9). The following studies showed that γ-synuclein was aberrantly expressed in various types of cancer, especially in advanced stages of the diseases, but rarely expressed in tumor-matched non-neoplastic adjacent tissues (NNAT) (10). Patients with γ-synuclein protein-positive breast cancer have a significantly shorter disease-free survival and overall

survival period compared to patients with no γ-synuclein expression (11,12). Furthermore, studies to date indicate that overexpression of γ-synuclein can stimulate proliferation, and induce invasion and metastasis of breast cancer cells *in vitro* assays as well as in animal models (13). γ-Synuclein has also been shown to compromise normal mitotic checkpoint controls, resulting in multinucleation as well as more rapid breast cancer cell growth (14,15). Overexpression of γ-synuclein also interferes with drug-induced apoptotic responses of breast and ovarian cancer cells (16).

α- and ß-synuclein also were expressed in some types of cancer. Seventy-nine percent (19/24) of ependymomas, 52% (16/31) of astrocytomas, 38% (3/8) of oligodendrogliomas, 76% (16/21) of medulloblastomas have immunoreactivity for either α- or ß-synuclein or both (17). Eighty-seven percent (39/45) of ovarian cancers were found to express at least one type of synuclein, and 42% (19/45) expressed all three synucleins simultaneously (18).

Recent reports demonstrate that colorectal cancer (CRC) is the third most common malignancy and the third leading cause of cancer-related deaths worldwide (19). The conventional therapies involving surgery and adjuvant therapy seem to give rise to improvements in progression-free and overall survival, nevertheless about 50% of patients die within 5 years owing to metastasis or recurrent disease. Patients with early stage CRC have an estimated 5-year survival rate of 91%, compared to only 6% for those with later stage disease. Early detection remains the most important factor in improving long-term survival. Furthermore, tumor invasion and regional lymph node metastasis are important factors for determining CRC prognosis (20-22). In a previous study, we have shown that γ-synuclein expression was up-regulated in CRC, which was primarily attributed to demethylation of CpG island in exon 1 of γ-synuclein gene, and aberrant expression and demethylation of γ-synuclein closely correlated with advanced clinical stage, lymph node involvement and distant metastasis (23).

The expression of synucleins (α and ß) in CRC has not been systematically examined. With demonstration of synucleins expression in ovarian cancer and γ-synuclein expression in CRC, we hypothesized that α-, ß-synuclein were also expressed in CRC and may be used as biomarkers to evaluate the disease progression. To address our hypothesis, we investigated the expression of α-, ß- and γ-synuclein in CRC, NNAT samples and CRC cell lines, and then analyzed the relationship between the expression of synucleins in CRC and clinicopathological factors.

Materials and methods

Tissue samples and cell lines. Fifty-one pairs of CRC and NNAT samples were obtained from patients undergoing CRC surgery between January 2007 and October 2008 at the Department of General Surgery, Ruijin Hospital, Shanghai, China. Washed with RNase-Free 0.9% NaCL to remove blood after surgery, one half of each sample was snap-frozen in liquid nitrogen immediately and stored at -80˚C for RNA extraction, and the other half was fixed in 10% formaldehyde for histological assessment. For tumor samples, non-tumor portions were trimmed off from the frozen tumor blocks and the selected areas had more than 80% tumor cells as shown by histological assessment for RNA extraction. Tumors were staged using the TNM and World Health Organization classification systems. The Ethics Committee at Ruijin Hospital approved the use of these tissues for research purposes. Eight CRC cell lines, COLO205 (ATCC no. CCL-222), HT-29 (HTB-38), HCT116 (CCL-247), LoVo (CCL-229), SW1116 (CCL-233), SW480 (CCL-228), SW620 (CCL-227) and Caco-2 (HTB-37) with different genetic background were cultured under appropriate conditions in our laboratory. COLO205, LoVo, SW1116 and SW480 cells were cultured in RPMI-1640 medium (Gibco BRL, Life Technologies Inc., USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO, USA). HT-29, HCT116 and Caco-2 cells were cultured in DMEM medium (Gibco BRL) supplemented with 10% FBS. SW620 cell was cultured in Leibovitz's L-15 medium (Gibco BRL) supplemented with 10% FBS.

Antibodies. The following antibodies were used: mouse anti-α-synuclein polyclonal antibody and rabbit anti-ß-synuclein polyclonal antibody were from Abcam (CA, USA), mouse anti-γ-synuclein monoclonal antibody, mouse anti-GAPDH polyclonal antibody, goat anti-mouse IgG-AP antibody, and goat anti-rabbit IgG-AP antibody were from Santa Cruz (CA, USA).

Total RNA extraction, cDNA synthesis and semi-quantitative PCR of synucleins mRNA. Cultured cells were washed twice with phosphate-buffered saline (PBS) and harvested, and tissues were ground into fine powder in liquid nitrogen before extraction of RNA. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis from 1 μg of RNA was performed with reverse transcription system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCR reactions were carried out in a thermal cycler (model PTC-225, MJ Research, Watertown, MA, USA) using Go Taq Green master mix (Promega, Madison, WI, USA) as follows: 94˚C for 5 min; 30 cycles of 94˚C for 1 min, 58˚C for 45 sec (60˚C, 45 sec for γ-synuclein), and 72˚C for 1 min; 72˚C for 10 min. The expression of GAPDH mRNA was taken as an internal loading control. H_2O was used as a negative control. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide. Table I provides the sequences of the primers used in this study.

Western blot analysis. Cells were harvested and lysed with mammalian protein extraction reagent (Pierce Rockford, IL, USA). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford). Samples containing 50 μg of protein were mixed with 2X sodium dodecyl sulfate (SDS) gel-loading buffer (100 mmol/l Tris-CL, 200 mmol/l dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), boiled for 5 min, loaded onto each lane of 15% acrylamide gel in a minigel apparatus (Bio-Rad, Richmond, CA, USA), and separated by SDS-PAGE. The separated proteins were electrophoretically transferred to Sequi-blot PVDF membrane (Bio-Rad Laboratories,

Table I. Sequences of gene-specific primers.

Primer	Sequence (5'-3')	Product size (bp)
α-synuclein-5'	ATGTAGGCTCCAAAACCAAGG	88
α-synuclein-3'	CCTCCAACATTTGTCACTTGCT	
ß-synuclein-5'	GTACAAGGTGTGGCTTCAGT	434
ß-synuclein-3'	GCGGGTAGGACAGACAGATGGA	
γ-synuclein-5'	GGTGTGGCATCCAAAGAGAAAG	153
γ-synuclein-3'	CATCCACGCTGGCCTGTAG	
GAPDH-5'	GAAGGTGAAGGTCGGAGTC	226
GAPDH-3'	GAAGATGGTGATGGGATTTC	

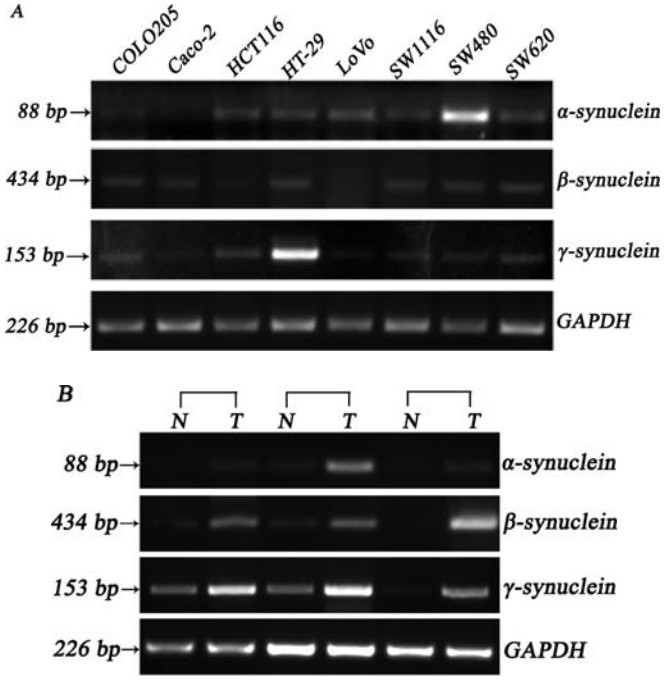

Figure 1. Detection of synucleins mRNA in CRC cell lines, CRC samples (T) and NNAT samples (N). (A) The results of RT-PCR in 8 CRC cell lines, COLO205, Caco-2, HT-29, HCT116, LoVo, SW1116, SW480, and SW620. (B) The results of RT-PCR in 3 representative pairs of samples.

Hercules, CA, USA). After being incubated with primary antibody (1:500) and AP-conjugated secondary antibody (1:5000) respectively, immune complexes were detected using BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, St. Louis, MO).

Tissue microarray construction. Tissue microarray recipient block was constructed containing paraffin-embedded 51 pairs of CRC and NNAT samples previously fixed in 10% formaldehyde. The most representative tumour or normal areas were carefully selected and marked based on the matched haematoxylin-eosin-stained slides. Altogether, 102 cores (diameter 1.8 mm) of test tissue were taken from the donor blocks with the tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA) and inserted into the recipient block.

Immunohistochemistry analysis. Unstained 4 mm sections were cut from the tissue microarray recipient block and deparaffinized in xylene, and the slides were bathed in 0.01 mol/l sodium citrate and heated in a microwave oven for 12 min. The sections were incubated with anti-synuclein antibody and kept at 4˚C overnight. Negative control slides were treated with only non-immunized mouse immunoglobulin fraction under equivalent conditions. For the secondary developing reagents, a labeled streptavidin-biotin kit (Dako, CA, USA) was used. Slides were developed with diaminobenzaminidine and counterstained with hematoxylin. Positive cases were defined by the presence of intracellular staining with red/brown color in epithelial cells. Negative cases were defined by the absence of specific intracellular staining as seen in negative control. Samples were evaluated under light microscopy independently by two pathologists without prior knowledge of the patients' clinical data.

Statistical analysis. Statistical analyses were performed using SPSS11.0 software (Shanghai Jiaotong University School of Medicine, Shanghai, China). Student's t-test was used to analyze expression of synucleins mRNA (mean ± standard deviation) in paired CRC and NNAT samples, Fisher' s exact test was used to analyze expression of synucleins protein in paired CRC and NNAT samples, and to assess the relationship between the synucleins protein expression and clinicopathological characteristics. $P<0.05$ was considered statistically significant.

Results

Expression of synucleins mRNA in CRC cell lines. The expression of α- and ß-synuclein in CRC has not been established. We first used RT-PCR to assess α-, ß and γ-synuclein mRNA in eight CRC cell lines, COLO205, HT-29, HCT116, LoVo, SW1116, SW480, SW620 and Caco-2, as shown in Fig. 1A. Under the 30-cycle conditions, synucleins mRNA expression was detected in almost all the cells. High levels of α-synuclein mRNA expression were identified in 7 of 8 cells. Very strong expression of α-synuclein mRNA was found in SW480 cells. Caco-2 showed very low expression of α-synuclein mRNA under the same PCR conditions. High levels of ß-synuclein mRNA expression were identified in 6 of 8 cells. HCT116 and LoVo expressed a trace amount of ß-

Table II. The expression of synucleins in colorectal cancer tissues (CRC) and tumor-matched non-neoplastic adjacent tissues (NNAT).

Group	CRC	NNAT	P-value
Synuclein mRNA expression ($\bar{x} \pm s$)			
α	0.62±0.32	0.41±0.27	0.034[a]
ß	0.61±0.32	0.40±0.25	0.026[a]
γ	0.72±0.37	0.45±0.25	0.007[a]
Positive synuclein protein expression			
α (%)	21/51 (41.2)	13/51 (25.5)	0.141[b]
ß (%)	28/51 (54.9)	19/51 (37.3)	0.112[b]
γ (%)	24/51 (47.1)	12/51 (23.5)	0.022[b]

[a]Calculated by the Student's t-test; [b]calculated by the Fisher's exact test.

synuclein under the same PCR conditions. Very strong expression of γ-synuclein mRNA was detected in HT-29 and HCT116, and the remaining six cell lines showed moderate levels of γ-synuclein mRNA expression. In contrast, RT-PCR detected equal expression of GAPDH mRNA in all the cells.

Expression of synucleins mRNA in CRC and NNAT samples. To gain initial knowledge, we chose 20 pairs of CRC and NNAT samples for the study of gene expression. The 20 tumors were diagnosed as CRC with 9 of TNM stage I/II, and 11 of stage III/IV. Fig. 1B shows the results of semi-quantitative RT-PCR in 3 representative pairs. The α-synuclein mRNA expression was detected in 16 of 20 (80%) CRC samples and 9 of 20 (45%) NNAT samples. The mean α-synuclein/GAPDH was 0.62±0.32 in CRC samples, while 0.41±0.27 in NNAT samples. The α-synuclein mRNA expression levels in CRC samples were significantly higher than those in NNAT samples (Table II, P=0.034). Similarly, ß-synuclein mRNA expression was detected in 18 of 20 (90%) CRC samples and 12 of 20 (60%) NNAT samples. The mean ß-synuclein/GAPDH was 0.61±0.32 in CRC samples, which was much higher than 0.40±0.25 in NNAT samples (Table II, P=0.026). The γ-synuclein mRNA expression was detected in 16 of 20 (80%) CRC samples with mean γ-synuclein/GAPDH 0.72±0.37, and in 12 of 20 (60%) NNAT samples with mean γ-synuclein/GAPDH 0.45±0.25; there was also a significant diference between tumor and normal samples (Table II, P=0.007), in line with our pevious report (23).

Expression of synuclein protein in CRC cell lines. Western blot analysis, using specific anti-α-, ß or γ-synuclein antibody, demonstrated very strong expression of synuclein protein was detected in the same cell lines that express very strong expression of synuclein mRNA (Fig. 2). SW480 showed

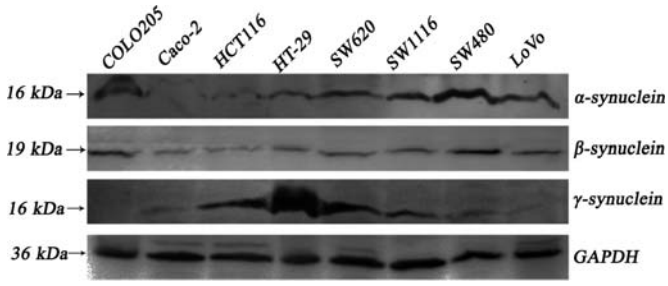

Figure 2. Western blot analysis of α-, ß and γ-synuclein protein expression in 8 CRC cell lines COLO205, Caco-2, HT-29, HCT116, LoVo, SW1116, SW480, and SW620.

very strong expression of α-synuclein protein, and very strong expression of γ-synuclein protein was detected in HT-29 and HCT116. However, some cells, which displayed very lower levels or a trace amount of synuclein mRNA also expressed synuclein protein, for instance, α-synuclein protein expression in Caco-2, and ß-synuclein protein expression in HCT116 and LoVo cells. On the other hand, there was undetectable γ-synuclein protein expression in COLO205 that expressed γ-synuclein mRNA.

Immunohistochemical staining of synuclein protein in CRC and NNAT samples. The expression of synuclein protein in the 51 pairs of tissues was also evaluated by IHC. Immunohistochemical staining showed that the subcellular localization of synuclein was cytoplasmic in epithelial cells of tumor or normal tissues (Fig. 3). Twenty-one of 51 (41.2%) CRC samples and 13 of 51 (25.5%) NNAT samples were immunohistochemically positive for α-synuclein, and 28 of 51 (54.9%) CRC samples and 19 of 51 (37.3%) NNAT samples for ß-synuclein. There was no significant difference in either α- or ß-synuclein protein expression between tumor and normal samples (Table II, P>0.05). Twenty-four of 51 (47.1%) CRC samples and 12 of 51 (23.5%) NNAT samples showed positive immunohistochemical staining for γ-synuclein. The result indicated that γ-synuclein protein expression was up-regulated in CRC samples compared to that in NNAT samples (Table II, P=0.022), which was in line with our pevious report (23). Immunohistochemical staining has also revealed that often more than one form of synuclein protein was expressed in one tumor sample, and some tumors expressed all three proteins (Table III). At least one type of synuclein was expressed in 41 of 51 (80.4%) CRC samples, compared in 29 of 51 (56.9%) NNAT samples (Table III, P=0.018). Similarly, the frequency of positive for either α or γ-synuclein expression was 31/51 (60.8%) for CRC samples, and 20/51 (39.2%) for NNAT samples (Table III, P=0.047); positive ß or γ-synuclein expression was observed in 40/51 (78.4%) for CRC samples, and 25/51 (49.0%) for NNAT samples (Table III, P=0.004); 14/51 (27.5%) CRC samples and 5/51 (9.80%) NNAT samples expressed both α and γ-synuclein (Table III, P=0.04). The synucleins protein expression was relatively more prominent in CRC samples than in NNAT samples.

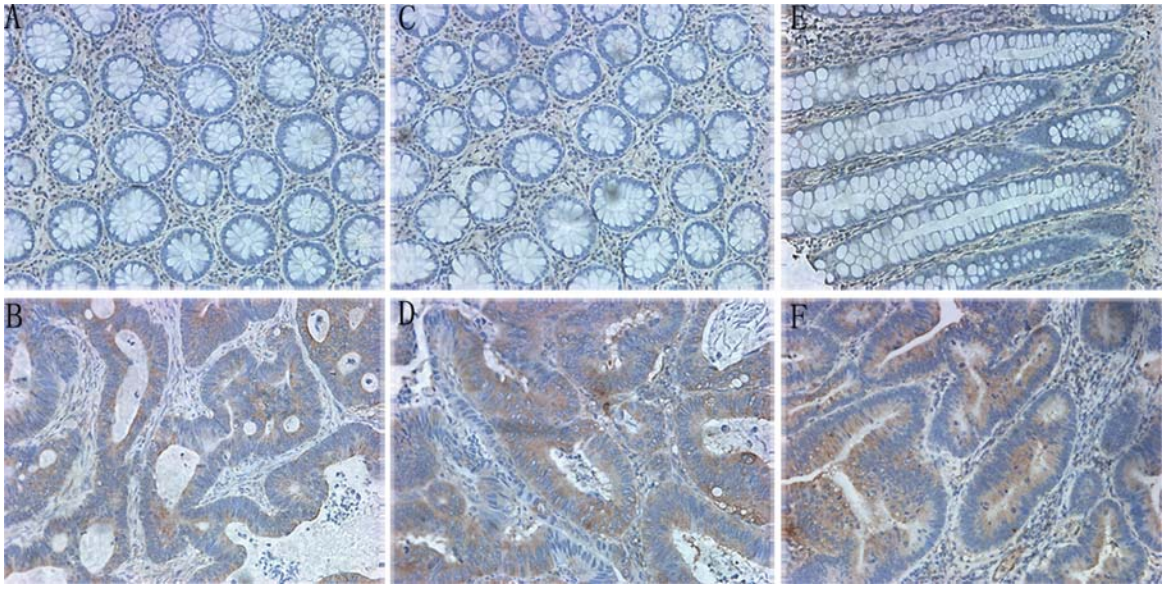

Figure 3. The expression of synuclein protein detected by IHC. Expression of (A) α-synuclein, (C) ß-synuclein and (E) γ-synuclein proteins in CRC tissue samples; expression of (B) α-synuclein, (D) ß-synuclein and (F) γ-synuclein proteins in NNAT samples. Strong cytoplasmic staining of α-, ß and γ-synuclein proteins were observed as brown stain in cancer cells, but negative or weak staining were observed in corresponding normal mucosal cells. Original magnification x400.

Table III. Coexpression of synuclein protein in CRC and NNAT.

Group	CRC	NNAT	P-value[a]
n	51 (100)	51 (100)	
α or ß (%)	35 (68.6)	25 (49.0)	0.070
α or γ (%)	31 (60.8)	20 (39.2)	0.047
ß or γ (%)	40 (78.4)	25 (49.0)	0.004
α and ß (%)	16 (31.4)	7 (13.7)	0.057
α and γ (%)	14 (27.5)	5 (9.80)	0.04
ß and γ (%)	13 (25.5)	6 (11.8)	0.126
α, ß and γ (%)	9 (17.6)	3 (5.88)	0.122
any synuclein (%)	41 (80.4)	29 (56.9)	0.018

Calculated by the Fisher's exact test.

Correlation between expression of synuclein protein and clinicopathological factors of CRC. To investigate clinical significance of synucleins, we analyzed the correlation between the expression of the three proteins and clinicopathological factors of CRC. The frequency of positive immunostaining of γ-synuclein for individual pathological stage was 2/13 (15.4%) for stage I, 5/10 (50%) for stage II, 14/24 (58.3%) for stage III, and 3/4 (75%) for stage IV. The γ-synuclein protein expression significantly correlated with clinical stage (Table IV, P=0.047). Similarly, there was a significant correlation between γ-synuclein protein expression and lymph node involvement (Table IV, P=0.048). There was no significant difference in either α- or ß-synuclein protein expression with respect to clinicopathological factors of CRC (age, sex, differentiation, stage, lymph node invision and distant metastasis) (Table IV, P>0.05). We further analyzed coexpression of synucleins in CRC samples of diverse clinicopathological characteristics (Table V). Compared with early stage and lymph node-negative tumors, more ratios of later stage and lymph node-positive tumors expressed a least one type of synuclein (Table V, P=0.04, P=0.03), and more ratios showed positive for either α or γ-synuclein expression (Table V, P=0.008, P=0.009). Similarly, more ratios of lymph node-positive tumors showed either ß or γ-synuclein expression (Table 5, P=0.04, P=0.03). Furthermore, it seemed more sensitive to predict advanced stage and lymph node invasion by detection of γ-synuclein protein combined with either α- or ß-synuclein protein or both than by detection of γ-synuclein only.

Discussion

Previous studies have indicated that γ-synuclein was up-regulated in a variety of cancers, including colorectal cancer (10). In our previous report, we also showed aberrant expression and demethylation of γ-synuclein in colorectal cancer, correlated with progression of the disease (23). In the present study, we report for the first time that multiple members of the synuclein family frequently are abnormally expressed in colorectal cancer.

Our results showed that α-, ß and γ-synuclein mRNA expression was up-regulated in CRC tissue samples and cell lines. Under the 30-cycle conditions of RT-PCR, synucleins mRNA expression was detected in almost all the cells studied. Very strong expression was detected in SW480 cells for α-synuclein mRNA, and in HT-29 and HCT116 for γ-synuclein mRNA. Under the same PCR conditions, the α-, ß and γ-synuclein mRNA expression levels in 20 CRC samples

Table IV. Correlation between synuclein protein expression and clinicopathological factors of colorectal cancer patients.

		Positive expression of synuclein protein					
Variable	n	α (%)	P-value	ß (%)	P-value	γ (%)	P-value
Age							
>65	28	15 (53.6)	0.085	17 (60.7)	0.407	14 (50.0)	0.780
≤65	23	6 (26.1)		11 (47.8)		10 (43.5)	
Sex							
Male	31	11 (35.5)	0.809	15 (48.4)	0.267	13 (41.9)	0.402
Female	20	10 (50.0)		13 (65.0)		11 (55.0)	
Histological type							
Differentiated	46	20 (43.5)	0.386	27 (58.7)	0.162	21 (45.7)	0.656
Undifferentiated	5	1 (20.0)		1 (20.0)		3 (60.0)	
Stage							
I	13	3 (23.1)	0.307	7 (53.8)	0.766	2 (15.4)	0.047
II	10	4 (40.0)		7 (70.0)		5 (50.0)	
III	24	11 (45.8)		12 (50.0)		14 (58.3)	
IV	4	3 (75.0)		2 (50.0)		3 (75.0)	
Lymph node invision							
Positive	28	26 (50.0)	0.253	14 (50.0)	0.573	17 (60.7)	0.048
Negative	23	15 (30.4)		14 (60.9)		7 (30.4)	
Distant metastasis							
Positive	4	4 (75.0)	0.293	2 (50.0)	1.000	3 (75.0)	0.331
Negative	47	37 (38.3)		26 (55.3)		21 (44.7)	

P-value was calculated by the Fisher's exact test.

were significantly higher than those in matched 20 NNAT samples (P<0.05).

We further examined the expression of synuclein protein in CRC cell lines by Western blot analysis. The results showed that the levels of synuclein protein expression in CRC cells approximately corresponded to the levels of synucleins mRNA expression. However, some cells, which displayed very lower levels or a trace amount of synucleins mRNA, also expressed synuclein protein, and there was undetectable γ-synuclein protein expression in COLO205 that expressed γ-synuclein mRNA. It appears that the levels of synuclein protein expression do not completely correspond to the levels of synuclein mRNA expression in CRC cell lines, although, no post-transcriptional regulation of synucleins has been found so far. There is a similar phenomenon in CRC and NNAT samples. Although, α-, ß and γ-synuclein mRNA expression was up-regulated in CRC samples compared in NNAT samples, there was no significant difference in either α- or ß-synuclein protein expression between tumor and normal samples (P>0.05). However, γ-synuclein protein expression was up-regulated in CRC samples compared to NNAT samples (P<0.05), and was significantly correlated with clinical stage and lymph node involvement of CRC (P<0.05), which was in line with our pevious study and supporting that γ-synuclein plays an oncogene role in CRC.

Although, there was no significant difference in either α- or ß-synuclein protein expression with respect to pathologic or clinical characteristics, based on γ-synuclein, α-, ß-synuclein may also be involved in the progression of CRC. Immunohistochemical staining also revealed that often more than one form of synuclein was expressed in one tumor sample, some tumors expressed all three proteins. We compared the α-, ß and γ-synuclein protein expression in CRC and NNAT samples, and found that there were significant differences in the expression of some protein combinations (the expression of either α or γ-synuclein, ß or γ-synuclein, both α and γ-synuclein, and any synuclein) between tumor and normal samples. The synuclein expression was more prominent in CRC samples than in NNAT samples. More ratios of later stage and lymph node-positive tumors expressed at least one type of synuclein protein, and more ratios showed positive either for α or γ-synuclein, as well as positive either for ß or γ-synuclein in more ratios of lymph node-positive tumors (P<0.05). Thus, it seemed more sensitive to predict advanced stage and lymph node invasion by detection of γ-synuclein protein combined with either α- or ß-synuclein protein or both than by detection of γ-synuclein only.

Clinical evidence from previous studies has shown the stage-specific expression of γ-synuclein in a wide range of cancer types, and experimental data from previous studies

Table V. Correlation between coexpression of synucleins protein and clinicopathological factors of colorectal cancer patients.

Variable	n	Positive expression of synuclein protein					
		Any synuclein (%)	P-value	α or γ (%)	P-value	ß or γ (%)	P-value
Age							
>65	28	23 (82.1)	0.739	19 (67.9)	0.388	23 (82.1)	0.565
≤65	23	18 (78.3)		12 (52.2)		17 (73.9)	
Sex							
Male	31	23 (74.2)	0.280	17 (54.8)	0.381	22 (71.0)	0.166
Female	20	18 (90.0)		14 (70.0)		18 (90.0)	
Histological type							
Differentiated	46	37 (80.4)	1.000	28 (60.9)	1.000	36 (78.3)	1.000
Undifferentiated	5	4 (80.0)		3 (60.0)		4 (80.0)	
Stage							
I	13	7 (53.8)	0.04	3 (23.1)	0.008	7 (53.8)	0.102
II	10	8 (80.0)		7 (70.0)		8 (80.0)	
III	24	22 (91.7)		17 (70.8)		21 (87.5)	
IV	4	4 (100)		4 (100)		4 (100)	
Lymph node invision							
Positive	28	26 (92.9)	0.03	22 (78.6)	0.009	25 (89.3)	0.048
Negative	23	15 (65.2)		9 (39.1)		15 (65.2)	
Distant metastasis							
Positive	4	4 (100)	0.573	4 (100)	0.145	4 (100)	0.565
Negative	47	37 (78.7)		27 (57.4)		36 (76.6)	

P-value was calculated by the Fisher's exact test.

have indicated that overexpression of γ-synuclein led to a significant increase in motility and invasiveness in cell culture and to a profound augmentation of metastasis in nude mice (10-13,24,25). α-, ß-synuclein were also expressed in some nervous system cancers, and breast and ovarian cancer (17,18). The results of this study on all members of synucleins further supported an oncogene role of γ-synuclein in colorectal cancer, and at the same time parallel a previous report on α-, ß-synuclein expression (18). However, there is not enough clinical and experiment evidence to support the positive role of α-, ß-synuclein in the process of tumorigenesis, except in the study of bladder cancer. Marta *et al* utilized oligonucleotide arrays to analyze the transcript profiles of bladder cancer. A genetic profile consisting of 174 probes including α-synuclein was identified in patients with positive lymph nodes and poor survival. Immunohistochemical analyses on tissue arrays sustained the significant association of α-synuclein with tumor staging and clinical outcome (26).

To understand the possible role of single α-, or ß-synuclein protein in tumorigenesis or progression of colorectal cancer, three possibilities can not be excluded. First, only 51 pairs of CRC and NNAT samples were brought in the experiment, and a significant correlation between single α-, or ß-synuclein protein and clinicopathological factors of CRC might be reached in the study on large quantities of samples. Second, synucleins may play different roles in different cancers with different genetic background, and α-synuclein may play a positive role in progression of bladder cancer but not in other cancer types including colorectal cancer. Third, pevious reports have shown that the fine balance of these synuclein proteins is important in maintaining normal brain function and the disturbance of this balance might lead to synucleinopathies (27). This raises a hypothesis that disturbance of this balance also might lead to tumorigenesis. α-, ß-, and γ-synuclein differ considerably in their acidic C-terminal domains, which is presumably where their functional differences arise, while share a characteristically conserved N-terminal domain, which arises presumably similar biological effects such as the chaperone activity and reversible binding to phospholipid membranes (4). All of these suggest that the relationship between α-, ß-, and γ-synuclein may be restriction or complementation, for instance, α-synuclein is the major pathological component of Parkinson's disease and Alzheimer's disease, and ß-, and γ-synuclein levels could reflect a neuroprotective role to inhibit the aggregation of α-synuclein proteins (7,8,28-30). On the other hand, the following situation might exist. α-, ß-synuclein are dispensable, and γ-synuclein plays a more important role in progression of colorectal cancer, when absent, the presence

and function of γ-synuclein is compensated by the other synucleins or inhibited by the other synucleins. Further study is needed to investigate the roles of synucleins, the relationship between synucleins and how it is implicated in colorectal cancer cell culture.

In conclusion, γ-synuclein expression is up-regulated in CRC, and is significantly correlated with the progression of CRC. α-, ß-synuclein are also expressed in a high percentage of CRC. Overall, the synuclein expression was relatively more prominent in CRC samples than in NNAT samples. It is more sensitive to predict advanced stage and lymph node invasion by detection of γ-synuclein protein combined with either α-, or ß-synuclein protein or both than by detection of γ-synuclein only. Further study is needed to prove the value of α-, ß-, and γ-synuclein as a tumor marker in large quantities of CRC tissue samples, and in patient prognosis evaluation. The functions of these small proteins in CRC cells are still unclear. Future research may encompass delineation of the interaction between γ-synuclein and α-, ß-synuclein and further identification of the relationship between synucleins.

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